SHANK ASSOCIATED RH DOMAIN-INTERACTING PROTEIN AS A NOVEL REGULATOR OF AMYLOID-BETA-MEDIATED INFLAMMATION AND PHAGOCYTOSIS INALZHEIMER'S DISEASE

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A THESIS PRESENTED BY

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FOR THE AWARD OF

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CERTIFICATE

I, **Dhanya Krishnan**, hereby certify that I had personally carried out the work depicted in the thesis entitled: **"SHANK Associated RH Domain-Interacting Protein as a Novel Regulator of Amyloid-Beta-Mediated Inflammation and Phagocytosis in Alzheimer's Disease"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

Dr. Srinivas Gopala

Date:

The thesis entitled

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Submitted by

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Abbreviations

ACE	Angiotensin- converting enzyme
ACh	Acetyl choline
AD	Alzheimer's disease
ANOVA	Analysis of variance
APH-1	Anterior pharynx- defective-1
ApoE/APOE	Apolipoprotein E
APP	Amyloid precursor protein
APS	Ammonium per sulfate
ASC	Apoptosis-associated Speck-like protein containing a caspase- recruitment domain (CARD)
Aβ	Amyloid-beta
BACE1	Beta-site APP cleaving enzyme 1
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BMDM	Bone marrow-derived microglia
C/EBP	CCAAT/enhancer-binding protein
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor type 2
CD163	Cluster of Differentiation 163
CD33	Cluster of Differentiation 33
CD36	Cluster of Differentiation 36
CD68	Cluster of Differentiation 68
cDNA	Complementary Deoxyribonucleic Acid
CDR	Clinical Dementia Rating
CNS	Central Nervous System
cpdm	Chronic proliferative dermatitis
CRP	C-Reactive Protein
CSF	Cerebrospinal fluid
CTFβ	C-terminal fragment of the amyloid β protein-precursor
CX3CR1	CX3C chemokine receptor 1

CXCL8	Chemokine (C-X-C motif) ligand 8
DCF	2', 7' –dichlorofluorescein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
ECE	Endothelin-converting enzyme
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
EOAD	Early onset Alzheimer's disease
EOFAD	Early onset familial Alzheimer's disease
EtBr	Ethidium Bromide
FACS	Fluorescence-activated cell sorting
FAD	Familial Alzheimer's disease
FAM	Fluorescein amidites
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
g	gram
H_2-DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICC	Immunocytochemishtry
IDE	Insulin degrading enzyme
IFN-γ	Interferon- gamma
IL-10	Interleukin- 10
IL-12	Interleukin- 12

IL-18	Interleukin- 18
IL-1β	Interleukin-1beta
iNOS	induced Nitric Oxide Synthase
IP	Immunoprecipitation
K_2HPO_4	Dipotassium phosphate
kDa	kiloDalton
KH_2PO_4	Potassium dihydrogenphosphate
LOAD	Late onset Alzheimer's disease
LPS	Lipopolysaccharide
LRP-1	Low density lipoprotein receptor-related protein 1
LUBAC	Linear ubiquitination assembly complex
M	Molar
MAP	Microtubule associated protein
MCI	Mild Cognitive Impairment
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
mg	milligram
MHC	Major Histocompatibility Complex
Min	minute
MIP	Macrophage inflammatory protein
ml	millilitre
mM	milli Molar
MMP	Matrix metalloproteinase
MMSE	Mini mental state examination
mRNA	messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
Na_2HPO_4	Sodium hydrogen phosphate
NAC	N-acetyl cysteine
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NCT	Nicastrin

NEP	Neprilysin
NFT	Neurofibrillary tangle
NF-κB	Nuclear Factor kappa-light chain-enhancer of activated B cells
NH_4OH	Ammonium hydroxide
NINCDS–ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NLR	Nod-like receptor
NLRP3	Nucleotide-binding domain (NOD)-like receptor protein-3 or Nucleotide binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing protein 3
nm	nanometre
nM	nanomolar
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PARP	Poly-ADP ribose polymerase
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate- buffered saline
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer 2
PHF	Paired helical filaments
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PRR	Pathogen recognition receptor
PS1/ PSEN1	Presenilin 1
PS2/ PSEN2	Presenilin 2
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RAGE-1	Receptor for advanced glycation endproducts-1

RANTES	Regulated on activation, normal T cell expressed and secreted
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
sAD	sporadic Alzheimer's disease
sAPP	soluble amyloid precursor protein
SCARA1	Scavenger receptor class A 1
scr	scrambled
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SHANK	SH3 and multiple ankyrin repeat domains
SHARPIN	Shank-associated RH domain-interacting protein
SIGLECS	Sialic acid-binding immunoglobulin-type lectins
siRNA	Small interfering Ribonucleic acid
SSP PCR	Sequence-specific amplification polymerase chain reaction
TBE	Tris borate EDTA
TBS	Tris- buffered saline
TBST	Tris- buffered saline Tween 20
TEMED	Tetramethylethylenediamine
TGF-β	Transforming Growth Factor-1beta
THP-1	Tohoku Hospital Pediatrics-1
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor-alpha
TREM2	Triggering receptor expressed on myeloid cells 2
VIC	Victoris (Aequorea victoria Green Fluorescent Protein)

µg	microgram
µl	microlitre
µM	micromolar

Synopsis

Background and Objectives

Alzheimer's disease (AD) is the most common neurodegenerative disease which manifests as a gradual loss in memory and cognition. Pathologically, AD is characterized by the presence of amyloid-beta (Aβ) plaques and neurofibrillary tangles formed by the accumulation of Aβ proteins and hyperphosphorylated tau proteins. During normal physiology, Aβ level in the brain is maintained through a homeostasis in production and degradation. This is effectuated mainly by immune cells, namely microglia in the brain and macrophages and monocytes in the peripheral system. Together, these cells play an important role in Aβ degradation through phagocytosis. Under certain conditions mostly associated with aging, these cells fail to phagocytose Aβ, leading to a dysregulation in the balance between Aβ production and degradation. In the long term, excessive Aβ accumulation accompanied by reduced degradation leads to chronic inflammatory activation and neuronal death resulting in the progression of AD. However, mechanisms that underlie inefficient Aβ phagocytosis and enhanced inflammation by macrophages remain insufficiently addressed.

The NLRP3 (nucleotide-binding domain (NOD)-like receptor protein 3) inflammasome, a protein complex composed of NLRP3, the adaptor protein Apoptosis-associated Speck-like protein containing a CARD (ASC) and the inflammatory caspase-1, is responsible for the cleavage and maturation of inflammatory cytokines like IL-1β and IL-18.Although NLRP3 has been linked to the progression of AD, studies focussing on the regulatory mechanism of the protein and its role in the progression of inflammation have not been reported. SHARPIN (SHANK-associated RH domain-interacting protein), a part of the LUBAC (linear ubiquitination assembly complex) has been proven to be controlling the expression of NLRP3 through NF-κB activation in chronic proliferative dermatitis. Nevertheless, its role in AD has not been well explored yet.

The objective of my study was to address the role of SHARPIN and its impact on macrophage function in AD scenario. Specifically, we hypothesized whether Aβ-mediated SHARPIN expression could impact Aβ phagocytosis, NLRP3 expression

and macrophage polarization to M1 (pro-inflammatory phenotype). To this end, utilizing differentiated THP-1 cell line and AD patient-derived macrophages, we present an evidence for the role of Aβ-induced SHARPIN in the expression and activation of NLRP3. Further, we show that SHARPIN plays a critical role in influencing Aβ phagocytosis and macrophage polarization in differentiated THP-1 cell line as an *in-vitro* model and that SHARPIN silencing in macrophages protects neurons from Aβ-induced inflammatory damage using differentiated SHSY5Y cell line.

Hypothesis

The hypothesis of the study was whether SHARPIN is involved in Aβ-phagocytosis by peripheral macrophages thereby activating inflammatory mechanisms that could contribute to heightened neuronal damage in Alzheimer's disease.

Methods

AD and MCI patients were recruited from the Memory & Neurobehavioral Clinic (MNC) at Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), after obtaining Institutional Ethical Clearance (IEC/234/2009). Informed consent was obtained from the subjects &/or their caregiver, generally a first-degree relative. Age-matched control samples were collected from the cognitively healthy caregivers/ spouses of patients (strictly non-consanguineous) and healthy volunteers. The study population comprised of 63 individuals in three groups of 31 Alzheimer's disease, 13 Mild Cognitively Impaired(MCI-preclinical stage of AD) and 19 cognitively unimpaired age- matched control subjects. Blood specimens (20 ml) were obtained from all subjects by venipuncture for isolation of monocytes, plasma and serum.

THP-1 acute monocytic leukemia cell line were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and the cells were differentiated

into macrophages by incubating with phorbol 12-myristate 13-acetate (PMA). Conditioned media experiments were carried out in differentiated SHSY5Y neuroblastoma cell line were cultured in RPMI 1640 medium supplemented with 10% FBS. The cells at 50% confluency were treated with 10 μM retinoic acid (RA) in 1% FBS for 3-4 days for differentiation into mature neurons and were characterized by morphological and immunocytochemical methods.

Differentiated THP-1 macrophages were stimulated with 10μM Aβ and N-acetyl cysteine (NAC), pharmacological Bay-110782 was used for inhibiting oxidative stress and NF-κB respectively. Western blotting, Taqman quantitative Real-time PCR analysis, Immunoprecipitation, Enzyme Linked Immuno Sorbent Assay (ELISA), flow cytometry, H2-DCFDA assay, FITC- Aβ uptake assay and gene knockdown by RNA interference were performed following standard protocols.

One way ANOVA with Dunnett's multiple comparisons test and student's *t*-test were used for statistical analysis. Pearson's correlation coefficient was used to correlate each parameter with SHARPIN expression in AD, MCI and age-matched control subjects. Results were represented as mean ± SEM and a p value<0.05 was considered as statistically significant.

Major findings

Aβ stimulates SHARPIN expression in macrophages

THP-1- derived macrophages were treated with 10μM Aβ for 6h and we have observed thatAβ enhanced the expression of SHARPIN by approximately two-fold in the cells.

SHARPIN regulates Aβ receptor expression and phagocytosis by macrophages

We observed that knockdown of SHARPIN in macrophages significantly reduced the Aβ phagocytic efficiency using FITC- Aβ uptake assay and flow cytometry, demonstrating a critical role for SHARPIN in modulating macrophage function in a context of AD. As Aβ phagocytosis is mediated by phagocytosis receptors, the

expression of receptors involved in Aβ phagocytosis wereanalyzed. SHARPIN knockdown attenuated Aβ -induced expression of the phagocytic receptors – Scavenger Receptor Class A1 (SCARA1), CD36,receptor for advanced glycation endproducts(RAGE-1) and low density lipoprotein receptor-related protein 1 (LRP-1), that are reported to mediate Aβ uptake in macrophages.

SHARPIN is required for Aβ- induced NLRP3 expression in macrophages

The link between SHARPIN and NLRP3 that comprise two principal mediators of pro-inflammatory signaling have not been analyzed in a setting of AD. Here, we show that Aβ-induced NLRP3 expression which was abolished by silencing SHARPIN in macrophages.

SHARPIN regulates Aβ- induced Macrophage Polarization

NLRP3 inflammasome controls the maturation and release of pro-inflammatory cytokines. Since we found NLRP3 expression to be under the regulatory control SHARPIN, we analyzed whether SHARPIN could dictate macrophage polarization to a pro-inflammatory (M1) phenotype or anti-inflammatory (M2) phenotype. We observed that SHARPIN knockdown resulted in a decrease in M1 markers: induced nitic oxide synthase (iNOS), IL-1β and TNF-α release, and mRNA expression of TLR2, CX3CR1 and CD68 and increase in M2 markers: TGF-β expression and release, IL-10 release and mRNA expression of TLR1, CCR2 and CD163, suggesting that Aβ polarizes the macrophages to M1 phenotype and SHARPIN knockdown reverses the polarization to M2 phenotype.

SHARPIN down- regulation prevents inflammation-mediated neuronal cell death

Differentiated SHSY5Y neuronal cells treated with conditioned media obtained from macrophages incubated with Aβ showed increased expression of apoptotic markers cleaved caspase 3 and cleaved PARP. Importantly, incubation with conditioned media derived from SHARPIN-silenced THP-1 macrophages was found to significantly reduce the expression of the pro-apoptotic markers cleaved caspase 3 and cleaved PARP.

Aβ-induced oxidative stress affects SHARPIN expression

Aβ is known to enhance ROS generation and oxidative stress in the AD brain and *in-vitro* cell cultures. In support of these previous studies, our findings show enhanced ROS generation in Aβ-treated macrophages compared to the control. To check the role of ROS in stimulating SHARPIN expression in macrophages exposed to Aβ, the cells were pre-incubated with NAC and SHARPIN expression was analyzed. NAC treatment attenuated SHARPIN expression, demonstrating the role of ROS in mediating Aβ- stimulated SHARPIN expression.

SHARPIN is controlled by NF-κB-mediated feedback regulation

Since NF-κB is a redox-sensitive transcription factor, its regulatory role on SHARPIN was investigated. Pharmacological inhibition of NF-κB was found to cause an increase in the molecular weight of SHARPIN. Ubiquitination is an important post-translation modification and also targets proteins for proteosomal degradation. Immunoprecipitation of SHARPIN and probing with anti-ubiquitin antibody showed a significant increase in ubiquitinylation, suggesting that the transcription factor NF-κB may function to ubiquitinate the protein SHARPIN.

SHARPIN expression is altered in Alzheimer's disease patient macrophages

We have observed that SHARPIN expression by macrophages was showing an increased expression in MCI subjects compared to AD and control subjects and were also showing a positive trend in the expression patterns with regard to Aβ42/40 levels, which corresponds to the fact that SHARPIN expression by peripheral macrophages, in the absence of peripheral infection, might be stimulated by the concentration of Aβ42/40 in the blood plasma.

Significance of the study:

Our study demonstrates, for the first time, a novel role for SHARPIN in the regulation of macrophage response to Aβ and its contribution to the progression of AD. SHARPIN was found to regulate Aβ phagocytosis and Aβ-stimulated inflammatory mechanisms in macrophages. Protein expression studies using AD and MCI- patient derived macrophages supports the findings. Further, Aβ-induced SHARPIN-mediated inflammation was found to induce neuronal cell death, demonstrating its role in promoting neurodegeneration via triggering neuronal cell death in AD. The findings clearly indicate that Aβ-induced neuronal apoptosis is primarily mediated through Aβ- induced pro-inflammatory mechanisms that involve SHARPIN. SHARPIN expression was found to be stimulated by Aβ- induced oxidative stress, which was further shown to be ubiquitinated by NF-κB- mediated signalling mechanism.

In short, our study proves SHARPIN as a critical protein that acts as a double-edged sword regulating phagocytosis and inflammation, where its downregulation reduces inflammation and protects inflammatory neuronal damage, but affecting immune-mediated phagocytosis and clearance of Aβ.

I. Introduction

I. 1. Alzheimer's disease: Clinical symptoms and pathological features

Alzheimer's disease is a devastating neurodegenerative disease clinically manifested by a gradual decline in memory and cognitive abilities. The estimated number of dementia patients worldwide accounts to 50 million and will reach to 82 million in 2030 (Dementia, n.d.). One new case of dementia is reported within every 3.2 seconds worldwide. Among them, Alzheimer's disease is the most common cause of dementia in the elderly population (Dementia statistics | Alzheimer's Disease International, n.d.).

AD is characterised by decline in memory, thinking and reasoning skills at a slow but progressive rate. The clinical symptoms start with difficulty in recollecting recent memory and words which gradually progresses to significant memory and cognitive impairment. Other signs include poor judgement and decision- making, missing things and unable to retrace, inability to have a social conversation, forgetting familiar names and places, etc. These early signs progress to severe loss of memory and cognition, affecting their daily life. The rate of progression of the disease varies in different patients based on their genetic compositions, polymorphisms and lifestyle factors.

Mild cognitive impairment (MCI) is considered as the initial clinical stage of AD, with a chance to progress to clinically diagnosable AD at a rate of about 12% per year (Petersen, 2009). MCI causes a slight but noticeable decline in memory and cognitive skills. These subjects do not meet the clinical criteria for dementia or AD, yet their degree of memory and cognitive impairment is beyond the expectations for age and education. MCI is further classified on the basis of the involvement of memory, into amnestic and non- amnestic MCI. The non- amnestic MCI patients have impairment in attention, concentration and visuospatial skills, with memory unaffected (Petersen, 2009).

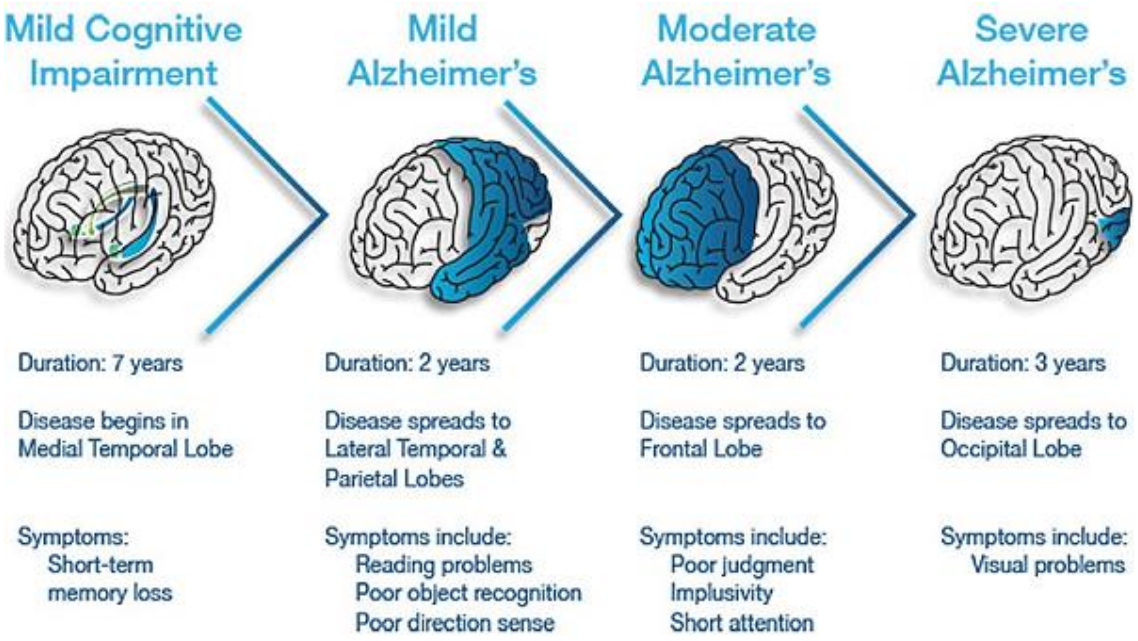


Figure I. 1: Symptoms of Alzheimer's disease (What are the Three Stages of Alzheimer's Disease | SeniorDirectory.com, n.d.)

Even though, the clinical symptoms of AD patients remain almost similar, based on the involvement of genetic mutations, AD is classified into two: familial AD/ early onset AD (FAD/EOAD/EOFAD) and sporadic AD/ Late onset AD (sAD/LOAD) (Duara *et al.*, 1993). Familial AD is further classified into AD1, AD3 and AD4 based on gene-specific mutations in Amyloid precursor protein (APP), Presinilin 1 (PSEN1), and Presinilin 2 (PSEN2) respectively. EOAD contributes to 1-5% of the total AD populations and among them, AD3 is the most frequent with 18-50% frequency and have an earlier onset among FAD, followed by AD1 with 5% and then AD4 with less than 1% (Bird, 2008). Pedigree analysis in a small number of families has shown that the mutations segregate in an autosomal dominant manner, however, studies in larger populations have shown an inconsistent data (Guerreiro *et al.*, 2012). Patients with familial mutations develop AD at an earlier age and progress at a higher rate than sporadic form of AD (Rossor *et al.*, 1996). However, no genetic mutations have been identified so far in the sporadic form of ADthat comprises 95-99% of the total AD population, in fact some genetic polymorphisms have been

identified that can be considered as risk factors rather than the cause. The most prominent among them is the polymorphism in Apolipoprotein E (ApoE), which is the main cholesterol transporter that plays a pivotal role in maintaining axon myelination and brain integrity. ApoE acts as a chaperone for Aβ uptake and degradation by glial cells, and the binding property of ApoE varies with the polymorphisms in the alleles (Liu *et al.*, 2013). ApoE isoform structure and the lipidation status also influence the activity of Aβ degrading enzymes and Aβ production since APP metabolism depends on membrane cholesterol levels. ApoEε4 has proven to be the risk allele for AD pathogenesis and rate of progression, followed by ApoEε3 being neutral and ApoEε2 being the protective allele against AD (M Liu *et al.*, 2014). Moreover, APOE due to its cholesterol binding property, also influence neuronal repair mechanisms and the maintenance of synaptic connections.

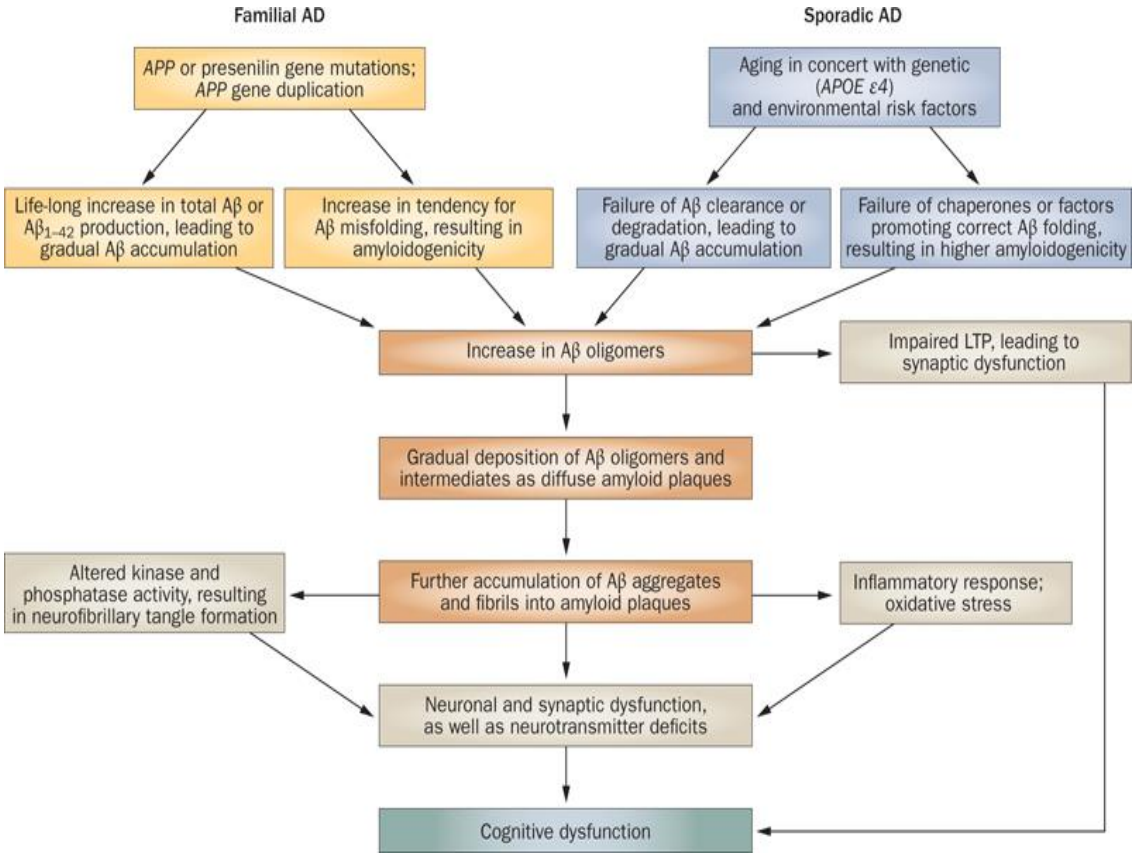


Figure I. 2: Difference between familial AD and sporadic AD (Blennow *et al.*, 2015)

The difference between familial and sporadic AD lies in the presence and absence of genetic mutation respectively. In familial AD, genetic mutations in the genes responsible for Aβ metabolism are responsible for the overproduction and accumulation of the protein, whereas in sporadic AD, unidentified factors cause a defective clearance of Aβ, leading to accumulation of the protein.

I. 2. Alzheimer's disease: past to present

Auguste.Deter, a 51-year-old woman from Frankfurt, was the first Alzheimer's patient officially declared to be admitted to Dr. Alois Alzheimer's asylum in 1901 with a severe decline in memory and cognition (Hippius and Neundörfer, 2003). He recognized that the memory and cognitive decline are symptoms of a disease rather than age-associated memory dysfunction and named the disease as Dementia Praecox. Alois Alzheimer, being a psychiatrist with a specialty in neuropathology, analyzed the autopsy brain samples and presented the findings in 1907, statingmiliary bodies and dense bundle of fibres as the pathological reason for her mental degeneration. These are now termed as the amyloid plaques and neurofibrillary tangles respectively (Schachter and Davis, 2000).Later in 1910, the disease was named as 'Alzheimer's disease' after Dr. Alois Alzheimer by his mentor Emil Kraeplin. But at that time, it was challenging for scientists to accept the involvement of molecular and cellular pathways in memory and cognition and senile dementia was considered as a part of ageing. It took several decades (1960s) to recognize AD as a distinct disease and in 1976, AD was recognized as the most common form of dementia (Ballenger, 2006).

In 1982, Bartus et al proposed the Cholinergic hypothesis for Alzheimer's disease pathogenesis. According to this hypothesis, a drastic decline in the concentration of acetylcholine (ACh) neurotransmitter and cholinergic neurons is the causal reason for AD(Bartus *et al.*, 1982). Cholinergic cell death was identified in AD brain samples and has a great influence on human brain function, specifically on memory and cognition. All the existing FDA approved drugs are based on this hypothesis and target the acetylcholine (ACh) esterases, thus preventing the recycling of ACh in the

synapses, increasing its concentration in the synapse and prolonging its action (Francis *et al.*, 1999). But this hypothesis is no longer accepted, since none of these drugs were found to reverse the symptoms of AD. Hence, the loss of cholinergic neurons was considered as an endpoint of the pathogenesis rather than the cause.

However, this was the most dominating hypothesis until 21 members of a German family affected with AD were examined at St. Mary's hospital in 1991, indicating a genetic cause of the disease.This led to identification of genetic mutations in APP with an autosomal dominant inheritance pattern (Goate *et al.*, 1991). Later, the amyloid cascade hypothesis of Alzheimer's disease was proposed by John Hardy and David Allsop in 1991 (Hardy and Higgins, 1992), after the structure of beta-amyloid was discovered by Glenner and Wong (Glenner and Wong, 1984). In 1988, Beyreuther and colleagues found that the Aβ is a protein fragment produced by the mismetabolic cleavage of a larger neuronal membrane protein called APP which is coded by a gene on chromosome 21 (Dyrks *et al.*, 1988). The observation that Down syndrome patients, with trisomy in chromosome 21 have excess copies of APP and thus have increased Aβ accumulation at an early age together with AD-like symptomsstrengthened the hypothesis (Glenner and Wong, 1984). While further studies on identifying APP mutations have failed, *in vitro* studies showing Aβ-mediated neuronal toxicity and *in vivo* studies using mice genetically-engineered to overexpress APP, showing AD-like brain pathology and memory impairment lent support to the hypothesis. Other mutations were also identified in Presinilin genes, the subunit of the γ-secretase enzyme that is required for the metabolism of APP (Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995)that further supported the amyloid cascade hypothesis.

I. 3. Amyloid-beta hypothesis

The most publicized and widely accepted theories about the etiology of Alzheimer's disease revolve around the neurotoxicity of Aβ peptide and its isoforms. According to amyloid cascade hypothesis, APP protein, normally cleaved by α- and γ-secretase enzymes sequentially, is cleaved instead by β- and γ-secretase, leading to

mismetabolism of APP, generating the hydrophobic Aβ peptide (Hardy and Allsop, 1991; Hardy and Higgins, 1992). These Aβ peptides, mainly $A\beta_{42}$,aggregate to form protofibrils and fibrils between neurons along with neuronal debris, tau proteins, immune cells and other proteins to form amyloid beta plaques that induce neurodegeneration resulting in the clinical symptoms typical of AD. Studies have shown that rather than the plaques, soluble Aβ proteins are the more toxic form and the plaque formation is hypothesized to be a way of protecting the brain from the toxic peptide (Cleary *et al.*, 2005).

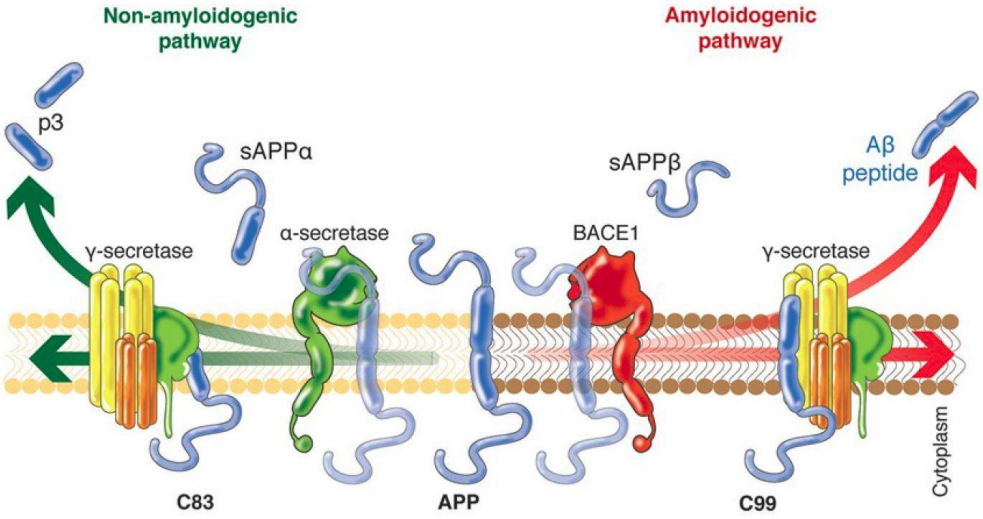


Figure I. 3: Amyloid-cascade hypothesis (MUDr. Dana Maňasková, n.d.)

