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Inflammatory changes parallel the early stages of Alzheimer disease

A. Parachikova [a,*], M.G. Agadjanyan [c], D.H. Cribbs [a,b], M. Blurton-Jones [a], V. Perreau [a], J. Rogers [d], T.G. Beach [d], C.W. Cotman [a,b]

[a] *Institute for Brain Aging & Dementia, University of California, 1113 Gillespie Neuroscience Research Facility, Irvine, CA 92697-4540, USA*
[b] *Department of Neurology, College of Medicine, University of California, Irvine, CA 92697, USA*
[c] *Institute for Molecular Medicine, Department of Immunology, Huntington Beach, CA 92647, USA*
[d] *L.J. Roberts Center, Sun Health Research Institute, Sun City, AZ 85351, USA*

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Abstract

Alzheimer disease (AD) is the most prominent cause of dementia in the elderly. To determine changes in the AD brain that may mediate the transition into dementia, the gene expression of approximately 10,000 full-length genes was compared in mild/moderate dementia cases to non-demented controls that exhibited high AD pathology. Including this latter group distinguishes this work from previous studies in that it allows analysis of early cognitive loss. Compared to non-demented high-pathology controls, the hippocampus of AD cases with mild/moderate dementia had increased gene expression of the inflammatory molecule major histocompatibility complex (MHC) II, as assessed with microarray analysis. MHC II protein levels were also increased and inversely correlated with cognitive ability. Interestingly, the mild/moderate AD dementia cases also exhibited decreased number of T cells in the hippocampus and the cortex compared to controls. In conclusion, transition into AD dementia correlates with increased MHC II$^+$ microglia-mediated immunity and is paradoxically paralleled by a decrease in T cell number, suggesting immune dysfunction.

Keywords: MHC class II; Microglia; Inflammation; Dementia; Amyloid beta; T cell

1. Introduction

AD is a chronic neurodegenerative disorder characterized by progressive memory deterioration. To elucidate the etiology of AD dementia, attempts have been made to correlate cognitive dysfunction with the classical neuropathological changes in AD, extracellular β-amyloid (Aβ) plaques, intraneuronal neurofibrillary tangles (NFTs), and synaptic/neuronal loss. However, some studies suggest that plaques and NFTs may not directly cause AD dementia since both pathologies can manifest in brains of patients that are not cognitively impaired [10,14,49,56]. Another potential mechanism underlying dementia is synaptic loss, since synaptic markers are decreased in AD brains [16,54,59,62]. However, evidence suggests that synaptic loss may occur only in moderate to severe clinical grades of dementia while early AD cases exhibit an increase in presynaptic markers [44]. Synaptic deficiency also correlates with the accumulation of soluble intraneuronal Aβ in AD vulnerable brain regions [34,45]. Thus in accordance with the amyloid cascade hypothesis [19,20], the accumulation of soluble Aβ with age may impair synaptic pathways associated with learning and memory.

Cognitive dysfunction in AD may also be critically influenced by Aβ-induced brain inflammation [3,13,37,41,61]. Specifically, Aβ accumulation leads to a site-specific activation of glia resulting in the secretion of pro-inflammatory cytokines [3,13]. The inflammatory response may be an attempt to clear Aβ deposits; however, the progressive accumulation of Aβ and its aggregation into insoluble plaques may induce a chronic pro-inflammatory response leading to compromised neuronal function [8]. In support of a role for neuroinflammation in AD dementia, one study found a higher

* Corresponding author. Tel.: +1 949 824 6071; fax: +1 949 824 2071.
E-mail address: aparachi@uci.edu (A. Parachikova).

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correlation between synapse loss and activated microglia than between Aβ deposits and NFTs [33]. Furthermore, some epidemiological studies demonstrate that non-steroidal anti-inflammatory drugs (NSAIDs) can prevent or retard AD cognitive decline [29,40,52,58], although other studies have found little improvement [1,50]. This slowing of cognitive decline may be attributed to decreased inflammation since NSAID therapy substantially reduces the number of activated microglia associated with plaques [22,36].

To better understand the factors contributing to AD dementia, this study aimed to identify critical changes in neuropathology and gene expression that occur during the transition into AD dementia, a very important and fundamental question in the field. Toward this goal, we compared the gene expression profiles followed by protein analyses in non-demented control patients and AD patients with mild to moderate clinical grades of dementia. Importantly, the majority of the cases comprising the control group were non-demented patients with high degree of AD-related pathology, thereby enabling us to identify factors that correlate with early cognitive decline in AD.

2. Materials and methods

2.1. Case selection

The study employed stringent criteria for case selection based on Mini Mental State exam scores (MMSE), plaque and tangle density, sex, age, post-mortem interval (PMI), and RNA quality. Dementia severity was evaluated using the MMSE with scores of 25–30 indicating unimpaired cognition, 17–22 indicating mild/moderate dementia and less than 10 indicating severe dementia. BRAAK staging was employed to characterize pathology with stages I–II being normal to mild, stages III–IV being moderate and V–VI being severe AD [9]. Plaque pathology was determined based on Aβ load quantification in the hippocampus [15].

A group of AD patients exhibiting mild to moderate memory impairment and extensive pathology ($N = 10$, MMSE 17–22, amyloid load 2.7–13.5%, BRAAK IV–V) was compared to a non-demented group with varied levels of pathology, including 10 cases with high plaque and/or tangle pathology ($N = 14$, MMSE 25–30, amyloid load 0–13.5%, BRAAK I–V) (Table 1). The two groups were matched for PMI (range 1.25–5.2 h), sex, and age. The clinical drug histories of the cases in the study are unknown and therefore we have controlled for this by using high sample numbers.

2.2. Tissue processing

At autopsy, brains were bisected; half frozen at −80 °C and half fixed in 4% paraformaldehyde. RNA was isolated from the frozen hippocampus and pre-frontal cortex (Brodmann's area 46) by the guanidium thiocyanate method [11] with TRIzol Reagent (LifeTechologies). After RNA isolation, proteins were extracted from the phenol phase following the manufacturer's recommendations (LifeTechologies).

Table 1
Clinical and pathological features of cases

Case id	Age	Sex	PMI[a]	Tangles[b]	Plaques[c]	MMSE[d]
Control group ($n = 14$)						
Non-demented controls						
C1	70	M	5.2	0	n/a	25–30[e]
C2	78	M	1.7	1	0	28
C3	74	F	2.7	2	1.5	30
C4	83	M	2.3	2	1.3	28
Non-demented high plaque and/or tangle pathology controls						
C5	95	M	3.7	1	9.41	25
C6	74	F	2.8	2	6.15	30
C7	91	F	2.5	3	1	27
C8	92	M	3.8	3	1.33	29
C9	86	F	2.5	3	0	27
C10	83	M	3	3	0.5	30
C11	86	F	2	3	0	29
C12	88	F	3	4	0	27
C13	77	M	2	4	13.5	26
C14	95	F	2.9	5	8.49	28
Average	83.71		2.86	2.57	3.32	28.00
S.D.	8.14		0.90	1.34	4.51	1.58
Experimental group ($n = 10$)						
Mild/moderate dementia						
D1	93	M	2.75	4	12.25	22
D2	93	F	1.4	4	12.25	21
D3	87	M	2	4	12.75	20
D4	80	M	1.25	4	19	19
D5	92	F	4	5	2.7	22
D6	77	F	3.7	5	5.88	22
D7	77	M	3.6	5	7.32	22
D8	84	M	1.5	5	10.3	17
D9	89	F	2.3	5	13	18
D10	81	M	3	5	13.5	20
Average	85.30		2.55	4.60	10.69	20.30
S.D.	5.08		0.93	0.00	3.37	2.28

[a] Post mortem interval (PMI; hours).
[b] Tangle density was assessed based on BRAAK staging criteria (0–VI range).
[c] Plaque density was determined based on Aβ load quantification in the hippocampus (percentage area of Aβ immunoreactivity).
[d] Mini-mental state exam (MMSE) score (0–30 range).
[e] C1 was verified as cognitively normal on the basis of retrospective next-of-kin interviews.

