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ORIGINAL RESEARCH

Apolipoprotein E Polymorphism and Oxidative Stress in Peripheral Blood-Derived Macrophage-Mediated Amyloid-Beta Phagocytosis in Alzheimer's Disease Patients

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Abstract

Peripheral blood-derived macrophages isolated from Alzheimer's disease (AD) patients have earlier been reported to demonstrate ineffective phagocytosis of amyloid-beta compared to the age-matched control subjects. However, the mechanisms causing unsuccessful phagocytosis remain unclear. Oxidative stress and the presence of ApoEε4 allele has been reported to play a major role in the pathogenesis of AD, but the contribution of oxidative stress and ApoEε4 in macrophage dysfunction leading to ineffective Aβ phagocytosis needs to be analyzed. Aβ phagocytosis assay has been performed using FITC-labeled Aβ and analyzed using flow cytometry and confocal imaging in patient samples and in THP-1 cells. Oxidative stress in patient-derived macrophages was analyzed by assessing the DNA damage using comet assay. ApoE polymorphism was analyzed using sequence-specific PCR and Hixson & Vernier Restriction isotyping protocol. In this study, we have analyzed the patterns of phagocytic inefficiency of macrophages in Indian population with a gradual decline in the phagocytic potential from mild cognitive impairment (MCI) to AD patients. Further, we have shown that the presence of ApoEε4 allele might also have a possible effect on the phagocytosis efficiency of the macrophages. Here, we demonstrate for the first time that oxidative stress could affect the amyloid-beta phagocytic potential of macrophages and hence by alleviating oxidative stress using curcumin, an anti-oxidant could enhance the amyloid-beta phagocytic efficacy of macrophages of patients with AD and MCI, although the responsiveness to curcumin might depends on the presence or absence of APOEε4 allele. Oxidative stress contributes significantly to decreased phagocytosis of Aβ by macrophages. Moreover, the phagocytic inefficiency of macrophages was correlated to the presence of ApoEε4 allele. This study also found that the Aβ-phagocytic potential of macrophage gets significantly enhanced in curcumin-treated patient-derived macrophages.

Keywords Alzheimer's disease · Mild cognitive impairment · Phagocytosis · Internalization · Colocalization · Amyloid beta · Macrophages · Monocytes · Curcumin · APO E

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Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease affecting nearly 50 million people worldwide (Dementia statistics | Alzheimer's Disease International 2008). Alzheimer's disease is pathologically characterized by the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles (Mohandas et al. 2009). The accumulation of cerebral amyloid beta has been attributed to a decreased clearance of the Aβ peptide by the resident brain microglia and peripheral monocyte-derived macrophages (Lai and McLaurin 2012). This compromised the ability of monocyte-derived macrophages to phagocytose Aβ has been causally linked to multiple factors. In addition to the conventional amyloid beta cascade hypothesis, polymorphisms in the Apolipoprotein E (APOE) gene encoding the protein which supports lipid transport and homeostasis in the brain has also been shown to be causally linked to the development of AD (Liu et al. 2013). Particularly, the presence of APOE ε4 allele has been associated with a decreased macrophage-mediated phagocytosis and macrophage function (Cash 2012). Earlier studies have implicated oxidative stress to contribute toward macrophage dysfunction (Kirkham 2007; Raley and Loegering 1999) and have demonstrated the therapeutic efficiency of curcumin through enhanced macrophage-mediated clearance of Aβ plaques (Fiala 2007). Although the relationships between APOE genotype and AD have been well established in various ethnicities, its relationship with respect to macrophage dysfunction in terms of decreased Aβ phagocytic ability has been relatively unexplored and warrants further investigation. Additionally, although the therapeutic effect of curcumin in enhancing macrophage-mediated clearance of Aβ has been demonstrated in Caucasians (Fiala 2007; Mishra and Palanivelu 2008), its therapeutic effect has been unexplored in the Indian population, who consume curcuminoids in their daily diet. In this study, we explore the association of APOE polymorphism and the macrophage function with respect to Aβ clearance. We also studied the effects of curcumin, a potent anti-oxidant, in its ability to modify macrophage functions in blood-derived monocytes derived from AD patients.

Materials and Methods

Inclusion of Study Subjects

Alzheimer's disease and MCI patients were recruited from the Memory & Neurobehavioral Clinic (MNC) at Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, Kerala, India, after obtaining Institutional Ethical Clearance. Informed consent was obtained from the subjects and/or their caregiver, generally a first-degree relative. Control samples were collected from the cognitively unimpaired non-consanguineous caregivers/spouses of patients and healthy volunteers. All participants were subjected to a structured interview which included inquiry into neurological symptoms and also for the presence of vascular risk factors such as hypertension, hyperlipidaemia, hypercholesterolemia, thyroid dysfunction, diabetes, cardiopathy or any history of cranial trauma. All subjects also underwent a series of blood biochemical tests for Vitamin B12 levels, thyroid function tests, lipid profile, VDRL and detailed neuropsychological tests as detailed in an earlier report from us (Mathuranath 2010). The diagnosis of AD was established using the diagnostic criteria of NINCDS-ADRDA (McKhann et al. 1984). The severity of AD was determined using the Clinical Dementia Rating Scale (Hughes et al. 1982). Preclinical AD cases were classified as MCI, using Petersen's criteria (Petersen et al. 1997) and their MMSE (Mini-Mental State Examination) and the Addenbrook's Cognitive Examination (ACE) (Mathuranath et al. 2000) scores. The study population comprised of 85 individuals in three groups of 36 AD, 22 MCI and 27 cognitively unimpaired controls. All subjects in the three groups were matched for age, gender and ethnicity. The blood specimens (6 ml) were obtained from all subjects by venipuncture; and collected in heparin-lysed vacutainers for isolation of monocytes and DNA. Subject demographics are presented in Table 1.

Table 1 Subject demographics

Sl No.	Flow cytometry study on monocytes			Macrophage amyloid internalisation assay		
	AD	MCI	Control	AD	MCI	Control
Number (No. of females)	12 (6)	4 (3)	7 (6)	24 (8)	18 (9)	20 (13)
MMSE score (mean ± SD)	17.2 ± 6.1	23.6 ± 2	28.2 ± 1.4	15.8 ± 5.2	20.3 ± 5	27.6 ± 2.2
Age (mean ± SD)	71.6 ± 9.25	68.2 ± 8.5	68.7 ± 9.5	70.65 ± 7.95	66.9 ± 9.4	66.82 ± 9.4

AD Alzheimer's disease, *MCI* mild cognitive impairment, *SD* standard deviation, *MMSE* Mini-Mental State Examination

Isolation of Monocytes from Blood Samples

Rosettesep protocol was used for the isolation of monocytes from anti-coagulated blood. The RosetteSep antibody cocktail (Stem Cell Technologies, Vancouver, Canada) cross-links all cells in human whole blood other than monocytes to multiple red blood cells (RBCs), forming immunorosettes. This increases the density of the unwanted (rosetted) cells, so that they pellet along with the free RBCs when centrifuged over a buoyant density medium such as Ficoll-Paque (Sigma Aldrich, St. Louis, MO, USA). The RosseteSep Cocktail was mixed at 50 µL/mL of whole blood and was incubated for 20 min at room temperature (RT). The blood sample mixed with the cocktail was diluted with an equal volume of phosphate-buffered saline (PBS) along with 2% fetal bovine serum (FBS) and 1 mM EDTA and layered on top of the Ficoll-Paque medium. The enriched monocytes at the interface between the plasma and the density medium was collected after centrifugation for 20 min at 1200×*g* at RT and washed in PBS.