The figure represents the metabolic pathways of APP in which Aβ is not generated in the non-amyloidogenic pathway, however, metabolism by the β-secretase (BACE1) and γ-secretase leads to the production of Aβ in the amyloidogenic pathway

Amyloid Precursor Protein (APP) is the parental protein from which Aβ is produced. Numerous studies suggest different roles of APP. It is reported to act as agrowth factor in fibroblasts (Schmitz *et al.*, 2002), promote cell adhesion (Schubert *et al.*, 1989), regulate calcium homeostasis (Crowther and Wischik, 1985), neural plasticity (Turner *et al.*, 2003), and regulation of synapse formation (Priller *et al.*, 2006). APP undergoes proteolytic processing through two pathways: a canonical and non-

canonical pathway. In the canonical pathway, APP undergoes sequential cleavage by α- and γ-secretase, resulting in the generation of two peptides; a larger soluble APP (αAPPs) peptide which has neuromodulatory functions and a smaller 3 kD peptide P3 with no identified biological roles. In the non-canonical pathway, APP is initially cleaved by β-secretase (BACE1) instead of α-secretaseproducing a large soluble APPβ (sAPPβ) peptide and a smaller C-terminal fragment (CTFβ). The CTFβ undergoes further cleavage by γ-secretase producing the Aβ peptide and P3 (O'Brien and Wong, 2011).

γ-secretase is a large protein complex consisting of presenilin 1 (PS1)/presenilin 2 (PS2), nicastrin (NCT), anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (Zhang *et al.*, 2014). Mutations in the genes coding for PS1 and PS2 subunits of the γ-secretase and APP resulting in enhanced Aβ production in familial AD strongly supports the amyloid cascade hypothesis. However, the mechanisms that cause the late onset sporadic Alzheimer's disease, are not well understood. Several factors and mechanisms stimulate BACE1 gene expression such as impaired intracellular calcium homeostasis (Cho *et al.*, 2008), oxidative stress mediated signalling pathways (Mouton-Liger *et al.*, 2012), endoplasmic reticulum stress (B Liu *et al.*, 2014) and $A\beta_{42}$(Tabaton *et al.*, 2010). A few studies suggest that the production rate of $A\beta_{40}$ and $A\beta_{42}$ remain the same in LOAD patients and controls. Incontrast, Aβclearance was significantly slower in AD patients than in cognitively normal individuals (Mawuenyega *et al.*, 2010) thus resulting in Aβ accumulation. In conclusion, the majority of the evidence at present suggests that Aβ is an important factor in the pathogenesis of Alzheimer's disease.

I. 4. Tau hypothesis

Tau is a microtubule- associated protein (MAP) that binds to tubulin and stabilizes microtubule assembly, thus participating in neuronal cargo and mitochondrial transport to and from the synapses. According to the tau hypothesis, the protein tau becomes hyperphosphorylated, resulting in conformational changes in the protein,

that then detaches from the microtubules and self-associates, forming the paired helical filaments (PHF) and neuro-fibrillary tangles (NFT) inside the neurons (Iqbal and Grundke-Iqbal, 2008). Destabilized microtubules fall apart resulting in failure of the axonal transport system, cause cytoplasmic dysfunctions and damage in mitochondrial integrity leading to cell death and neurodegeneration. Tau gene is located in chromosome 17 and expresses six isoforms in the brain through alternative splicing (Goedert *et al.*, 1989), all of which are found to be hyperphosphorylated in the PHFs and NFTs which appears first in the entorhinal cortex spreading to the limbic region and neocortical areas in AD brain. In contrast to the amyloid pathology, the occurrence of tau pathology in the brain and CSF correlates well with the degree of neurodegeneration and progression of clinical symptoms of AD (Bejanin *et al.*, 2017; Okamura and Yanai, 2017). However, tau pathology is not specific to AD, it is also observed in the brain sections of patients with other neurodegenerative diseases like parkinsonism, frontotemporal dementia, encephalopathies, etc with different tau morphologies (Irwin, 2016).

I. 5. Aβ clearance mechanisms in Alzheimer's disease

Even though Aβ isregarded as a toxic peptide, it is interesting to note that this protein is produced constitutively in the brain throughout life. This fact suggests that Aβ has physiological functions in the brain. Although the exact role for Aβ in the brain is still elusive, several studies found that the protein is involved in mediating long-term potentiation in the hippocampus (Puzzo et al., 2008), exhibits antioxidant activity through chelating redox metal ions (Atwood et al., 2003) and other neurotrophic properties (Fonseca et al., 2013) at picomolar concentrations. However, once the concentration of Aβ exceeds the threshold, they promote oxidative stress and neuronal cell-death. A study on the dose-dependent effects of Aβ in the brain reported that Aβ infusion at physiological picomolarconcentrationsdid not activate inflammatory responses in the brain, however, infusion with pathological nanomolarconcentrations of Aβ induced neurovascular inflammation in mice (Deane et al., 2003). Hence, maintenance of physiological Aβ levels is essential towards

protecting the brain from excessive Aβ-induced neuronal damage. For this purpose, different levels of clearance mechanisms exist in the brain that aid in maintaining the physiological level of Aβ.

- Clearance into the peripheral circulation through the BBB

  The BBB is critical for maintaining Aβ homeostasis through the efflux and influx of Aβ via two receptors LRP1 and RAGE respectively(Deane *et al.*, 2009). The soluble form of LRP1 (sLRP1) sequesters more than 70% of Aβ in the peripheral circulation, preventing its reentry into the brain(Zlokovic, 2011). However, in AD, the expression of LRP1 was found to be downregulated and RAGE1upregulated thus leading to impairment of Aβ homeostasis. Further, LRP-1 was found to be oxidized in AD, compromising its binding with Aβ due to oxidative modifications in the protein, thus reducing Aβ efflux from the brain(Kanekiyo and Bu, 2014). The presence of ApoE allele variants also hinder interaction between LRP-1 and Aβ as ApoEε4 allele which is a risk factor for AD blocks LRP-1-mediated Aβ clearance (Zlokovic, 2011).

- Proteolytic degradation by enzymes secreted by the brain cells

  A diverse array of Aβ protein degrading enzymes classified as Aβ- degrading proteases (AβDPs) have been identified. This includes Insulin Degrading Enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme (ECE), Angiotensin converting enzyme (ACE), Matrix metalloproteinases, Plasmin, cathepsin D, tissue plasminogen activator, BACE1, the Aβ generating enzyme itself, and the list is still growing. These proteinases and peptidases are secreted by various cell types in the brain including neurons and glial cells, pericytes and endothelial cells (Saido and Leissring, 2012). However, during aging and in AD, the expression and activity of these enzymes have been found to be diminished, thus affecting the clearance of Aβ from the brain, leading to dysregulation in the maintenance of physiological concentration of Aβ in the brain. Overexpression of some of these enzymes have shown to cause a significant reduction in Aβ load in the brain of AD transgenic mouse models(Baranello *et al.*, 2015).

- Receptor-mediated uptake by glial cells

  Glial cells play an important role in maintainingAβ homeostasis by aiding in degradation of Aβ. One mechanism of degradation is by the secretion of Aβ degrading enzymes IDE, NEP, and MMPs. The second and the most important clearance mechanism isthrough the phagocytic degradation of Aβ by astrocytes and microglia. These cells express receptors that bind with and phagocytose Aβ, and the phagocytosed Aβ protein istargeted to the lysosomes for degradation. Further, these cells in response to Aβ secrete chemokines for recruiting blood-derived monocytes, which cross the BBB and surround the accumulated plaques to initiate phagocytosis (Ries and Sastre, 2016). However, these cells are reported to be inefficient phagocytes. It has been reported that at initial stages, they are efficient at phagocytosing Aβ, however, as the disease progresses, Aβ load inside the cells, surroundings and lysosomes reaches saturation levels such that the cells are no longer able to degrade the phagocytosed Aβ and become activated to an inflammatory phenotype in response to the continuous exposure to Aβ(Fiala *et al.*, 2005; Bolmont *et al.*, 2008; Hickman *et al.*, 2008). This in turn affects Aβ homeostasis, leading to Aβ accumulation and damage to the surrounding neurons induced by the inflammatory environment created by the glial cells through the failed process of clearing Aβ(Wyss-Coray and Mucke, 2002; Fiala, Cribbs, *et al.*, 2007).

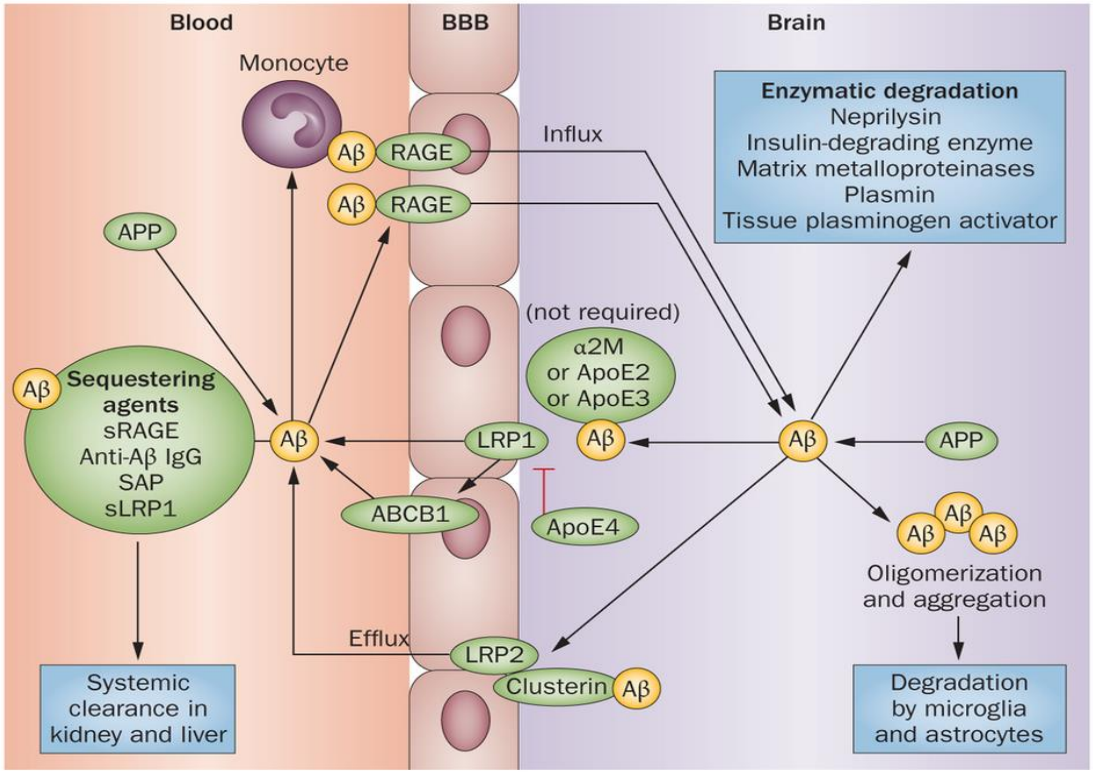


Figure I. 4:Aβ clearance mechanisms in the brain and the peripheral system(Tarasoff-Conway *et al.*, 2015)

Three major mechanisms in maintaining the physiological level of Aβ are depicted in the figure. In the brain, Aβ is cleared by enzymatic degradation and phagocytosis. The excess Aβ is shuttled into the circulatory system through LRP. The Aβ once in the circulation undergo phagocytic clearance and systemic clearance. Aβ in the circulation will be sequestered with Aβ receptors and Aβ antibody, thus inhibiting its re-entry into the brain.

Among these clearance mechanisms, phagocytic clearance by glial cells are the most explored. Despite being more efficient phagocytes than the brain resident microglia, macrophages were also shown to display frustrated phagocytosis, where they fail to degrade the phagocytosed Aβ leading to regurgitation of the ingested Aβ, but still, the exact mechanism that alters the immune cell phenotype leading them to be inefficient phagocytes is still unclear. Chronic activation of these cells by prolonged

exposure to Aβ is considered as a possible reason, where the immune cells are programmed to polarize into an inflammatory phenotype. These pro-inflammatory cells then secrete inflammatory cytokines and ROS, that change the cellular environment, mimicking senescence, which might be considered as one reason for the phagocytic inefficiency of these cells (Wilkinson and El Khoury, 2012). In this regard, linking inflammatory mechanisms and phagocytosis in response to Aβ stimulus and the molecular players involved in the regulation of phagocytosis and inflammatory mechanisms is an interesting areathat warrants further investigation.

II. Review of Literature

II. 1. Aβ metabolism in astrocytes, microglia and peripheral macrophages

II. 1.1. Astrocytes

Astrocytes are the most essential glial cell in the brain that perform numerous functions neurotransmitter release and recycling, synaptic remodeling, ion homeostasis, and regulation of metabolism, to name a few. Moreover, they also respond quickly to pathological changes in the brain by getting activated into reactive astrocytes with morphological and functional changes (Sofroniew and Vinters, 2010).

Reactive astrocytes have been found to surround Aβ deposits in the AD brain and in transgenic mouse models forming glial scar tissue thus providing a barrier between healthy brain tissue and damaged tissue (Rodríguez *et al.*, 2009). Astrocytes are recruited to the Aβ plaques by the chemotactic factor MCP-1, which also recruits the peripheral blood-derived monocytes to the Aβ plaques, and the reactive astrocytes surrounding the Aβ plaques were found to be expressing Aβ receptors including RAGE, LRP-1, membrane-associated proteoglycans, and scavenger receptor-like receptors (Wyss-Coray *et al.*, 2003). These cells are also capable of degrading Aβ through the expression of insulin degrading enzyme (IDE) (Leal *et al.*, 2006), neprilsin, matrix metalloproteinases (MMPs), Endothelin-converting enzymes (ECE-1 and ECE-2), plasmin and angiotensin converting enzyme (ACE) (Yin *et al.*, 2006; Iram and Frenkel, 2012). These reactive astrocytes will further respond to Aβ by the overexpression of inflammatory factors like IL-1β, TNF-α, INF-γ, IL-12, iNOS, and NO production, which will in turn induce damage the surrounding neurons (Abbas *et al.*, 2002; White *et al.*, 2005). NF-κB and C/EBP transcription factor activation were also found to be altered in astrocytes in response to Aβ which further upregulates the release of inflammatory cytokines, chemokines and adhesion molecules, further promoting the invasion of brain by peripheral immune cells in AD (Bales *et al.*, 1998; Li *et al.*, 2004; Moynagh, 2005).

II. 1.2. Microglia

Microglia are the resident immune cells of the CNS and are extremely essential for normal development, function and maintenance of the CNS (Dheen *et al.*, 2007). These cells are the earliest responders to external stimuli in the CNS and when encountered with a pathological stimulus, they get transformed from a ramified morphology to an active state with an amoeboid appearance and initiate immediate immune responses with an increased expression of MHC and other markers (Rock *et al.*, 2004; Perry and Teeling, 2013). They express numerous pathogen recognition receptors (PRRs) like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Doens and Fernández, 2014).

In Alzheimer's disease, the role of these cells has been a topic of controversy and its precise role is still unknown. It has been reported that these cells get activated in presence of Aβ, however, it is still unclear whether these cells are effective in clearing these Aβ deposits in the brain (Paresce *et al.*, 1996; Chung *et al.*, 1999; Wegiel *et al.*, 2001), even though they were showing effective Aβ degradation *in vitro*(Majumdar *et al.*, 2008). Some studies were showing complete Aβ degradation while others were demonstrating incomplete processing of Aβ termed as 'frustrated phagocytosis' (Zuroff *et al.*, 2017). Further research have shown that depletion of microglia had no effect on Aβ accumulation, proving that microglia are not the only cell types responsible for Aβ clearance in AD (Grathwohl *et al.*, 2009).

Interestingly, it has been reported that healthy microglia were found to act as the first line of defence against accumulating Aβ (Michell-Robinson *et al.*, 2015), however, the efficiency of these cells in clearing these toxic peptides decline progressively with age and due to accumulated damage associated with inflammatory mechanisms in the brain. Microglia express Aβ-degrading enzymes like NEP, IDE, ACE and MMP-9, but the expression and activity of these enzymes was shown to be reduced in aged APP/PS1 mice, which might be one reason behind the reduced clearance of Aβ by these cells in the later stages of AD (Hickman *et al.*, 2008). These cells were also indicated to show an altered and damaged phenotype in response to the changing environment in the AD brain (Michell-Robinson *et al.*, 2015). This altered

environment in the AD brain renders the microglia in a chronically activated stage which in turn results in reduced phagocytosis ability and enhanced inflammatory response leading to an overproduction of inflammatory cytokines, chemokines and ROS, causing heightened neuronal damage (H. Akiyama *et al.*, 2000; Wyss-Coray and Mucke, 2002).

In spite of being ineffective phagocytes and activating chronic inflammatory response, these cells, on the other hand, have been shown to secrete enzymes that clear cellular debris and recruit the peripheral immune system to regulate the immune responses in the brain (Lai and McLaurin, 2012). Based on all these evidences, microglia cannot be regarded as either neuroprotective or neurotoxic. Microglia cells are extremely vital for maintaining a healthy brain however, in the process of protecting the brain from toxic insults, especially in neurodegenerative diseases, they were reported to be causing more neuronal damage rather than protection mainly in account of age-induced ineffective microglial activity and chronic inflammation (Meyer-Luehmann *et al.*, 2008).

II. 1.3. Macrophages

Macrophages are the professional phagocytic immune cells in the peripheral system, the counterpart of the brain resident microglia, derived from the bone marrow and are also termed as bone marrow- derived microglia (BMDM) once they cross the blood-brain barrier (BBB). The role of these peripheral macrophages in AD has been controversial for decades. The major controversy is based on whether these cells can cross the highly selective BBB. Using several different techniques, it has been finally proven that these cells are capable of entering the brain especially in AD (Zuroff *et al.*, 2017). Recent studies using transgenic AD mouse models have established that a subset of the cells surrounding the plaques in the AD brain are the peripheral macrophages or BMDMs (Malm *et al.*, 2005). Further, it is also interesting to note that these cells are more efficient phagocytes than the brain-resident microglia and BMDMs, not microglia, clear amyloid deposits in AD (Simard *et al.*, 2006).

Several mechanisms exist to recruit peripheral monocytes to the brain in Alzheimer's disease. Damage in the vascular blood vessels induced by Aβ deposition permits the recruitment of monocytes to the brain parenchyma (Perlmutter, 1994). Further, the presence of a leaky blood-brain barrier, caused due to ageing process and induced by Aβ itself through the degradation of tight junction proteins like zona-occludins, will also allow the passage of monocytes through the BBB (Algotsson and Winblad, 2007). The microglia, chronically activated by the Aβ, will itself recruits peripheral monocytes to aid in Aβ degradation by secreting chemokines like MCP-1 (Conductier *et al.*, 2010). Studies in AD transgenic mouse demonstrated that blocking of CCR2 or MCP-1 signaling(Naert and Rivest, 2011) or selective ablation of blood-derived monocytes accelerates Aβ accumulation (Butovsky *et al.*, 2007). Contrariwise, induction of monocyte recruitment into the brain by LPS stimulation, immunization or monocyte engraftment reduced Aβ accumulation in AD transgenic models (Zuroff *et al.*, 2017). Patrolling monocytes also gets recruited to Aβ depositions in blood vessels, phagocytoze Aβ and recirculate into the bloodstream, thus maintaining an equilibrium of Aβ in the brain and the peripheral system, a process termed as 'peripheral sink effect' (Hawkes and McLaurin, 2009). All these observations strongly prove that peripheral blood-derived monocytes are recruited to the brain under amyloid pathology and these cells are more efficient in clearing these toxic peptides than the resident microglia.

However, several studies has shown that these macrophages also exhibit symptoms of senescence like poor differentiation, reduced phagocytic capacity and enhanced inflammatory mechanisms in response to Aβ (Fiala *et al.*, 2005; Fiala, Liu, *et al.*, 2007; Saresella *et al.*, 2014; Tian *et al.*, 2014; Tiribuzi *et al.*, 2014). Whether this microglial and macrophage senescence and altered function is the reason or the consequence for LOAD is still needs to be understood.

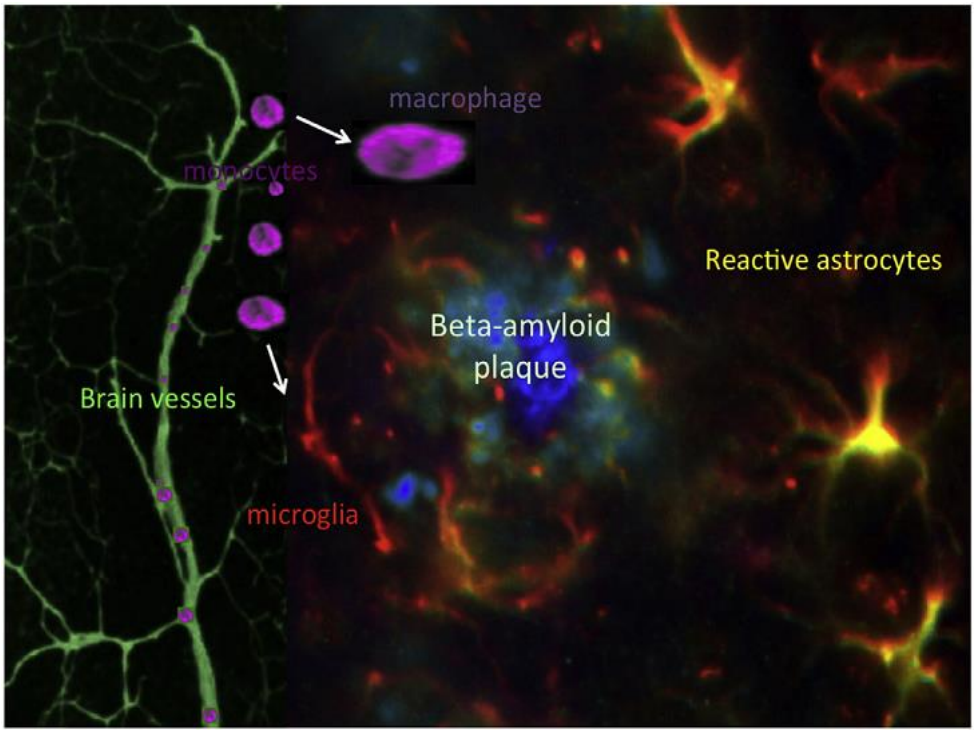


Figure II. 1: Migration of peripheral monocytes to Aβ plaques in the AD brain

(Hohsfield and Humpel, 2015)

A schematic rendering of fluorescent staining of Aβ core containing aggregated Aβ in the blood vessels in brain, surrounded by reactive astrocytes and activated microglia. Monocytes migrate into the brain and may differentiate into macrophages or microglia.

II. 2. Aβ phagocytic receptors

Macrophage scavenger receptor 1 (SCARA1) is one of the primary Aβ receptor expressed by microglia, monocytes and macrophages and the lack of this single receptor has been shown to affect Aβ phagocytosis by 50-65% and caused increased mortality in AD transgenic mouse models (El Khoury *et al.*, 1996; Frenkel *et al.*, 2013). CD36 is another Aβ receptor which is a type B scavenger receptor and is expressed by monocytes, microglia, macrophages, astrocytes and neurons. Microglia surrounding Aβ plaques have been shown to have an increased expression of SCARA1 and CD36 in transgenic AD mouse models and human AD brain (Wilkinson and El Khoury, 2012). Both these receptors also promote inflammatory mechanisms, inflammasome activation and pro-inflammatory cytokine release

(Bornemann *et al.*, 2001; Sheedy *et al.*, 2013). Thus microglia and macrophages itself create the toxic environment leading to their own dysfunction and senescence, which in turn downregulates or induce oxidative damage to these receptors leading to reduced Aβ phagocytosis in AD (Pan *et al.*, 2011). Toll-like receptors (TLRs) are a family of pattern recognition receptors and these receptors, particularly, TLR2 and TLR4 form receptor complexes with CD14 thus indirectly binds with Aβ promoting phagocytosis (Reed-Geaghan *et al.*, 2009).

Other Aβ receptors expressed by microglia and macrophages are TREM2 (triggering receptor expressed on myeloid cells 2), a variant of which is recognized as a risk factor for AD; CD33, a sialic acid-binding immunoglobulin-like lectins (SIGLECS) family protein; RAGE (receptor for advanced glycationendproducts), which is also involved in the shuttling of Aβ from the blood to brain and activates NF-κB, thus enhancing Aβ accumulation in the brain and inflammation respectively; LRP-1 (Lipoprotein Receptor-related Protein-1), which is also involved in shuttling Aβ from the brain to blood, thus reducing Aβ load in the brain, however, the expression of LRP-1 is downregulated in AD and also reported to be dysfunctional due to oxidative damage (Zuroff *et al.*, 2017).

II. 3. Inflammation and AD

AD is a multifactorial disorder in which neurodegeneration is induced by a combination of different factors including protein mismetabolisms, vascular and genetic factors, oxidative and endoplasmic stress, immune dysfunction and neuroinflammatory mechanisms and other neurotoxic agents. Among them, inflammatory pathways stimulated by the abnormal protein deposits play a prior role in neuronal death which contributes significantly to AD pathology. All the factors leading to neurodegeneration finally acts as an alarm signal for stimulating the otherwise less active immune cells in the brain, namely the brain- resident microglia and the astrocytes. Further, even before these toxic protein accumulations, the

presence of inflammatory mediators has been reported to be detected in AD brain (Sastre *et al.*, 2011).

Unlike the peripheral macrophages, microglia remain inactive to protect the brain from unwanted inflammatory damage and instead they continuously survey the neurons and synapses for any unsuitable stimuli and inactive synapses. In AD brain cortex, the microglia were identified to be major histocompatibility complex type II (MHC II) positive presenting the first clue that these cells are immunologically active in AD brain (Luber-Narod and Rogers, 1988). Even though there are multiple stimuli that activates the microglia in the AD brain into an inflammatory phenotype, Aβ plaques are reported to be the most potent one, indicated by the accumulation of MHC II and other inflammatory marker expressing microglia surrounding the plaques (Luber-Narod and Rogers, 1988; McGeer *et al.*, 1988; Rogers *et al.*, 1988). These cells thus under chronic inflammatory stimuli produces inflammatory cytokines like IL-1β, IL-6, TNF-α, and interferon γ (IFN-γ) (Lue *et al.*, 2001); chemokines like macrophage inflammatory protein 1α (MIP1α), MIP1β, CXCL8, RANTES, and monocyte chemotactic protein 1 (MCP1) (Thériault *et al.*, 2015) and reactive oxygen species (Von Bernhardi *et al.*, 2015) affecting the surrounding neurons leading to apoptotic neuronal death. They also express cytokine receptors (Haruhiko Akiyama *et al.*, 2000), chemokine receptors (Cartier *et al.*, 2005), receptor for advanced glycationendproducts (RAGE) (Walker and Lue, 2005), scavenger receptors (El Khoury and Luster, 2008) and TLRs (Toll-like receptors) (Landreth and Reed-Geaghan, 2009) that are associated with inflammatory mechanisms and phagocytosis. Activation of these receptors namely RAGE will further stimulate feedback oxidative and inflammatory mechanisms thus leading to chronic inflammatory condition in the brain (Lue *et al.*, 2009; Yan *et al.*, 2012). Increased expression of all these factors has been reported in *in-vitro* studies, animal models and also in AD brain, prominently in the pathologically vulnerable regions (Haruhiko Akiyama *et al.*, 2000; Rogers *et al.*, 2007; Landreth and Reed-Geaghan, 2009). Further, transcription factors associated with inflammatory stimuli like NF-κB(Granic *et al.*, 2009), PPARγ(Jiang *et al.*, 2008), Sp1 (Citron *et al.*, 2008) were also found to be altered in microglial cultures and AD brain. All these factors in turn

leads to overproduction of pro-infammatory cytokines and ROS that trigger signalling mechanisms in neurons deregulating many factors including protein kinases and phosphatases, which in turn leads to tau hyperphosphorylation and other deleterious effects leading to neuronal death (Maccioni *et al.*, 2010). Neuronal apoptosis will release the intracellular accumulated NFT which will further reactivate microglia and inflammatory cascade causing feedback chronic stimulation of the immune cells and neuronal apoptosis (Maccioni *et al.*, 2010).

Neurons are known to express a wide range of proteins to protect themselves against inflammatory damage including CD22 (Mott *et al.*, 2004), CD200 (Walker *et al.*, 2009), fractalkine(Beech *et al.*, 2007), TREM2 (Hsieh *et al.*, 2009), and the complement defense protein CD59 (Singhrao *et al.*, 1999). However, some of the protein expressions are found to be deficient in AD like selective downregulation of fractalkine(Duan *et al.*, 2008), CD59 (Yang *et al.*, 2000) and CD200 (Walker *et al.*, 2009) in the brain regions vulnerable to AD. These factors combined with an increased inflammatory milieu in the brain promote inflammatory damage to the neurons thus significantly enhancing neurodegeneration in AD.

II. 3.1. Macrophage polarization in Alzheimer's disease

Based on the surrounding micro-environment and the stimuli, macrophages can attain different phenotypes by the process known as polarization. The main two macrophage subsets are M1 or pro-inflammatory or the classically activated macrophages and M2 or anti-inflammatory or alternatively activated macrophages. The stimuli for M1 polarization are LPS, IFN-γ, GM-CSF and TLR ligands and are associated with immune response to acute infections. M1 polarized macrophages clear infectious agents and aggregated toxic peptides and create an inflammatory response by secreting pro-inflammatory cytokines like IL-1β, TNF-α, IL-6, IL-12; chemokines like CCL2; nitric oxide (NO) and ROS. In contrast, M2 polarized macrophages are stimulated by parasitic infections and Th2-derived cytokines like IL-4 and IL-13 and in response they secrete anti-inflammatory cytokines like IL-10

and TGF-β and growth factors and contributes to vasculogenesis, tissue repair and remodeling. The M2 macrophages are further subdivided into M2a, M2b, M2c, and M2d based on the cytokine profile and the markers they express. The two subsets of macrophages use the aminoacid arginine, where it is converted by inducible Nitric Oxide Synthase (iNOS) enzyme into NO and citrulline in M1 macrophages for cytotoxicity and by arginase enzyme into ornithine and urea in M2 macrophages for tissue repair (Thériault *et al.*, 2015; Ley, 2017). The brain-resident microglia also show the similar polarization states in response to diverse stimuli.