2.3. Gene chip

Total RNA samples were purified using RNeasy quick spin columns (Qiagen) and quantified using a UV spectrophotometer. RNA quality was assessed by Bioanalyzer on DNA 500 chips (Agilent Technologies). High quality RNA samples (mean 28S/18S = 1.4 ± 0.3 S.D.) were hybridized to the high-density human oligonucleotide microarray U95Av2 GeneChip (Affymetrix). Cases were processed on individual GeneChips using 5 μg of total RNA. In both, the hippocampus and the cortex, we employed 10 GeneChips. An additional 14 GeneChips were used to validate the hippocampus

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data. First strand cDNA was synthesized using the SuperScript Choice System (Life Technologies) and an HPLC-purified T7-(T)24 DNA primer encoding poly(dT) and T7 RNA polymerase promoter sequence (Integrated DNA Technologies, Inc.). In vitro transcription (IVT) reactions were carried out using the Enzo High Yield RNA Transcript Labeling kit (Affymetrix and Enzo Diagnostics). cRNA was phenol–chloroform extracted on Phase-Lock Gels (Eppendorf), ethanol (100%) precipitated, purified using RNA easy spin columns (Qiagen) and fragmented by heating the samples to 94 °C in fragmentation buffer. Biotin labeled cRNA was hybridized to the GeneChips for 16 h, washed on a fluidics station (Affymetrix). Probe arrays were scanned on a Hewlett Packard Genearray Scanner at 540 nm. The extracted image files (.dat) were imported into DChip for data analysis.

2.4. Statistical analysis of gene expression data

Two statistical methods, DChip and CyberT, were used in conjunction with GeneSpring to identify significant changes in gene expression level in cases with mild/moderate dementia compared to control. Expression values were generated in DChip employing a model-based expression algorithm and the perfect match/mismatch model (PM/MM). DChip performed a Student *t*-test with probe sets filtering criteria of $P < 0.05$, fold change > 1.2, and a 90% confidence bound. DChip filtering incorporated an error value for each probe set allowing for the identification of poorly performing probe sets. The expression values were also imported into the Web-based CyberT program for further analysis without considering the DChip generated standard error. CyberT incorporated a Baysian algorithm and took into account the variance of 101 probe sets expressed at a similar level and used that to estimate the variance of each probe set. Filtering criteria in CyberT were $P < 0.01$, $\ln P < 0.01$, and fold change >1.2. CyberT is a statistical program shown to enhance the identification of genes expressed at low levels [5]. The lists of significantly changed genes generated with DChip and CyberT were enter into GeneSpring (Silicon Genetics). Venn diagrams were employed to compare the DChip and CyberT lists of significantly altered probe sets and to generate a common list representing the genes with heightened statistical confidence and significance.

2.5. Immunohistochemistry

2.5.1. Plaque/tangle pathology

The hippocampus was dissected from the paraformaldehyde-fixed hemispheres stored in phosphate buffered saline (PBS) with .02% sodium azide at 4 °C. Tissue blocks were sectioned at 50 μm using a vibratome. Sections were double immunostained for 6E10 (Signet) and AT8 (Endogen) to identify plaque and tangle histopathology respectively. 6E10 is a monoclonal antibody reactive to amino acid residue 1–17 of the human beta amyloid (Aβ) peptide. AT8 is a monoclonal antibody which recognizes a phosphate-sensitive epitope on PHF-tau at serine residue 202.

Sections were pre-treated with formic acid (90%) for a period of 4 min. Following pre-treatment, sections were washed in Tris and treated with hydrogen peroxide to quench endogenous peroxidase activity [57]. Next, sections were blocked using normal goat serum and 3% bovine serum albumin (BSA) then incubated with anti-$A\beta_{1-17}$ (6E10) primary antibody (1:5000; overnight at 4 °C). On the following day, sections were washed in Tris containing 0.1% Triton X-100 (TBS-A) then blocked in TBS-A with 3% BSA (TBS-B) for 1 h and placed in biotinylated secondary anti-mouse antibody (1:150; 1 h; Vector Laboratories). After the same sequences of washes and blocking, sections were incubated with avidin-biotinylated horseradish peroxidase complex (ABC) using the vectastain Elite ABC kit (Vector Laboratories). ABC was followed by visualization using 3,3′-diaminobenzidine tetrahydrochloride (DAB) reaction (Vector Laboratories). Since the double label is performed using two primary antibodies raised in the same species, before the AT8 antibody was added, sections were pre-treated in 37% formalin at 37 °C for 2 h then washed in Tris and treated with hydrogen peroxide [57]. Sections followed the same staining procedure as for the first label and were incubated with the AT8 primary antibody (1:500; overnight at 4 °C) then the antigen was visualized by incubating in biotinylated anti-mouse secondary (Vector Laboratories) followed by an ABC step with a blue SG substrate kit (Vector Laboratories). Elimination of either primary or secondary antibodies yielded negative immunoreactivity.

2.5.2. MHC II and microglia

Fluorescent double-label immunohistochemistry for MHC class II and microglia employed a monoclonal CR3/43 mouse antibody against the MHC class II that recognizes HLA-DR, DP and DQ sub-regions (DAKO) and the polyclonal rabbit antibody IBA1 (WAKO) as a marker of microglia. IBA1 is a calcium-binding protein, which is restrictedly expressed in macrophages/microglia [24], and IBA1 expression is up-regulated in activated microglia [26]. Hippocampal sections from mild/moderate dementia and control cases were washed in PBS, blocked with 5% normal goat and horse serum for 1 h, and incubated overnight at 4 °C with IBA1 and CR3/43, both at a 1:100 dilution. On the following day, sections were washed and incubated in secondary antibody goat anti-rabbit Alexa 488 for IBA1 (Molecular Probes) and anti-mouse Alexa 555 for CR3/43 (Molecular Probes) (1:200; 1 h). Following three washes in Tris, sections were mounted and coverslipped with Fluoromount-G (Southern Biotechnology). Controls were performed to rule out non-specific immunostaining by eliminating the primary or secondary antibody. Controls were negative.