Flow Cytometry

Monocytes isolated from whole blood were seeded at a density of 1.6×10^5 cells/mL in 35 mm culture dishes and were exposed to HiLyte Flour 488-labeled Amyloid β1–42 (1 µg/mL) (Anaspec, CA, USA). After overnight incubation at 37 °C in a 5% CO_2 humidified incubator, monocytes collected from the dish were washed with PBS and 100,000 monocytes per sample were subjected to flow cytometry to determine fluorescein isothiocyanate (FITC) fluorescence. BD fluorescence activated cell sorter (FACS) Aria benchtop flow cytometer (Becton and Dickinson, Franklin Lakes, NJ, USA) was employed for the analysis. Monocytes were gated according to side scatter (SSC)/forward scatter (FSC) and at least 10,000 cells were analyzed in FL1 (FITC) and FL2 phycoerythrin (PE) using BD FACS can with a 488 nm argon laser and the FL1 filter 530 ± 15 nm and the FL2 filter 585 ± 21 nm. Aβ phagocytosis rate was calculated as the mean fluorescent intensity (MFI) times percentage of cells.

Macrophage Aβ Internalization Assay

Monocytes isolated were cultured in RPMI 1640 medium supplemented with 10% autologous serum for 14 days until complete differentiation. Differentiated macrophages were exposed to HiLyte Flour 488-labeled Amyloid β 1–42 (1 µg/mL) and incubated overnight, washed with PBS and examined by fluorescence and confocal microscopy for analyzing Aβ uptake. Lysosomal marker Lysotracker Red (Life Technologies, Carlsbad, CA, USA) was used to analyze the extent of intra-lysosomal localization of phagocytosed Aβ. Carl Ziess Confocal Microscope was used for acquiring images. Image analysis was performed using Zeiss LSM imager/Image J software packages. MFI over six different fields per sample were subjected to analysis. Image J Colocalization finder was employed for analysis of colocalization of the two signals, and means of values of colocalization coefficient were calculated for all study samples.

Comet Assay

PBMCs were isolated from fresh heparinized blood samples, washed with 1X PBS and resuspended in 0.5% low-melting point agarose (LMPA). The cells were encapsulated in a layer of LMPA covered with a coverslip, spread over a frosted glass slide which is pre-coated with 1.5% agarose. This was allowed to solidify over a tray placed on ice-packs for 10 min. The coverslip was gently removed and a third layer of 0.5% LMPA was added, and a coverslip placed on top again to spread the layer evenly. After solidification by placing the slide over ice-packs, the cells were subjected to alkali lysis by immersing the comet slide in lysis buffer for 2 h. The slide was then subjected to electrophoresis, after equilibrating the slide in running buffer for 20 min. Electrophoresis was conducted for 24 min at 25 V/300 mA constant current. DNA with strand breaks will move extensively from 'head' through the gel like a 'comet tail'. After run, slides were neutralized and stained with ethidium bromide solution and were visualized using fluorescent microscope (IX-51, Olympus, Melville, NY). Image analyses were performed using Comet Score software. The extent of DNA damage was calculated as tail moment and that of cases and controls were compared.

Treatment of Curcumin on Macrophages

Curcuminoid stock solution (100 mM) was prepared by dissolving 36.8 mg curcumin C3 complex in 1 ml DMSO (Dimethyl SulOxide). Curcumin C3 complex was supplied generously from Sabinsa Corporation (Piscataway, NJ). Substocks (1 mM) were prepared with sufficient quantity of sterile deionized water. Differentiated macrophages were treated with varying concentrations of curcuminoids (Curcumin C3 complex) ranging from 2 µM to 20 µM in the medium overnight and were then exposed to FITC-Aβ 1–42, incubated for 24 h and examined by fluorescence or confocal microscopy post exposure to Lysotracker Red to visualize the effect of curcuminoids on the phagocytic potential of macrophages.

Genetic Analyses

ApoE Genotyping: Hixson and Vernier Restriction isotyping protocol (RFLP) was performed on all samples and the results were confirmed through SSP-PCR on the same samples. RFLP using HhaI Restriction Enzyme revealed

all homozygous/ heterozygous combinations of APOE genotypes (Hixson and Vernier 1990), (Jairani et al. 2016). The sizes of fragments were estimated by comparison with known size marker, MspI-digested pUC18 DNA Ladder. In Sequence-Specific Primer PCR (SSP PCR), 3 PCR reactions were carried out for each sample and the presence of a 173 bp band indicated the presence of the specific APOE haplotypes (Jairani et al. 2016), (Pantelidis et al. 2003).

THP-1 Cell Culture and Differentiation

THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% serum and the cells were differentiated into macrophages by incubating with 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. Treatments were carried out in the PMA-induced differentiated THP-1 cells. Dichlorofluorescin diacetate (H_2DCFDA) assay was used to detect the intracellular reactive oxygen species (ROS) levels after incubating the cells with different concentrations of hydrogen peroxide (H_2O_2) ranging from 10 to 1000 µM. The differentiated cells were preincubated with 10 µM H_2DCFDA solution for 1 h and then treated with different concentrations of H_2O_2 for 30 min. After washing the cells with Hank's Balanced Salt Solution, the fluorescence was read at maximum excitation and emission spectra of 495 nm and 529 nm, respectively, using a fluorescent microscope (IX-51, Olympus, Melville, NY).

Statistical Analyses

AD and MCI subjects were compared with controls for every parameter under analysis. Two-tailed *t* test and Fisher's exact test were used to compare the features of patients and controls, and APOE ε4 carriers and non-carriers. Results were represented as mean ± SD and a p value < 0.05 was considered as statistically significant.

Results

Efficiency of Aβ Phagocytosis is Significantly Impaired in Patient's Monocytes in Comparison to Age-Matched Controls

Patient-derived macrophages showed mild internalization of Aβ within 1 h, however, there was no lysosomal localization. Differentiated THP-1 cell lines was also found to phagocytose FITC-labelled Aβ within 1 h of incubation, however, the intensity reached its maximum after 12 h of incubation. To get clear localization of Aβ within the lysosomes, we analysed after overnight incubation with FITC- Aβ.

Monocytes incubated with HiLyte Flour 488-labeled amyloid β 1–42 overnight were subjected to FACS analysis to determine the phagocytic potential of freshly isolated monocytes for internalizing Aβ-42 in vitro. Blood samples of AD patients ($n = 12$), MCI ($n = 4$) and age matched controls ($n = 7$) were subjected to FACS (Table 1). Monocytes were gated according to SSC/FSC and at least 10,000 cells were analyzed in FL1 (FITC) and FL2 (PE) using BD FAC Scan analyzer (Fig. 1).

The mean FITC-Aβ uptake by AD patients' monocytes (27 ± 6.4 MFI units) and the mean uptake by MCI patients' monocytes (32.9 ± 9.4 MFI units) were both significantly lower ($p < 0.005$) than the mean uptake by controls' monocytes (47.5 ± 3.9 MFI units) on Student's t test. However, when AD was compared to MCI, $p = 0.1868$, no statistically significant difference was observed.

Phagocytosis of Aβ is Significantly Reduced in AD and MCI Patients' Macrophages Compared to Controls

Differentiated macrophages from AD ($n = 24$), MCI ($n = 18$) and controls ($n = 20$) (Table 1) were subjected to amyloid β internalization assay and their phagocytic potential were estimated and compared between groups. On the 14th day of culture, mature macrophages were exposed overnight to HiLyte Flour 488-labeled amyloid β 1–42. Fluorescent microscopy and confocal imaging were performed over cells after treatment and images were analyzed through image analysis softwares of LSM and Image J (Fig. 2). For each sample, 100 cells from different fields were scored for finding out MFI.

On Student's *t* test, AD patients showed statistically significant reductions in mean MFI on FITC-Aβ uptake by differentiated macrophages compared to that of controls ($p < 0.0001$). However, the difference in MFI between the macrophages of MCI and control was not statistically significant ($p = 0.2540$). On F test, significant difference in variance was obtained between AD & Control and AD & MCI groups.