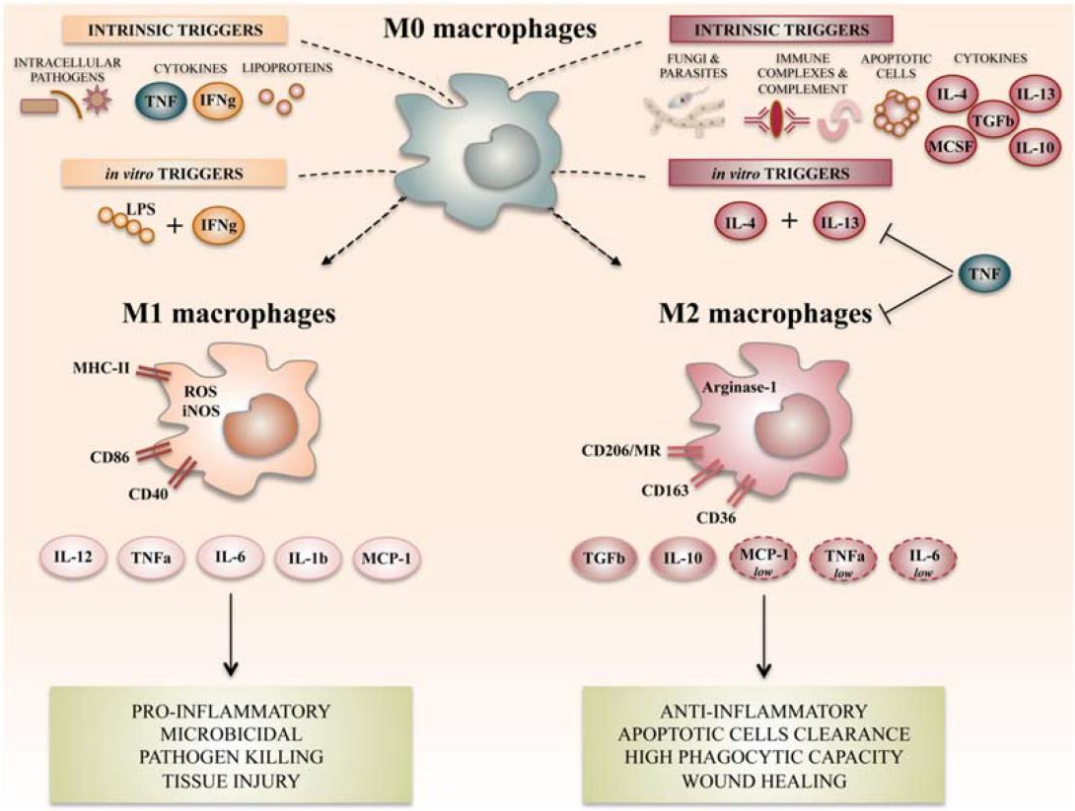


Figure II. 2: Polarization of macrophages into M1 and M2 phenotype (Atri *et al.*, 2018)

The figure shows polarization of M0 (macrophages without any stimuli) macrophages into M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. The M1 macrophages are cytotoxic and cause tissue injury, whereas the M2 macrophages are anti-inflammatory and are responsible for tissue repair and remodelling.

To maintain a homeostasis between tissue repair and protection against infections, a balance between M1 (neurotoxic) and M2 (neuroprotective) subsets is critical to avoid tissue damage and organ failure in many disease conditions including Alzheimer's disease (Lund *et al.*, 2014; Famenini *et al.*, 2017). In AD, these cells are well known to be polarized into M1 pro-inflammatory macrophages and microglia. The monocytes that are recruited to the brain through CCR2 signalling are also pro-inflammatory in nature and studies have found a significant reduction in the M2 macrophages in AD subjects compared to age-matched MCI and control subjects (Saresella *et al.*, 2014). The interaction of Aβ and tau with pattern recognition receptors (PRRs) in microglia stimulates these cells into pro-inflammatory stage and studies have shown that the increase in the activation state of microglia and the presence of inflammatory markers in the brain correlates with increase in cognitive impairment in AD subjects (Thériault *et al.*, 2015; Chitre *et al.*, 2016; Fani Maleki and Rivest, 2019) suggesting the role of macrophage M1 polarization in AD. Several studies and clinical trials administered immunotherapy against Aβ by early stimulation of immune cells, which were proven to be an effective method of clearing Aβ. However, stimulation of phagocytosis is accompanied by a pro-inflammatory activation of these cells, which in turn was responsible for the side effects in these trials, like vascular damage duo to the interaction with pro-inflammatory cytokines and ROS. Based on this, studies also aimed at inhibiting the transition of macrophages to M1 phenotype and promoting M2 polarization thus clearing Aβ without inflammatory damage. Molecules like tuftsin and ghrelin has been identified, which could promote an anti-inflammatory switch in macrophages and co-administration of immunotherapy for activating immune cell-mediated clearance of Aβ along with these molecules thus reducing inflammatory damage has been a possible therapeutic strategy (Baatar *et al.*, 2011; Wu *et al.*, 2012; Chitre *et al.*, 2016). Based on all these observations, maintaining an M1/M2 transition of microglia and macrophages in AD is essential to prevent further neuronal damage and disease progression.

II. 3.2. NLRP3 inflammasome in Alzheimer's disease

Inflammasomes are intracellular multiprotein complexes that under activation are responsible for the initiation of inflammatory mechanisms. There are different types of inflammasomes, namely NLRP1, NLRP3, NLRC4, NALP3, AIM2, and new inflammasomes like NLRP6, NLRP12 are considered putative (Sharma and Kanneganti, 2016). Among them, the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome is the most studied inflammasome and was first identified in Muckle-Wells autoinflammatory disorder (Agostini *et al.*, 2004).

The NLRP3 inflammasome is composed of NLRP3 protein; ASC, the adaptor protein; and pro-caspase-1, the effector molecule (Mangan *et al.*, 2018). A diverse array of stimuli was found to be triggering NLRP3 inflammasome activation, including low intracellular K+ concentration, mitochondrial DNA, cathepsin release into the cytosol induced by lysosomal destabilization, cardiolipin from damaged mitochondria, Ca^{2+} signalling and ROS (Song *et al.*, 2017). Once a change in cellular homeostasis is detected, the expression of NLRP3 and pro-IL-1β will be enhanced by NF-κB mediated signalling termed as the priming signal for NLRP3 inflammasome activation. This is followed by an activating signal where the components of the inflammasome assemble together to form the NLRP3 inflammasome. Once activated, the pro-caspase-1 in the inflammasome is converted into the active caspase-1 which cleaves and activates the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into its active form (Bauernfeind *et al.*, 2009). The release inflammatory cytokines will in turn trigger inflammatory mechanisms and pyroptosis, a distinct form of programmed cell death.

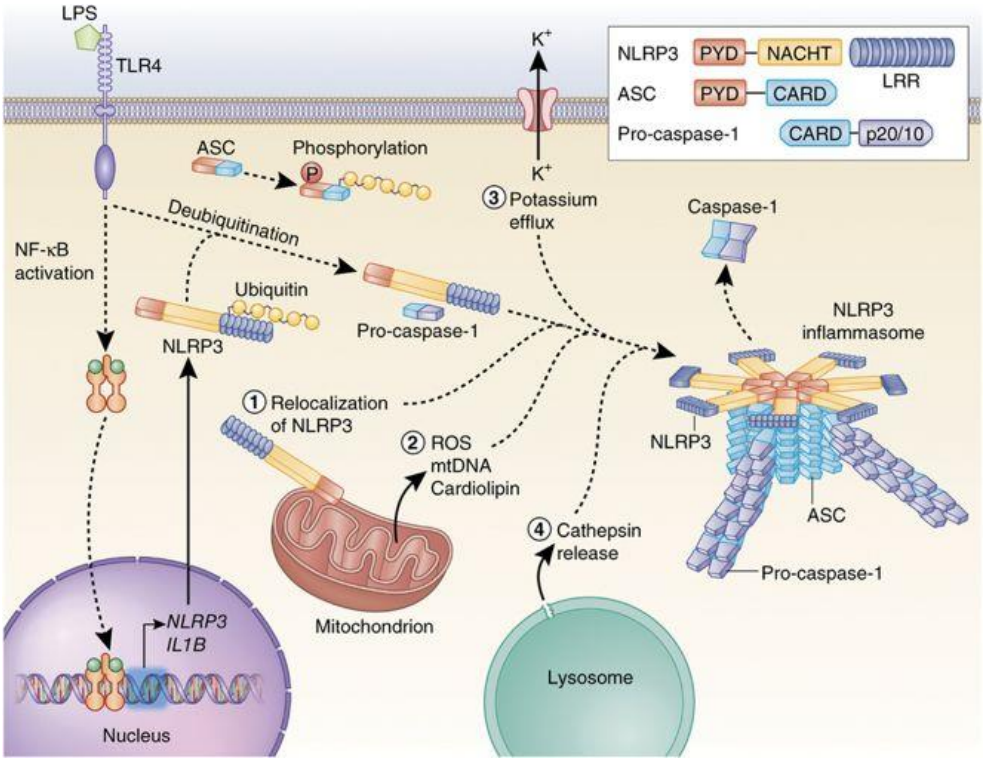


Figure II. 3: Mechanisms involved in NLRP3 expression and inflammasome activation(Guo *et al.*, 2015)

The figure depicts the diversity and complexity of mechanisms that enhance NLRP3 and pro-IL-1β expression (priming) as well as NLRP3 deubiquitination and the activation signal stimulating the formation of the NLRP3 inflammasome complex by the assembly of the inflammasome components NLRP3, ASC and pro-caspase-1. The inflammasome formation then activates caspase-1 by cleaving pro-caspase-1 which then cleaves pro-IL-1β and pro-IL-18 into its mature and active form, thus inducing inflammatory mechanisms and pyroptosis.

In Alzheimer's disease, higher levels of NLRP3, caspase-1 and their downstream effectors, IL-1β and IL-18 has been detected in patient brain sections and AD transgenic mouse models (Heneka, Kummer, Stutz, Delekate, Schwartz, Saecker, *et al.*, 2013; Saresella *et al.*, 2016). Aβ, by inducing the production of ROS, has been identified as the primary triggering factor for NLRP3 activation in microglia and astrocytes in AD brain (Parajuli *et al.*, 2013). The subsequent release of IL-1β and IL-18 will induce autocrine signalling in microglia and astrocytes and they will also

bind to receptors in neurons and endothelial cells, triggering the secondary expression of pro-inflammatory genes such as IL-6 and TNF-α thus enhancing the neurotoxic inflammatory mechanism in the brain. IL-1β aids in the infiltration of immune cells from the peripheral circulation into the brain by modulating BBB integrity (Alvarez *et al.*, 2011) and by also enhancing the expression and release of chemokines(Gosselin and Rivest, 2007). IL-18 stimulates NK (natural killer), Th (T-helper) and B cell mediated inflammatory response by inducing the expression and release of pro-inflammatory cytokines and chemokines. IL-18 also induces Fas-mediated neuronal death by increasing the expression of Fas ligand in glial cells (Bossù *et al.*, 2010). The final outcome of NLRP3 inflammasome activation is pyroptosis, where the cells are destined to undergo a distinct type of inflammatory programmed cells death. Pyroptosis occurs in neurons and glia through the rapid rupturing of plasma membrane thereby releasing an excessive amount of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and chemokines which will further cause severe inflammatory damage in the brain (Bergsbaken *et al.*, 2009).

Studies with NLRP3 or caspase-1 deficient AD transgenic mice have shown significant increase in Aβ clearance and spacial memory (Heneka, Kummer, Stutz, Delekate, Schwartz, Saecker, *et al.*, 2013). Another study has found the presence of two SNPs in the NLRP3 gene have an added effect as a risk factor for LOAD (Tan *et al.*, 2013).

II. 4. SHARPIN in Alzheimer's disease

SHANK-associated RH domain-interacting protein or SHARPIN or SIPL1 is a ubiquitously expressed protein that is conserved among mammalian species, from mice to humans (Wang *et al.*, 2012). SHARPIN is a cytosolic protein that is also recently found to be localized in nucleus and membrane ruffles (Lim *et al.*, 2001; Rantala *et al.*, 2011). The protein consists of multiple functional motifs: the amino-terminal coiled-coil domain (CC) required for protein-protein interaction, the carboxy terminal ubiquitin-like domain (ULD) and the NPL4 zinc finger domain

(NZF) are required for the functional role of SHARPIN in binding with ubiquitin and HOIP to form LUBAC (Stieglitz *et al.*, 2012). Eventhough the protein was initially identified in the neurons as a post-synaptic density protein, its functional role as a neuronal protein has not been explored till now (Lim *et al.*, 2001). However, the protein has been highly implicated in inflammatory diseases and in cancer. Genetic mutations has been identified in SHARPIN which leads to chronic proliferative dermatitis (cpdm) which is a chronic inflammatory disease characterized by uncontrolled inflammation (Wang *et al.*, 2012). The protein upregulation and genetic mutations has also been identified in many types of cancers (Li *et al.*, 2015; Melo and Tang, 2015; Tanaka *et al.*, 2016; Yang *et al.*, 2017).

The primary function of SHARPIN has been identified as a functional component of the linear ubiquitin chain assembly complex (LUBAC) along with HOIL-1L and HOIP, where the complex adds linear ubiquitin chains to the NF-κB essential modulator (NEMO) in response to NF-κB activating signals like inflammation and oxidative stress. This will target the NEMO protein for proteosomal degradation, thus phosphorylating and activating the IκB kinase (IKKα/β). This will leads to the phosphorylation of inhibitor of NF-κB (IκBα) which will initiate ubiquitination and target IκBα for degradation thus releasing the NF-κB subunits p65/p50 thus promoting the nuclear translocation of the active transcription factor NF-κB(Ikeda *et al.*, 2011; Wang *et al.*, 2012). The binding of TNF-α and IL-1β to their respective receptors can activate autocrine signalling for SHARPIN mediated NF-κB activation, thus maintaining an inflammatory state (Tokunaga and Iwai, 2012). SHARPIN is also essential for the expression and activation of NLRP3 inflammasome, which further proves its role in inflammation (Gurung and Kanneganti, 2015; Gurung *et al.*, 2015). Hence maintenance of an optimum level of SHARPIN is essential for prevention of many inflammatory diseases and cancer, where its downregulation or upregulation are detrimental for cells and organs. The role of SHARPIN in inflammatory diseases and targeting SHARPIN as a therapeutic strategy in inflammation- associated diseases including neurodegenerative diseases has to be explored in detail.

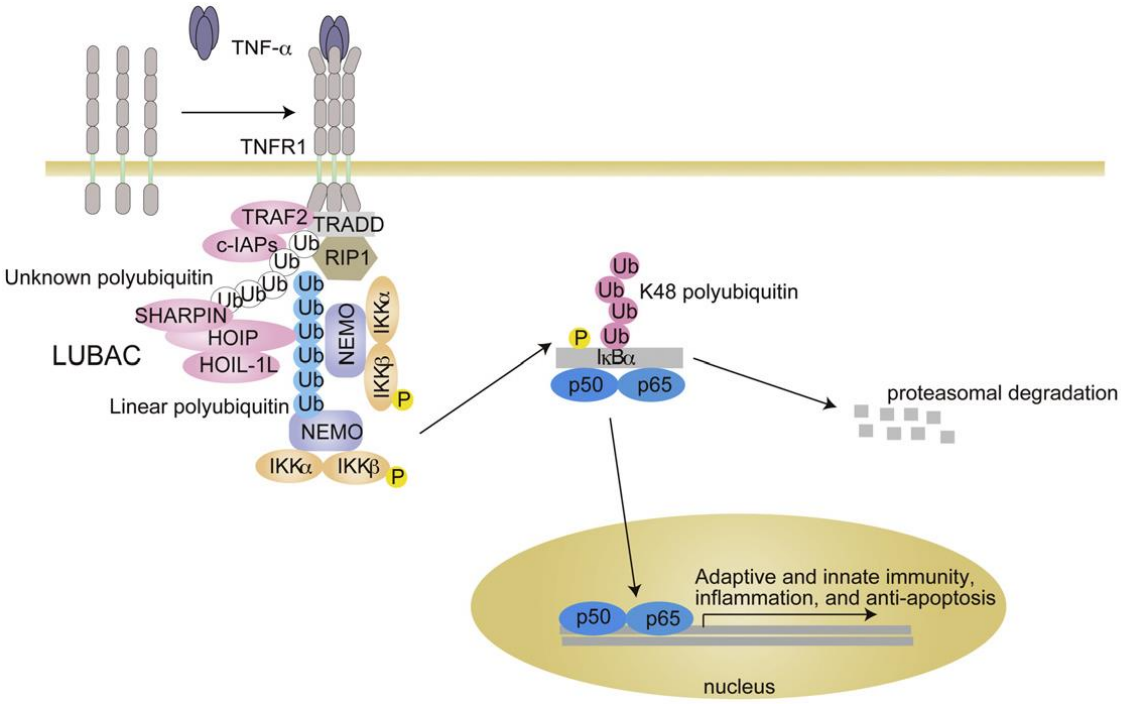


Figure II. 4: LUBAC-mediated NF-κB activation (Tokunaga and Iwai, 2012)

Signals that promote inflammation (TNF-α) and cell survival will stimulate the assembly of LUBAC complex (SHARPIN, HOIL-1L and HOIP), which ubiquitinate and degrade NEMO initiating signalling pathways leading to NF-κB activation and nuclear translocation.

In 2018, a genome wide association study (GWAS) in Alzheimer's disease predicted the involvement of novel genes that are possible risk factors for AD and SHARPIN was one among them (Lancour *et al.*, 2018). Recently, Asanomi et al identified a rare functional variant of SHARPIN as a genetic risk factor for late onset Alzheimer's disease. The study analysed genetic mutations in LOAD patients without the ApoEε4 risk allele using whole exome sequencing analysis and found that the presence of this genetic mutation (rs572750141, NM_030974.3:p.Gly186Arg) leads to the expression of a functional variant of SHARPIN which on preliminary analysis was shown to have affected NF-κB activity by reducing its nuclear translocation thereby attenuating inflammatory response in AD. The mutation was found to enhance the risk for AD by 6.1 times, higher than the known AD risk factor TREM2 (Asanomi *et al.*, 2019). An online preprint version of another study identified that a

nonsynonymous mutation (rs34173062: p.Ser17Phe) in SHARPIN correlates with reduced cortical thickness in entorhinal region and enhanced amygdalar atrophy, the most vulnerable area in AD (Soheili-Nezhad *et al.*, 2019).

Eventhough only three studies, focused on the genetic role of SHARPIN in AD, have been reported yet, the findings in these studies pointed out the significant role of this protein in the pathogenesis and the progression of Alzheimer's disease. Hence, it is critical to explore the functional role of SHARPIN in the pathogenesis of Alzheimer's disease.

II.5. Rationale of the study

Late onset or sporadic form of Alzheimer's disease (LOAD) is characterized by the accumulation of amyloid-beta (Aβ) protein in the brain, in the absence of genetic mutations, leading to neuronal cell-death and neurodegeneration. Although the reason for pathogenic accumulation of Aβ is elusive, ineffective clearance of Aβ is regarded as a major factor. Brain-resident microglia and macrophages from the peripheral circulation are the key cell-types involved in phagocytosis of Aβ. However, prolonged exposure to Aβ in the long term leads to cellular senescence and dysfunction, leading to ineffective degradation and accumulation of Aβ. Further, continuous stimulation of these immune cells by Aβ render these cells in a chronically activated state that promotes pro-inflammatory signalling, causing bystander damage to neurons and neuronal cell-death. Together, the inability of macrophages and microglia to phagocytose Aβ and persistent activation of pro-inflammatory signalling promotes neurodegeneration, leading to pathogenesis of AD. Hence, there is a need to identify mechanisms that mediate Aβ phagocytosis and inflammation in macrophages.

The SHANK-associated RH domain-Interacting protein (SHARPIN) has been widely explored with regard to its involvement in inflammatory mechanisms. A genetic mutation in SHARPIN which leads to the synthesis of a functional variant of the protein has been recently identified as a risk factor for LOAD. These reports offer strong grounds for investigating the role of SHARPIN in Aβ phagocytosis and inflammation.

II. 6. Hypothesis

SHARPIN plays a significant role in Aβ-phagocytosis by peripheral macrophages and in the resolution of inflammation in macrophages in response to Aβ stimulation.

II. 7. Objectives of the study

1. To investigate whether SHARPIN plays a role in:
   a. Aβ-phagocytosis via the regulation of expression of Aβ receptors in macrophages
   b. Aβ-induced inflammation via the regulation of NLRP3 expression
   c. Aβ-mediated macrophage polarization to M1 (pro-inflammatory) phenotype
   d. Inflammation- mediated neuronal apoptosis
2. To identify the mechanisms that regulates SHARPIN expression in macrophages in response to Aβ.
3. To analyze whether the *in vitro* findings correlate with the protein expression patterns in macrophages isolated from study subjects (control, MCI and AD).

III. Materials and Methods

III. 1. Materials

III. 1.1. Chemicals

Hydrogen peroxide, N-acetyl cysteine (NAC), Bay 11-7082, Retinoic acid (RA), Phorbol 12-myristate 13-acetate (PMA), 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA), 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT), Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4, skimmed milk, Bovine serum albumin (BSA), Oligonucleotide primers for Single Specific Primer-Polymerase Chain Reaction (SSP PCR), Low Melting Point Agarose, Ethidium Bromide, Di Methyl Sulphoxide (DMSO), Hoescht 33342 stain, SDS, Trizma base, Glycine, Sodium acetate, Acrylamide, Bis-acrylamide, Mercaptoethanol, TEMED, APS, Ponceau S and all the routine chemicals used for the preparation of buffers and for experiments were purchased from Sigma Aldrich (St. Louis, MO, USA). Consumables/plasticwares were purchased from Tarsons (Kolkata, India) and Axygen Scientific (Union City, CA, USA). Plain (Red-capped) and EDTA treated (Lavender- capped) vacutainers and cryovials, used for blood collection and storage of biological samples, were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). DNA isolation kit, cell culture media RPMI-1640, 10X antibiotic-antimycotic solution, Fetal Bovine Serum (FBS), Ficoll density- gradient solution were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), EmeraldAmp® GT PCR Master Mix were purchased from Takara Bio Inc. (Kusatsu, Shiga Prefecture, Japan). Culture wares were ordered from Thermofisher Scientific (Waltham, MA, USA) and Becton Dickinson (Franklin Lakes, NJ, USA).

All the ELISA kits (Human Aβ 1-42, Human Aβ 1-40, Human IL-1β, Human TNF-α, Human IL-10, Human TGF-β), anti-human CD68 and CD14 antibodies and Anti-fade mounting medium for immunocytochemistry (ICC) were purchased from G-Biosciences (St. Louis, MO, USA). Lysosomal tracker dye Lysotracker Red was bought from Life Technologies (Carlsbad, CA, USA). Antibodies for human Aβ, PARP and CD36 were purchased from Santa Cruz Biotechnology Inc (Paso Robles, CA, USA); SHARPIN, NLRP3, iNOS and human Aβ protein from Abcam

(Cambridge, MA, USA); SCARA1, RAGE-1, LRP-1, TGF-β, Cleaved caspase-3 and caspase- 3 from Cell Signalling Technology (Danvers, MA, USA). Secondary anti-rabbit and anti- mouse antibodies and SHARPIN- siRNA were purchased from Cell Signalling Technology (Danvers, MA, USA). Chemiluminescent reagents used for western blots were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and Pierce Biotechnology (Rockford, Illinois, USA) and PVDF membrane were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RIPA, Protease and Phosphatase inhibitors, BCA reagent, DyLight Fluor-488- labelled anti-rabbit and DyLight Fluor-650- labelled anti-mousesecondary antibody were purchased from Pierce Biotechnology (Rockford, Illinois, USA) and RNA isolation kit and cDNA synthesis kit from Invitrogen (Carlsbad, CA, USA). jetPRIME® transfection reagent for siRNA transfection was purchased from Polyplus-transfection® SA (Graffenstaden, France). HiLyte Flour 488-labeled Aβ was bought from Anaspec (Fremont, CA, USA). Realtime PCR mastermix, TaqMan primers and Protein-A coated magnetic beads were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

III. 1.2. Equipments

PCR Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), ELISA plate reader (Bio-Tek Instruments, Winooski, Vermont, USA), UV-visible spectrophotometer (Shimadzu, Kyoto, Kyoto Prefecture, Japan), Electrophoresis unit (Bio-Rad Laboratories, Hercules, CA, USA), Waterbath (Julabo, Seelbach, Germany), Weighing balance (Sartorius, Gottingen, Germany), pH meter (Eutech, Singapore), Magnetic Stirrer (Schott, Mainz, Germany), Good precision Pipettes (Eppendorf, Hamburg, Germany), -80^{o}C Freezer (New Brunswick Scientific, Edison, NJ, USA), -20^{o}C Freezer (Vest frost, Falkevej, Denmark), Cooling centrifuge (Eppendorf, Hamburg, Germany), Centrifuge (REMI, Mumbai, India), CO_2 incubator (Sanyo, Osaka, Osaka Prefecture, Japan), Laminar Air Flow Hood (MicroFilt, Pune, India), Mini-PROTEAN Tetra Cell, Powerpac universal, Semi Dry Blot Apparatus and Wet-transfer Apparatus (Bio-Rad Laboratories, Hercules, CA, USA), BD FACSJazz Cell Sorter (Becton and Dickinson, Franklin Lakes, NJ, USA), Fluorescent Microscope (Olympus, Shinjuku, Tokyo, Japan), Gel Documentation

apparatus (Bio-Rad Laboratories, Hercules, CA, USA), 7500 Real-Time PCR System and PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

III. 1.3. Media, Buffers and Reagents

III. 1.3.1. PCR reagents

Emerald GTPCR mastermix,

III. 1.3.1.1. Oligonucleotide Primers:

ApoE primer 1(CGG ACA TGG AGG ACG TGT)

ApoE primer 2(CTG GTA CAC TGC CAG GCG)

ApoE primer 3(CTG GTA CAC TGC CAG GCA)

ApoE primer 4(CGG ACA TGG AGG ACG TGC)

HLA primer 1(TGC CAA GTG GAG CAC CCA A)

HLA primer 2(GCA TCT TGC TCT GTG CAG AT)

III. 1.3.2. Reagents for Agarose Gel electrophoresis

III. 1.3.2.1. TBE Buffer 5X:

1.1M Tris, 900 mM Borate & 25 mM EDTA, pH 8.3. TBE (0.5X) is used as working buffer

III. 1.3.2.2. 1% agarose gel:

Agarose (1%) molten in 1X TBE

III. 1.3.2.3. 3% ethidium bromide (EtBr):

3% EtBr dissolved in distilled water was dissolved in molten agarose for visualization of DNA under UV- light and Gel Documentation system.

III. 1.3.3. Reagents for Immunoprecipitation (IP)

III. 1.3.3.1. IP buffer:

150 mMNaCl, 10 mMTrisHCl (pH- 7.4), 1 mM EDTA, 1 mM EGTA (pH- 8), 0.2 mM PMSF (protease phosphatase inhibitor), 1% Triton X-100, 0.5% NP-40 in deionized water.

III. 1.3.3.2. Phosphate Buffered Saline (PBS) (1X):

137 mMNaCl, 2.7 mMKCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4 dissolved in distilled water, pH 7.4

III. 1.3.3.3. Protein- A coated magnetic beads

III. 1.3.3.4. 3X SDS sample loading buffer:

2% (w/v) SDS, 0.03% Bromophenol blue, 0.3% β-mercapto ethanol, 10% (v/v) Glycerol and 0.067 M Tris in distilled water, pH 6.8. Dilute this 6X dye to 3X in distilled water.

III. 1.3.4. Reagents for Western Blotting

III. 1.3.4.1. Running Buffer (10X):

0.25 M Tris base, 1.92 M Glycine and 1% SDS in deionized water.

III. 1.3.4.2. Blotting Buffer (10X):

0.25 M Tris base and 1.92 M Glycine in deionized water. 1X blotting buffer constitutes 100 ml 10X buffer & 200 ml methanol, made up the volume to 1 L.

III. 1.3.4.3. TBS (Tris Buffered Saline) (10X):

12.1 g Tris base and 84.8 g NaCl dissolved in deionized water (1 L), pH 7.9

III. 1.3.4.4. TBST (Tris Buffered Saline with Tween-20):

1X TBS containing 0.1% Tween-20 .

III. 1.3.4.5. Resolving Gel Buffer for SDS PAGE (8X) (10%):

1.5 M Trizma base (18.165 g) in 100 ml deionized water, pH 8.8

III. 1.3.4.6. Stacking Gel Buffer for SDS PAGE (4X) (5%):

1M Trizma base (18.165 g) 150 ml deionized water, pH 6.8

III. 1.3.4.7. Loading Dye for SDS PAGE:

2% (w/v) SDS, 0.03% Bromophenol blue, 0.3% β-mercapto ethanol, 10% (v/v) Glycerol and 0.067 M Tris in distilled water, pH 6.8.

III. 1.3.4.8. Resolving Gel (10%):

Acrylamide/ Bis-acrylamide solution (3.3ml), Resolving buffer (2.5 ml), 10% APS (4 µl), TEMED (100 µl) mixed in deionized water (4 ml).

III. 1.3.4.9. Stacking Gel (5%):

Acrylamide/ Bis-acrylamide solution (0.670 ml), Stacking buffer (0.5 ml), 10% APS (4 µl), TEMED (40 µl) mixed in deionized water (2.7 ml).

III. 1.3.4.10. BSA for antibody dilution:

3% BSA in 1X TBST

III. 1.3.4.11. Blocking solution:

5% Skim milk in 1X TBST

III. 1.3.5. Reagents for Primary Macrophage Isolation and Culture

III. 1.3.5.1. Phosphate Buffered Saline (PBS) (1X):

137 mMNaCl, 2.7 mMKCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4 dissolved in distilled water, pH 7.4

III. 1.3.5.2. HiSep™ LSM 1077 (Ficoll- histopaque) density gradient medium

III. 1.3.5.3. RPMI- 1640 Medium:

RPMI- 1640 was reconstituted with sterile water, supplemented with 10% autologous serum, 1X antibiotic-antimycotic solution, pH adjusted to 7.4 using $NaHCO_3$ (2g). Autologous sera samples were collected by centrifugation of coagulated blood (6 ml), were complement-inactivated and filtered through Millipore 0.22 μm filters and stored at 4^oC for short term and -80^oC for long term use.

III. 1.3.5.4. β Amyloid 1-42, HiLyte Flour 488-labeled working solution:

0.1 mg peptide reconstituted with 50 μl NH_4OH (1%) and diluted with 1X PBS to substocks (1 μg/μl), stored at -20^oC till use.

III. 1.3.5.5. Lysotracker Red working solution:

Lysotracker red dye (0.1 mg/1ml) in 1X PBS.

III. 1.3.6. Reagents for THP-1 culture and Differentiation

III. 1.3.6.1. Phosphate Buffered Saline (PBS) (1X):

137 mMNaCl, 2.7 mMKCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4 dissolved in distilled water, pH 7.4

III. 1.3.6.2. RPMI- 1640 Medium:

RPMI- 1640 was reconstituted with sterile water, supplemented with 10% fetal bovine serum, 1X antibiotic-antimycotic solution, pH adjusted to 7.4 using $NaHCO_3$ (2g).