2.5.3. MHC II and Aβ

Fluorescent immunohistochemistry was performed for MHC class II and $A\beta_{1-42}$ using the monoclonal CR3/43

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(DAKO) and polyclonal rabbit $A\beta_{1-42}$ (BioSource) antibodies respectively on hippocampal sections from mild/moderate AD cases and controls. Briefly, sections were washed in Tris and incubated for 1 h in block containing 5% normal goat and horse serum. Sections were then placed in primary antibodies CR3/43 and $A\beta_{1-42}$ diluted in block (1:100; overnight at 4 °C). Following Tris rinses, sections were incubated with biotinylated anti-mouse secondary antibody (1:150; 1 h; Vector Laboratories). Lastly, sections were placed in streptavidin-conjugated Alexa 488 and anti-rabbit Alexa 555 (1:200; 1 h, Molecular Probes). Controls were performed to rule out non-specific immunostaining by eliminating the primary or secondary antibody. Controls were negative.

2.5.4. T cell

Hippocampal and cortical sections from mild/moderate dementia and control cases were immunostained for T cells using a mouse anti-human monoclonal CD3 antibody (Chemicon). Sections were washed in Tris, treated with hydrogen peroxide [57], blocked using normal goat serum and 5% bovine serum albumin (BSA) and incubated with the primary CD3 antibody (1:1000; overnight at 4 °C). On day 2, sections were washed and incubated with biotinylated goat anti-mouse secondary antibody (1:150; 1 h; Vector Laboratories). Following Tris washes, sections were placed in an avidin-biotinylated horseradish peroxidase complex (ABC) using the vectastain Elite ABC kit (Vector Laboratories) and visualized using the diaminobenzidine tetrahydrochloride (DAB) reaction (Vector Laboratories). Quantification of T cell number was conducted independently by three blind scorers on hippocampal sections from 12 control and 7 mild/moderate AD cases. T cell numbers were obtained by counting T cells in the entire hippocampus from sections at the same level. T cell quantification in the cortex of eight controls and six mild/moderate AD cases was based on T cell counts in a 1 cm square area from comparable cortical regions. Statistical analysis employed a Student *t*-test.

Sections from control cases were additionally stained for CD8 using the monoclonal CD8 antibody (1:100; Chemicon) to confirm T cell specificity.

2.5.5. Confocal and light microscopy

Light-level labeled sections were imaged on an Olympus BX61 microscope using an Olympus U-TV1X digital camera. Fluorescently labeled sections were imaged on a Bio-Rad Radiance 2100 confocal system using Argon and Krypton lasers and appropriate filter sets. Images were scanned using lambda-strobing to avoid non-specific cross-excitation or detection. Images represent either confocal Z-stacks or individual confocal slices.

2.6. Western blot analyses

The isolated protein pellet was sonicated in non-ionic detergent-based lysis buffer (BD Biosciences). Protein quantification was performed using the bicinchoninic acid (BCA) assay (BioRad). Five micrograms of total protein was resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. Membranes were blocked in 5% milk, washed, then incubated with the monoclonal CR3/43 primary antibody against HLA-DP, DQ, DR (1:100; overnight at 4 °C). On the following day, the membrane was washed and incubated with HRP-conjugated goat anti-mouse secondary (1:1000; 1 h; BioRad). Membranes were visualized using enhanced chemiluminescence (Pierce). Subsequently, membranes were stripped and re-probed with a polyclonal β-actin antibody (Abcam) as a loading control. Quantification of MHC II protein levels was performed using Scion Image Software (NIH). Three measurements were taken for each band and MHC class II protein levels (monomer + dimer) were obtained relative to each β-actin or average β-actin level across all cases.

2.7. Dot blot analyses

Dot blot analyses were performed using the polyclonal rabbit CD3 antibody (Dako). Briefly, 10 μg of protein sample, in equal volumes, was directly added onto the nitrocellulose membrane and left to dry. The membrane was then blocked in 5% milk and incubated with the primary CD3 antibody (1:1000; overnight at 4 °C). On the second day, the membrane was washed and incubated with HRP-conjugated goat anti-rabbit secondary (1:1000; 1 h; BioRad). Membranes were visualized using enhanced chemiluminescence (Pierce). Quantification of CD3 protein levels was performed using Scion Image Software (NIH).

3. Results

We aimed to identify gene expression and neuropathological changes in mild to moderate AD dementia cases ($N = 10$, MMSE 17–22, amyloid load 2.7–13.5%, BRAAK IV–V) compared to non-demented controls including 10 high plaque and/or tangle pathology controls ($N = 14$, MMSE 25–30, amyloid load 0–13.5%, BRAAK I–V) (Table 1). Importantly, the groups were chosen based on MMSE scores of cognitive functioning since plaque and tangle pathology does not always correlate with memory impairment.

The low MMSE group (experimental group, $N = 10$) consisted of clinically characterized AD cases with mild to moderate degree of dementia (MMSE 17–22). In the hippocampus, the plaque load ranged from 2.7 to 13.5% and the tangle pathology was determined to be BRAAK stage of either IV or V.

The high MMSE group (control group, $N = 14$) consisted of clinically normal non-demented cases (MMSE 25–30) with a range of AD pathology. Specifically, four control cases (C5, C6, C13, C14) exhibited significant Aβ load (6.15–13.5%); eight cases (C7–C14) had significant NFTs (BRAAK III–V); two cases (C13, C14) were characterized

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by significant plaque and tangle pathology (see Table 1 for case demographics).

3.1. Changes in gene expression in mild/moderate AD dementia cases compared to non-demented controls

Gene expression in the hippocampus and pre-frontal cortex of mild/moderate AD dementia and non-demented cases was compared using high-density oligonucleotide microarrays. Genes that were differentially expressed between the two groups were identified using DChip and CyberT statistics. Genes flagged as significantly altered by both methods (DChip and CyberT) were of primary focus for further analysis since they increase statistical confidence in the data.

3.1.1. Regional differences in mRNA levels with mild/moderate dementia

More sequences were significantly altered in the pre-frontal cortex compared to the hippocampus in AD cases with mild to moderate dementia (MMD) relative to non-demented controls (NDC) (Fig. 1A). Specifically, four times as many changes in gene expression were observed in the pre-frontal cortex compared to the hippocampus, 217 and 53 sequences respectively. A complete list of significantly altered mRNAs can be found in Supplementary data. In cases with mild to moderate dementia, trend analysis revealed that the majority of sequences in the pre-frontal cortex were up-regulated while most in the hippocampus were down-regulated (Fig. 1B). Thus, the number of differentially expressed sequences and the direction of the change were region-specific during the early stages of dementia in AD.

3.1.2. Inflammatory markers are up-regulated in the early stages of AD dementia

After examining the pattern of changes at the expression level, the identity of these sequences was determined. Most sequences were functionally different but included numerous mRNAs associated with inflammation. Moreover, markers of inflammation were higher in both the pre-frontal cortex and hippocampus in cases with mild/moderate dementia compared to controls. It is important to note that while most sequences were lower in the hippocampus, those associated with inflammation were increased.