AD Macrophages Showed Weak Lysosomal Localization of the Internalized Aβ

Localization of Aβ into cellular compartments was studied in detail using the lysosomal tracker dye, Lysotracker Red. Compared to the macrophages of AD group, that of the MCI and control groups showed good to excellent lysosomal trafficking of the internalized Aβ (Fig. 3). Student's *t* test was performed to compare the means of colocalization coefficients between groups. The difference in the mean colocalization between AD and controls were found to be statistically significant ($p = 0.0134$), but that between MCI and control were not significantly different ($p = 0.53$) in localization of intracellular Aβ into lysosomes.

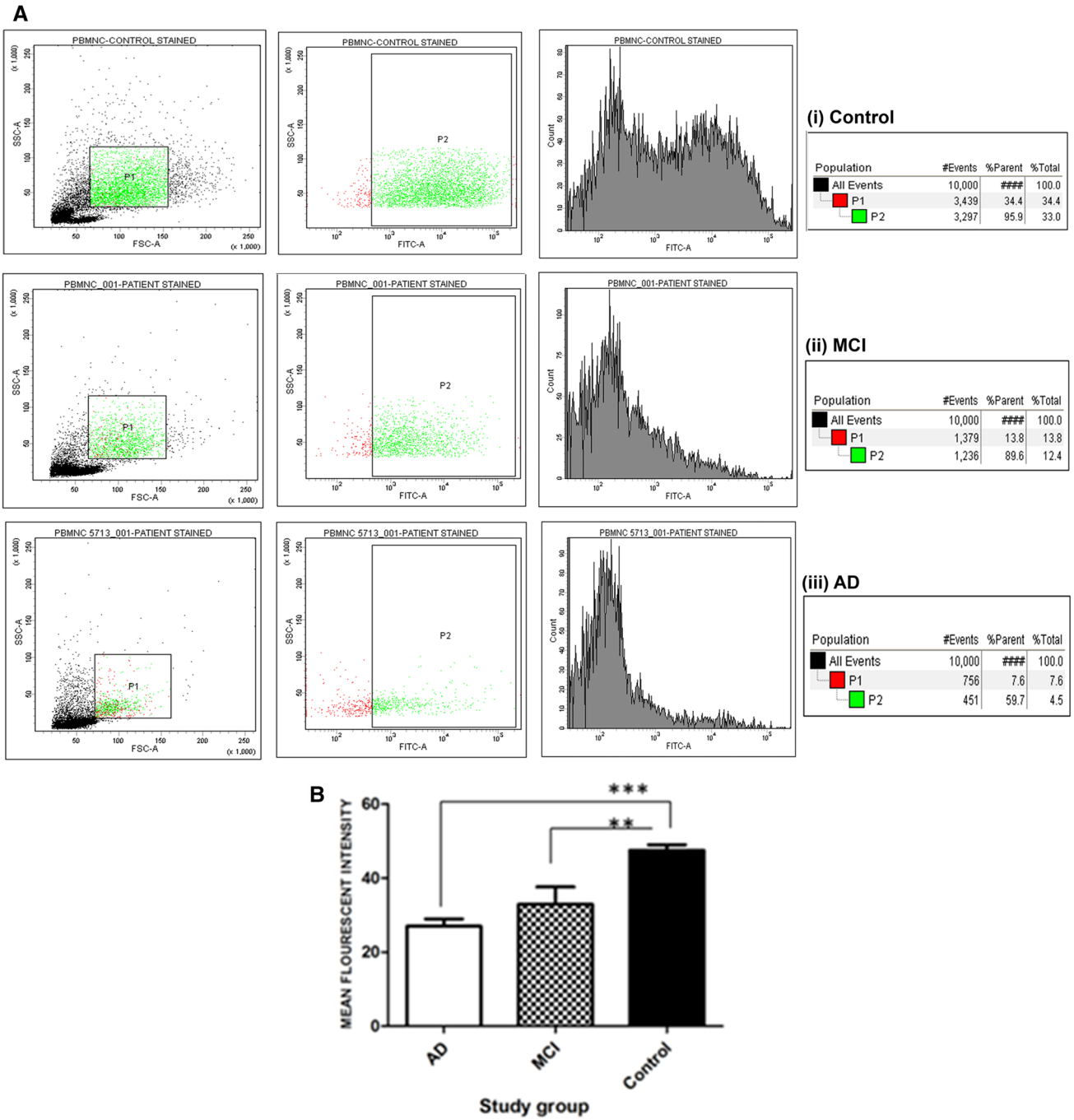

Fig. 1 **a** Representative flow cytometry histograms of cognitively normal control, MCI and AD patient (Histogram shown is from ungated population). Phagocytosis of Aβ = Mean fluorescence intensity (MFI) of FITC Aβ in upper right corner times % cells upper right corner. Top panel (i) (Control) showing higher proportions of fluorescent cells compared to that of MCI and AD patient (ii and iii). This further illustrates that the impairment of Aβ phagocytosis is evident in peripheral cells supporting the hypothesis in different clinical stages of AD. **b** Monocytes of disease groups (AD & MCI) showed statistically significant reductions on MFI compared to controls. $^{**}p < 0.005$ and $^{***}p < 0.0001$ on Student's t test. MFI shown in the graph are from multiple experiments

Further evidence for the inefficiency of Aβ phagocytosis, internalization and colocalization into periplasmic vacuoles/lysosomal compartments in AD macrophages were obtained through confocal imaging. Z imaging and overlays of slices clearly distinguished phagocytic properties of AD and MCI from that of controls (Fig. 4).