III. 1.3.6.3. Phorbol 12-myristate 13-acetate (PMA):

PMA was dissolved in DMSO (1mg/ml) and a concentration of 100nM was used for differentiation of THP-1 into macrophages.

III. 1.3.6.4. Hydrogen peroxide solution:

1M H_2O_2 was prepared from 8.82M (30% w/v) H_2O_2 by adding 1/133ml of H_2O_2 in 8.867ml 1X PBS. 500 µM H_2O_2 in RPMI-1640 media without FBS was used to induce oxidative stress.

III. 1.3.6.5. Human $A\beta_{1-42}$ protein:

Aβ (1mg powder) reconstituted in 80 µl of 1% NH_4OH and 920 µl of 1 PBS was prepared (1mg/ml or 221.529 mM) and aliquots of 10 mM Aβ was used as stock solution by diluting the stock in 1X PBS and a working solution of 10 µM Aβ was prepared by diluting the stock solution in RPMI-1640 media supplemented with 1% FBS.

III. 1.3.6.6. N-acetyl Cysteine (NAC):

NAC (163 mg) dissolved in 1ml deionised sterile water at a concentration of 1M was used as stock solution and a working solution of 10 mM NAC was prepared by diluting the stock solution in RPMI-1640 medium supplemented with 1% FBS.

III. 1.3.6.7. Bay 11-7082:

2.0725 mg of(E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile or Bay 11-7082 was dissolved in 1 ml DMSO under sterile condition to prepare 10 mM stock solution and was stored at 4^oC till use.

III. 1.3.7. Reagents for Immunocytochemistry

Methanol, 1X PBS, blocking solution (2% FBS and 2% BSA in 1X PBS), primary antibody diluted in 1X PBS containing 3% BSA, fluorescent labelled secondary antibody diluted in 1X PBS, anti-fade mounting medium.

III. 2. Methods

III. 2.1. Inclusion of study subjects

Case control study design was adopted to meet the objectives of the study. Alzheimer's disease (AD) and Mild Cognitively Impaired (MCI) patients were recruited from the Memory & Neurobehavioral Clinic (MNC) at SreeChitraTirunal Institute for Medical Sciences and Technology (SCTIMST), Kerala. All subjects/first degree relatives gave signed informed consent approved by the SCTIMST Institute

Ethics Committee for Human Studies. Control samples were collected from the cognitively normal caregivers/ spouses of patients and healthy volunteers. All the recruited subjects were tested for hypertension, hyperlipidaemia, hypercholesterolemia, Vitamin B12 deficiency, thyroid dysfunction, diabetes, cardiopathy or any history of cranial trauma or other neurological disorders. Subjects with high plasma CRP level were excluded to avoid the possibility of peripheral infection or inflammation- mediated alteration of protein expression. The diagnostic criteria of NINCDS–ADRDA (McKhann *et al.*, 1984) were used to confirm AD and MCI pathology. The severity of AD was determined using the Clinical Dementia Rating Scale (Hughes *et al.*, 1982). The preclinical AD cases were classified as MCI using Petersen's criteria (Petersen, 2004), if they scored between 1.5 and 2.0 standard deviations below the corresponding age group's population mean score on the memory tests (Mathuranath *et al.*, 2007) on the Addenbrook's Cognitive Examination (Mathuranath *et al.*, 2004) administered in their native language of Malayalam. The study population comprised of 63 individuals with 31 AD, 13 MCI and 19 cognitively unimpaired control subjects.

III. 2.2. Separation of blood into components for assays

III. 2.2.1. Serum: Serum was separated from freshly collected blood samples through centrifugation. Blood samples drawn into plain vacutainers (coagulated blood) were centrifuged at 2500 rpm for 15 min and the supernatant (serum) was collected, complement inactivated at 56^{o}C for 30 min, filtered through 0.22 μm syringe filter and stored at 4^{o}C for short term use and -80^{o}C for long term use in cryovials.

III. 2.2.2. Plasma: Anti-coagulated blood was layered on Ficoll-Paque medium and centrifuged at 2000 rpm for 20 min. The upper layer of plasma was collected and stored at -80^{o}C until analysis.

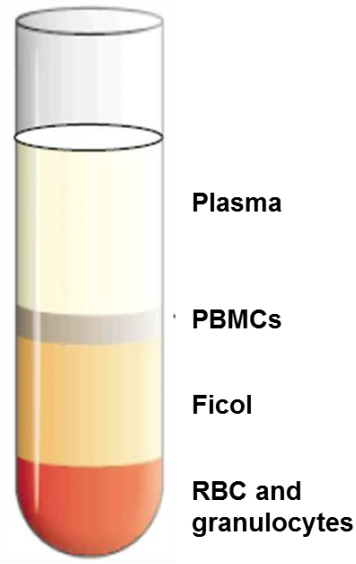


Figure III. 1: Separation of blood into its components using Ficoll- gradient method

III. 2.2.3. Macrophages: The middle buffy coat was collected for isolating and culturing macrophages. Peripheral Blood Mononuclear Cells (PBMCs) obtained from the buffy coat were washed twice with 1X PBS, seeded and cultured for 14 days until complete differentiation into macrophages in RPMI-1640 medium supplemented with 10% autologous serum.

III. 2.3. DNA Isolation and Quantification

DNA isolation from 200 µl blood samples were carried out using Column- based DNA isolaton kit with the kit specific protocol. The procedure involves cell lysis, Proteinase K and RNase- A treatment and eluting the DNA bound to the silica resin in the spin-column. The isolated DNA was quantified using Nanodrop and was stored at -20^{o}C till use.

III. 2.4. APOE Genotyping Protocol

Sequence Specific Primer (SSP) PCR:

In this method, sequence- specific forward and reverse primers were combined to raise three haplotype-detecting reaction mixtures such as APOE ε2 (ApoE primers 1+3 & HLA primers 1+2), APOE ε3 (ApoE primers 1+2 & HLA primers 1+2) and

APOE ε4 (ApoE primers 2+4 & HLA primers 1+2). Here, presence of APOE band indicates that sample is positive for that allele. Hence a pair of control primers (HLA) was also incorporated. PCR mixtures were subjected to initial denaturation for 1 min at 96°C; followed by 10 cycles of 20 sec at 96°C, 45 sec at 70°C, and 25 sec at 72°C; 21 cycles of 25 sec at 96°C, 50 sec at 65°C, and 30 sec at 72°C; 4 cycles of 30 sec at 96°C, 60 sec at 55°C, and 120 sec at 72°C. The PCR products are analyzed by agarose gel electrophoresis. For all PCR reactions, the presence of a 173 bp band indicates the presence of specific APOE haplotype along with any of the HLA bands at 785 bp or 1598 bp.

ApoE	**ε2**	**ε3**	**ε4**
ε2	**ε**2/ **ε**2 (1,3/1,3)		
ε3	**ε**2/ **ε**3 (1,3/1,2)	**ε**3/ **ε**3 (1,2/1,2)	
ε4	**ε**2/ **ε**4 (1,3/2,4)	**ε**3/ **ε**4 (1,2/2,4)	**ε**4/ **ε**4 (2,4/2,4)

Table III. 1: ApoE primer combinations (1, 2, 3 and 4) and polymorphism analysis using SSP-PCR

III. 2.5. Cell culture and differentiation

Acute monocyticleukemia cell lines (THP-1) purchased from National Centre for Cell Sciences (NCCS), Pune, was used as an *in vitro* model for establishing the study objectives. The cells were maintained in suspension culture in RPMI-1640 medium supplemented with 10% FBS in 37ºC 5% CO_2 incubator. THP-1 monocytes were differentiated into macrophages by incubating the cells with 100nM phorbol 12-myristate 13-acetate (PMA) for 48 h. All the treatments were performed in PMA-induced differentiated macrophages. The cells were frozen in freezing media

containing 50% FBS, 45% RPMI-1640 and 5% DMSO and stored at -80^{o}C or liquid nitrogen.

SHSY5Y cell line (obtained from CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum) were cultured in RPMI-1640 medium supplemented with 10% FBS. The cells were differentiated into mature neurons using 10 μM retinoic acid (RA) in RPMI supplemented with 1% FBS for 3-4 days.

III. 2.6. Characterization of Differentiated Cells

Characterization of cell differentiation was done using morphological analysis using phase- contrast microscopy and by the expression of cell specific markers using immunocytochemical staining technique.

III. 2.6.1. THP-1 cells: Undifferentiated THP-1 cells are non-adherent cells with round morphology with an increased expression of monocyte marker CD14 and decreased expression of macrophage marker CD68. PMA- differentiated THP-1 macrophages are adherent with the characteristic fried egg morphology with a decreased expression of CD14 and an increased expression of CD68.

III. 2.6.2. SHSY5Y cells: Undifferentiated SHSY5Y cells are a mixture of both adherent and suspension cells with a smaller cell size and increased expression of stem cell lineage marker Nestin. Differentiated SHSY5Y neurons are adherent with a larger cell size with higher density of axons and synapses and a decreased expression of Nestin.

III. 2.7. Immunocytochemistry

III. 2.7.1. Adherent cells: The adherent cells were washed with ice-cold 1X PBS thrice to remove cellular debris. The cells were then fixed and permeabilized using ice-cold methanol for 10 minutes at -20^{o}C. The cells were then washed with 1X PBS thrice and the fixed cells were blocked with blocking buffer containing 2% BSA and

2% FBS in 1X PBS for 1 h. The cells were washed and incubated with primary antibody in 1:200 dilutions in 3% BSA in 1X PBS overnight at 4^{o}C. After overnight incubation, the cells were washed thrice with 1X PBS and incubated with respective DyLight Fluor - labelled secondary antibody in 1:1000 dilutions in 1X PBS at room temperature for 90 minutes. The cells were washed thrice with 1X PBS after the incubation and mounted with anti- fade mounting medium and were visualized under fluorescent microscope.

III. 2.7.2. Suspension cells: The cells were obtained from the flask and centrifuged at 2500 rpm for 10 min to remove the media and washed twice with 1X PBS by centrifugation. The cell pellet was then dissolved in methanol and incubated at 20^{o}C for 10 min, centrifuged and removed the methanol. The cell pellet was then washed twice in 1X PBS and was dissolved in blocking buffer containing 2% FBS and 2% BSA in 1X PBS. After incubating with blocking buffer for 1 h, the cells were pelleted and washed in 1X PBS twice and was dissolved in primary antibody in 1:200 dilutions in 1X PBS containing 3% BSA. After overnight incubation at 4^{o}C, the cells were pelleted and washed twice with 1X PBS and incubated with DyLight Fluor - conjugated secondary antibody for 90 min at room temperature. The cells were pelleted after incubation, washed and dissolved in anti- fade mounting medium and seeded in glass slide and mounted before visualizing through the fluorescent microscope.

III. 2.8. Aβ preparation

Lyophilized $A\beta_{1-42}$ and HiLyte Flour 488-labeled $A\beta_{1-42}$ (FITC-Aβ) were reconstituted in 1% NH_4OH to 2 mg/ml concentration and was further diluted to 1 mg/ml in 1X PBS. Aβ thus prepared was analysed using western blotting and confirmed that Aβ prepared was in the cytotoxic soluble oligomeric form.

III. 2.9. siRNA transfection

The cells were seeded and differentiated at 60-80% confluency. After 48 hours of differentiation, differentiated THP-1 cells were transfected with siRNA using jetPrimePolyPlus transfection reagent as per the protocol. Required volume of

siRNA (100 nM) was diluted in jetPRIME buffer (200 µl for 6-well plate), vortexed for 10 sec and added jetPRIME reagent (4 µl for 6-well plate), vortexed and incubated at room temperature for 10 min. The transfection mix was added to the cells in fresh RPMI-1640 media supplemented with 10% FBS and incubated for 24 h. The cells were then incubated for 48 h after which Aβ treatment was carried out. Transfection efficiency was confirmed using western blotting.

III. 2.10. Cell Treatments

III. 2.10.1. H_2O_2: For analysing oxidative stress mediated protein expression and FITC-Aβ phagocytosis studies, the cells were treated with 500 uM H_2O_2 for 6 h and 18 h respectively in RPMI-1640 medium.

III. 2.10.2. Aβ: After 48 h of PMA induced differentiation, the differentiated macrophages were treated with 10 µM Aβ for a period of 6 h or 12 h for protein expression studies and 40 µM for 12 h for flow cytometry to analyse Aβ-mediated oxidative stress.

III. 2.10.3. Bay 11-7082:10 mM of(E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile or Bay 11-7082 was prepared by dissolving 2.0725 mg in DMSO under sterile condition and was stored at 4ºC till use. Pre-treatment with 10 µM Bay-117082 for 1 h pre-treatment and 12 h co-treatment with Aβ was carried out to analyse NF-κB-mediated protein expression after inhibiting NF-κB activity.

III. 2.10.4. NAC: 1 M N-acetyl cysteine was prepared by dissolving 163.2 mg of NAC in deionized RNA-se free water and was stored at 4ºC till use. Pre-treatment with 10 mM NAC for a period of 1 h and 6 or 12 h co-treatment with Aβ was carried out to analyse oxidative stress and protein expression after inhibiting reactive oxygen species (ROS) production.

III. 2.11. MTT assay

MTT assay was used to standardize the concentration of $H_2O_2$and Aβ required for cell treatments without causing cell death. MTT colorimetrically measures the reduction of yellow colored 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to an insoluble, dark purple coloured formazan product by mitochondrial succinate dehydrogenase once the MTT enters live cells thus allowing the measurement of cell viability. The cells were treated with different concentrations of H_2O_2 and Aβ for 24 h in a 96 well plate. After removing the media, 100 µl of 5mg/ml MTT in 1X PBS was added in dark and the cells were incubated in 37^oC incubator for 3-4 h till violet colour precipitates were visible. The cells were the solubilized by removing 75 µl of MTT and adding 50 µl of DMSO, mixed well and incubated for 10 min to release the formazan product. The absorbance was read at 540 nm and the percentage of cell viability was calculated and normalized with the non-treated control cells.

III. 2.12. H2-DCFDA assay and FACS

Oxidative stress induced by H_2O_2 and Aβ were determined by analysing the levels of ROS using H2- DCFDA assay. H2-DCFDA when incubated with the cell will get internalized and will be deacetylated by cellular esterases to a non-fluorescent compound, which is further oxidized by ROS into the fluorescing compound 2', 7' – dichlorofluorescein (DCF). So this assay allows the detection of ROS and cellular viability at the same time since only live cells can deacetylate the compound to the active form. The intracellular ROS levels were quantified after pre-incubation with 10 mM NAC, an ROS scavenger, for 1 h and 40 µM Aβ for 12 h. 10mM stock solution of DCFDA was prepared in DMSO and a working solution of 50 µM and 10 µM in HBSS was used for DCFDA spectrophotometric assay and flow cytometry, respectively. The cells were incubated with different concentrations of H_2O_2 in black 96 well plate, to determine the optimum concentration of H_2O_2 required to induce oxidative stress without affecting the viability in differentiated THP-1 cell lines. After removing the media and washing the cells with HBSS, DCFDA was added and incubated at 37^oC in dark for 1 h. After incubation, the cells were washed with HBSS

and the fluorescence was read at an excitation of 485-495 nm and emission of 517-528nm or was analysed using flow cytometry.

III. 2.13. RNA Isolation

RNA isolation was carried out using spin-column based separation from lysed cells with the kit specific protocol. The cells were lysed and treated with RNase inhibitor and the RNA bound to the resin in the spin column was eluted using RNAase free water. The yield and purity of the isolated RNA were determined spectrophotometrically. Intactness of RNA was ascertained by 1% agarose gel electrophoresis, aliquoted and stored at -80^{o}C till use. Repeated freeze-thaw was avoided to minimize RNA degradation.

III. 2.14. cDNA Synthesis

cDNA was synthesized from the isolated and quantified RNA using the cDNA synthesis kit specific protocol. Reverse transcriptase enzyme, dNTP mix, MgCl2, required amount of RNA were mixed and were incubated at temperatures 25^{o}C, 45^{o}C and 95^{o}C for 10 min, 30 min and 5 min respectively. The cDNA thus isolated was aliquoted and stored at -20^{o}C till use.

III. 2.15. Realtime PCR

The mRNA expression was analysed using Realtime quantitative PCR using TaqMan Primers. Taqman quantitative Real-time PCR analysis was carried out using 100 ng of cDNA, Taqman master mix, Taqman primers and specific FAM- or VIC-labelled probes. PCR reactions were performed under the following thermal cycling conditions: 95°C for 10 min followed by denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min for each of 40 cycles. Gene expression was quantified by ΔΔCt method, with human tubulin as internal control using the following formula:

$$\Delta Ct = Ct\ target - Ct\ reference\ gene$$

ΔΔCt was calculated as:

$$\Delta\Delta Ct = (Ct\ target - Ct\ reference)\ sample - (Ct\ target - Ct\ reference)\ control$$

Relative fold of target mRNA levels of treated with respect to control = $2^{-\Delta\Delta Ct}$

III. 2.16. Protein Isolation

The culture dishes were placed on ice immediately after removing from the incubator and the media was removed and washed twice with ice cold 1X PBS. 1X RIPA buffer with 1X proteinase and phosphatase inhibitors were added to the cells and incubated for 1-2 min. The cells were scrapped using a cell scrapper and the isolated cells were collected and vortexed for 1 h at 4^{o}C. After 1 h, the tube containing the lysed cell contents were centrifuged at 12000 rpm for 20 min at 4^{o}C and the supernatant was collected, aliquoted and stored at -80^{o}C till use.

III. 2.17. Protein Quantification

Protein quantification was carried out using BCA (Bicinchoninic acid) protein assay. In the first step, cupric ions are chelated by protein in alkaline environment containing sodium potassium tartrate to form a light blue chelate complex known as the biuret reaction. In the second step, two molecules of bicinchoninic acid react with one molecule of reduced cuprous ion to form an intense purple-colored product. Protein diluted in water was mixed with BCA reagents A and B (50:1 v/v) and incubated at 37^{o}C for 30 min and the absorbance was read at 562 nm.

III. 2.18. Immunoprecipitation

The cells were washed 1X PBS and was lysed using cold IP buffer (low-ionic isolation buffer) and incubated for 30 min at 4^{o}C with constant agitation. The cells were then scrapped and sonicated in ice for 5 sec 4 times. The total lysate was the centrifuged at 4^{o}C for 5 min at 13000 rpm and the supernatant was collected and stored at -80^{o}C till use.

The isolated protein was quantified and reconstituted to 1mg/ml concentration using IP buffer. 25 µl of protein-A coated magnetic beads were added to 200 µl of protein and incubated at 4^{o}C for 1 h to avoid non-specific binding. After incubation, the magnetic beads are pulled down by applying magnetic field and the supernatant was then incubated with 1-2 µg of antibody and incubate at 4^{o}C for 1 h in a rocker. The protein was then incubated with Protein-A coated magnetic beads for 1 h at 4 in a

rocker and the magnetic beads bound to our protein of interest was pulled down by applying magnetic field, washed 2 times with IP buffer and the resuspended in 30 µl of 3X SDS sample loading buffer. Then the protein, along with the magnetic beads, was incubated at 70^{o}C for 5 min and the supernatant was collected after applying magnetic field. The supernatant, which is our protein of interest in SDS loading buffer was stored at -20^{o}C till use.

III. 2.19. Western blotting

The isolated protein samples were mixed with gel loading dye at a ratio of 6:1 and were heated for 5 min at 95^{o}C on a dry bath. The constituent proteins were electrophoretically fractionated on a 10% SDS PAGE gel along with a protein marker. Separated proteins were transferred onto a Polyvinylidenedifluoride (PVDF) membrane at 100 V using a wet transfer apparatus. Membranes were stained with Ponceau S solution for 30 sec to visualise and confirm the transferred bands. The membranes were washed and blocked in 5% non-fat skim milk for 1 h and incubated with primary antibodies at 1:1000 dilution in 1X TBST overnight at 4^{o}C. After incubation, primary antibody was removed and the membranes were washed in 1X TBST (3 X 10 min) and then incubated with HRP- conjugated secondary antibody for 1 h in 5% skimmed milk. The membranes were again washed with 1X TBST (3 X 10 min) and subjected to the Enhanced Chemiluminescence (ECL) detection system to detect the antigen- antibody complex. The ECL substrate was applied on the membrane and the signals thus developed were exposed to an X-ray film in the dark room to develop corresponding bands. The developed bands were quantified densitometrically using ImageJ software.

III. 2.20. Enzyme Linked Immuno Sorbent Assay

The release of pro- inflammatory (IL-1β and TNF-α) and anti- inflammatory (IL-10 and TGF-β) cytokines and $A\beta_{40}$ and $A\beta_{42}$ in plasma samples and THP-1 cell conditioned media were quantitatively analysed using Enzyme-Linked Immuno-Sorbent Assay. The samples (blank, standards and unknown samples) were pre-treated and diluted as per the kit protocol and incubated in specific antibody pre-coated wells. The wells were washed and incubated with biotin- labelled primary

antibody, horseradish peroxidase-labeled (HRP) and streptavidin- conjugated secondary antibody and TMB substrate sequentially with intermittent washing steps. After the final incubation step, stop solution was added to stop the reaction, the absorbance was read at 450 nm and unknown concentration was calculated from standard plot.

III. 2.21. Macrophage FITC-Aβ internalisation assay

Differentiated macrophages were exposed to HiLyte Flour 488-labeled Amyloid β 1-42 (1 μg/ml) and incubated overnight, washed with 1X PBS and examined by fluorescence or confocal microscopy for visualising Aβ uptake. Lysosomal marker Lysotracker Red was used to find out the extent of intra- lysosomal localisation of phagocytosed Aβ. Image analysis was performed using Image J software. Mean Fluorescent Intensity over three different fields per sample were analysed using ImageJ software and calculated by taking the ratio of fluorescent intensity to the total number of cells in each field.

III. 2.22. Conditioned media experiments

Differentiated SHSY5Y cells were treated with the conditioned media from differentiated THP-1 cells treated with Aβ with/without SHARPIN siRNA for 48 h and the expression of apoptotic markers were analysed.

III. 2.23. Statistical Analyses

One way ANOVA and student's *t*-test were used to compare control parameters with treatment groups. Pearson's correlation coefficient was used to correlate each parameter with SHARPIN expression in AD, MCI and control subjects. Results were represented as mean ± SEM and p value <0.05 was considered as statistically significant. GraphPad Prism software was used to calculate and plot graphs.

IV. Results

IV. 1. Characterization of THP-1 - derived macrophages

IV. 1.1. Morphological characterization

THP-1 human acute monocytic leukemia cell lines were used to establish the objectives of the study. The cells were spherical, proliferative and non-adherent in nature. For the study, the monocytic cells were differentiated into mature macrophages by treating the cells with 100 nM PMA for 48 h and the differentiated cells were adherent, non-dividing and showed the morphological characteristics of macrophages as depicted in Figure IV. 1.

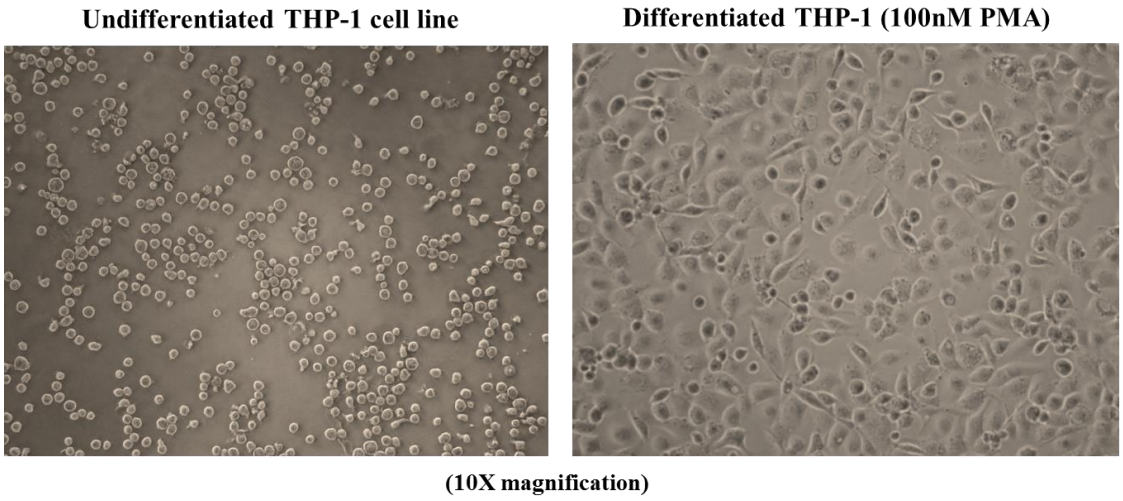


Figure IV. 1: Phase contrast micrograph of undifferentiated and differentiated THP-1 cells (10X magnification)

THP-1 cells were treated with 100 nM PMA as described under the methods section and were observed under phase contrast microscope before and after differentiation. The undifferentiated cells were morphologically round characteristic of monocytic phenotype and after differentiation, the cells were showing well-attached and distinct morphological features of macrophages.

IV. 1.2. Immunocytochemical characterization

The cells before and after PMA treatment were fixed and stained for monocyte specific marker CD14 and macrophage marker CD68 to confirm the differentiation into macrophages. The cells before PMA treatment were showing higher expression of CD14, the monocytic marker and lower expression of CD68, the macrophage marker (Figure IV. 2). The cells after differentiation induced by PMA treatment showed lower expression of monocytic marker CD14 and higher expression of CD68 (Figure IV. 2), thus confirming that PMA induces complete differentiation of THP-1 monocytes to macrophages.

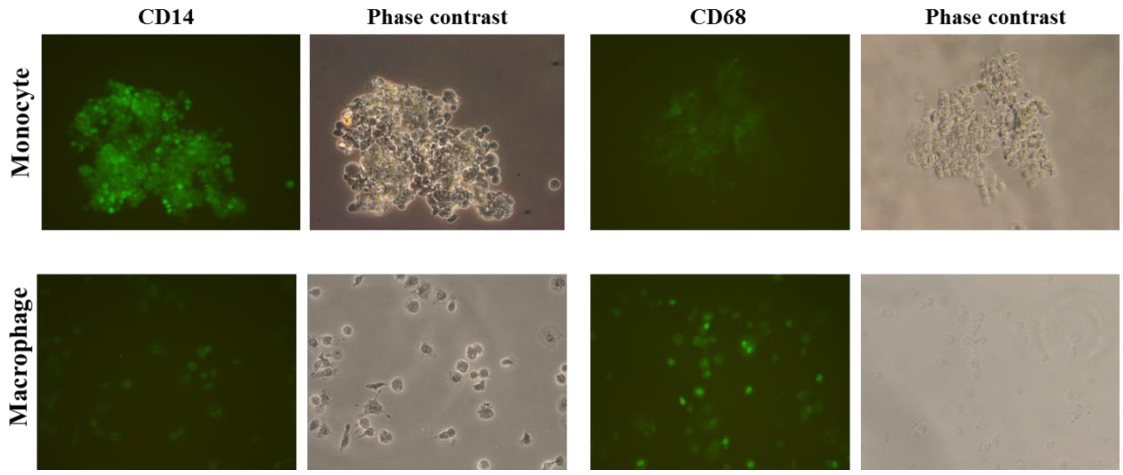


Figure IV. 2: Fluorescent microscopic image of THP-1 cells before and after differentiation (10X magnification)

THP-1 cells before and after PMA treatment were stained with monocytic and macrophage specific antibodies CD14 and CD68 respectively. The undifferentiated monocytes were showing higher expression of CD14 and lower expression of CD68 and the differentiated macrophages were showing increased expression of CD68 and decreased expression of CD14.(N=3).

IV. 2. Characterization of Aβ

Human $A\beta_{1-42}$ was prepared as described in the methods section by dissolving sequentially in 1% NH_4OH and 1X PBS to obtain a final concentration of 1 mg/ml. The aggregative property of the Aβ prepared was analyzed based on the molecular weight of the protein prepared using western blotting (Cerf *et al.*, 2009; Pryor *et al.*, 2012; Wang *et al.*, 2013). It has been reported that the soluble Aβ-oligomer is the toxic form of Aβ. Based on the analysis of molecular weight using western blot analysis, figure IV. 3 show that the Aβ prepared as per the protocol was primarily oligomeric in nature with trimeric and tetrameric aggregates.

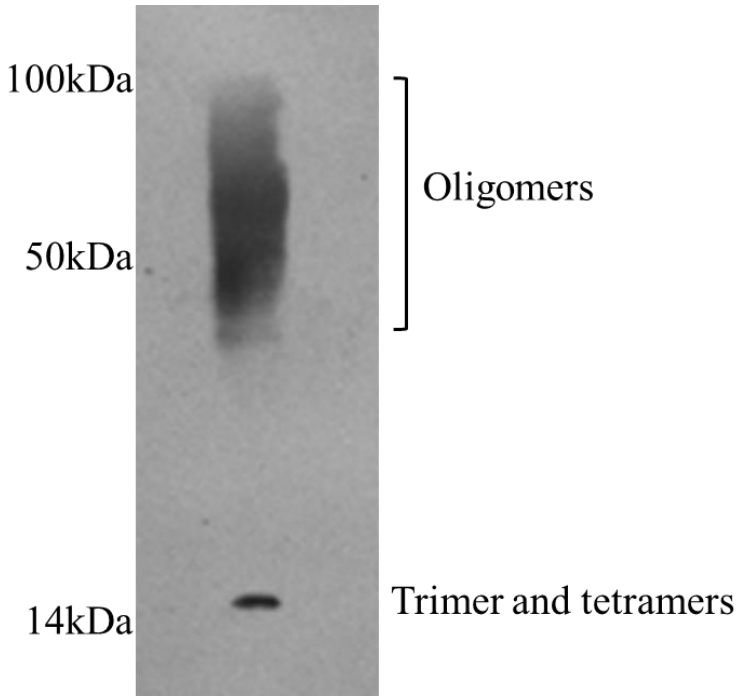


Figure IV. 3: Characterization of Aβ oligomers using western blot analysis

Aβ prepared was analyzed for the nature of the protein aggregates by western-blot analysis. The data confirms that the Aβ prepared was in the oligomeric form based on the molecular weight of the protein.