In the pre-frontal cortex of AD patients with mild to moderate dementia, MHC class I and II sequences were increased, in addition to a number of innate immune response markers including the LPS-receptor (CD14), toll-like receptor 2 and mac-1β compared to controls (Table 2). Also, anti-inflammatory sequences including transforming growth factor (TGF) β and the IL-10 receptor were higher in cases with mild/moderate dementia. In comparison, inflammation in the hippocampus was very specific, with 86% of the inflammatory markers being sequences of MHC class II haplotypes

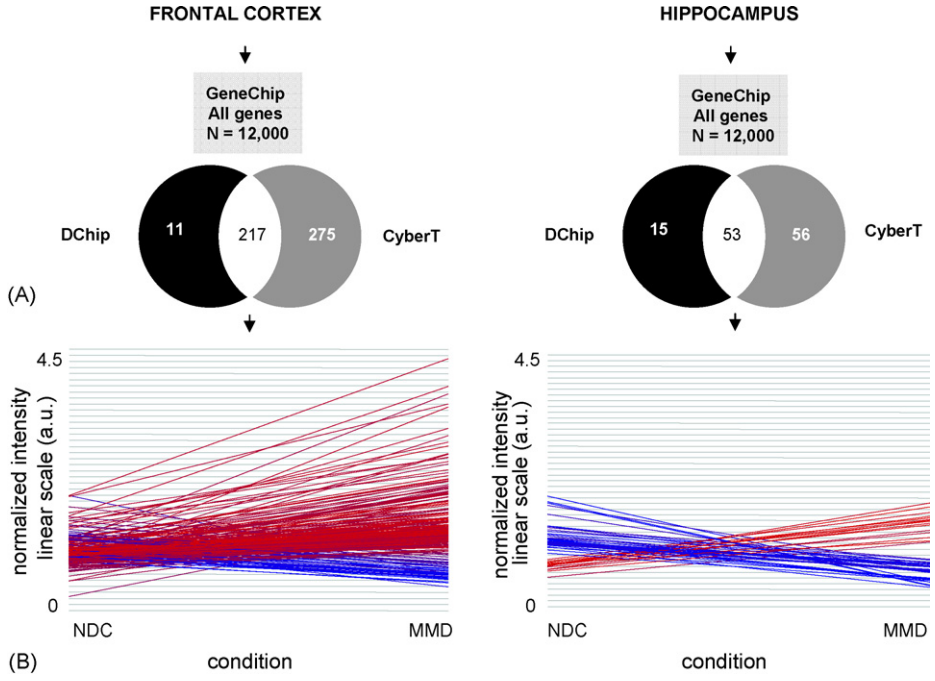

Fig. 1. Statistical analysis of GeneChip data in the hippocampus and pre-frontal cortex comparing mild/moderate dementia cases (MMD) to non-dementia control (NDC). (A) DChip and CyberT statistics generated lists of significantly altered sequences from the 12,000 total probe sets on the GeneChip. The DChip and CyberT lists were subsequently compared for each brain region yielding a final common gene list. The common list included 217 sequences in the frontal cortex and 53 in the hippocampus. (B) Trend analysis of the common gene lists in the hippocampus and the pre-frontal cortex. On the *y*-axis is the normalized probe set intensity; the condition (MMD or NDC) is represented on the *x*-axis. For each significantly altered gene, the average intensity level is plotted to yield a trend of expression between MMD and NDC. Genes increased in mRNA level with mild/moderate dementia are in red; decreased in blue. In the frontal cortex, the majority of changed sequences are increased in expression from non-dementia to dementia whereas the opposite holds true for the hippocampus.

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Table 2
Inflammatory markers in the frontal cortex

CyberT[a]		DChip[a]		Affymetrix[b]	
P	ln *P*	*P*	Fold change[c]	Sequence annotation	id
1.58E−03	2.86E−03	2.12E−02	4.80	Interleukin 15 receptor, alpha	41677_at
1.79E−04	2.55E−03	2.72E−02	3.16	Pro-platelet basic protein (chemokine (C–X–C motif) ligand 7)	39208_i_at
1.68E−06	6.49E−06	2.11E−03	3.10	Lymphocyte cytosolic protein 1 (L-plastin)	37023_at
1.11E−06	1.64E−06	4.39E−03	3.01	Fc fragment of IgG, low affinity IIIb, receptor for (CD 16)	37200_at
2.23E−05	1.90E−05	3.18E−03	2.38	Fc fragment of IgG, high affinity Ia, receptor for (CD64)	37220_at
2.05E−05	1.19E−05	2.66E−02	2.36	Fc fragment of IgG binding protein	39014_at
2.72E−05	2.38E−05	7.48E−03	2.36	Interferon, gamma-inducible protein 16	1456_s_at
8.22E−05	1.06E−04	4.34E−02	2.35	Major histocompatibility complex, class II, DR beta 3	41723_s_at
2.34E−04	1.31E−03	4.72E−02	2.27	Lymphocyte antigen 96	33956_at
1.91E−04	4.92E−04	2.43E−02	2.27	Toll-like receptor 2	40310_at
7.68E−05	3.14E−04	6.13E−03	2.27	Intercellular adhesion molecule 2	38453_at
6.03E−05	2.63E−04	3.03E−02	2.27	chemokine (C–X3–C) receptor 1	40646_at
1.22E−04	1.86E−04	3.54E−02	2.25	Oxidised low density lipoprotein (lectin-like) receptor 1	37233_at
6.56E−06	1.28E−06	8.49E−03	2.24	Major histocompatibility complex, class I, A	41237_at
5.51E−05	1.54E−04	2.28E−02	2.19	Interferon induced transmembrane protein 1 (9–27)	675_at
5.26E−05	3.47E−05	2.25E−02	2.15	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	35016_at
4.70E−05	1.89E−04	2.45E−02	2.15	Transforming growth factor, beta RII	1815_g_at
1.21E−04	1.93E−04	4.08E−02	2.14	Fc fragment of IgG, low affinity IIIb, receptor for (CD 16)	31499_s_at
3.98E−05	2.76E−05	2.77E−02	2.11	CD 14 antigen	36661_s_at
4.77E−06	5.05E−06	2.21E−03	2.03	CD53 antigen	38378_at
4.83E−05	6.60E−05	4.20E−03	2.00	Intercellular adhesion molecule 2	38454_g_at
9.50E−05	5.34E−05	2.69E−02	1.98	Macrophage antigen 1 (mac-1) beta	37918_at
1.02E−03	1.33E−03	4.44E−02	1.95	Interleukin 10 receptor, beta	33227_at
2.66E−04	3.35E−04	4.34E−02	1.90	CD37 antigen	31870_at
1.75E−05	1.79E−05	3.15E−03	1.88	Transforming growth factor, beta 1	1830_s_at
5.89E−04	9.87E−04	4.00E−02	1.84	Major histocompatibility complex, class II, DP beta 1	38095_i_at
5.58E−04	1.03E−03	1.54E−02	1.79	Interleukin 1 receptor, type I	1368_at
8.40E−04	1.14E−03	2.18E−02	1.74	Chemokine (C–C motif) 2	875_g_at
3.06E−04	1.58E−04	1.72E−02	1.69	Major histocompatibility complex, class I, C	37383_f_at
3.37E−04	4.44E−04	6.04E−03	1.63	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1461_at
4.65E−04	3.30E−04	1.11E−02	1.58	Major histocompatibility complex, class II, DR beta 3	3326 1_at
8.45E−04	8.63E−04	4.05E−03	1.57	Chemokine (C–C motif) ligand 2	34375_at
8.20E−04	8.60E−04	1.25E−02	1.55	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)	39170_at
9.48E−04	1.20E−03	2.02E−02	1.54	Major histocompatibility complex, class I, E	3232 1_at
7.39E−04	1.01E−03	8.58E−03	1.53	Major histocompatibility complex, class I, F	37420_i_at
1.72E−03	1.56E−03	8.54E−03	1.47	CD59 molecule, complement regulatory protein	3935 1_at
1.90E−03	1.73E−03	9.68E−03	1.46	HLA-G histocompatibility antigen, class I, G	40369_f_at
6.32E−04	1.06E−03	7.86E−03	1.46	Interferon, gamma-inducible protein 30	925_at
3.33E−03	5.24E−03	5.18E−03	1.44	Intercellular adhesion molecule 2	590_at