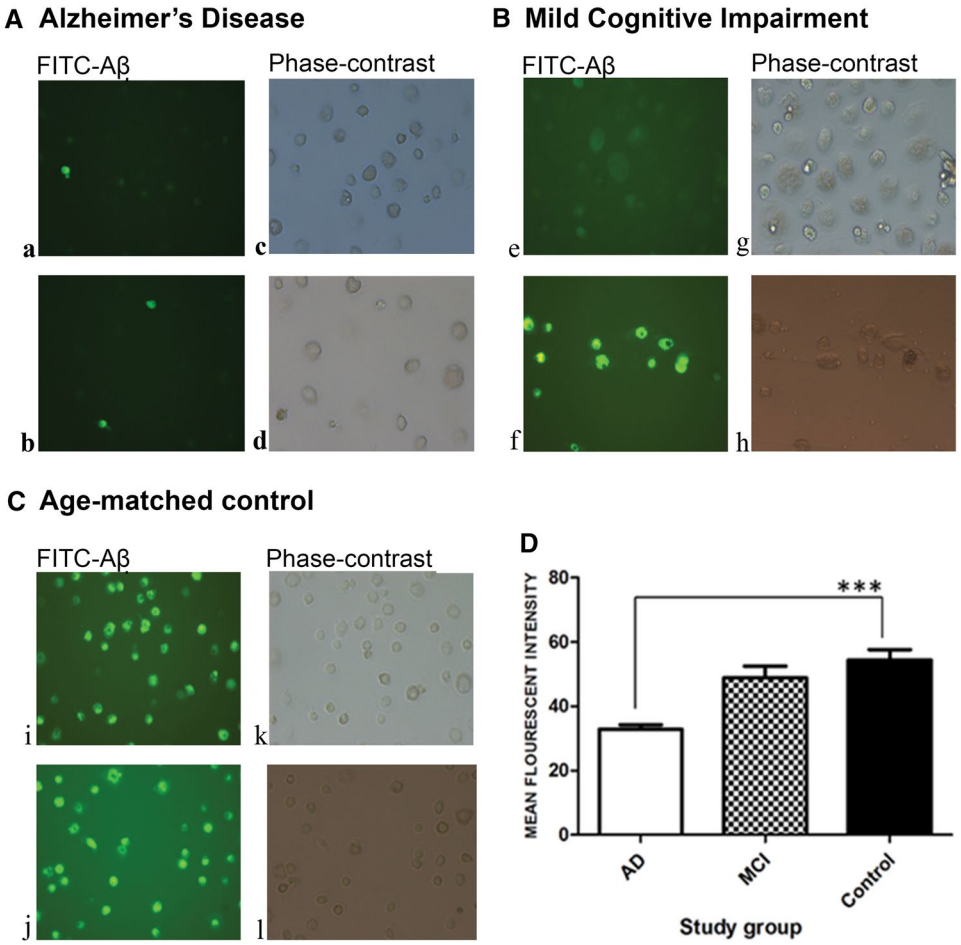

Fig. 2 MFI for macrophage amyloid β internalisation assay. Fluorescent microscopic images: mature macrophages, after overnight exposure to FITC-Aβ, were observed by fluorescent microscope. Representative images at ×40 magnification on fluorescent microscope are given along with respective phase contrast images of the same fields [AD (**A**c, **A**d), MCI (**B**g, **B**h), and Control (**C**k, **C**l)]. Control macrophages showed vigorous phagocytosis of fluorescent Aβ as seen by fluorescence microscopic images (left panels in **C**i and **C**j). In comparison, AD macrophages showed least degree of internalization of Aβ (left panels in Fig **A**a and **A**b). MCI macrophages showed wide variations in mean uptake, as some subjects showed good to excellent internalization (**B**f) (which was similar to controls) and in others, weak internalization potential was observed (**B**e). **D** Graphical representation on MFI (Mean ± SEM) for the three groups, showing highest phagocytic potential in control group compared to patients. ***$p < 0.0001$

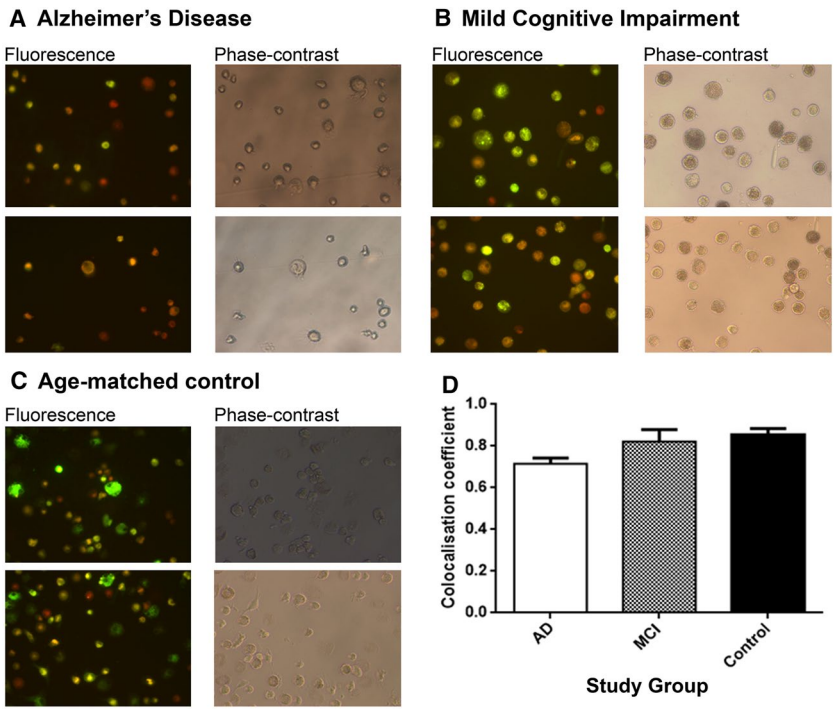

Fig. 3 Differentiated macrophages of an AD patient (**a**), MCI subject (**b**) and a control subject (**c**) were undergone overnight exposure to Aβ (1 µg/mL) and stained by Lysotracker red, examined by fluorescent microscopy (×40 magnification). **d** Degree of colocalization was determined using image Jcolocalization finder software and results are presented in graph. *$p < 0.05$

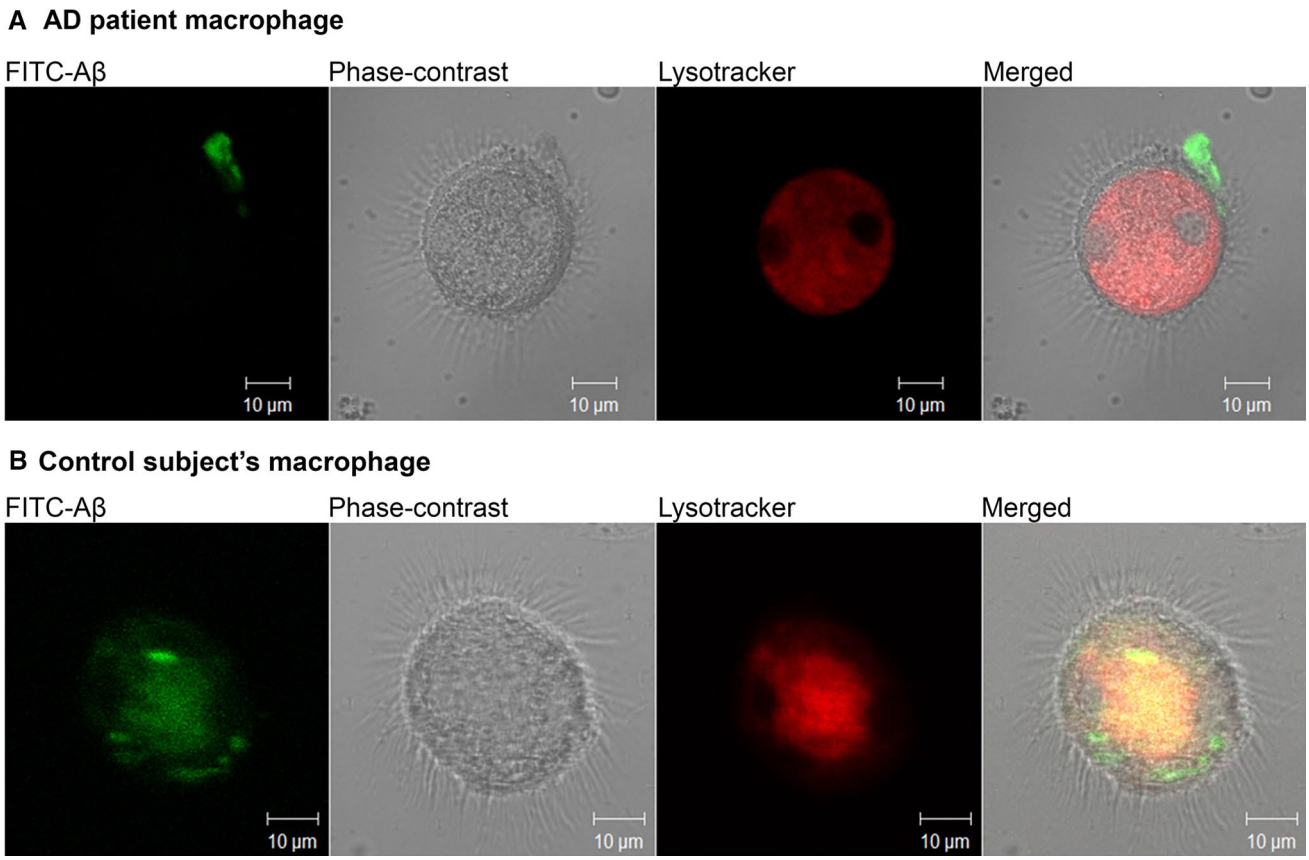

Fig. 4 Confocal images of AD and control macrophages after overnight exposure to fluorescent tagged Aβ. **a** Activated AD macrophage (with processes) being unsuccessful in internalizing Aβ, magnified image (×40) split XY into three channels and merged view showing only surface binding. **b** Control subjects' macrophage has efficiently internalized Aβ and transported into inner lysosomal compartment where the two colour channels red (lysosomal marker Lysotracker-Red) and green (phagocytosed FITC-labelled Aβ) merges to yellow (indicating colocalization of phagocytosed Aβ with lysosomes)