IV. 3.Aβ induces SHARPIN expression in macrophages

Differentiated THP-1 cells were treated with different concentrations of Aβ (0 to 10 µM) and incubated for 6 h in RPMI-1640 medium supplemented with 10% FBS in a CO_2 incubator. Total protein was isolated from the cells after the incubation and the expression of SHARPIN was analyzed using western blotting technique. Figure IV. 4 shows the expression levels of SHARPIN at different concentrations of Aβ and at 10 µM concentration, the data showed a two-fold increase in the expression of SHARPIN when the cells were treated with Aβ compared to the control, proving that SHARPIN expression is upregulated in macrophages in response to Aβ.

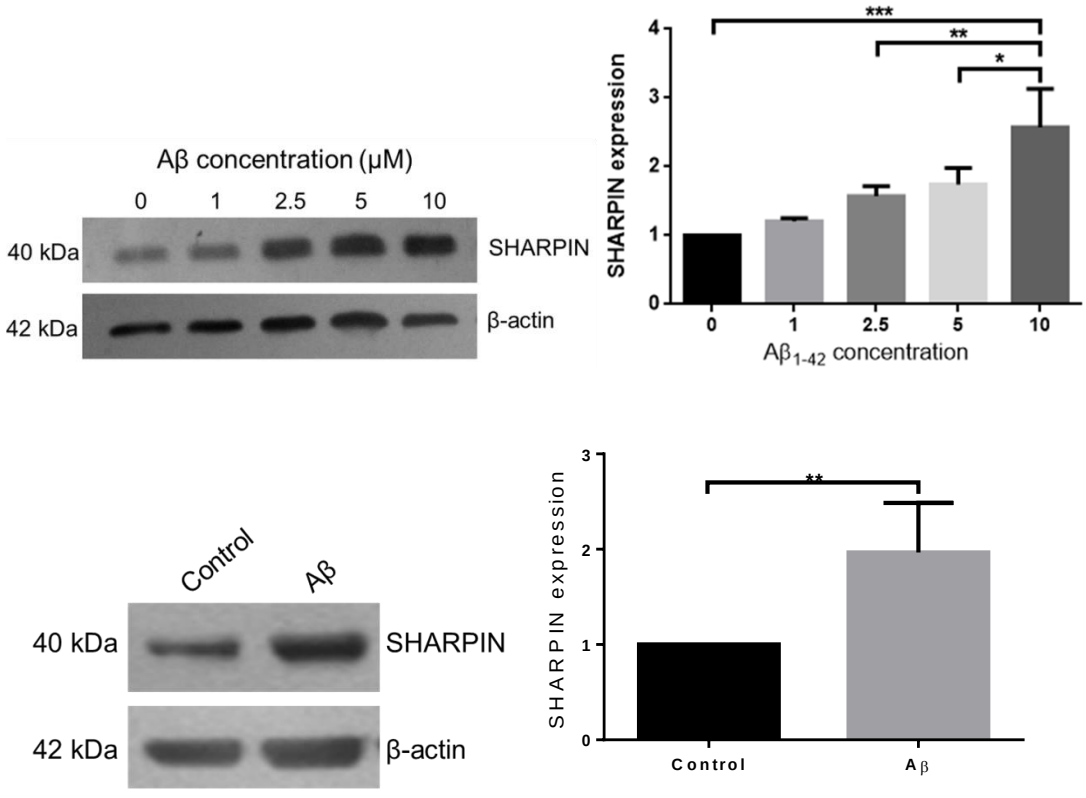


Figure IV. 4: Aβ enhances SHARPIN expression in macrophages

PMA-differentiated THP-1 cells were treated with different concentrations of Aβ and the expression of SHARPIN was analysed with respect to control using western blotting as described in the methods section. The figure shows enhanced expression

*of SHARPIN in the THP-1-derived macrophages in response to Aβ where the protein expression increases with an increase in concentration of Aβ. The expression shows a two-fold increase after incubating the cells with Aβ at a concentration of 10 μM. β-actin was used as the endogenous control. (*p>0.05, **p>0.01, ***p>0.001; N=5)*

IV. 4. SHARPIN enhances Aβ phagocytosis via upregulation of Aβ-phagocytic receptor expression

IV. 4.1. Time-dependent phagocytosis of Aβ by macrophages

Differentiated THP-1 macrophages were subjected to FITC- labelled Aβ, prepared as described in the methods section. The phagocytic efficiency of THP-1 macrophages exposed to FITC-labelled Aβ was analysed using fluorescent microscopy at various time points of 1.5, 3, 6, 12 and 24 h to determine the time-point at which Aβ-phagocytosis by macrophages is maximum. Lysotracker- red was used as a counter stain to mark the lysosomes in macrophages. Colocalization of FITC with Lysotracker was checked to identify phagocytosed Aβ localized in lysosomes. The figure IV.5 shows fluorescent-microscopy images of macrophages phagocytosing FITC-labelled Aβ (green) at different time points and the lysosomal colocalization of the phagocytosed Aβ. The MFI (Mean Fluorescent Intensity) was calculated and represented graphically. The data showed that Aβ phagocytosis increased gradually until 12 h of incubation with FITC-labelled Aβ. Hence, all the Aβ phagocytosis -related studies were performed at a time point of 12 h of incubation with Aβ.

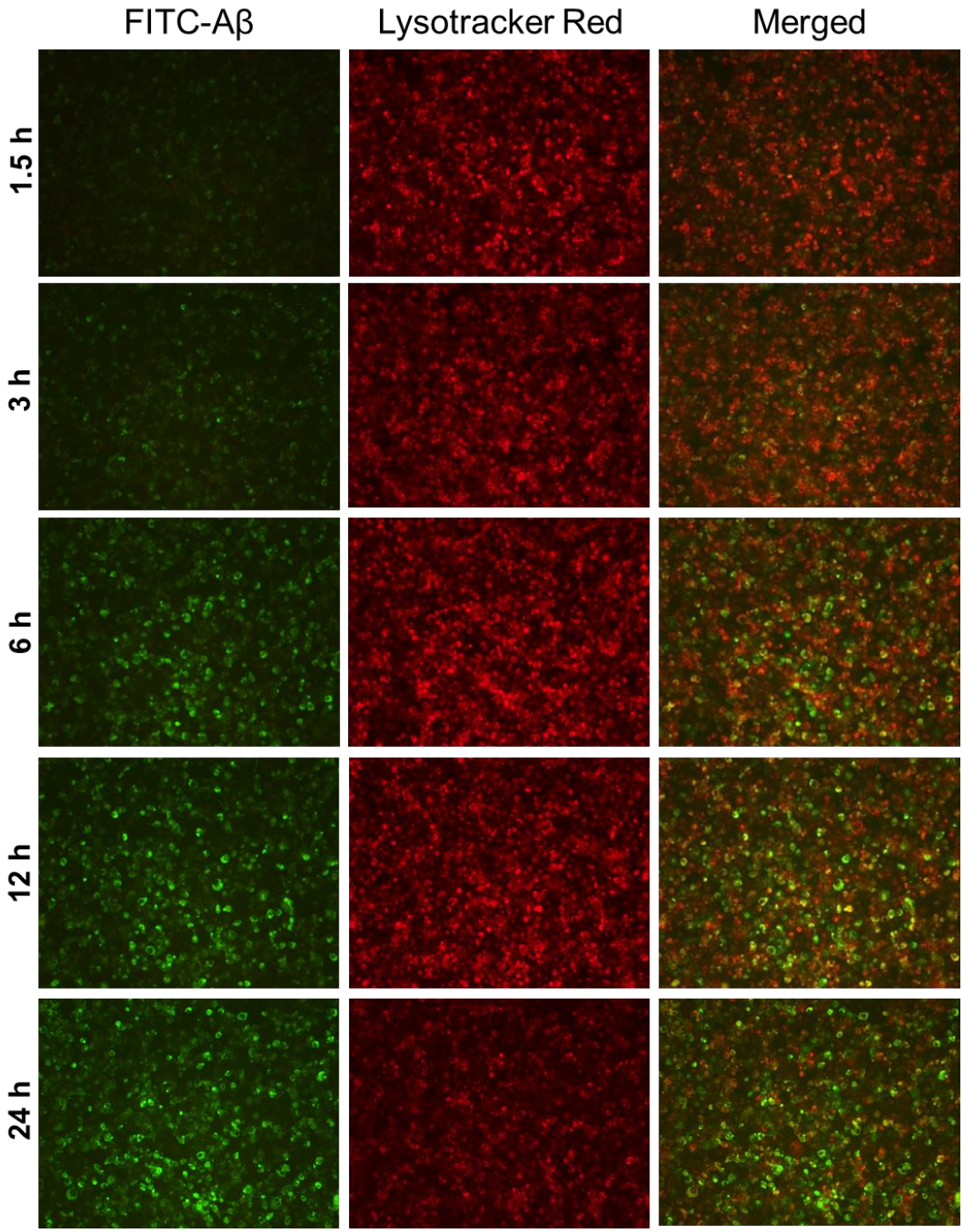


Figure IV. 5: Analysis of Aβ phagocytosis efficiency by macrophages

Differentiated THP-1 cells were exposed to FITC-Aβ for different time periods and the phagocytosis efficiency was observed using fluorescent imaging. Lysotracker-red is used as counter stain.

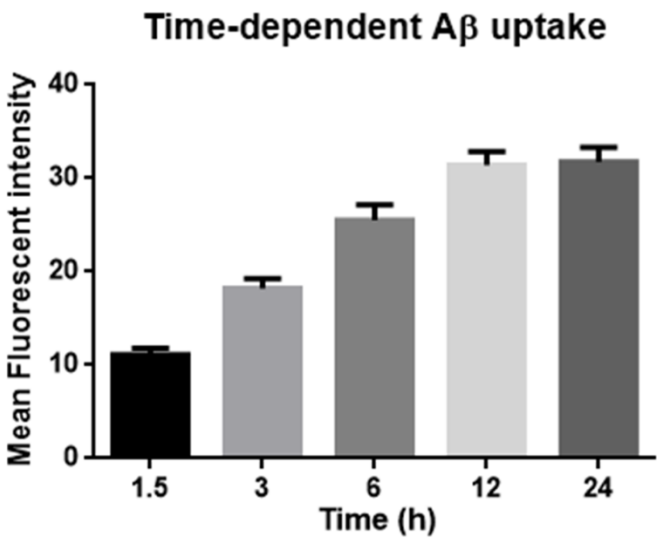


Figure IV. 6: Graphical representation of phagocytosis efficiency of Aβ by macrophages

The phagocytosis efficiency of FITC-Aβ was analyzed at different time points (Figure IV.5). Three images from different field were obtained and the MFI was calculated by taking the ratio of the fluorescent intensity to the total number of cells in the field.The MFI obtained was quantified and represented graphically.(N=3)

IV. 4.2. SHARPIN silencing reduces Aβ uptake by macrophages

In order to identify the functional role of SHARPIN in response to Aβ, SHARPIN gene was silenced using siRNA (siRNA validation shown in Fig IV.7) and the Aβ phagocytosis efficiency of differentiated THP-1 macrophages was analyzed by incubating the cells with FITC-labeled Aβ overnight. After incubation, the phagocytic efficiency of cells was analyzed by fluorescent microscopy and flow cytometry analysis. Macrophages transfected with SHARPIN siRNA showed a significant decrease in phagocytosis efficiency at 12-16 hr compared to the cells transfected with scrambled siRNA (Fig IV.8), clearly showing that SHARPIN is involved in regulating Aβ phagocytosis by macrophages.

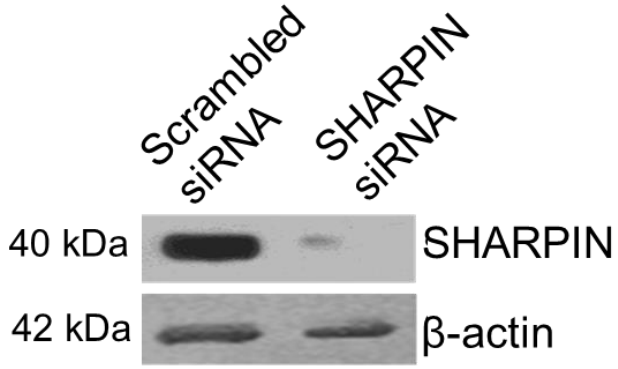
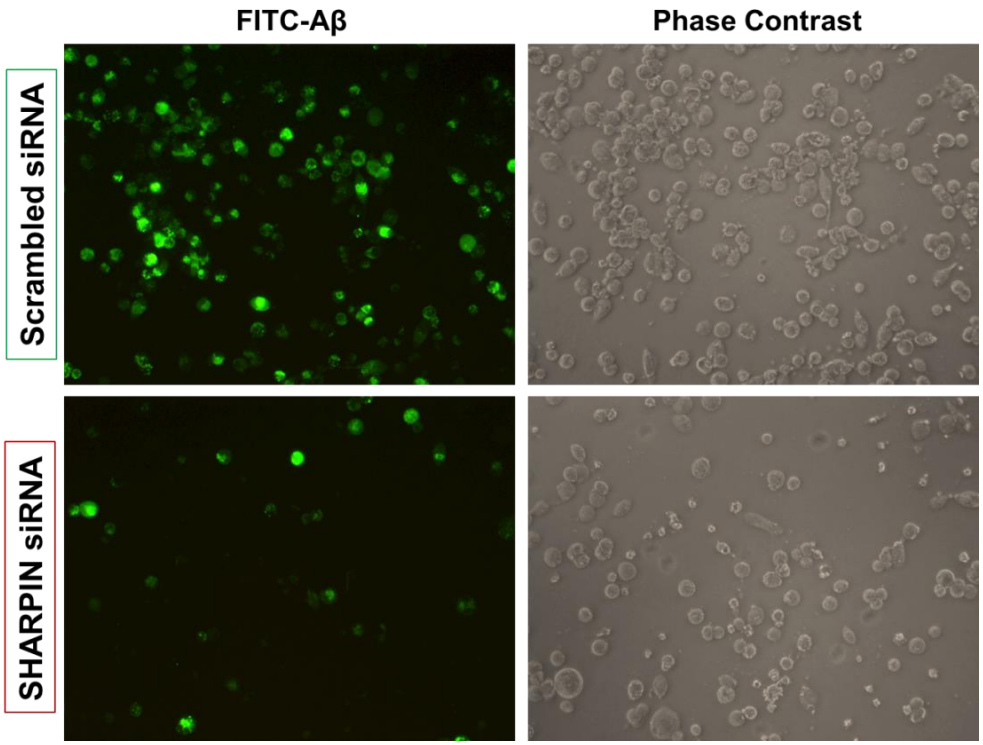


Figure IV. 7: Validation of SHARPIN siRNA transfection efficiency

Differentiated THP-1 cells were transfected with scrambled siRNA or SHARPIN siRNA (100nM) as described in the methods section and the transfection efficiency was validated using western blotting with β-actin as internal control.



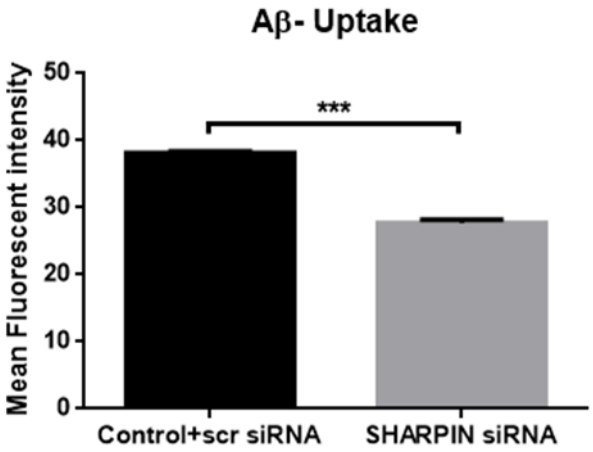
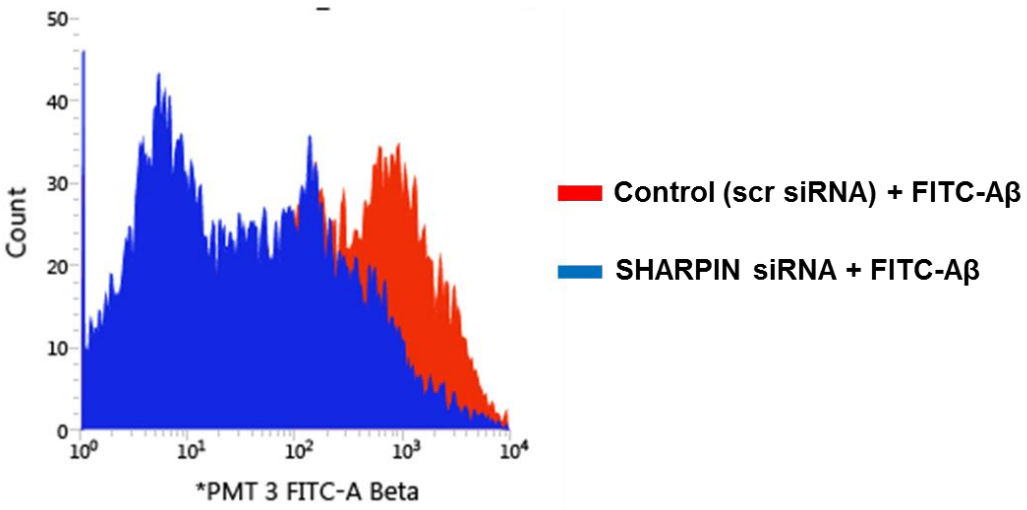


Figure IV. 8: Fluorescent image showing decreased uptake of Aβ by SHARPIN-silenced macrophages

*SHARPIN gene was silenced and the phagocytosis efficiency was analyzed by incubating THP-1 macrophages with FITC-Aβ compared to control (scrambled siRNA) using fluorescent imaging following overnight exposure to FITC-Aβ. MFI from 3 different fields was calculated using ImageJ software and was represented graphically. (*p>0.05, **p>0.01, ***p>0.001; N=4)*



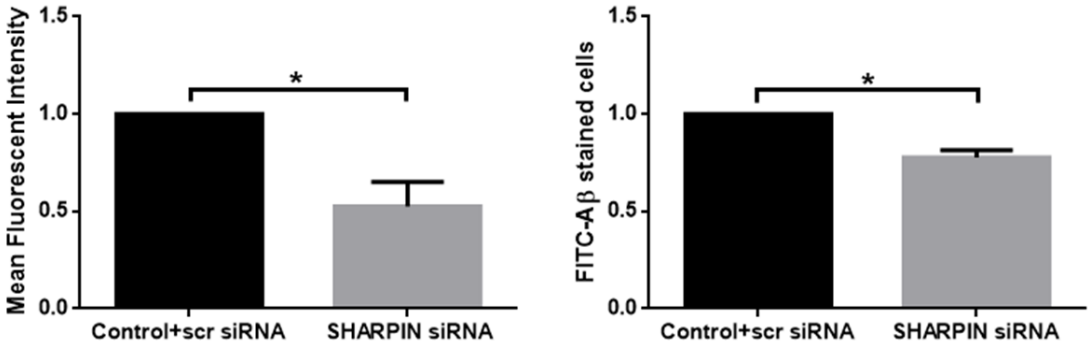


Figure IV. 9: Flow cytometry data showing decreased uptake of Aβ by SHARPIN-silenced macrophages compared to control

*THP-1 macrophages treated with SHARPIN siRNA or control siRNA were exposed to FITC-Aβ for 12 hr. SHARPIN gene was silenced and the cells were subjected to flow cytometry after incubating the cells with FITC- Aβ. MFI and the number of cells that have phagocytosed FITC-Aβ was calculated and represented graphically compared to cells transfected with scrambled (scr) siRNA.(*p>0.05, **p>0.01, ***p>0.001; N=3)*

IV. 4.3. SHARPIN silencing reduces Aβ phagocytic receptor expression in macrophages

Differentiated THP-1 cells were transfected with SHARPIN or scrambled (scr) siRNA and incubated with Aβ to analyze whether SHARPIN is regulating Aβ uptake through regulating the expression of Aβ phagocytic receptors. SCARA (Scavenger Receptor class A-1) and CD36 (Scavenger Receptor class B3) are the two major receptors involved in Aβ phagocytosis. Additionally, RAGE-1 (Receptor for Advanced Glycation endproducts 1) and LRP-1 (Lipoprotein Receptor related Protein-1) are reported to participate in receptor-mediated phagocytosis of Aβ (Doens and Fernández, 2014; Jarosz-Griffiths *et al.*, 2016). The expression of these receptors involved in Aβ phagocytosis was analyzed using western blotting. siRNA-

mediated silencing of SHARPIN significantly attenuated Aβ-stimulated expression of SCARA1, CD-36, RAGE and LRP-1 in THP-1-derived macrophages (Figure IV. 11-14), suggesting that SHARPIN regulates the expression of Aβ phagocytic receptors and thus modulates Aβ phagocytosis by macrophages.

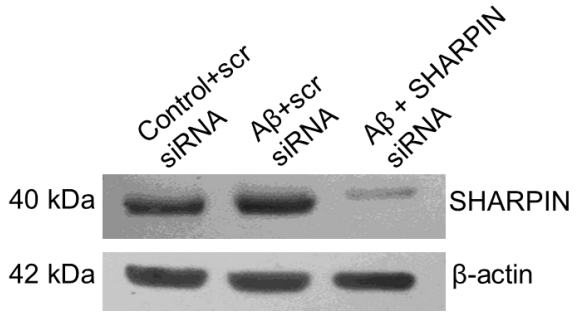


Figure IV. 10: Validation of SHARPIN siRNA transfection efficiency in differentiated THP-1 cells

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ to study the expression levels of Aβ phagocytic receptors. Transfection efficiency of SHARPIN siRNA was analyzed using western blotting technique and was depicted in the figure. β-actin was used as the endogenous control. (*p>0.05, **p>0.01, ***p>0.001; N=5)*

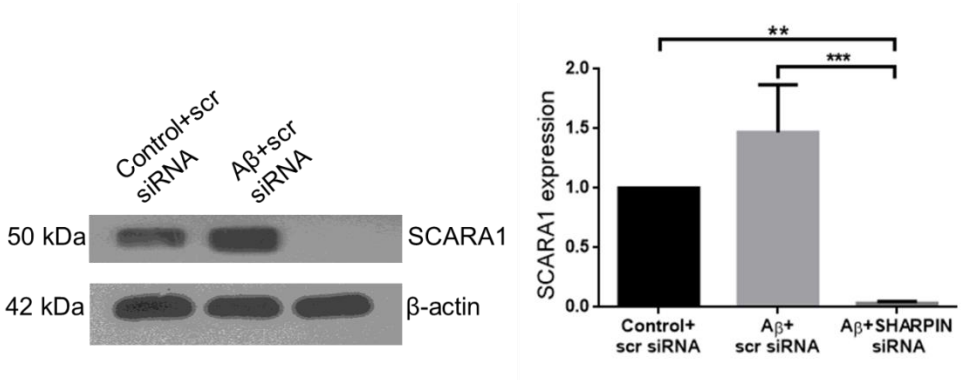


Figure IV. 11: SHARPIN knockdown reduced the expression of SCARA1 receptor in macrophages exposed to Aβ

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ to study the expression level of Aβ phagocytic receptor, SCARA1. β-actin was used as the endogenous control. (*p>0.05, **p>0.01, ***p>0.001; N=4)*

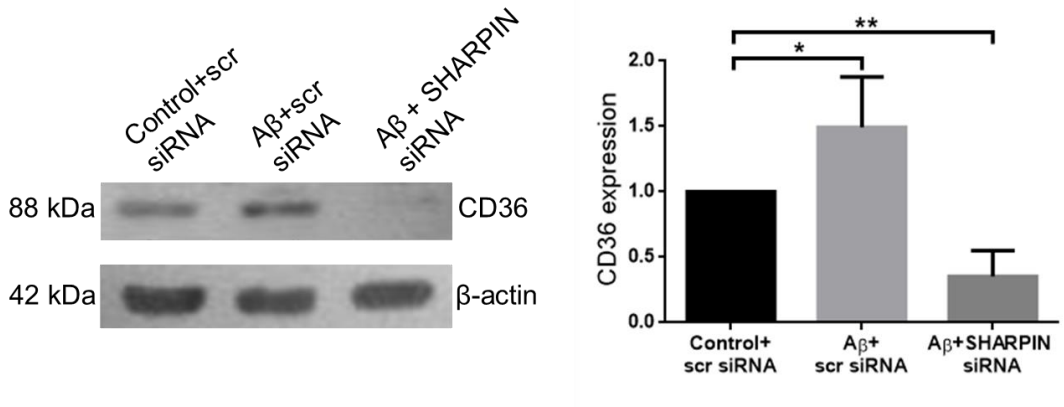


Figure IV. 12: SHARPIN knockdown reduced the expression of CD36 receptor in macrophages exposed to Aβ

*Differentiated THP-1 cells transfected with SHARPIN siRNA or scrambled siRNA were exposed to Aβ. The expression Aβ phagocytic receptor, CD36 was analyzed by western blotting. β-actin was used as loading control.(*p>0.05, **p>0.01, ***p>0.001; N=3)*

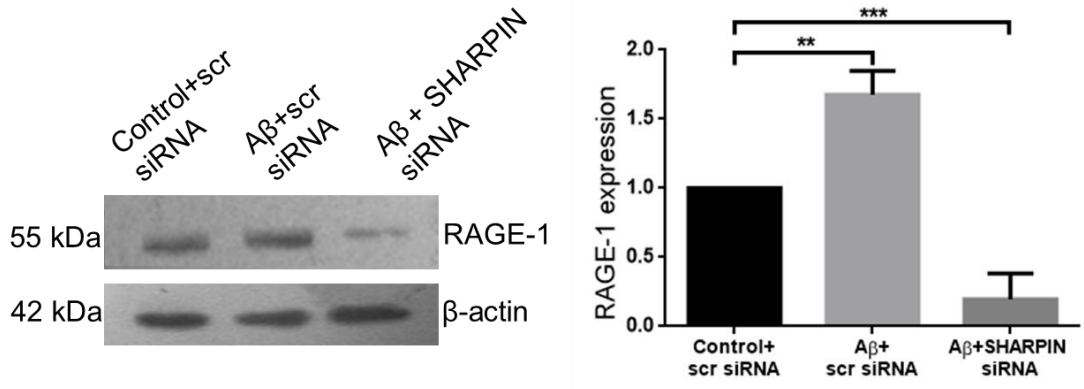


Figure IV. 13: SHARPIN knockdown reduced the expression of RAGE-1 receptor in macrophages exposed to Aβ

*Differentiated THP-1 cells were transfected with SHARPIN siRNA or scrambled siRNA and then exposed to Aβ. The expression of RAGE-1 was analyzed by western blot analysis. β-actin was used as loading control.(*p>0.05, **p>0.01, ***p>0.001; N=5)*

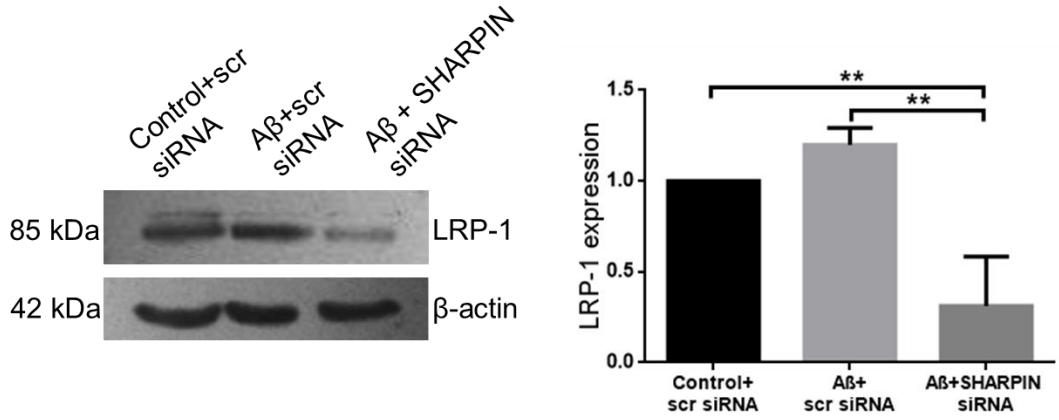


Figure IV. 14: SHARPIN knockdown reduced the expression of LRP-1 receptor in macrophages exposed to Aβ

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ to study the expression levels of Aβ phagocytic receptor, LRP-1. β-actin was used as the endogenous control. LRP-1 expression was upregulated in response to Aβ and was significantly downregulated when SHARPIN was silenced in macrophages. (*p>0.05, **p>0.01, ***p>0.001; N=4)*

IV. 5. SHARPIN upregulates Aβ-mediated NLRP3 expression

The role of SHARPIN in regulating the expression of the inflammasome components, especially the NLRP3 protein through NF-κB signaling pathway has been reported to promote chronic proliferative dermatitis (cpdm) (Gurung *et al.*, 2015). Although SHARPIN and NLRP3 are critical regulators of inflammation, their role in AD, a disease whose pathophysiology is manifested as a consequence of inflammation-dependent neuronal cell-death, has not been explored. Additionally, although NLRP3 expression has been reported to promote AD pathogenesis, the

underlying molecular mechanisms that promote NLRP3 expression and consequent pro-inflammatory signaling in macrophages remain inadequately explored.

Aβ is reported to upregulate expression and activation of the NLRP3 inflammasome (Heneka, Kummer, Stutz, Delekate, Schwartz, Saecker, *et al.*, 2013). To analyze the role of SHARPIN in mediating Aβ-stimulated NLRP3 expression, SHARPIN-silenced macrophages were incubated with Aβ for 6 h and the expression of NLRP3 was analyzed using western blotting. Presence of Aβ increased the expression of NLRP3 and the Aβ-induced NLRP3 expression was abolished by silencing SHARPIN in macrophages, demonstrating that SHARPIN mediates Aβ-stimulated expression of the NLRP3 inflammasome (Figure IV.15).