Markers of inflammation significantly changed with mild/moderate dementia compared to control based on the common DChip + CyberT gene list. Table sorted by fold change. DChip criteria of $P < 0.05$, fold change >1.2 and 90% confidence bound and CyberT criteria of $P < 0.01$ and fold change >1.2.

[a] CyberT *P* and ln *P*-values + DChip *P*-value.

[b] Reference sequence annotation and probe set id from U95Av2 GeneChip.

[c] Fold change with mild/moderate dementia compared to control.

(Table 3A) compared to 14% for the pre-frontal cortex. None of the innate immune response markers or MHC class I, which were increased in the pre-frontal cortex, were differentially expressed in the hippocampus. Next, the nearly exclusive increase in MHC class II mRNA expression in the hippocampus was further characterized by examining the gene lists that were found to be significant with either CyberT- or DChip-only. The CyberT-only list showed that additional MHC class II probe sets were greater in mild/moderate dementia cases compared to controls (Table 3B).

3.2. Changes in protein levels

3.2.1. Increased MHC class II mRNA is paralleled by increased MHC class II protein in AD cases with mild/moderate dementia

Altered levels of MHC class II expression in the hippocampus were further investigated since the hippocampus is considered integral for learning and memory. To determine if the increase in MHC class II mRNA corresponded to protein changes, MHC class II protein levels were studied by

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Table 3
Inflammatory markers in the hippocampus

CyberT[a]		DChip[a]		Affymetrix[b]	
P	ln *P*	*P*	Fold change[c]	Sequence annotation	id
(A)					
2.80E−05	8.50E−05	2.06E−03	2.15	CD37 antigen	31870_at
1.30E−05	9.00E−06	1.13E−02	2.09	Major histocompatibility complex, class II, DP alpha 1	38833_at
1.16E−04	2.01E−04	2.65E−02	1.94	Major histocompatibility complex, class II, DR beta 1	41723_s_at
1.73E−04	4.18E−04	1.18E−02	1.83	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	35016_at
3.56E−04	5.72E−04	9.72E−03	1.78	B-cell linker	38242_at
3.05E−04	2.70E−04	2.42E−02	1.76	Major histocompatibility complex, class II, DR alpha	37039_at
3.16E−03	3.61E−03	1.27E−02	1.45	Major histocompatibility complex, class II, DO alpha	31728_at

Sequence annotation	id
(B)	
Major histocompatibility complex, class II, DM alpha	37344_at
Major histocompatibility complex, class II, DQ alpha 1	32773_at
Major histocompatibility complex, class II, DR beta 3	3326 1_at
Major histocompatibility complex, class II, DQ beta 1	36878_f_at
Major histocompatibility complex, class II, DM beta	41609_at

(A) Markers of inflammation significantly changed with mild/moderate dementia compared to control based on the common DChip + CyberT gene list. Table sorted by fold change. DChip criteria of $P < 0.05$, 1.2-fold change and 90% confidence bound and CyberT criteria of $P < 0.01$ and 1.2-fold change. (B) Markers of inflammation significantly changed with mild/moderate dementia compared to control based on the CyberT only list ($P < 0.01$ and 1.2-fold change excluding sequences from the common DChip + CyberT list).

[a] CyberT *P* and ln *P*-values + DChip *P*-value.

[b] Reference probe set id from U95Av2 GeneChip.

[c] Fold change with mild/moderate dementia compared to control.

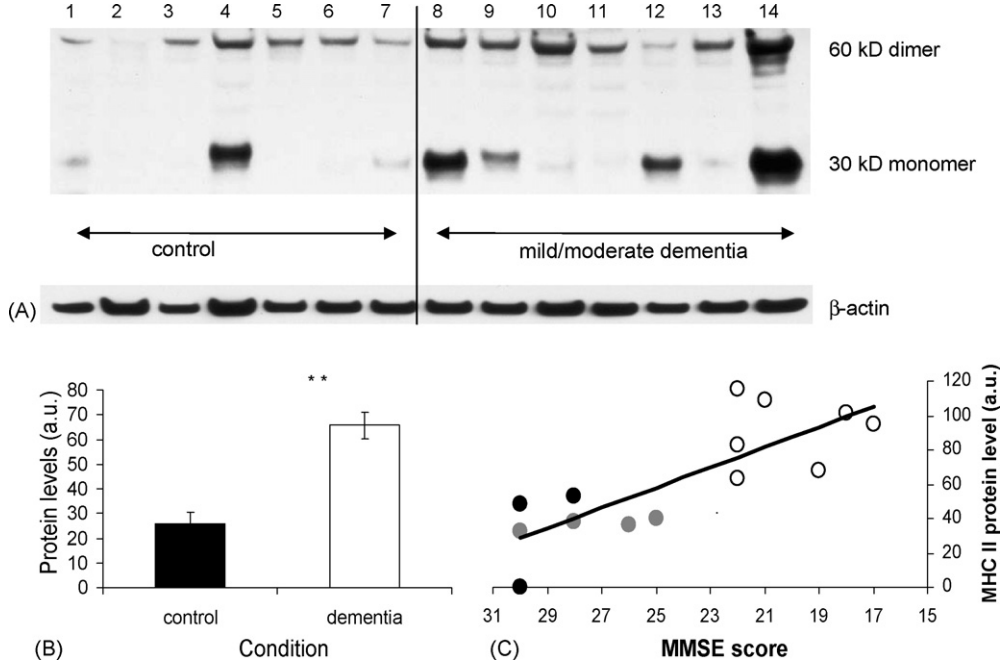

Fig. 2. MHC class II protein levels in the hippocampus as assessed via Western blot in seven controls including four high plaque and/or tangle pathology controls and seven mild/moderate dementia cases. (A) Western blot for CR3/43 and β-actin. (B) Bar graph representation of MHC class II protein levels relative to β-actin control. Error bars representing standard error. MHC class II protein levels are significantly increased with mild/moderate dementia compared to control ($P < 0.01$). (C) Scattergram of MHC class II protein level and MMSE scores. MHC II protein levels inversely correlate with scores of cognitive function. Mild/moderate AD (white) compared to non-demented controls (black), including non-demented high-pathology controls (gray).