Defective Internalization of Aβ Might be Influenced by APOE Genotype of Subjects

APOE genotyping was performed in the DNA samples isolated from monocytes to delineate any possible correlations between defective internalization and possession of the risk factor APOE isoforms; APOE ε4. All subjects were stratified according to their possession of APOE ε4. Although the MFI for the total samples showed an expected gradation in phagocytic potential, as AD < MCI < Control, there were wide variability in this property within group (Table 2). On Student's *t* test, the difference in MFI in AD patients with at least one APOE ε4 allele those with no APOE ε4 allele ($p = 0.1322$) did not meet statistical significance. For MCI, a p value of 0.7227 was obtained and the difference in MFI in those with or without APOE ε4 allele was not statistically

Table 2 Presentation of MFI on study groups as per possession of APOE ε4

	AD ($n = 24$)	MCI ($n = 18$)	Control ($n = 20$)
MFI for subjects with two APOE ε4 allele	29.56 ± 3.9 ($n = 10$)	46.94 ± 14.5 ($n = 6$)	43.80 ± 9.88 ($n = 4$)
MFI for subjects with one APOE ε4 allele	30.68 ± 2.7 ($n = 8$)	48.58 ± 20.8 ($n = 4$)	45.68 ± 5.60 ($n = 4$)
MFI for subjects with no APOE ε4 allele	34.5 ± 7.8 ($n = 6$)	50.54 ± 8.01 ($n = 8$)	58.93 ± 13.96 ($n = 12$)
p value	1E4/2 E4 = 0.83 1E4/no E4 = 0.45 2E4/no E4 = 0.26	1E4/2 E4 = 0.53 1E4/no E4 = 0.02 2E4/no E4 = 0.04	1E4/2 E4 = 0.59 1E4/no E4 = 0.32 2E4/no E4 = 0.0001

significant. However, in the control group, a statistically significant difference was found between the MFIs of the two groups ($p = 0.03$). In AD and MCI subjects, the MFI value is lower with possession of at least one APOE ε4 allele, and in controls, the difference met statistical significance.

Oxidative Stress Influences Aβ Phagocytosis

THP-1 cell line derived from human acute monocytic leukemia was used as a model for establishing the role of oxidative stress in AD macrophages. The cells were differentiated to macrophages using phorbol-12-myristate-13-acetate. Hydrogen peroxide (H_2O_2) was used as the oxidative stress inducer at a concentration of 500 µM. The optimum concentration of H_2O_2 to induce oxidative stress in the differentiated cell lines were estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay and H_2DCFDA assay. The effect of H_2O_2-induced oxidative stress on the phagocytic efficiency of macrophages was studied using the Fluorescein isothiocyanate-conjugated Aβ internalization assay. The presence of H_2O_2 has significantly reduced the phagocytic potential of FITC-labeled Aβ by differentiated THP-1 cell lines suggesting that oxidative stress plays a major role in the phagocytic inefficiency of macrophages in Alzheimer's disease patients (Fig. 5).

Oxidative Stress and DNA Damage in Alzheimer's Disease Patients

Comet analysis was performed in a subset of the study population comprising 5 AD and two MCI and five controls and comet scores were compared. Visualisation of comets representing the three study groups is presented in Fig. 6 and their scoring of comet tail lengths on Cometscore software and corresponding tail moments are given in Table 3. Cells undergone immobilisation and alkali-denaturation of DNA when subjected to electrophoresis, cellular DNA with damage migrates from the position of the nucleus in the gel towards the positive electrode. The migrated DNA fragments were visualised as comets and the extent of DNA damage could be quantified using Cometscore analysis

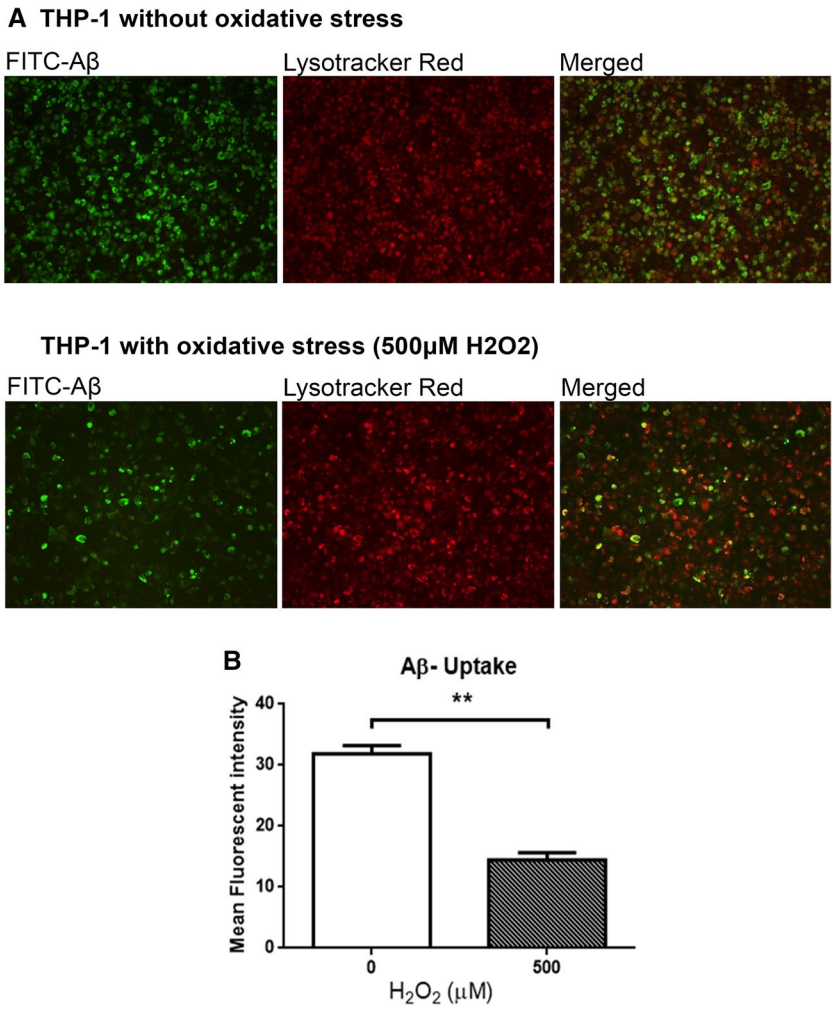

Fig. 5 **a** After pretreatment with 500 µM H_2O_2 for 30 min, the cells were incubated with FITC-Aβ overnight. Total fluorescence was analyzed using fluorescent microscope (×10 magnification). The cells were stained with lysosomal marker Lysotracker Red to visualize lysosomal colocalization of phagocytozed Aβ. **b** Graphical representation of **a**; quantified using ImageJ software and the difference in phagocytosis efficiency in H_2O_2-treated cells compared to control is statistically significant with a p value 0.0055