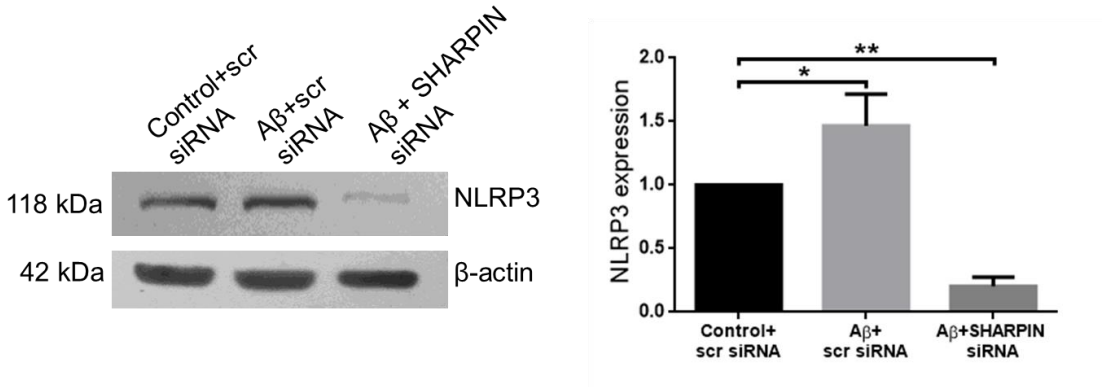


Figure IV. 15: SHARPIN knockdown abolished NLRP3 expression in macrophages exposed to Aβ

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ to study NLRP3 expression. NLRP3 expression was upregulated in response to Aβ and was significantly downregulated when SHARPIN was silenced in macrophages. β- actin was used as endogenous control. (*p>0.05, **p>0.01, ***p>0.001; N=6)*

IV. 6. SHARPIN promotes Aβ-induced macrophage polarization to M1 (pro-inflammatory) phenotype

Exposure to Aβ promotes pro-inflammatory signaling in macrophages which is a characteristic of M1-polarized macrophages that promote a pro-inflammatory signaling in response to inflammatory stimuli (Rogers *et al.*, 2007; Perry and Teeling, 2013). Since silencing of SHARPIN reduced Aβ phagocytosis, consequently reducing the exposure of macrophage cells to Aβ, the status of macrophage polarization to an M1 phenotype was examined.

SHARPIN was silenced in differentiated THP-1 cells and incubated with Aβ for 12 h to analyze the role of SHARPIN in regulating macrophage polarization in response to Aβ. Total protein was isolated and the expression of M1 and M2 markers - iNOS and TGF-β were analyzed. We observed that siRNA-mediated knockdown of SHARPIN reduced the expression of M1 marker iNOS in THP-1 cells exposed to Aβ (Figure IV.16). Additionally, SHARPIN silencing reduced the mRNA expression of M1 markers TLR-2, CX3CR1 and CD68 in these cells (Figure IV.19). TGF-β1 has been reported as a marker for M2 macrophages. Upon analyzing its expression we found an increase in TGF-β1 levels in SHARPIN silenced THP-1 cells (Figure IV.16), suggesting that a relative reduction in SHARPIN levels would polarize macrophages primarily to an M2 phenotype while it presence would promote polarization to an M1 phenotype in macrophages exposed to Aβ.

Since M1 macrophages exhibit enhanced pro-inflammatory cytokine synthesis, the concentration of pro-inflammatory cytokines IL-1β and TNF-α in the conditioned media of SHARPIN-silenced cells exposed to Aβ was analyzed by ELISA. In agreement with our finding that SHARPIN promotes polarization to a pro-inflammatory M1-macrophage phenotype, the concentration of pro-inflammatory markers were significantly less in the conditioned media of SHARPIN-silenced macrophages exposed to Aβ (Figure IV.17). Further, in support of our observation that SHARPIN silencing promoted macrophage polarization to an anti-inflammatory-M2 phenotype, there was a concomitant increase in the concentration of anti-

inflammatory cytokines in the conditioned media of SHARPIN-silenced macrophages exposed to Aβ (Figure IV.17).

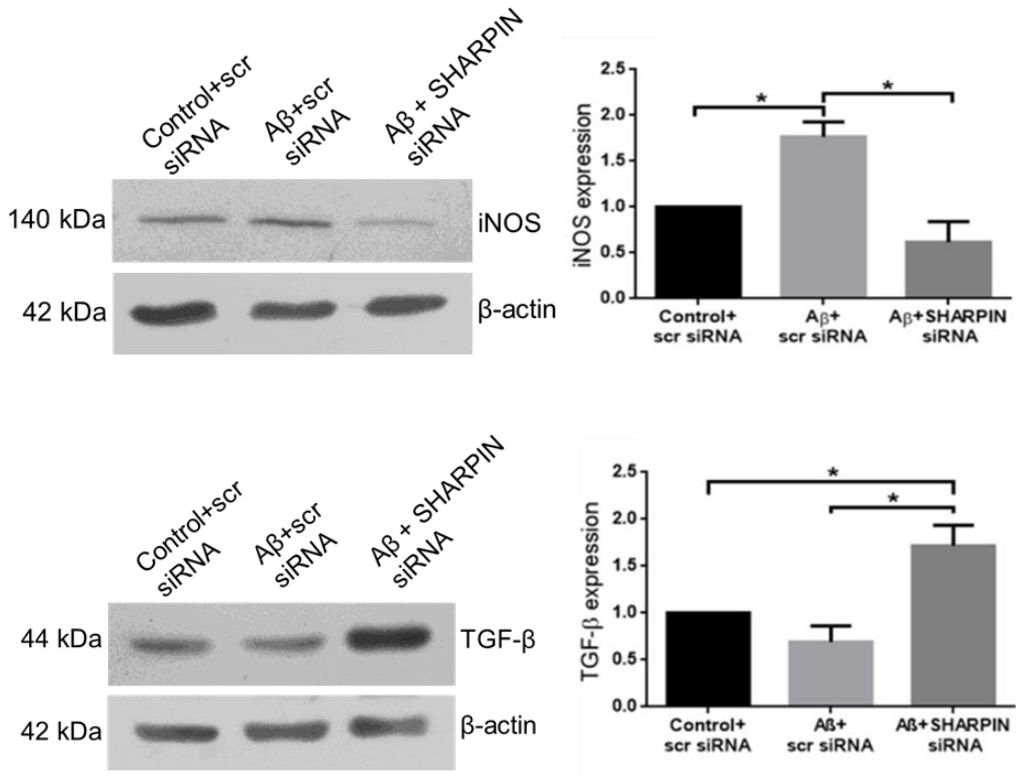


Figure IV. 16: SHARPIN knockdown in presence of Aβ polarized macrophages to M2 phenotype as demonstrated by expression levels of M1 and M2 markers

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ for 12 h to study the role of SHARPIN in regulating macrophage polarization. The expression of iNOS, an M1 marker and TGF-β, an M2 marker were analyzed using western blotting and were represented graphically. β-actin was used as the loading control. (*p>0.05, **p>0.01, ***p>0.001; N=4)*

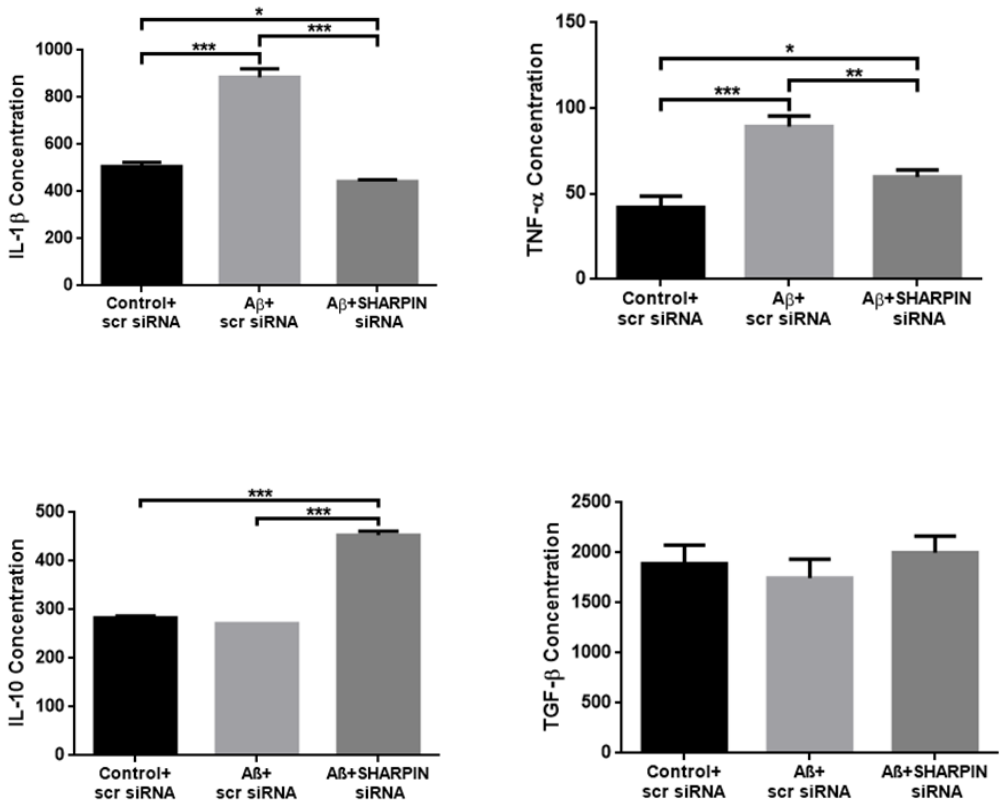


Figure IV. 17: SHARPIN knockdown in presence of Aβ polarized macrophages to M2 phenotype marked by the release of M1 and M2 macrophage-specific cytokines in the conditioned media

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ for 12 h. After incubation, the conditioned media was collected and analyzed for the concentration of IL-1β and TNF-α (M1 macrophage specific- cytokines) and IL-10 and TGF-β (M2 macrophage specific cytokines) using ELISA and normalized with total protein concentration isolated from the cells. (*p>0.05, **p>0.01, ***p>0.001; N=3)*

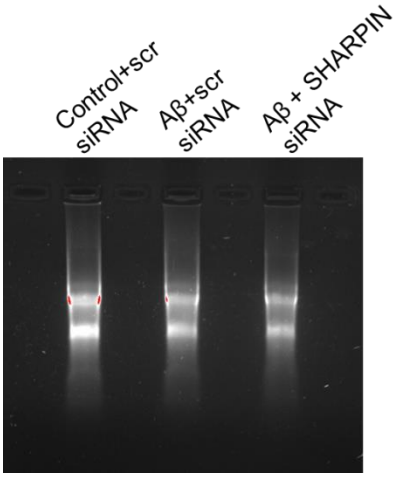


Figure IV. 18: Analysis of RNA integrity using agarose gel electrophoresis

Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ for 12 h. Total RNA was isolated and the integrity of RNA isolated was analyzed using agarose gel electrophoresis.

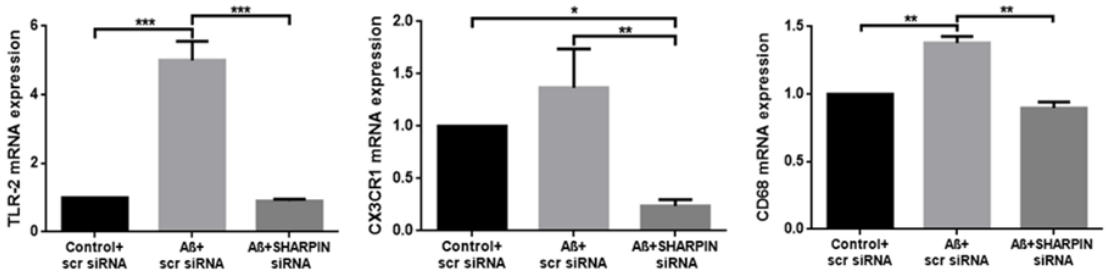


Figure IV. 19: SHARPIN knockdown in presence of Aβ attenuated the polarization of macrophages to M1 phenotype

*Differentiated THP-1 cells were transfected with SHARPIN siRNA and then exposed to Aβ for 12 h. Total RNA was isolated, quantified and real-time PCR was performed using TaqMan probes for M1 markers TLR-2, CX3CR1 and CD68. (*p>0.05, **p>0.01, ***p>0.001; N=3)*

IV. 7. SHARPIN silencing in macrophages prevents Aβ-induced inflammatory damage in neurons

IV. 7.1. Characterization of SHSY5Y-derived neurons

SHSY5Y neuroblastoma cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS and were used as an *in-vitro* model for studying the effect of inflammation-mediated neuronal cell-death. The cells at 50% confluency were treated with 10μM retinoic acid (RA) in RPMI-1640 medium supplemented with 1% FBS and maintained in culture for 3-4 days until complete differentiation into mature neurons. The differentiated cells showed increased cell volume, number of synaptic connections and axonal length that are characteristic features of mature neurons as shown in Figure IV. 20.

The cells before and after differentiation were further analyzed for the expression of neuronal stem cell marker Nestin using immunocytochemistry to confirm differentiation of SHSY5Y to mature neurons. Undifferentiated neurons express Nestin whose expression is gradually reduced upon differentiation to mature neurons. The cells before RA treatment showed higher expression Nestin, which reduced after 4 days of treatment with RA as shown in Figure IV. 21.

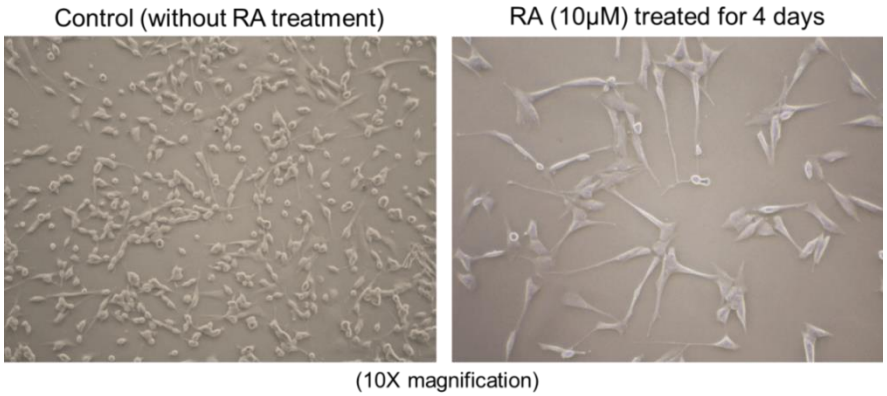


Figure IV. 20: Phase contrast micrograph of undifferentiated and differentiated SHSY5Y cells (10X magnification)

SHSY5Y cells were treated with 10μM retinoic acid as described under the methods section and were observed under phase contrast microscope before and after differentiation. The undifferentiated cells were small with mixed cell populations of adherent and suspension cells. After differentiation, the cells displayed visible increases in cell volume, synaptic connections and axonal length.

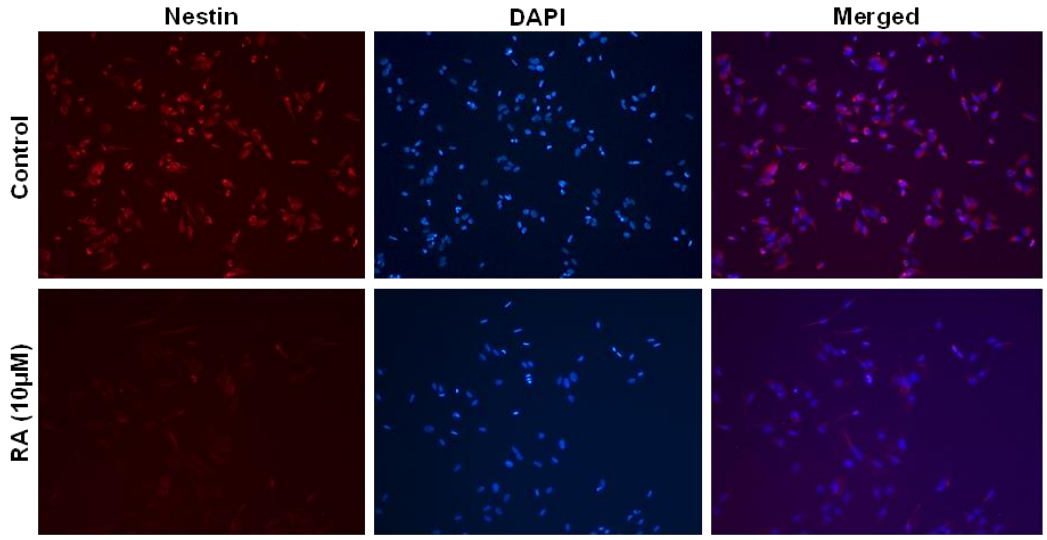


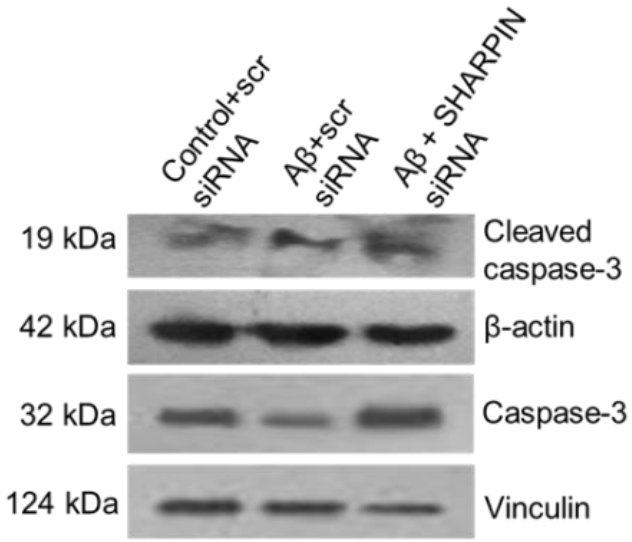
Figure IV. 21: Fluorescent microscopic image of SHSY5Y cells before and after differentiation (10X magnification)

SHSY5Y cells before and after RA treatment were stained with neuronal stem cell marker, Nestin. Nuclear marker DAPI (blue) was used as counter stain.(N=3)

IV. 7.2. Analysis of apoptotic markers in SHSY5Y-derived neurons treated with conditioned-media from SHARPIN-silenced THP-1 macrophages

Macrophages are reported to secrete inflammatory cytokines that cause bystander damage to neurons, causing neuronal cell-death (Wyss-Coray and Mucke, 2002). Since our results demonstrate SHARPIN to regulate expression of pro-inflammatory cytokines in macrophages, the role of SHARPIN in mediating inflammation-induced

neuronal cell-death was examined. Differentiated THP-1 cells were treated with Aβ with or without knocking down SHARPIN and the conditioned media from the cells were collected after 6 h of Aβ incubation. Differentiated SHSY5Y cells were then treated with the conditioned media for 24 h and total protein was isolated and analyzed for the expression of apoptotic markers cleaved caspase-3 (Figure IV. 22) and cleaved PARP (Figure IV. 23) to analyze whether inflammatory factors secreted from Aβ-stimulated macrophages would activate pro-apoptotic mechanisms in neurons and whether this mechanism is mediated by SHARPIN. Consistent with our findings on the role of SHARPIN in mediating expression of pro-inflammatory cytokines in Aβ-stimulated macrophages, neurons treated with Aβ underwent apoptosis as shown by a significant increase in expression of cleaved caspase-3 and cleaved PARP. Importantly, this effect was abrogated in neuronal cultures exposed to conditioned media derived from SHARPIN-silenced macrophages. This finding provides evidence that SHARPIN- mediated inflammatory mechanisms activated by the presence of Aβ in macrophages is a major determinant of neuronal apoptosis in Alzheimer's disease.



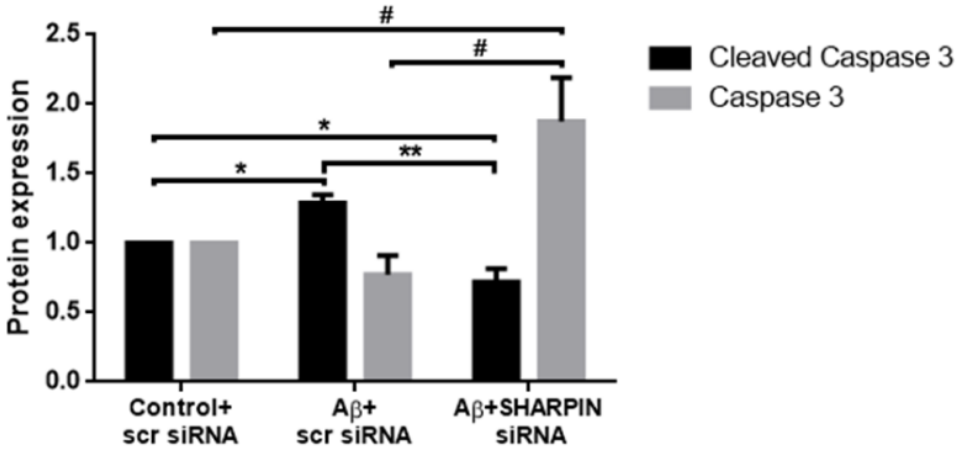


Figure IV. 22: Expression of apoptotic marker - cleaved caspase-3 in differentiated SHSY5Y neurons

*Differentiated SHSY5Y cells were treated with conditioned media obtained from differentiated THP-1 cells incubated with Aβ with or without SHARPIN knockdown for 24 h. Total protein was isolated and the expression levels of cleaved caspase-3 with respect to caspase-3 was analyzed using western blotting and was normalized with β-actin and was represented graphically. (*p>0.05, **p>0.01, ***p>0.001; N=3)*

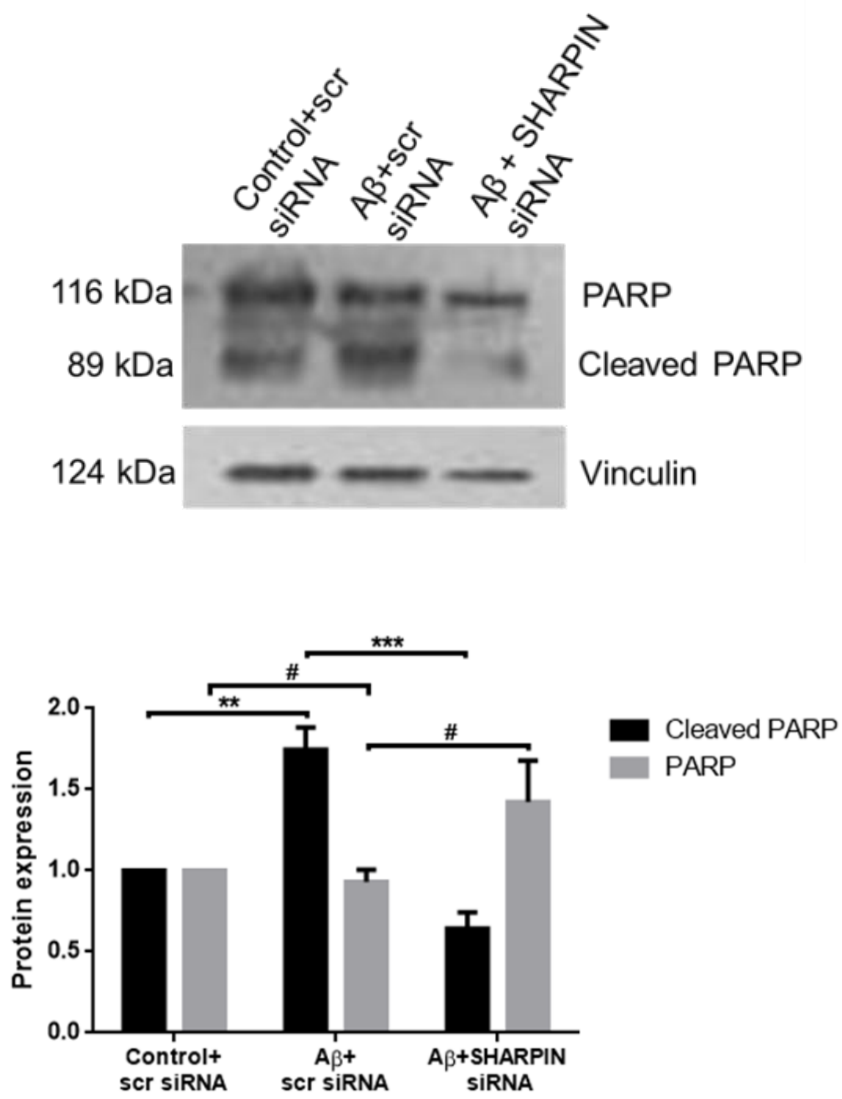


Figure IV. 23: Expression of apoptotic marker - cleaved PARP in differentiated SHSY5Y neurons

*Differentiated SHSY5Y cells were treated with conditioned media obtained from differentiated THP-1 cells incubated with Aβ with or without SHARPIN knockdown for 24 h. Total protein was isolated and the expression levels of cleaved PARP with respect to total PARP was analyzed using western blotting and was normalized with β-actin and was represented graphically. (*p>0.05, **p>0.01, ***p>0.001; N=3)*

IV. 8. Aβ-induced oxidative stress stimulates SHARPIN expression in macrophages

Since SHARPIN was found to be a critical determinant of macrophage function, regulating Aβ phagocytosis and activation of pro-inflammatory pathways, the mechanism underlying Aβ-dependent stimulation of SHARPIN expression in macrophages was explored.

Preliminary evidence on the role of oxidative stress in inducing SHARPIN expression was analyzed using cultures of THP-1-derived macrophages exposed to H_2O_2 (500μM).

IV. 8.1. SHARPIN expression is upregulated in the presence of H_2O_2-induced oxidative stress in THP-1 derived macrophages

To analyse the role of oxidative stress in SHARPIN expression, the optimum concentration of H_2O_2 was determined using MTT assay and H_2-DCFDA assay. Differentiated THP-1 cells were treated with different concentrations of H_2O_2 in a 96 well plate and MTT assay was carried out to determine the maximum concentration of H_2O_2 where the cell viability was least affected. Further, to determine the optimum concentration of H_2O_2 that induces maximum oxidative stress in macrophages, differentiated THP-1 cells were treated with different concentrations of H_2O_2 and H_2-DCFDA assay was carried out in a 96- black well plate. After analysis using the two assays, 500 μM concentration of H_2O_2 was found to be the optimum concentration at which the cells were under maximum oxidative stress with least effect on cell viability (Figure IV.24).

After determining the concentration of H_2O_2, the differentiated THP-1 cells were pre-treated with 10 mM NAC for 1 h and then incubated with 500 μM H_2O_2 for 6 h with or without the presence of NAC. The expression of SHARPIN was analyzed using western blotting. A significant increase in the expression of SHARPIN in H_2O_2

treated cells compared to the control and H_2O_2 + NAC groups was found (Figure IV.25).

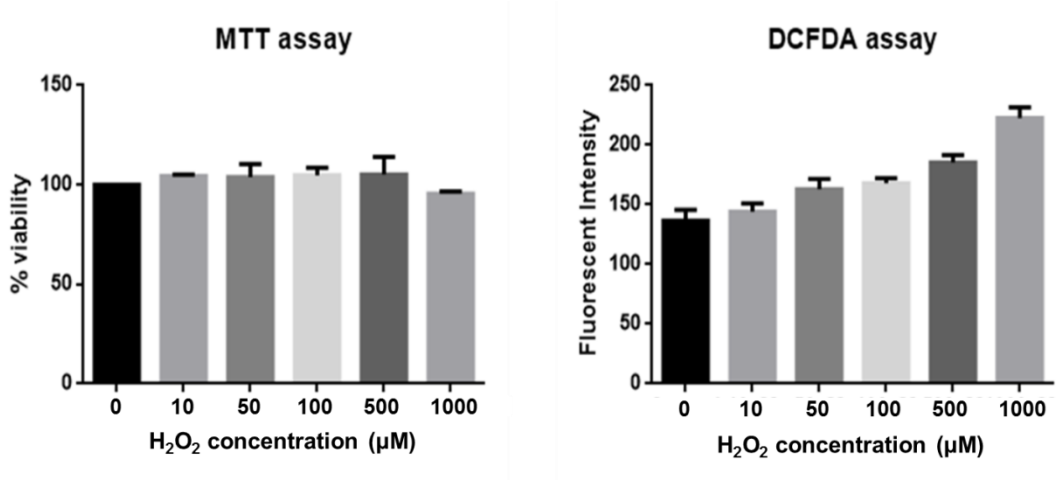


Figure IV. 24: Determination of optimum H_2O_2 concentration in THP-1-derived macrophages

Differentiated THP-1 cells were treated with different concentrations ofH_2O_2 and analyzed for the optimum concentration at which the cells were under significant oxidative stress without affecting the cell viability, using MTT assay and H2-DCFDA assay as described in the methods section. The absorbance was read and plotted graphically after normalizing with the control.(N=3)

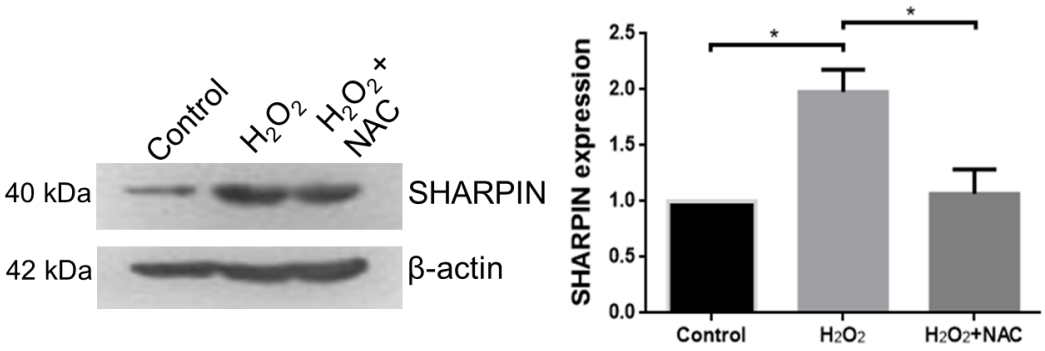


Figure IV. 25: SHARPIN expression is altered in macrophages in the presence of oxidative stress

*Differentiated THP-1 cells were exposed to H_2O_2 for 6h with or without pretreatment with NAC and the expression of SHARPIN was analyzed using western blotting, normalized and represented graphically. The cells were showing significant increase in the expression of SHARPIN in presence of oxidative stress.(*p>0.05, **p>0.01, ***p>0.001; N=5)*

After confirming a role for oxidative stress in mediating SHARPIN expression, a specific role for Aβ-induced oxidative stress in enhancing SHARPIN expression was analyzed.

IV. 8.2. Aβ induces oxidative stress in macrophages

Aβ is known to enhance Reactive Oxygen Species (ROS) generation and oxidative stress in the AD brain and in-vitro cell cultures of macrophages (Butterfield *et al.*, 2013; Cheignon *et al.*, 2017). To analyze whether Aβ induces oxidative stress in macrophages, the cells were pre-treated with the ROS scavenger N-acetyl cysteine (10mM), followed by treatment with Aβ (40μM) for 12 h. H_2-DCFDA assay was used to quantify ROS levels produced as a result of treatment with Aβ. The cells incubated with Aβ alone (red) showed a significant shift in the fluorescence intensity compared to the control which was reduced in the NAC-treated group suggesting that Aβ induces oxidative stress in macrophages. The mean fluorescent intensity (MFI) was calculated, normalized to control and was represented graphically.