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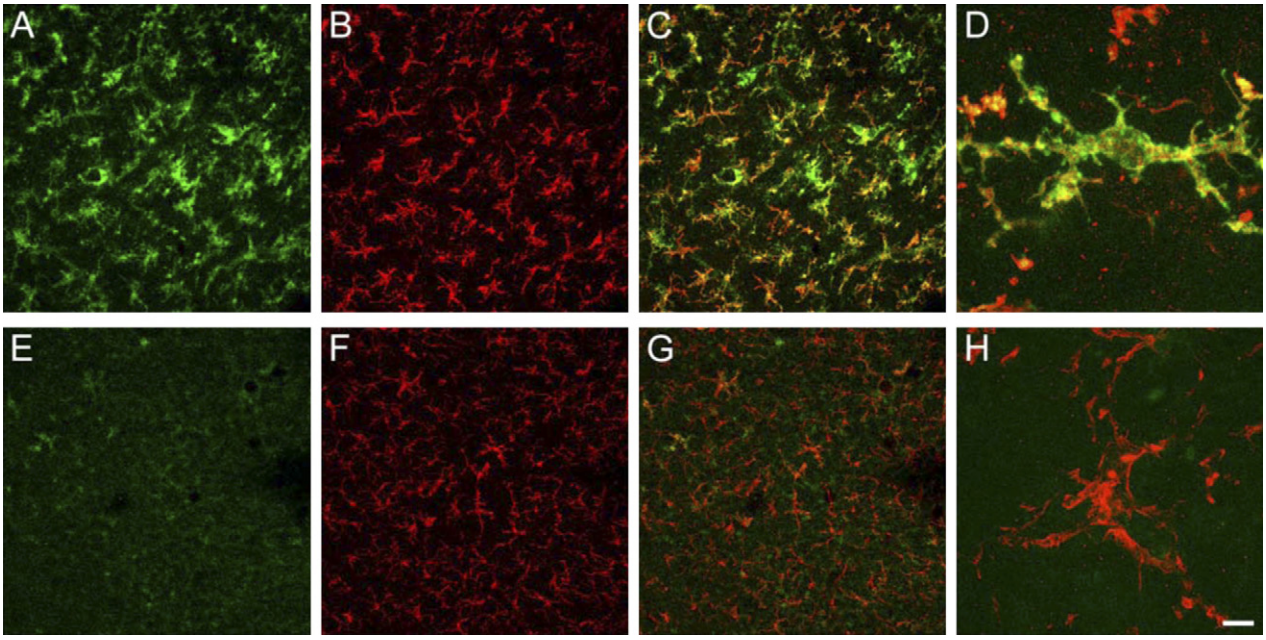

Fig. 3. Double immunofluorescence for microglia (red) and MHC class II (green) in the hippocampus with mild/moderate dementia (A–D) and control (E–H). The majority of microglia were double labeled in the mild/moderate AD case (C and D). The control case exhibited minimal MHC II staining and diminished microglia activation (E and F). Scale bar equals 55 μm (A–C and E–G) and 7 μm (D and H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Western blot. Normalized to the loading control (β-actin), MHC class II protein levels were significantly greater ($P < 0.01$) in cases with mild to moderate dementia compared to controls including four high plaque and/or tangle-pathology controls (Fig. 2A and B). The 1.8-fold greater mRNA expression corresponded to a 2.6-fold greater protein load. The regulation of protein translation or degradation may be affected in cases with AD dementia, which may account for this difference in mRNA versus protein expression levels. Furthermore, MHC class II protein levels were inversely correlated with cognitive function as measured by MMSE ($R^2 = 0.648$, $P < 0.01$, Fig. 2C).

3.2.2. *MHC class II co-localizes with reactive microglia in the hippocampus*

A highly specific up-regulation of MHC class II was noted in the hippocampus with mild to moderate dementia in contrast to a broader inflammatory response in the prefrontal cortex. Since MHC class II expression is restricted to antigen presenting cells (APCs) including dendritic cells, B cells, and monocytes/microglia, the relation between MHC class II and microglia was examined in the hippocampus of control and early AD dementia cases. As shown in Fig. 3, microglia (red) and MHC class II (green), as assessed with immunostaining for IBA1 and CR3/43 respectively, were frequently co-localized as visualized using high-resolution confocal microscopy in mild/moderate AD cases (Fig. 3A–C). These microglia appeared reactive as they were highly ramified (Fig. 3D). MHC II-positive cells that were IBA1-negative were absent, suggesting that MHC class II is restricted to microglia in the hippocampus during the early stages of AD dementia (Fig. 3). Control non-demented cases exhibited minimal MHC class II labeling, supportive of the Western data, as well as less intense IBA1 labeling, suggestive of diminished microglia activation (Fig. 3E–H).

3.2.3. *MHC class II and the adaptive immune response in AD*

MHC class II molecules, which are located on the surface of APCs, function to present portions of an ingested antigen to specific T cells, thereby initiating an adaptive immune response. To determine if adaptive immunity has been initiated, it is important to demonstrate that an antigen, such as Aβ, is presented in the context of MHC class II and that T cells are present. Therefore, hippocampal sections were immunostained for both MHC II (CR4/3) and $A\beta_{1–42}$ or for CD3, which is a marker of both T cell subtypes: $CD8^+$ (cytotoxic) and $CD4^+$ (helper). As shown in Fig. 4, numerous clusters of cells which were immunopositive for both MHC II (green) and $A\beta_{1–42}$ (red) were observed in mild/moderate AD (Fig. 4A–D) and high pathology control (Fig. 4E–H) cases as visualized with confocal imaging. MHC II immunostaining appeared localized to $A\beta_{1–42}$ regions in the high-pathology controls whereas mild/moderate dementia cases exhibited a more widespread MHC II staining. Higher magnification imaging showed co-localization of MHC II and Aβ (Fig. 4I–L).

CD3-positive T cells were present in the hippocampus of both non-demented controls and mild/moderate dementia