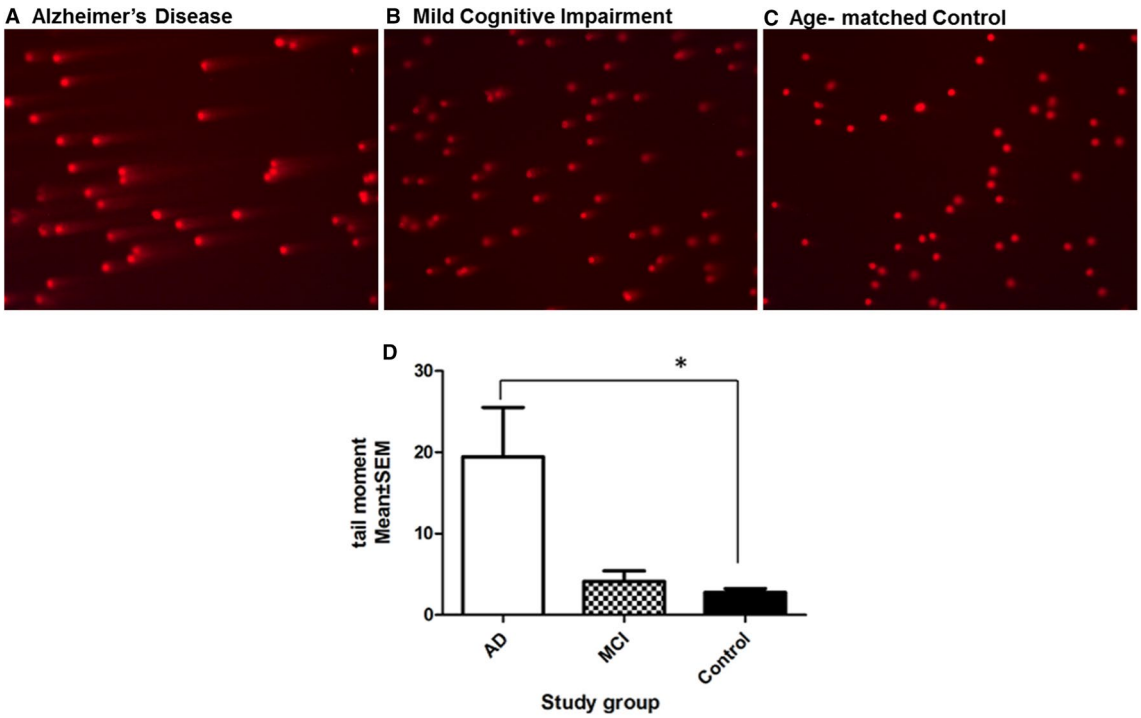

Fig. 6 Representative images of comets on peripheral blood cells of AD patient (**a**), MCI subject (**b**) and age-matched control (**c**) after electrophoresis under UV microscopic observation. **d** Graphical representation of mean tail moments of AD, MCI and controls on comet assay. *$p < 0.05$

Table 3 Results of comet assay

Sl No	% of DNA in tail	Tail moment	Tail moment (Mean ± SEM)
Patients			
AD subjects			
1	27.42	6	19.43 ± 6.071
2	27.75	8.51	
3	29.16	16.32	
4	44.21	38.18	
5	35.09	28.16	
MCI subjects			
1	19.9	2.8	4.1 ± 1.305
2	26.8	5.41	
Age-matched controls			
1	19.1	2.71	2.766 ± 0.4711
2	17.88	4.34	
3	10.28	1.37	
4	15.55	2.73	
5	13.25	2.68	

software that measures several damage parameters. The tail length (TL) was measured from the centre of the nucleus towards the end of the tail and using the percentage of DNA in the tail (TD) Olive Tail-Moment (OTM = TL × TD) was calculated. Student's t test (2 tailed) was performed to compare means of two groups under comparison, and obtained a p value = 0.0256 with CI −30.71 to −2.627, showing a statistically significant difference between AD and Control group. F test yielded a p value of 0.0002, with a statistically significant difference in variance.

Curcumin Treatment Enhances Aβ Uptake by Macrophages In Vitro

In a subset of study subjects (7 AD and 4 controls), the macrophages were treated with curcuminoids in vitro and the Aβ uptake was measured using confocal microscopy and image J software. Replicate macrophage cultures of AD and controls were established on eight chamber culture slides. At baseline, the intensity of Aβ uptake by AD macrophages was significantly lower in comparison to control macrophages and involved surface binding but no intracellular uptake. After treatment of the macrophages with curcuminoids, Aβ uptake by macrophages of four of the seven AD patients increased significantly ($p < 0.0001$). In the remaining three AD patients, no such increase was found. Confocal microscopy of AD macrophages responsive to curcuminoids showed surface binding in untreated macrophages but co-localization with lysotracker red in intracellular compartment after curcumin treatment (Figs. 7, 8).

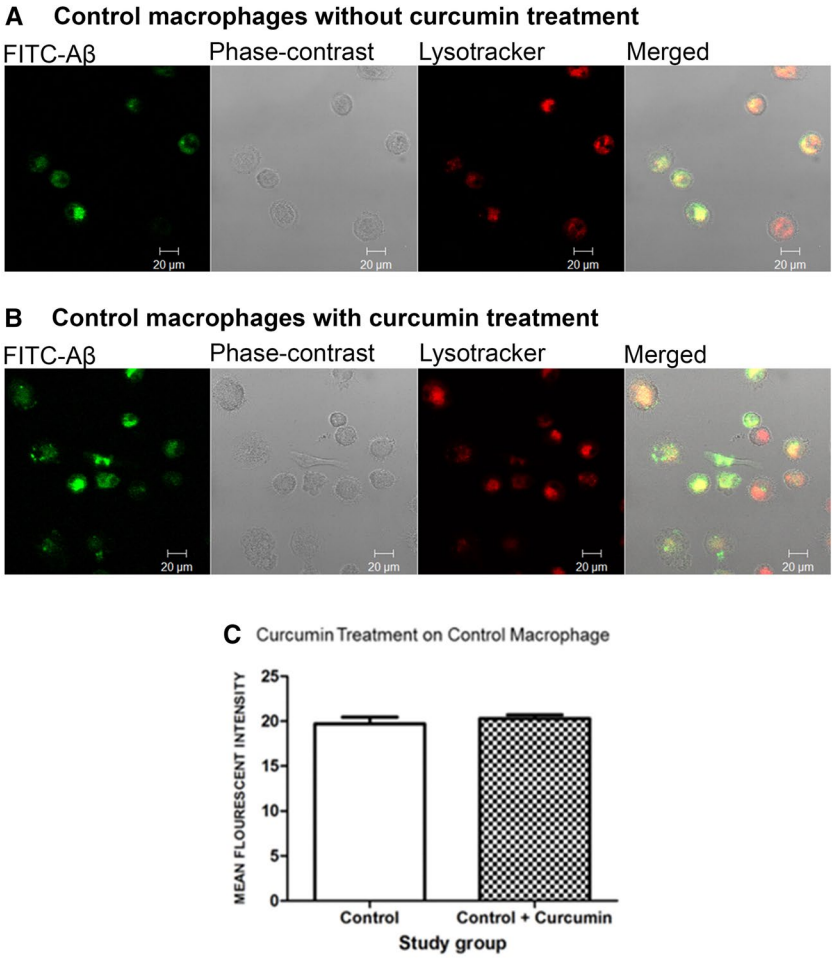

Fig. 7 **a** Control macrophages Showing Baseline Internalization of Fluorescent Aβ. **b** Confocal image of macrophages derived from control subjects showing no significant difference in the phagocytosis efficiency after curcumin treatment. **c** Graphical representation of **a** and **b**

Unpaired 2-tailed t test revealed that a statistically significant difference exists in the AD macrophages between the MFI's of curcumin treated and untreated cells on fluorescent Aβ exposure ($p = 0.0025$) (Table 4). However, in the macrophages of controls, curcumin treatment did not result in statistically significant improvement on internalization of Aβ. Curcumin treatment on AD macrophages resulted in statistically significant increase in MFI on exposure to FITC-Aβ ($p < 0.005$).

Is the Enhancement of Aβ Uptake by Curcumin Influenced by APOE Genotype?

A subset of samples chosen for curcumin treatment study was proved to be unresponsive to treatment with curcumin. The property of responsiveness toward curcumin treatment is probably correlated well to the subject's APOE status. For AD patient's macrophages, excellent improvement in Aβ internalization on curcumin exposure is confined in 4 subjects out of 7, who are bearing APOE ε3 rather than APOE ε4. Similarly, in controls, highest MFI variations were observed in individuals bearing APOE ε3 compared to APOE ε4. However, with this data, we cannot substantially state that APOE ε4 negatively regulates Aβ internalization even in the presence of an inducer (curcumin) due to lack of effective sample size for the analysis (Table 5).