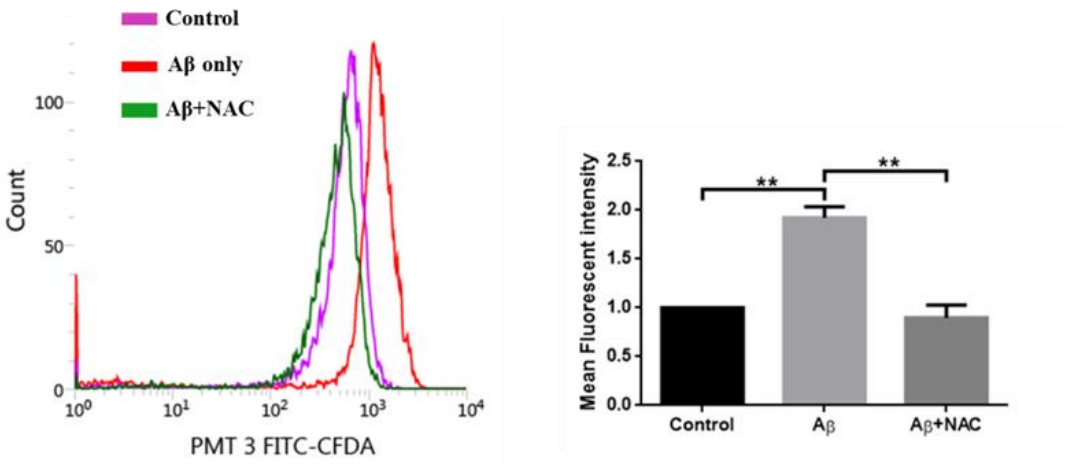


Figure IV. 26: H_2-DCFDA FACS data showing Aβ induces oxidative stress in macrophages

*Differentiated THP-1 cells were pre-incubated with NAC and then treated with Aβ for 12 h and were subjected to H2-DCFDA assay. The cells were then subjected to FACS analysis and the fluorescence intensity (MFI) was calculated and represented graphically. (*p>0.05, **p>0.01, ***p>0.001; N=3)*

IV. 8.3. SHARPIN expression is stimulated by Aβ-induced oxidative stress

Since Aβ was shown to increase ROS, we analyzed whether Aβ-induced ROS would stimulate SHARPIN expression. Differentiated THP-1 cells were pre-incubated with 10 mM NAC and then with Aβ for 6 h. after incubation, total protein was isolated and subjected to protein expression analysis using western blotting technique as described in the methods section. Treatment with NAC reduced Aβ-stimulated increase in SHARPIN suggesting that SHARPIN expression is stimulated by Aβ-induced oxidative stress.

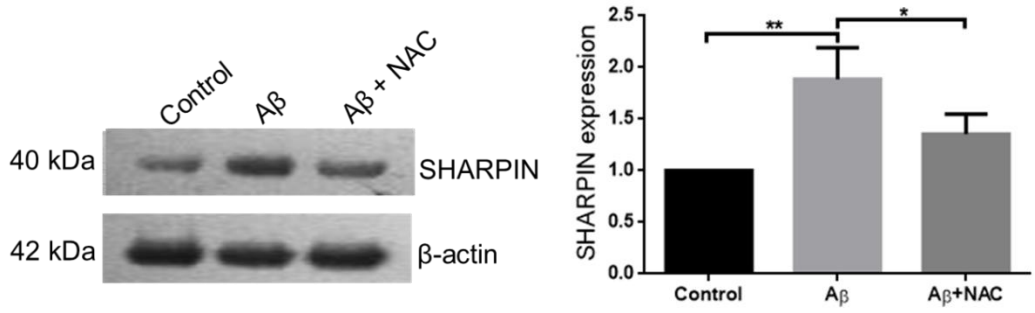


Figure IV. 27: Aβ-induced oxidative stress stimulated SHARPIN expression in macrophages

*Differentiated THP-1 cells were treated with Aβ with or without the presence of NAC and the expression levels of SHARPIN was analyzed using western blotting. The expression was normalized with β- actin and the fold change was calculated and represented graphically.(*p>0.05, **p>0.01, ***p>0.001; N=5)*

IV. 8.4. NLRP3 expression and pro-inflammatory signaling in macrophages are stimulated in response to Aβ-induced oxidative stress

Since Aβ-induced oxidative stress stimulates SHARPIN expression in macrophages, the expression of downstream effectors of SHARPIN - NLRP3 and macrophage polarization markers iNOS and TGF-β were analyzed.Differentiated THP-1 cells were pre-incubated with NAC and then with Aβ and the total protein was isolated. The protein expression was analyzed and compared with THP-1 incubated with Aβ alone and with control (untreated cells). NLRP3 expression was found to be downregulated (Figure IV. 28) when Aβ-induced ROS was scavenged using NAC. Further, macrophage polarization markers iNOS (M1 marker) was downregulated and TGF-β (M2 marker) was upregulated (Figure IV. 29), suggesting a role for Aβ-induced oxidative stress in regulating NLRP3 expression and macrophage polarization in downstream of SHARPIN in THP-1 macrophages exposed to Aβ.

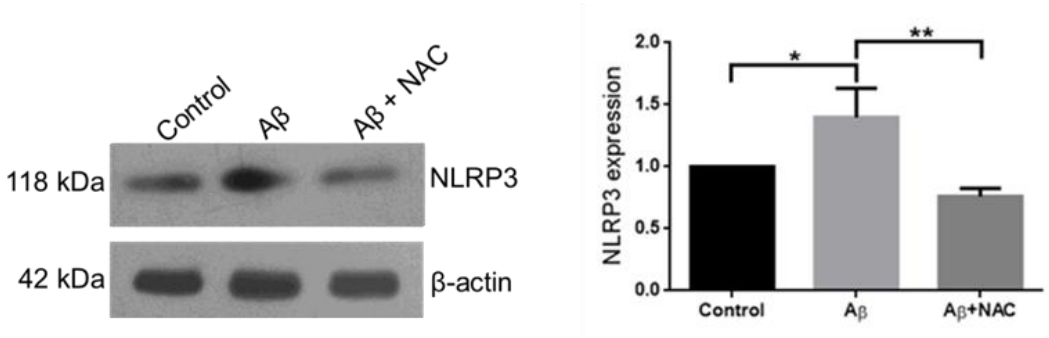


Figure IV. 28: NLRP3 expression is regulated by Aβ-induced oxidative stress in macrophages

*Differentiated THP-1 cells were treated with Aβ with or without the presence of NAC and the expression levels of NLRP3 was analyzed using western blotting. The expression was normalized with β- actin and the fold change was calculated and represented graphically.(*p>0.05, **p>0.01, ***p>0.001; N=3)*

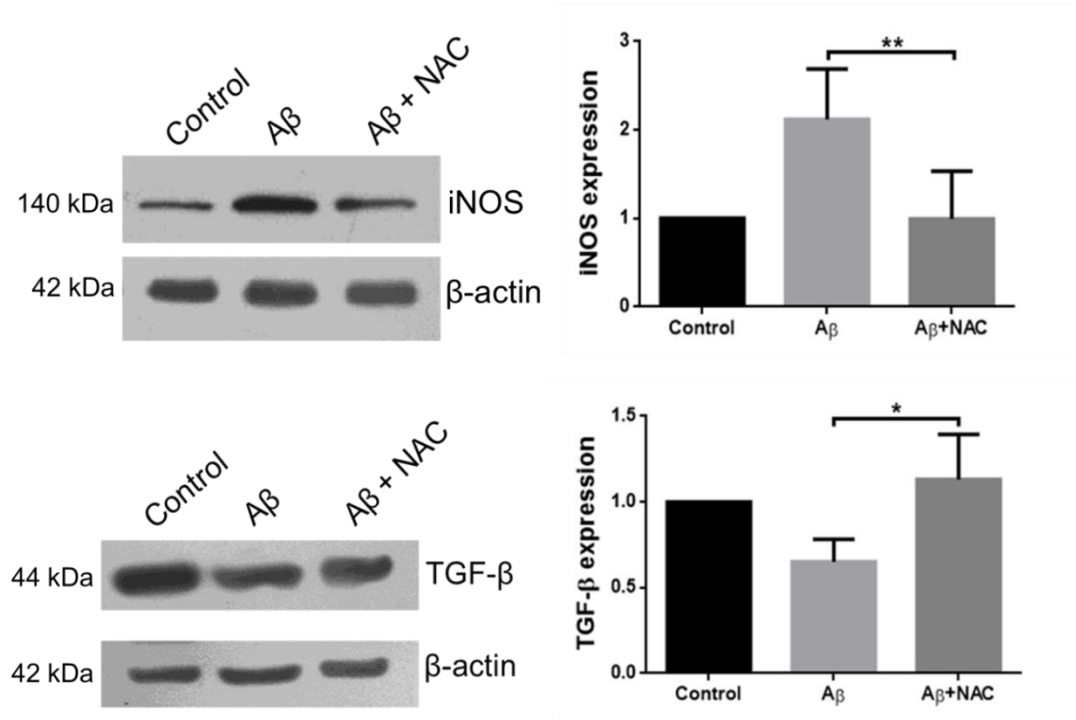


Figure IV. 29: Aβ-induced oxidative stress stimulated macrophage polarization to M1 phenotype

*Differentiated THP-1 cells were treated with Aβ with or without the presence of NAC and the expression levels of iNOS and TGF-β was analyzed using western blotting. The expression was normalized with β- actin and the fold change was calculated and represented graphically.(*p>0.05, **p>0.01, ***p>0.001; N=3)*

IV. 9. SHARPIN is ubiquitinated by NF-κB-mediated signaling mechanism in macrophages exposed to Aβ

IV. 9.1. NF-κB inhibition causes post-translational modification in SHARPIN

Since NF-κB is a redox-sensitive transcription factor known to play a major role in promoting expression of pro-inflammatory cytokines, its regulatory role on SHARPIN was investigated. Differentiated THP-1 cells were pre-treated with 10 μMBay-117082 for 1 h and then exposed to Aβ for 12 h to study the role of NF-κB in regulating SHARPIN expression. Pharmacological inhibition of NF-κB using Bay-117082 was found to cause an increase in the molecular weight of SHARPIN compared to the control and Aβ groups, suggesting that NF-κB-mediated signaling may lead to post-translational protein modifications in SHARPIN that would cause an increase in its molecular weight.

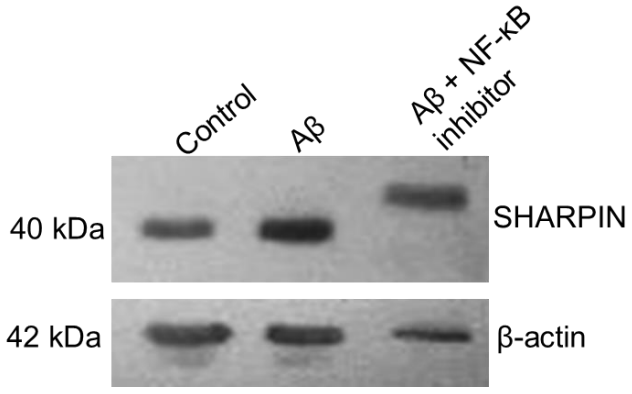


Figure IV. 30: SHARPIN protein is post-translationally modified by an NF-κB-mediated signaling mechanism

Differentiated THP-1 cells were treated with Aβ with or without the NF-κB inhibitor Bay-117082 and the expression of SHARPIN was analyzed using western blotting. Cells incubated with Bay-117082 were showing an increase in the molecular weight of SHARPIN when compared to Control group and Aβ group. β-actin was used as endogenous control.(N=5)

IV. 9.2. Inhibition of NF-κB leads to ubiquitination of SHARPIN

To identify the type of post-translational modification in SHARPIN protein by NF-κB, THP-1 macrophages were pre-treated with BAY-117082 for 1 hour and then exposed to Aβ. Total protein was isolated and SHARPIN was immunoprecipitated (Figure IV. 31) using anti-SHARPIN antibody. IgG antibody was used as the negative control. Immunoprecipitated SHARPIN was then probed with anti-ubiquitin antibody. The data show that SHARPIN in the Aβ+Bay-117082-treated group showed a significant increase in ubiquitinylation (Figure IV. 32), suggesting that the transcription factor NF-κB may act to ubiquitinate SHARPIN.

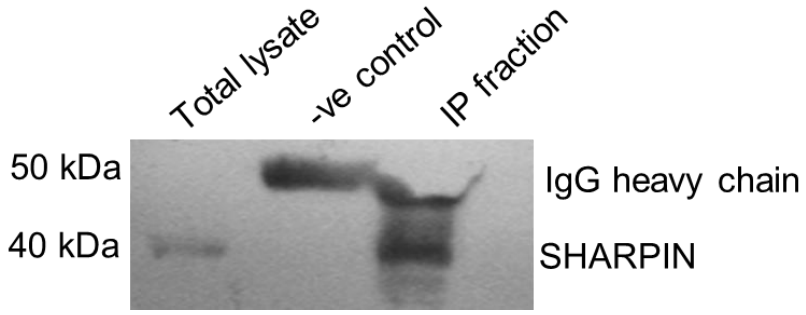


Figure IV. 31: Western blot image showing specificity of SHARPIN immunoprecipitaion

SHARPIN was immunoprecipitated from the total protein lysate using anti-SHARPIN antibody. The purity of the immunoprecipitation method was analyzed using western blotting technique where the IP fraction shows the immunoprecipitated SHARPIN at 40 kDa.

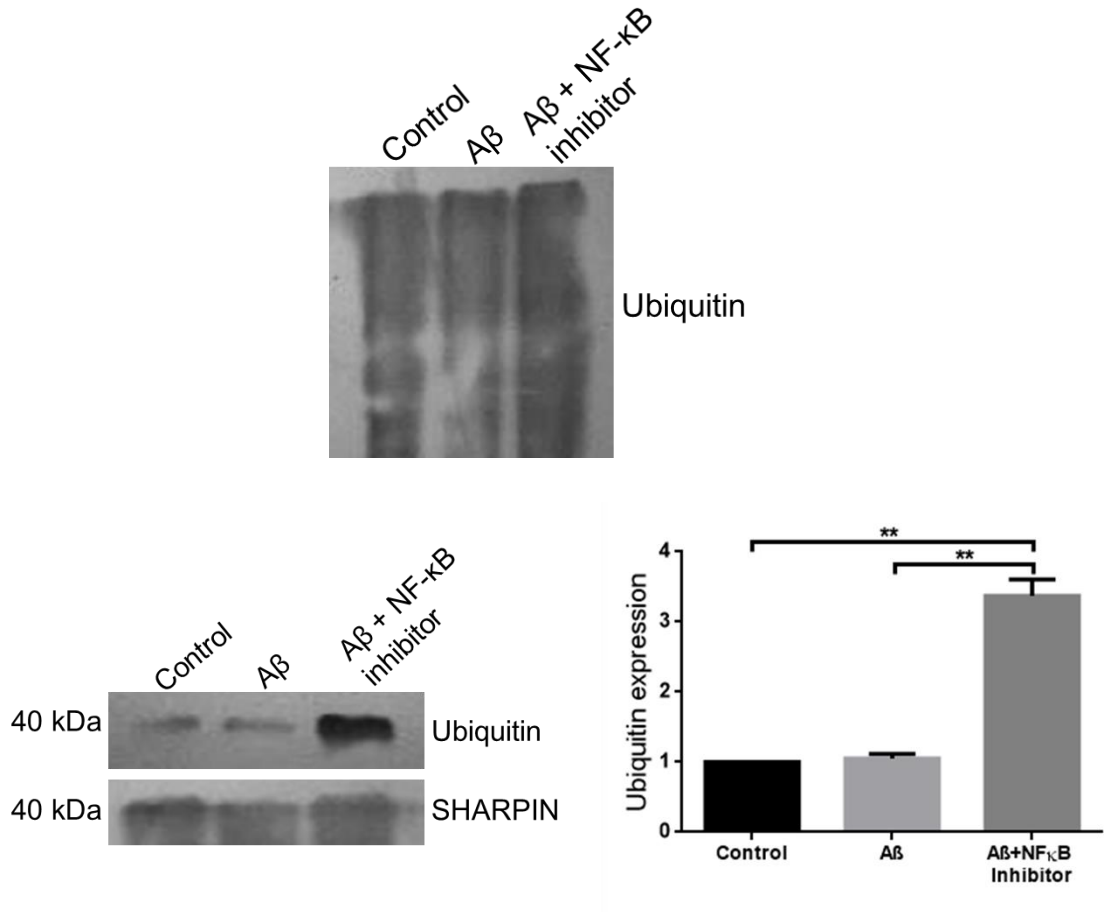


Figure IV. 32: SHARPIN protein is ubiquitinylated by an NF-κB- mediated signaling mechanism

*Immunoprecipitated SHARPIN protein was probed for ubiquitination using anti-ubiquitin antibody and the ubiquitination of SHARPIN protein and total protein ubiquitination was analysed using western blotting, normalised with control and represented graphically. Compared to control and Aβ, the SHARPIN protein immunoprecipitated from cells treated with Aβ+NF-κB inhibitor showed a higher degree of ubiquitination.(*p>0.05, **p>0.01, ***p>0.001; N=3)*

IV. 10. SHARPIN expression is altered in blood-derived macrophages

IV. 10.1. Categorization of study subjects

All the study subjects (patients) were recruited from the Memory & Neurobehavioral Clinic (MNC) at the SreeChitraTirunal Institute for Medical Sciences and Technology (SCTIMST), Kerala, after obtaining Institutional Ethical Clearance. The control subjects were healthy volunteers. Informed consent was obtained from all the participants.

All the recruited subjects were tested for hypertension, hyperlipidaemia, hypercholesterolemia, Vitamin B12 deficiency, thyroid dysfunction, diabetes, cardiopathy, cranial trauma or other neurological disorders. Subjects were tested for CRP level in blood plasma and those subjects with high plasma CRP level were excluded from the study to avoid the possibility of systemic inflammation- mediated alteration of protein expression and inflammatory status in the peripheral circulatory system. The diagnosis and severity of AD and MCI was determined as described in the methods section.

The study population comprised of 63 individuals with 31 AD, 13 MCI and 19 cognitively unimpaired control subjects. ApoE polymorphism in the study subjects were analyzed and depicted in Table IV. 2, however, the subjects were not categorized on the basis of ApoE polymorphism due to the small sample size in the study. $A\beta_{42}$ and $A\beta_{40}$ concentrations in the plasma of the study subjects were quantified using ELISA as described in the methods section and the $A\beta_{42/40}$ ratio was calculated and represented as scatter plot in Figure IV. 33. Details of the study subjects were represented in Table IV. 1.

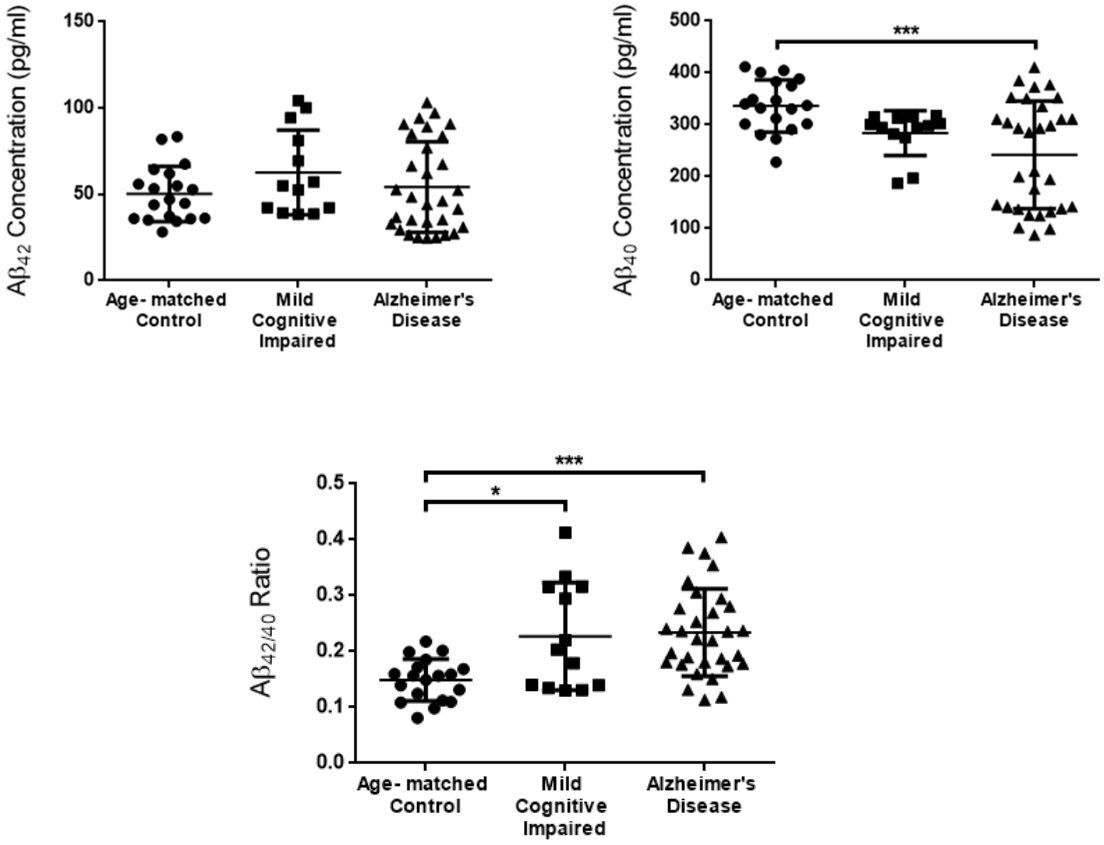


Figure IV. 33: Scatter plot of the concentration of Aβ42, Aβ40 and Aβ42/40 in the plasma of study subjects

*Plasma samples were isolated from the blood of study subjects and were subjected to ELISA for analysis of Aβ42 and Aβ40 concentration in the peripheral circulation. Aβ40 is the most abundant form of Aβ produced and thus directly correlates with the total Aβ metabolism in the study subject. Aβ42 is the toxic form of Aβ in AD and thus the ratio of Aβ42 to Aβ40 determines the concentration of the toxic protein in the subject. Aβ42 and Aβ40 in the blood plasma were analyzed and the ratio of Aβ42 to Aβ40 was calculated and represented as scatter plot.(*p>0.05, **p>0.01, ***p>0.001)*

Study Subjects	Total number	MMSE scores	ACE scores	Age	Average $A\beta_{42/40}$ ratio
Control	19	-	-	54.7 ± 10	0.149
MCI	13	27 ± 3	82 ± 10	71.3 ± 5.5	0.227
AD	31	22 ± 5	67 ± 6	69.5 ± 8.6	0.234

Table IV. 1: Study subject demographics

The study subjects were categorized into healthy controls, Mild cognitively impaired (MCI) and Alzheimer's disease (AD) based on their performance in the neuropsychological tests and the test scores on MMSE, ACE, the study subjects age and average $A\beta_{42/40}$ concentrations were depicted in the table.

The presence of Apolipoprotein E4 allele has been identified as the greatest genetic risk factor for AD. The role of ApoE protein in phagocytosis has been well studied. Aβ oligomers have been shown to have higher affinity to ApoE3 than ApoE4 suggesting that ApoE3 might be more efficient in Aβ clearance preventing accumulation (Tai *et al.,* 2014). 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 and microglial migration than ApoE3 suggesting reduced clearance of Aβ and increased neurotoxicity through M1 (pro-inflammatory) polarization (Jofre-Monseny *et al.,* 2007; Cudaback *et al.,* 2011). 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 (Tachibana *et al.,* 2019). 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 (Jairani *et al.*, 2019a).

Hence, we tried to group the study subjects on the basis of ApoE polymorphism which classified each study group into further sub-groups. However, since the number of study subjects is very low in each group, subdividing the group further into sub-groups will leave each set with very few numbers of subjects. Therefore, even though we have done ApoE analysis to group the patients, the classification of each group into further subgroups with SHARPIN expression, inflammation and phagocytosis was not done.

Study Subjects	**ApoEε 2/2**	**ApoEε 2/3**	**ApoEε 3/3**	**ApoEε 3/4**	**ApoEε 4/4**
Control	0	0	50	50	0
MCI	0	0	50	43	7
AD	0	2.9	22.9	42.9	31.3

Table IV. 2: Percentage of ApoE polymorphisms in the study subjects

DNA was isolated from blood samples of the study subjects and ApoE polymorphisms were analyzed using SSP-PCR as described in methods section. The percentage of each ApoE genotype in each group of study subjects was represented. As expected, the percentage of the presence of the risk allele ApoEε4is higher in the AD group as compared to the control and MCI groups.

IV. 10.2. Isolation and characterization of blood-derived macrophages

PBMCs were isolated from the blood samples collected from the study subjects as described in the methods section and were cultured in RPMI-1640 media supplemented with 10% autologous serum. The cells were given a media change after 24 h so that all the non-adherent cells in the PBMCs will be washed off and only the attached monocytes will be retained. The cells were then maintained for 14 days until complete differentiation of monocytes into macrophages as represented in Figure IV. 34.

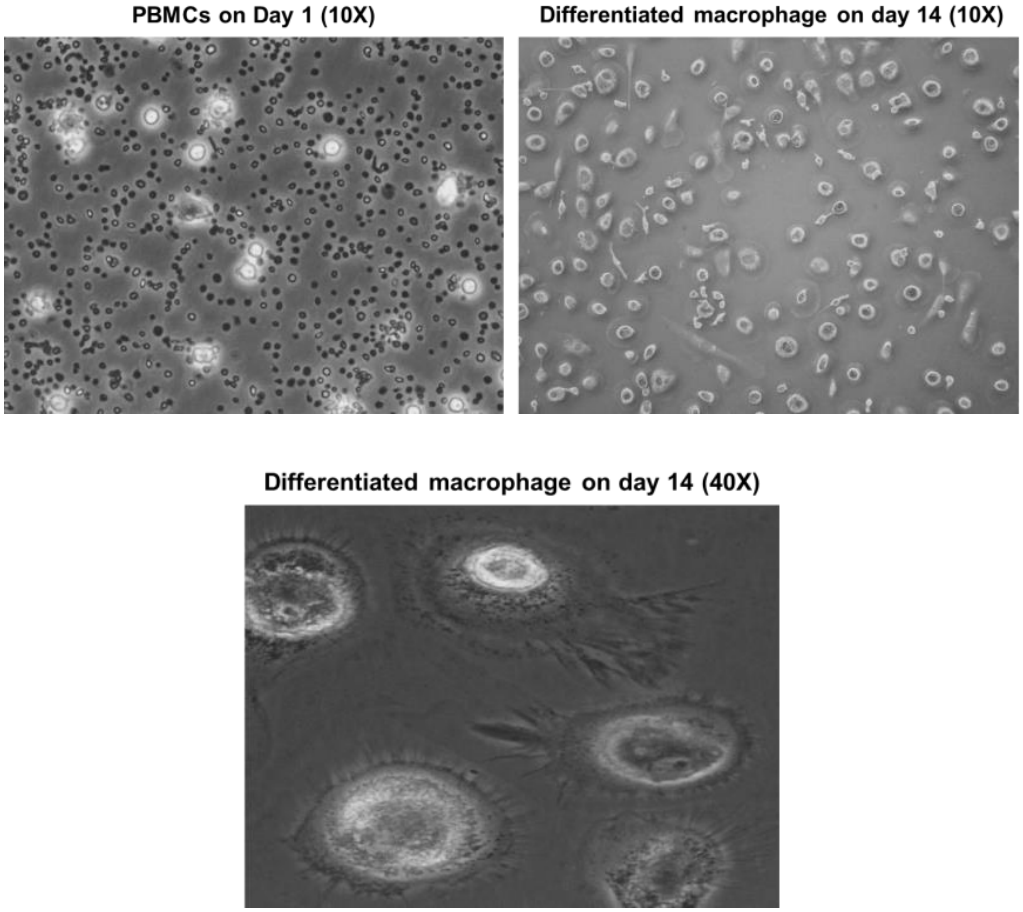


Figure IV. 34: Phase contrast micrograph of PBMCs and differentiated macrophages derived from blood samples of study subjects

PBMCs were isolated from the blood of study subjects as described under the methods section and were observed under phase contrast microscope after seeding the PBMCs and after differentiation into macrophages on 14th day of differentiation.

The undifferentiated monocytes in the PBMC culture were morphologically round, characteristic of monocytic phenotype and after differentiation, the attached cells showed distinct morphological features of macrophages.

IV. 10.3. Analysis of SHARPIN expression in blood-derived macrophages

PBMCs were isolated from the blood samples of study subjects and were cultured in RPMI-1640 medium supplemented with 10% autologous serum for 14 days until complete differentiation into macrophages. Total protein was isolated from the macrophages at the 14th day and was analyzed for the expression of SHARPIN using western blotting. SHARPIN expression was found to be higher in MCI and AD subjects compared to the control subjects and among MCI and AD, the MCI subjects were showing showed significantly higher expression of SHARPIN. This results suggested that SHARPIN expression by peripheral blood-derived macrophages, in the absence of systemic inflammation, is higher in MCI and AD subjects and this corresponds to the observed higher concentration of $A\beta_{42/40}$ in the plasma of AD and MCI subjects in figure IV. 33.

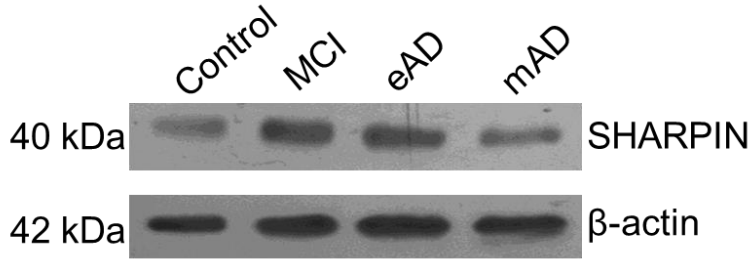


Figure IV. 35: SHARPIN expression in peripheral blood-derived macrophages

SHARPIN expression was analyzed in protein isolated from macrophages derived from the study subjects' peripheral blood using western blotting. β-actin was used as loading control. The figure shows a representative blot for SHARPIN expression in blood-derived macrophages from control subjects, MCI, early stage AD (eAD) and moderate stage AD (mAD).