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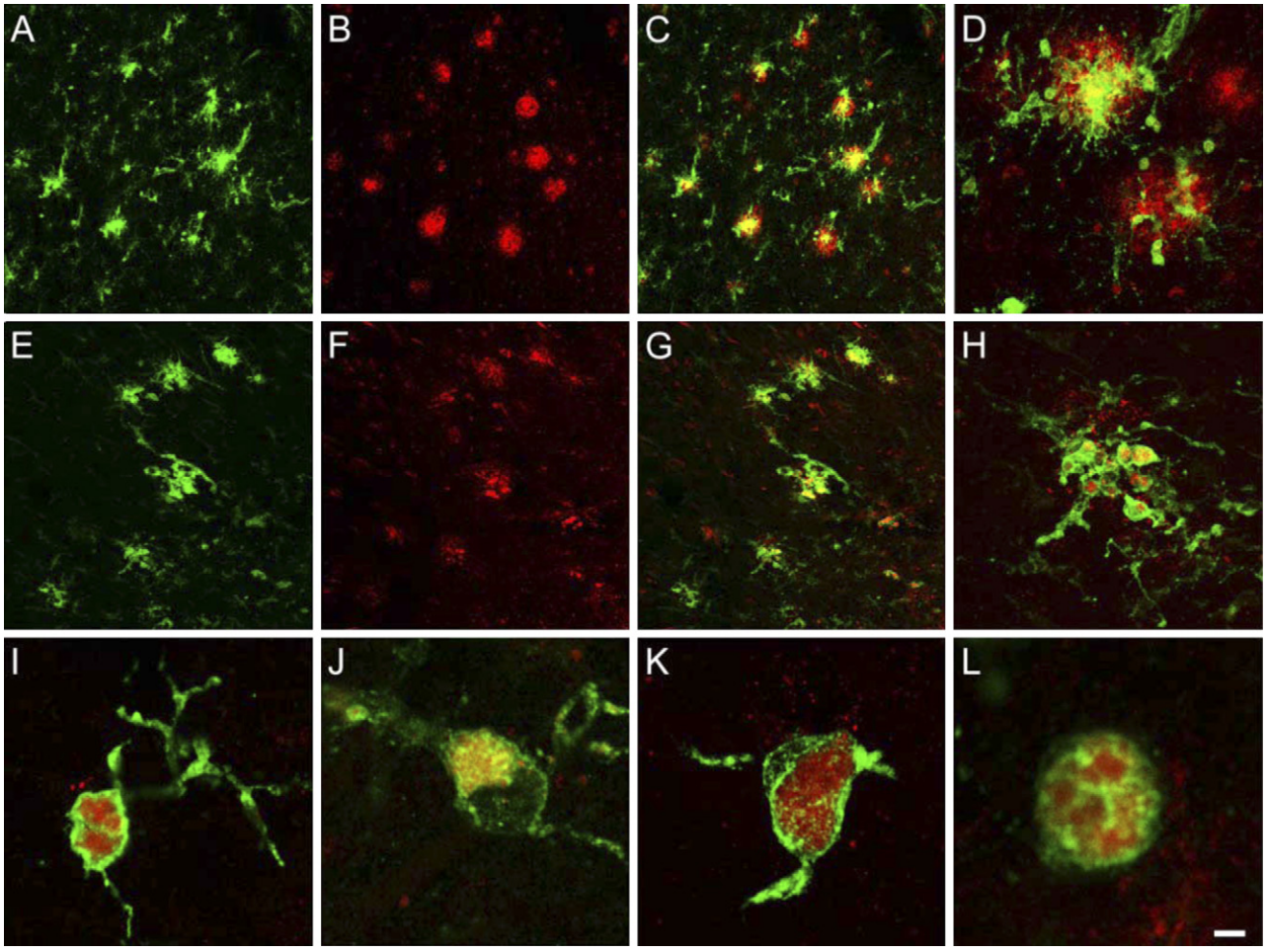

Fig. 4. Aβ (red) and MHC class II (green) immunofluorescence in the hippocampus of mild/moderate AD (A–D) and high pathology control (E–H). Low power images show Aβ-positive and MHC class II-positive clusters. High power imaging reveals co-localization of Aβ and MHC class II. Scale bar equals 60 μm (A–C and E–G), 15 μm (D and H), 7 μm (I), 5 μm (J) and 4 μm (K and L). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cases (Fig. 5A and B). The T cells were distributed in a non-homogeneous fashion. Some regions showed occasional and scattered T cells while other fields showed clusters of cells, as illustrated in Fig. 5. In order to obtain a measure of total T cells, we counted the number of CD3-positive cells in sections of the hippocampus. Quantification of these T cell numbers revealed that T cells are significantly decreased in cases with mild/moderate AD dementia compared to controls ($P = 0.04$, Fig. 5C). Further quantification of CD3 steady state levels in hippocampal brain homogenates of control and demented cases was assessed by dot blot (Fig. 5E). Likewise to CD3-positive T cell counts, demented cases showed a significant decrease in CD3 steady state levels ($P = 0.03$, Fig. 5F). The presence of T cells in the hippocampus was further confirmed by staining for an alternative T cell marker, CD8, as shown in Fig. 5D. Analyses of frontal cortex from control and mild/moderate dementia cases paralleled the hippocampal findings with decreased T cell numbers with dementia ($P = 0.04$, Fig. 5G) as counted from a 1 cm square area from comparable cortical areas. Together these results are suggestive of a dysfunctional adaptive immune response induced by Aβ or another mechanism.

4. Discussion

This study suggests that immune responses in the CNS may be involved in the transition to AD dementia. Our goal was to identify factors which correlate with cognitive decline by comparing non-demented controls, including high-pathology controls, to cases with mild/moderate clinical grades of AD dementia. We demonstrated that inflammatory molecules, most prominently MHC II, a marker of microglia activation, were increased in AD patients with early stage dementia. One prominent consequence of MHC II up-regulation is the potential for antigen presentation to activated T cells thereby inducing an adaptive immune response. However, mild/moderate AD dementia cases had reduced numbers of T cells compared to non-demented controls, suggesting that adaptive immune responses may be dysfunctional

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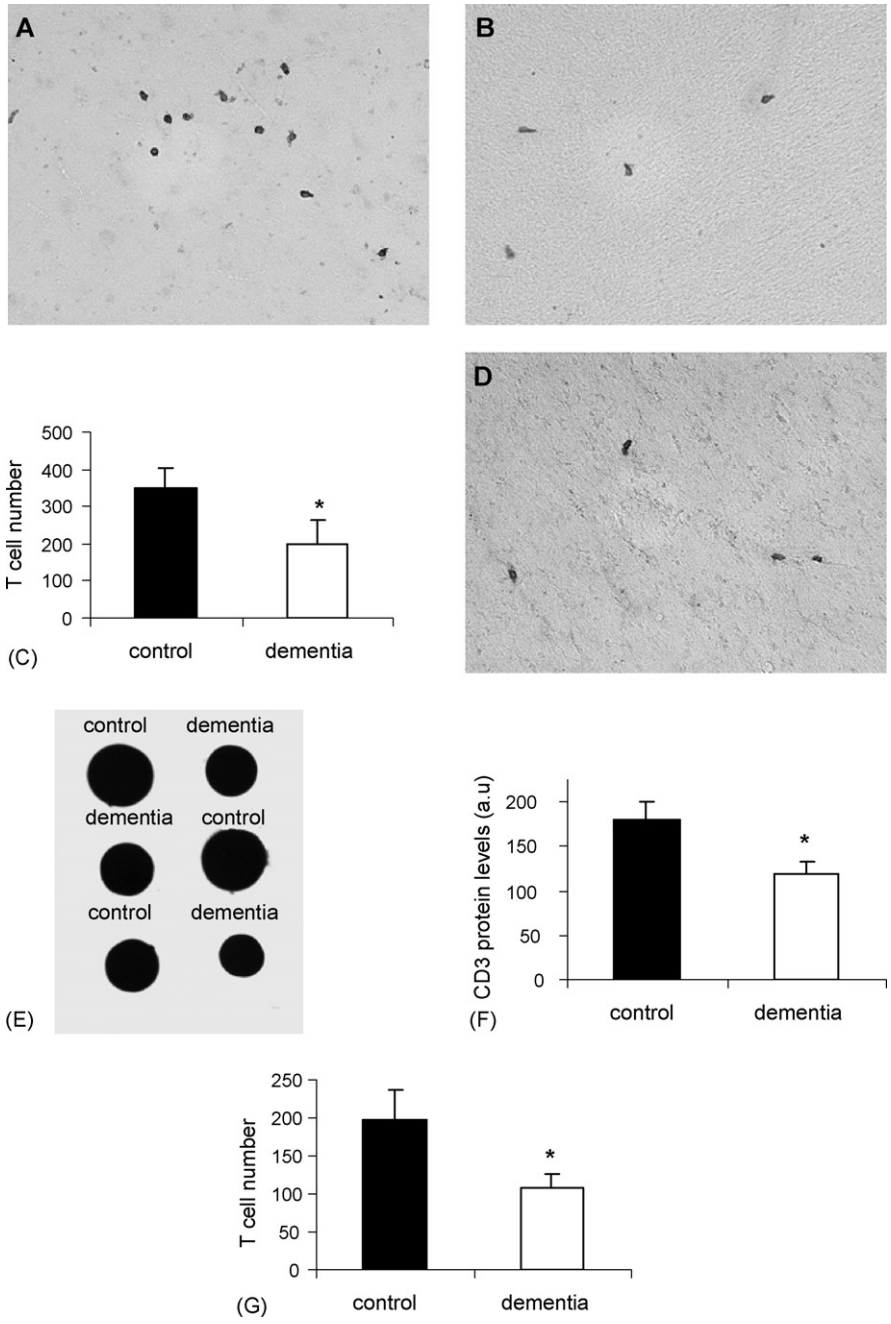