Discussion

Aβ1–42 peptide secretion increases in brain with age. When stressed by these noxious peptide and age-associated oxidative stress, neurons secrete chemo-attractants to make MMs (Monocyte- Macrophages) migrate into brain crossing the Blood-Brain-Barrier (BBB) which gets 'leaky' (Fiala 1998). In normal subjects, MM's migrate and phagocytize Aβ at a physiologic pace and thus forestall accumulation of Aβ. However, in AD patients, these MM's are defective and, instead of providing help, disrupt BBB, produce neurotoxic cytokines and ineffectively phagocytize Aβ (Zuroff et al. 2017). Thus, one of the contributing factors resulting in AD neuropathology is this ineffective of Aβ clearance by immune cells.

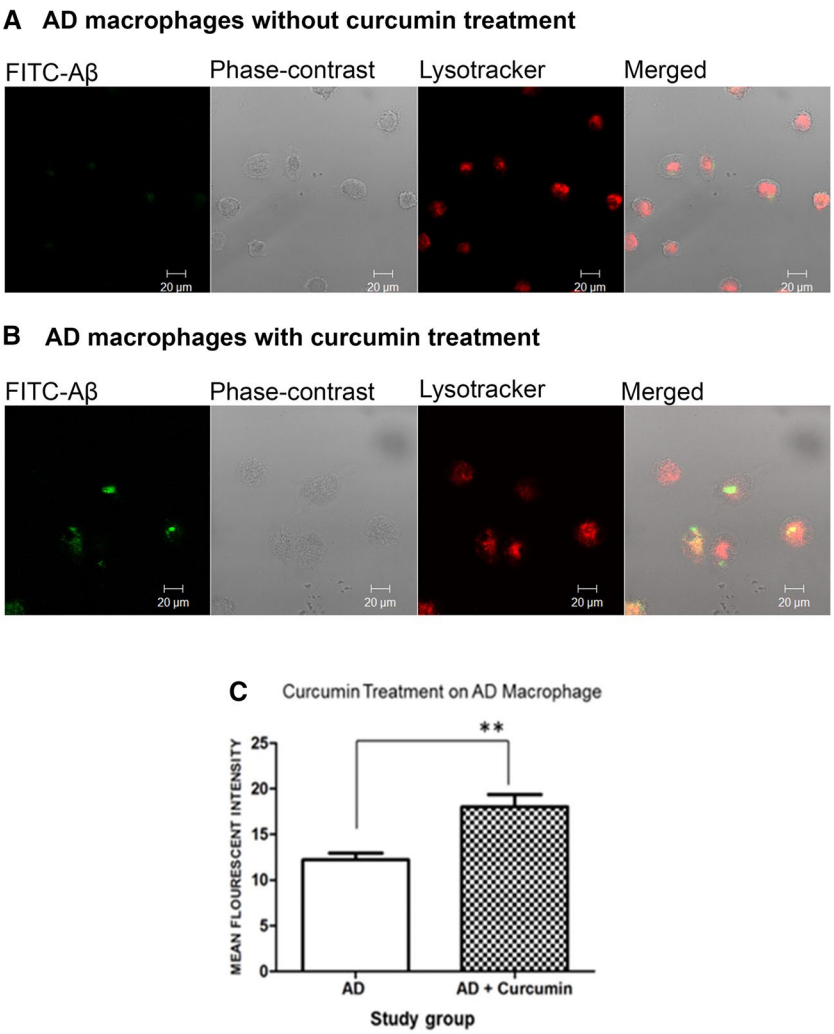

Fig. 8 **a** Confocal images showing significant reduction in phagocytosis efficiency of AD macrophages compared to macrophages derived from age-matched control. **b** Confocal image of AD macrophages showing enhancement of phagocytic efficiency after curcumin treatment when compared to untreated macrophages. **c** Graphical representation of MFI of all samples analysed. *p* value 0.0025

Table 4 Presentation of MFI and colocalisation coefficients in untreated versus treatment group

	AD patients ($n=7$)		Age-matched controls ($n=4$)	
	Without curcumin treatment	Treatment with 10 µM curcumin	Without curcumin treatment	Treatment with 10 µM curcumin
MFI (mean ± SD)	12.2 ± 1.9	18.03 ± 3.53	19.72 ± 1.45	20.3 ± 0.8
Colocalisation coefficient	0.72	0.93	0.87	0.92

While analyzing the macrophage amyloid internalization assay, we could distinctly differentiate AD and control macrophages in culture, in their morphology, adherence properties and relative survival up to 14th day differentiating into macrophages. On exposure to Aβ, different types of responses were observed among patient's macrophages, such as either no surface binding, weak surface binding, strong surface binding forming a halo around reactive macrophages, lesser degree of internalization or persistence inside cells after internalization. In macrophages of healthy controls, cells effectively internalized Aβ, and actively translocated the peptide into lysosomal compartments. This internalization and starting of localization into intracellular vesicles was evident even at 1 h post exposure in control's macrophages (unpublished observation).

We have observed for the first time that the phagocytic rate in MCI macrophages were much heterogenous, showing a wide range of variations in MFI. This could be explained by the clinical course of the MCI subjects, who comprised of those who were potential converters to AD, those who would remain stable at MCI and those who would revert back to normal cognition in future. On staining

Table 5 Presentation of MFIs of selected AD patients and controls with their APOE status

Study subject	MFI of Aβ internalisation without curcumin	MFI of Aβ internalisation with curcumin	Change in MFI (mean ± SD) for responsive patients	APOE Genotype
AD patient 1	10.4	**20**	12.21 ± 1.9 to 18.02 ± 3.53	E3E3
AD patient 2	12.8	**22.4**		E3E3
AD patient 3	10.2	**21.5**		E3E4
AD patient 4	10	**19**		E3E4
AD patient 5	14	14.4	NR	E3E4
AD patient 6	13.9	14	NR	E4E4
AD patient 7	14.2	14.9	NR	E4E4
Control 1	20.5	20.5	NR	E3E4
Control 2	19.7	**21.3**	19.72 ± 1.45 to 20.3 ± 0.8	E3E3
Control 3	17.7	**19.4**		E3E3
Control 4	21	20	NR	E3E4

Statistically significant improvement in MFI on curcumin exposure is represented in bold letters

NR Not responsive to curcumin treatment

with Lysotracker Red and FITC-Aβ, MCI macrophages often stained heterogeneously even in a single field, having cells fluorescing green only, red only and a small percentage of yellow (colocalization of two stains). Possibly due to this heterogeneity, we could not get statistically significant reductions in colocalization of MCI subjects when compared to controls, whereas AD patients were shown to have significant reduction in Aβ internalization. However, confocal microscopy and Z imaging could differentiate the surface bound FITC-Aβ signal from internalized signals which get localized to Lysotracker Red (Fig. 4). Our data provides supporting evidence to the Immune Hypothesis of Fiala and colleagues for AD, and is in agreement with their findings (Fiala 2007) over the phagocytic dysfunction of AD blood-derived macrophages.

The role of ApoE protein in phagocytosis has been well studied (Grainger et al. 2004; Lee et al. 2012). Aβ oligomers has been shown to have higher affinity to apoE3 than apoE4 (Petrlova 2011) suggesting that apoE3 might be more efficient in Aβ clearance preventing accumulation (Castellano 2011). Microglial activation has also been shown to be modulated by ApoE. ApoE protein can polarize macrophages to the neuromodulatory M2 (anti-inflammatory) phenotype and also promote microglial migration. However, ApoE4 isoform is less effective in inducing M2 polarization (Zhu 2012) and microglial migration (Cudaback et al. 2011) than apoE3 suggesting reduced clearance of Aβ and increased neurotoxicity through M1 (pro-inflammatory) polarization. ApoE4 isoform also disrupts the interaction of Aβ with cell surface receptors mainly LRP-1 thus affecting the clearance mechanisms through phagocytosis and through blood brain barrier to the circulation (Kline 2012). We have provided evidences for the heterogenous phagocytic dysfunction in MCI. The possibility of correlation of this dysfunction with pathogenic isoform of APOE locus has also been analyzed. In patients with MCI with and without possession of APOE ε4 allele we found no significant difference in MFI values. However, in control subjects, mean MFI is significantly lower in those possessing even one APOE ε4 allele compared to those with no APOE ε4 allele, suggesting thereby that the possession of APOE ε4 allele could play a role in the phagocytic functioning of macrophages. Although the absence of this difference in MCI patients could possibly suggest these results need to be reproduced in other studies on a larger subject samples to determine if there are other factors also contributing to this difference.