Fig. 5. Light level immunohistochemistry for $CD3^+$T cells depicting a cluster in the hippocampus of control (A) and mild/moderate dementia cases (B). Scale bar equals 100 μm. T cell quantification in the hippocampus of 7 MMD and 12 non-demented controls documents a significant decrease in T cell number with dementia ($P = 0.04$) (C). CD8-positive T cells in control cases are depicted in (D). Dot blot analysis of CD3 levels in control and dementia cases (E). Quantification of (E) confirms the decreased CD3 protein load with dementia ($P = 0.03$) (F). Analysis of CD3 levels in the frontal cortex of 6 MMD and 8 non-demented control cases also shows a significant decrease in $CD3^+$T with dementia ($P = 0.04$) (G).

during the early stages of AD dementia and may contribute to the cognitive decline observed in these individuals. This work complements a number of prior studies which have also demonstrated immune responses in AD, both in the CNS [2,7,12,32] and also from peripheral lymphocytes [27,46,51]. However as we have focused on the dementia component of AD, rather than the pathology, we highlight that this immune response may be directly involved in cognitive decline.

In the hippocampus, a primary area implicated in learning and memory, several members of the MHC class II family are significantly increased, in both mRNA and protein levels, at early stages of AD dementia and this increase is inversely correlated with MMSE scores of cognitive function. The comparison with control cases that were cognitively intact but had high AD pathology supports the notion that increased MHC class II levels are correlated to the dementia

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component of the disease. Previous studies have highlighted abnormal MHC II expression in AD [30,31,39,64] as well as in transgenic mouse models of AD [18,42,60], which suggests that these effects on MHC II are mediated by increased APP fragments including Aβ. In addition, severe late stage AD dementia has been correlated with increased MHC class II levels in the brain [4,35,67]. The current study provides evidence showing that the increase in MHC class II occurs earlier than previously described, is already present in cases with mild to moderate AD dementia and correlates inversely with MMSE scores.

In addition to increased MHC class II molecules, two other lines of evidence presented here indicate that T cell-mediated adaptive immunity may be involved in the transition to AD dementia. First, MHC class II expression was restricted to microglia cells and these cells co-localized with Aβ, suggesting that Aβ may be an antigen being presented. MHC class II expression in activated microglia associated with plaques has been previously established [53,38,48]. Second, the presence of T cells in the hippocampus of mild to moderate AD dementia cases, as well as controls, suggests a potential for a T cell response. Although the central nervous system (CNS) has been thought to be immunologically privileged, T cells have been shown to routinely penetrate the blood–brain barrier (BBB) and enter the CNS [23,66] and they are present in the aged human hippocampus [63] as well as the AD brain [25,53]. We hypothesize that since in AD patients Aβ can cross the BBB and enter into the bloodstream, it is likely that the immune system can recognize this self-antigen and induce specific immune responses. In support, Monsonego and colleagues demonstrated the presence of Aβ-reactive T cells restricted to MHC class II in the brains of AD patients with moderate to severe grades of dementia [43]. These Aβ-reactive T cells may have been recruited by microglia, since microglia have been shown to serve as antigen presenting cells that stimulate T cells in vitro [6]. However, the exact role of T cells in the AD brain remains ambiguous. This ambiguity stems from evidence that T cells may actually die during inflammatory cascades [17,47]. Monsonego and colleagues documented that T cell apoptosis, caused by nitric oxide (NO) production at sites of Aβ plaques [21,55], occurs prior to T cell activation by microglia [43]. In the current study, we show that early stage AD dementia is associated with a decreased number of T cells in the hippocampus as compared to non-demented controls, suggesting that the T cell response in AD is dysfunctional. In addition to T cell death, lack of co-stimulatory molecules is known to induce T cell anergy. In the current study, CD80 and CD86 co-stimulatory molecules are not found to be altered at the mRNA level between non-demented control and mild/moderate dementia cases (data not shown), suggesting that proper activation of cell-mediated immunity is not taking place, although this may be due to the small sample size that T cells constitute in the brain homogenates. Therefore, it is unclear whether the antigen presentation capacity of MHC II-positive microglia cells to T cells in the AD brain is a functional process leading to Aβ removal, or if removal is inhibited by the death and/or anergy of T cells.

Importantly, there is also an established positive link between T cells and cognition. More specifically, T cell deficiency has been associated with deficits in spatial learning and memory [28]. In addition, a recent study demonstrated that CNS-specific T cells maintain neurogenesis and are required for spatial learning and memory in adulthood [68]. Immune deficient SCID mice show memory deficits and the addition of CNS-specific T cells restored neurogenesis and improved cognition [68]. Our studies show a decrease in T cells in AD. Therefore, it is interesting to speculate that decreased T cells contribute to the cognitive deficits observed in transitional AD cases as compared to non-demented controls including individuals with high plaque and/or tangle pathology.

In conclusion, increased microglia activation, paralleled by a decrease in T cell number, correlates with the initial stages of AD dementia. In particular, the progression into AD-type dementia coincides with a specific MHC class II increase in the hippocampus, the primary brain area related to learning and memory. Thus, MHC class II-positive microglia-mediated adaptive immunity may be fundamental to the cognitive decline that occurs in AD. But the findings of decreased T cells in the same brain region suggest that cognitive decline in AD is associated with immune dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2006.08.014.

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