In normal physiology, microglia and astrocytes are the major participants in Aβ clearance in brain, but they are dysfunctional in Alzheimer's disease. Analysing the functional parameters of brain-derived immune cells contribute better understanding to the immune hypothesis of Alzheimer's disease. However, this work was performed in macrophages isolated from the peripheral blood of Alzheimer's disease, mild cognitively impaired and normal subjects. Our results support the reliability of defective Aβ internalisation as a blood monocyte biomarker for AD. FACS on peripheral blood monocytes has the potential of being a less invasive and rapid method to easily identify individuals with preclinical or clinically overt AD helping in early diagnosis and starting therapeutic interventions. The declining immune function in flow cytometric test can serve as marker of impending neurodegeneration and can serve as an early predictor of dementia. This is further supported by the novel observation that the mean MFI of MCI group was intermediate between that of AD and controls. However, a study with larger sample size needs to be performed to arrive into a conclusion whether a combination of APOE polymorphism and macrophage phagocytosis inefficiency can be used as a

possible biomarker to predict if MCI patients could progress to a clinical stage of AD.

Our observations have shown that oxidative stress serves as a major contributor in Aβ phagocytosis inefficiency of peripheral macrophages. There has been several debates regarding whether increased ROS and oxidative stress alters phagocytic property of immune cells (Splettstoesser and Schuff-Werner 2002). However, most studies have been focused on increased ROS production after phagocytosis. In Alzheimer's disease, the effect of oxidative stress in brain (Butterfield et al. 2007) and the peripheral cells (Coppedè and Migliore 2015) has been widely reported. Chronic oxidative stress affects the cellular organelles including mitochondria and also causes DNA damage. We have the comet assay data which shows increased DNA damage in AD patients compared to the controls, possibly caused by increased oxidative stress. Studies have shown that oxidative stress also modifies proteins which involves in Aβ clearance mechanisms like LRP-1 which affects its Aβ-binding activity (Owen 2010). These oxidative modifications of the phagocytic receptors might explain the reduced phagocytosis of FITC-Aβ by macrophages subjected to oxidative stress.

Curcumin has been extensively regarded to show antioxidant and free radical scavenging properties (Butterfield et al. 2007; Ak and Gülçin 2008). The protective effects of curcumin in Alzheimer's disease have been widely studied (Butterfield et al. 2007; Coppedè and Migliore 2015; Reddy et al. 2016). In vitro studies have shown increased phagocytosis in Alzheimer's macrophages and microglia treated with curcumin (Fiala 2007, b), however, no in vivo report is available as on date. Apart from the lipophilic nature of curcumin, it rarely cross the Blood–Brain–Barrier, but a few studies has shown the effects of curcumin on glial cells in transgenic mice models (Lim et al. 2001; Garcia-Alloza et al. 2007). In vivo demonstrations on APP TG mice have shown that 7-day curcumin supplementation in diet reduced the senile plaque density in brain (Lim et al. 2001). In Alzheimer's disease patients, BBB has been proven to be leaky due to amyloid beta-induced vascular damage and tight junction disruption (Montagne et al. 2017). Thus, in Alzheimer's disease patients, the leaky BBB could provide the beneficial effects of curcumin on glial cells and neurons through the BBB even though conclusive evidence is lacking. Since oxidative stress has been found to have a huge impact in effective phagocytosis of Aβ by macrophages, the well-known naturally existing antioxidant curcumin has been used as a treatment modality to enhance the phagocytosis property of AD macrophages. Thus, as an extension to macrophage amyloid internalization assay, we also analyzed the influence of curcumin in a subset of study subjects. Vast numbers of people in India and other countries take curcuminoids in their diet either as a dietary habit or with preventive or therapeutic goal not only against AD but also against cancer (Owen 2010). It was considered important to check whether the monocyte cells, which are already exposed to curcuminoids, will show any further response to it when cultured in vitro. The responsiveness to curcumin was also analyzed based on the presence or absence of the risk allele ApoEε4 and we have analyzed that both patients and control macrophages with ApoEε4 show reduced response to curcumin treatment compared to those without the allele.

Research on immune hypothesis of AD has reported the two distinct types of macrophages, Type I and Type II. This differentiation is based on the degree of regulation of phagocytosis related genes in the cells such as β-1,4-mannosyl-glycoprotein 4-β-*N*-acetylglucosaminyltransferase (MGAT-III) for Type I and toll-like receptors (TLRs) for Type II (Fiala 2007). These genes were found to be strictly downregulated in AD patients, and conversely, upregulated in healthy controls. However, upon treatment with Bis-demethoxy curcumin (BDC), MGAT-III transcriptional activation and enhancement of phagocytic potential were observed in Type I AD patients. Type II AD patients were not sensitive to curcumin, and downreglulation of MGAT-III and non-responsiveness to curcumin treatment in terms of phagocytic activation were observed in this group (Fiala 2007). Our patient group might possibly be comprised of both Type I and Type II classes, having 57% Type I (based on the responsiveness to curcumin) patients (Table 5) which might explain the heterogeneity of the patients in response to curcumin. As we have not categorized patients by gene expression analysis, question of Type 1 or Type II patients remains speculative. Neverthless, the differences in phagocytic responses between Type I and Type II patients observed in this study may be important for the design of immunotherapeutic studies with curcuminoids.

Conclusions

Ineffective clearance of Aβ by peripheral macrophages has been regarded as one of the major reason behind the amyloid pathology in sporadic Alzheimer's disease patients (Lai and McLaurin 2012). Reduced expression of Aβ receptors has been reported in AD macrophages and microglia (Reddy et al. 2018; Doens and Fernandez 2014). Even though several factors including inflammation have been identified as contributing factors for this reduced Aβ-receptor expression and decreased phagocytosis, the exact mechanism leading to this defective clearance is yet to be revealed. Our study has proven that oxidative stress could significantly affect the phagocytosis process of Aβ by macrophages in vitro. This study has provided a novel association between the presence of APOE ε4 allele and decreased macrophage phagocytosis of Aβ from Alzheimer's disease patients in Indian population. Further, we

have also shown progressive defect in phagocytosis from mild cognitive impairment to AD. Moreover, the macrophages from AD patients are not only unsuccessful in effectively phagocytosing Aβ, but also fail to degrade and clear the phagocytosed Aβ, which has been shown by reduced colocalization of the internalized Aβ with lysosomal content. Curcumin has been well regarded as a naturally occurring antioxidant and the protective role of curcumin against Alzheimer's disease prevention has been well studied (Mishra and Palanivelu 2008). We have also found significant increase in the phagocytosis efficiency of Aβ by macrophages derived from a group of AD patients, possibly by alleviating oxidative stress, which might probably prolong the pace of disease progression. We have also analyzed that the responsiveness to curcumin treatment varies depending upon the presence or absence of the APOEε4 allele. Although antioxidant therapies has shown effective in transgenic Alzheimer's disease mice models, no therapies has been proven to be effective in treating AD patients once the pathological features has been developed.

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Author Contributions MPS, RNM, JV and SG conceived and designed the study. JPS, DK, APM performed the experiments, acquired the results. JPS, APM, DK, MPS, RNM and SG analyzed, interpreted the results and drafted the manuscript. All authors reviewed and approved the manuscript. All authors verify that the data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Cellular and Molecular Neurobiology.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in the above study were in accordance with the ethical standards of the Institutional Human Ethical Committee and with the 1964 Helsinki declaration and its later amendments or comparable standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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