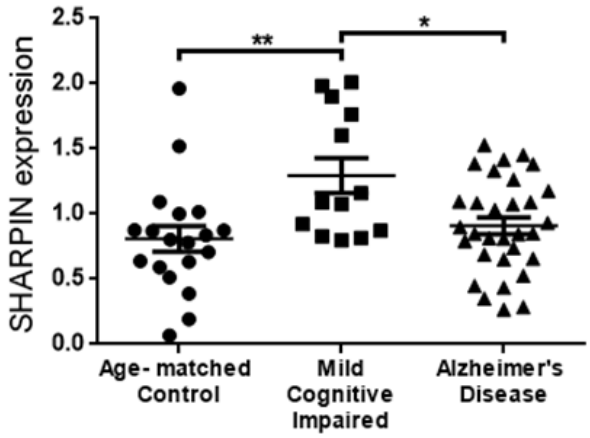


Figure IV. 36: Scatter plot for SHARPIN expression in peripheral blood-derived macrophages

*SHARPIN expression was analyzed in protein isolated from macrophages derived from the study subjects' peripheral blood using western blotting technique (Figure IV.35). Due to the small sample size of mAD subjects, early stage AD and moderate stage AD were combined as Alzheimer's disease and the SHARPIN expression by all the study subjects were represented using a scatter plot. SHARPIN expression was found to be significantly higher in the MCI subjects compared to AD and control subjects.(*p>0.05, **p>0.01, ***p>0.001)*

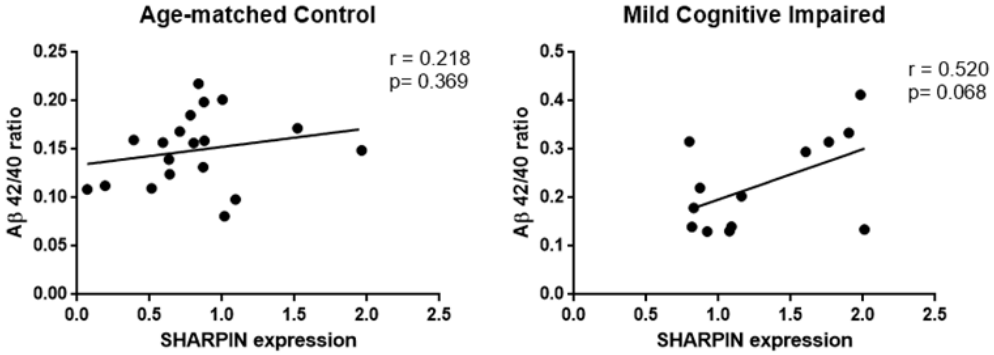
Our *in-vitro* studies point to a role for SHARPIN in regulation of Aβ phagocytosis and inflammation in THP-1-derived macrophages. Further, SHARPIN expression was found to be upregulated in response to Aβ and oxidative stress. Therefore, a correlation between these factors and SHARPIN expression in peripheral blood-derived macrophages of study subjects was analyzed. SHARPIN expression in the peripheral-blood-derived macrophages was correlated with:

- $A\beta_{42/40}$ level in the plasma of the study subjects
- Age of the study subjects
- Aβphagocytosis efficiency by the macrophages of the study subjects

- The release of pro-inflammatory cytokines IL-1β and TNF-α in the plasma of the study subjects
- The release of anti-inflammatory cytokines IL-10 and TGF-β in the plasma of the study subjects

IV. 10.4. SHARPIN expression in macrophages correlated with $A\beta_{42/40}$ in the plasma of study subjects

SHARPIN expression by the blood-derived macrophages from the study subjects were analyzed using western blotting and $A\beta_{42/40}$ level in the plasma of the same study subjects was analyzed using ELISA. In the absence of systemic inflammation, the presence of $A\beta_{42/40}$ could be the contributing factor for an increased expression of SHARPIN by the macrophages in the peripheral circulation. Hence, SHARPIN expression in peripheral blood-derived macrophages of each study subject was correlated with $A\beta_{42/40}$ in the plasma of the respective study subjects. The results showed mild to moderate positive correlation of SHARPIN expression in macrophages with $A\beta_{42/40}$ in the plasma, suggesting that the concentration of Aβ in the plasma or in the peripheral circulation of study subjects have a significant role in stimulatingSHARPIN expression in macrophages in the peripheral circulation, which supports the *in-vitro* cell culture study.



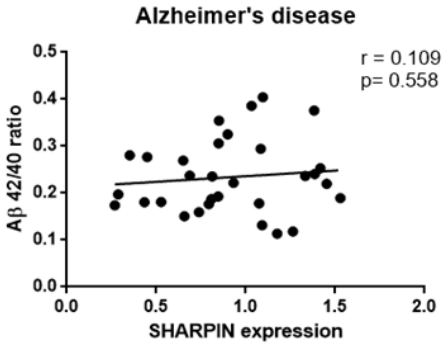


Figure IV. 37: Scatter plot showing correlation of SHARPIN expression in macrophages with $A\beta_{42/40}$ release in the plasma of control, MCI and AD subjects

SHARPIN expression in peripheral blood-derived macrophages (Figure IV.36) was correlated with the concentration of $A\beta_{42/40}$ in the respective study subjects. The two factors were correlated in each study subjects and are represented as control MCI and AD groups separately.

IV. 10.5. SHARPIN expression in macrophages is dependent on the age of study subjects

Increase in age and presence of diabetes are strong risk factors for AD pathogenesis. Our *in-vitro* studies demonstrated a role for oxidative stress, an important factor associated with increasing age, to enhance SHARPIN expression. Hence the expression of SHARPIN as a function of age and presence of diabetes was analyzed. Additionally, due to the small sample size and a heterogeneous population (diabetes, age, life style factors, genetic factors, drug interventions, etc), the subjects were further grouped on the basis of age and the presence of diabetes.

IV. 10.5.1. SHARPIN expression in blood-derived macrophages positively correlated with increasing age in study subjects

In support of the *in-vitro* data demonstrating a role for oxidative stress, an important component of increasing age, in regulating SHARPIN expression, a correlation between increasing age and expression of SHARPIN was analyzed in the study subjects. Macrophages were isolated from peripheral blood of the study subjects as per the protocol described in the methods section and the expression of SHARPIN was analyzed using western blotting. Then the study subjects (control, MCI and AD) were further regrouped into subjects below and above 65 years of age and the expression of SHARPIN was represented as scatter plot in Figure IV. 38. The study subjects (mainly the AD and control groups) showed an increase in expression of SHARPIN in the age group of greater than 65 years compared to the subjects with age group below 65. MCI, due to the smaller sample size compared to the control and AD, did not show a difference in SHARPIN expression between age groups.

Further, SHARPIN expression was correlated with the age of each study subject in each group and represented as a scatter plot. The data also shows a mild positive correlation between age and SHARPIN expression in each group. These data clearly shows that age-associated oxidative stress also plays a significant role in regulating the expression of SHARPIN by macrophages in the peripheral circulation.

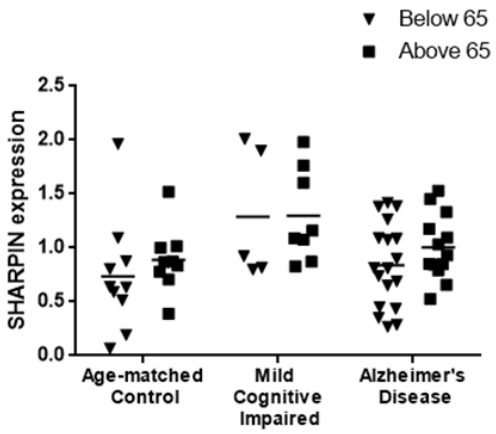


Figure IV. 38: Scatter plot showing SHARPIN expression in macrophages in different age groups of control, MCI and AD subjects

SHARPIN expression by peripheral blood-derived macrophages was analyzed using western blotting (figure IV.36). The study subjects were grouped into age groups below 65 and above 65 and the expression of SHARPIN was analyzed and represented as scatter plot.

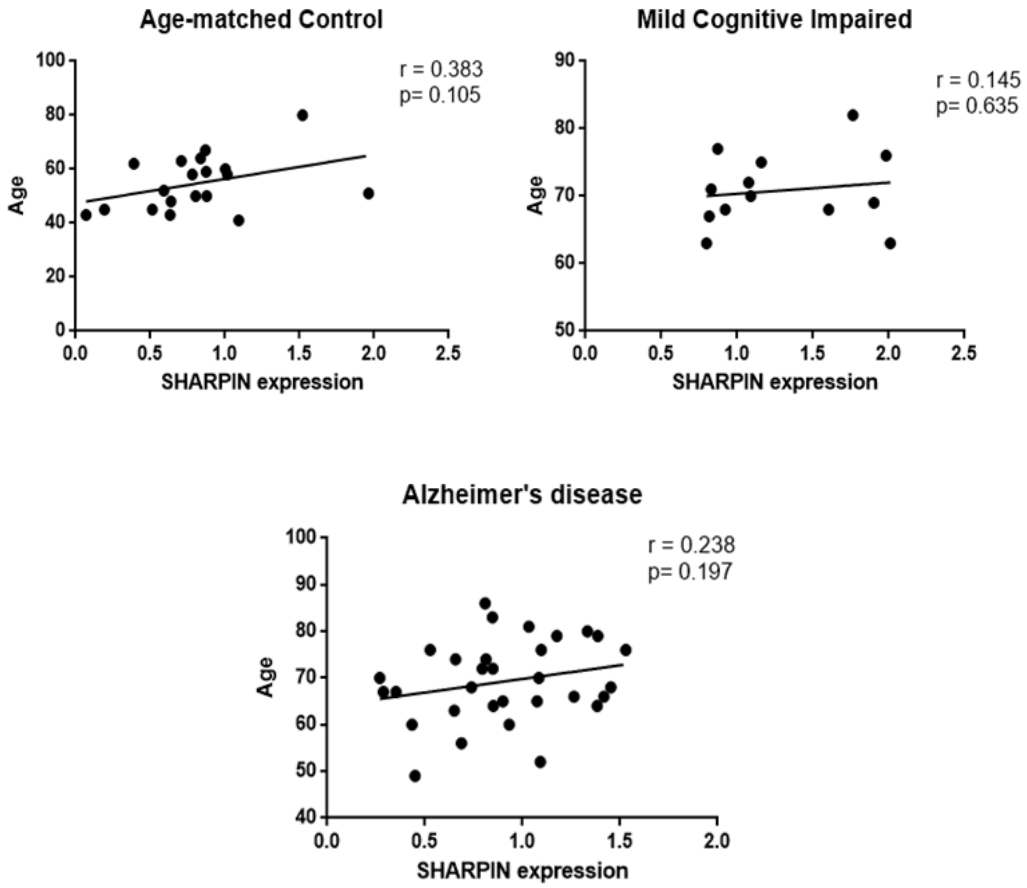


Figure IV. 39: Correlation of SHARPIN expression in macrophages with the age of study subjects in control, MCI and AD groups

SHARPIN expression by peripheral blood-derived macrophages (figure IV.36) was correlated with age of the study subjects and represented graphically. The study subjects were showing a mild positive correlation between SHARPIN expression and age.

IV. 10.5.2. SHARPIN expression by macrophages is altered with the presence of diabetes in the study subjects

Due to the unavailability of sufficient number of study subjects in a homogenous population, there are higher chances of alteration of protein expression by the macrophages in the peripheral circulation in response to many factors. Age, which is the greatest risk factor for AD, had been found to be a significant factor affecting SHARPIN expression. Since diabetes has been already identified as another risk factor in the pathogenesis of AD, and since the study subjects comprises of a mixture of diabetic and non-diabetic subjects, the subjects were further classified on the basis of the presence or the absence of diabetes and the expression of SHARPIN was analyzed and represented graphically. The graph clearly shows an increased expression of SHARPIN in diabetic subjects compared to non-diabetic.

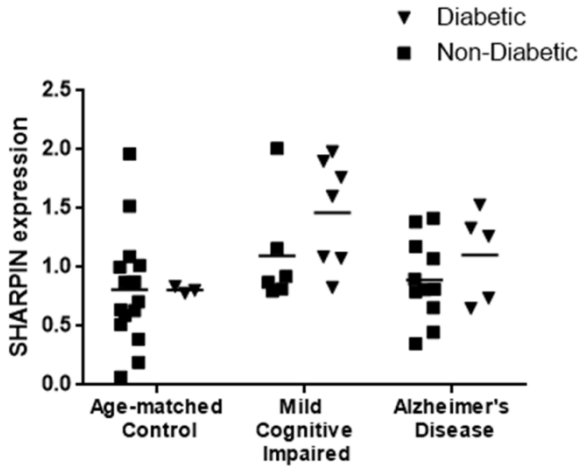
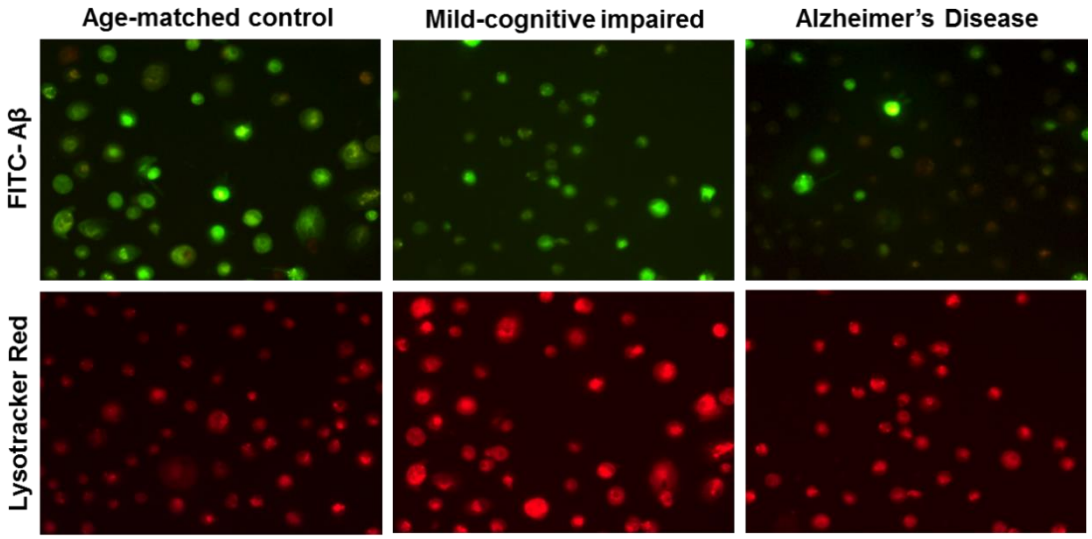


Figure IV. 40: Scatter plot showing SHARPIN expression in macrophages in diabetic and non-diabetic subjects in control, MCI and AD groups

The study subjects were grouped on the basis of the presence of diabetes and the expression of SHARPIN was analyzed (Figure IV.36) and represented as scatter plot.

IV. 10.6. Correlation of SHARPIN expression with Aβphagocytosis in peripheral blood-derived macrophages of study subjects

SHARPIN expression was found to be involved in regulating Aβ phagocytic receptor expressions and Aβ phagocytosis by macrophages using differentiated THP-1 cells. To analyze whether SHARPIN expression can also participate in regulating Aβ phagocytosis in patient-blood-derived macrophages, the macrophages were isolated from the study subjects and total protein was isolated at the 14th day of culture and the expression of SHARPIN was analyzed using western blotting. The macrophages at the 14th day of culture were further incubated with FITC- Aβ overnight and the phagocytosis efficiency was quantified using fluorescent imaging and ImageJ software as described in the methods section. Then the SHARPIN expression by the macrophages was correlated with the MFI of FITC uptake by the macrophages of the same study subjects. The results did not show a significant difference in phagocytosis efficiency among control MCI and AD subjects (Figure IV.41) and there was found to have no significant correlation between SHARPIN expression and phagocytosis efficiency in the study subjects (Figure IV.42)



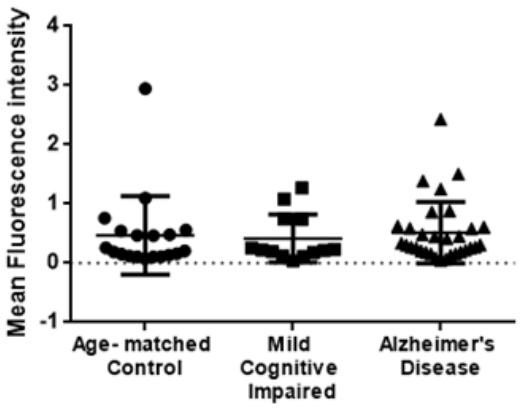
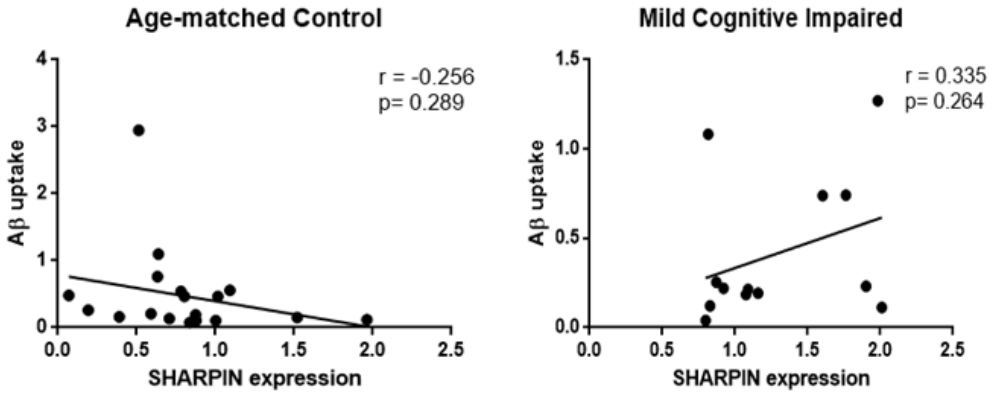


Figure IV. 41: Analysis of Aβ phagocytosis efficiency by macrophages in control, MCI and AD subjects

Aβphagocytosis efficiency was analyzed using FITC-Aβ uptake assay as described in the methods section in each study subject. A representative fluorescent image of FITC-Aβ phagocytosis in peripheral blood-derived macrophages from control, MCI and AD is shown in the figure. Lysotracker Red was used as counter stain. Aβ phagocytosis efficiency was analyzed in all the study subjects, the MFI was quantified from three different fields in each subject and was represented using scatter plot. There was no significant difference in the phagocytosis efficiency of Aβ by macrophages among the groups.



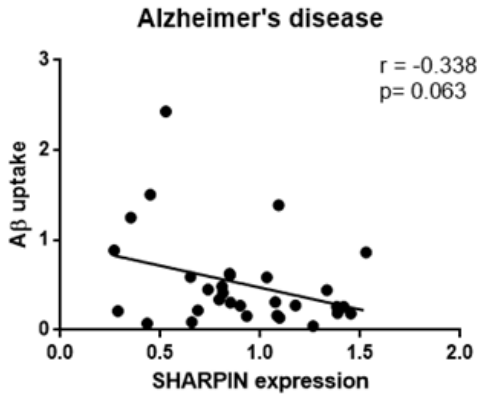


Figure IV. 42: Scatter plot showing correlation of SHARPIN expression with Aβ phagocytosis efficiency by macrophages in control, MCI and AD subjects

SHARPIN expression by peripheral blood-derived macrophages was analyzed using western blotting (Figure IV.36) and the Aβphagocytosis efficiency was analyzed using FITC-Aβ uptake assay as shown in figure IV.41. Correlation between these variables was analyzed.

IV. 10.7. SHARPIN expression in macrophages correlated with inflammatory status in the plasma of the study subjects

SHARPIN was found to regulate macrophage polarization to M1 pro-inflammatory phenotype in response to Aβ in differentiated THP-1 macrophages. To analyze whether SHARPIN is also involved in regulating macrophage polarization and thus the inflammatory status of the study subjects, the expression of SHARPIN protein by the peripheral-blood derived macrophages was analyzed as described in the previous sections. The inflammatory status of the study subjects was analyzed by quantifying the concentration of pro-inflammatory cytokines (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF-β) in the plasma of the study subjects using ELISA. Then each cytokine concentration was correlated with SHARPIN expression in each study subjects to determine whether SHARPIN is regulating the inflammatory status of the study subjects.

IV. 10.7.1. Correlation of SHARPIN expression in macrophages with IL-1β concentration in the plasma of study subjects

The pro-inflammatory cytokine IL-1β release in the plasma of the study subjects were analyzed using ELISA and were then correlated with SHARPIN expression by the peripheral blood- derived macrophages in the study subjects. The release of the cytokine was found to be higher in MCI and AD subjects compared to the control subjects similar to the pattern of SHARPIN expression by the macrophages between the groups. A correlation analysis of SHARPIN expression with the cytokine release was found to be showing a mild to moderate correlation among all the study groups (Figure IV.44).

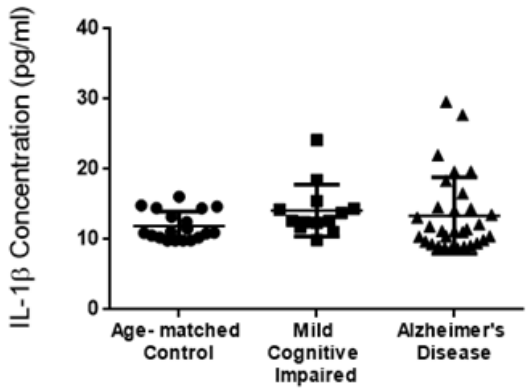


Figure IV. 43: Scatter plot showing the concentration of IL-1βin the plasma of study subjects

Plasma samples were isolated from the blood of the study subjects and were subjected to ELISA for analysis of the concentration of IL-1β in the peripheral circulation, quantified and represented graphically. The MCI subjects were showing higher concentration compared to AD, which was higher when compared to control subjects.

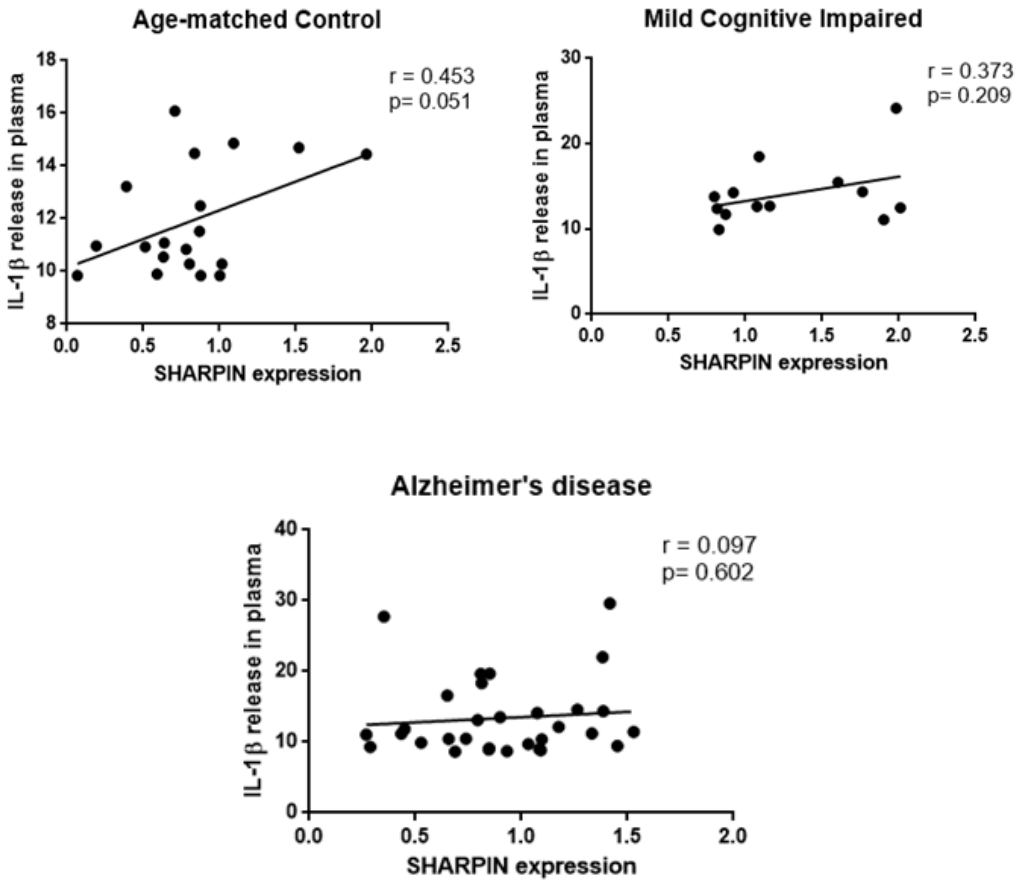


Figure IV. 44: Scatter plot showing correlation of SHARPIN expression in macrophages with IL-1β concentration in the plasma of control, MCI and AD subjects

SHARPIN expression by peripheral blood-derived macrophages was analyzed using western blotting (Figure IV.36) and the concentration of IL-1β was analyzed using ELISA (Figure IV.43) as described in the methods section in each study subject. The two factors were correlated in each study subjects and are represented as control MCI and AD groups separately. SHARPIN showed a positive trend in the expression patterns with IL-1βrelease in the plasma of control, MCI and AD subjects.

IV. 10.7.2. Correlation of SHARPIN expression in macrophages with TNF-α concentration in the plasma of study subjects

The pro-inflammatory cytokine, TNF-α release in the plasma of the study subjects were analyzed using ELISA and were then correlated with SHARPIN expression by the peripheral blood- derived macrophages in the study subjects. The release of the cytokine was found to be higher in control subjects compared to MCI and AD subjects as contrary to the pattern of SHARPIN expression by the macrophages between the groups. A correlation analysis of SHARPIN expression with the cytokine release was found to be showing a mild positive correlation among control and MCI groups whereas a moderate negative correlation in the AD groups.

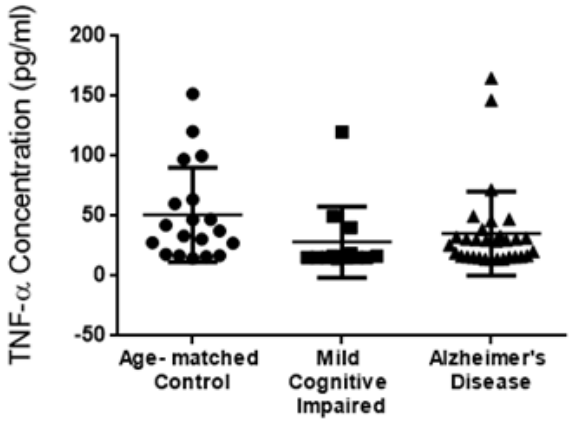


Figure IV. 45: Scatter plot showing the concentration of TNF-αin the plasma of study subjects

Plasma samples were isolated from the blood of the study subjects and were subjected to ELISA for analysis of the concentration of TNF-α in the peripheral circulation, quantified and represented graphically. The MCI subjects were showing a lower concentration compared to AD, which was lower when compared to control subjects.

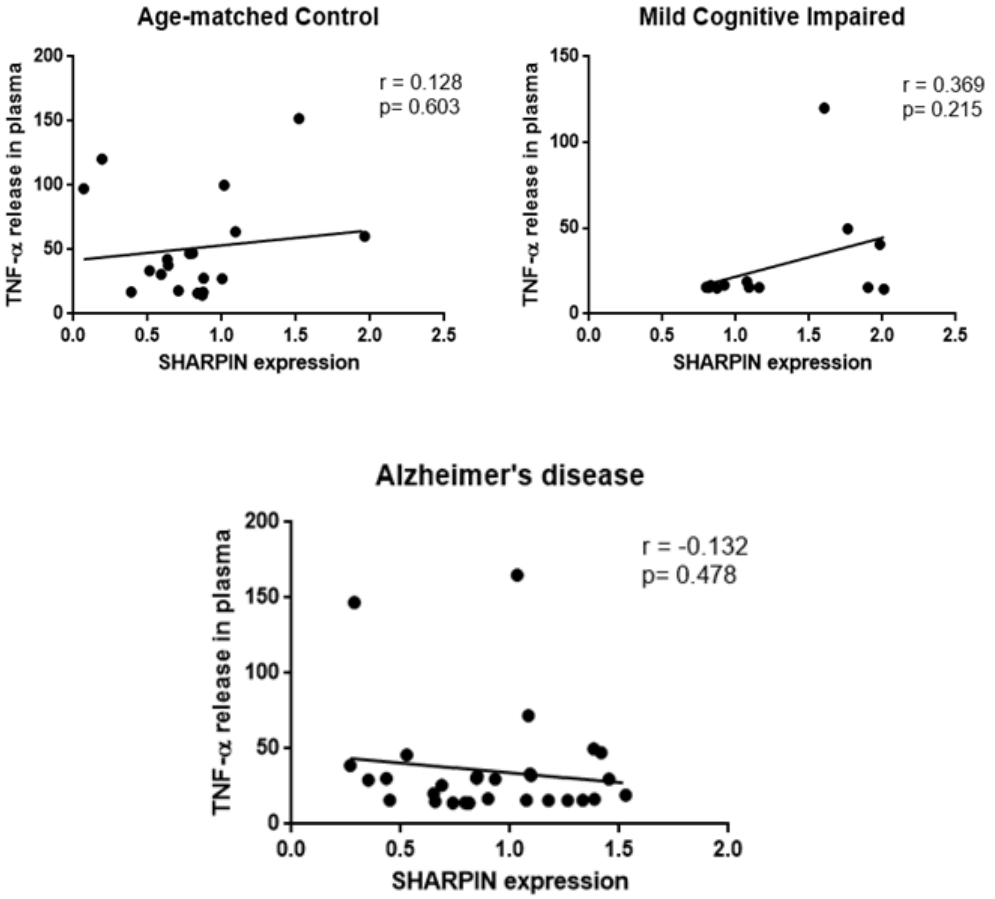


Figure IV. 46: Scatter plot showing correlation of SHARPIN expression in macrophages with TNF-α concentration in the plasma of control, MCI and AD subjects

SHARPIN expression by peripheral blood-derived macrophages was analyzed using western blotting (Figure IV.36) and the concentration of TNF-α was analyzed using ELISA (Figure IV.45) as described in the methods section in each study subject. The two factors were correlated in each study subjects and are represented as control MCI and AD groups separately. SHARPIN showed a positive trend in the expression patterns with TNF-αrelease in the plasma of control and MCI subjects and a negative correlation in AD subjects.

IV. 10.7.3. Correlation of SHARPIN expression in macrophages with IL-10 concentration in the plasma of study subjects

The concentration of anti-inflammatory cytokine IL-10 in the plasma of the study subjects was analyzed using ELISA and correlated with SHARPIN expression in the peripheral blood- derived macrophages of the study subjects. The release of the cytokine was found to be slightly higher in control subjects compared to MCI and AD subjects. A correlation analysis of SHARPIN expression with the cytokine release was found to be showing a very mild positive correlation among control group and a mild to moderate negative correlation in MCI and AD groups respectively.

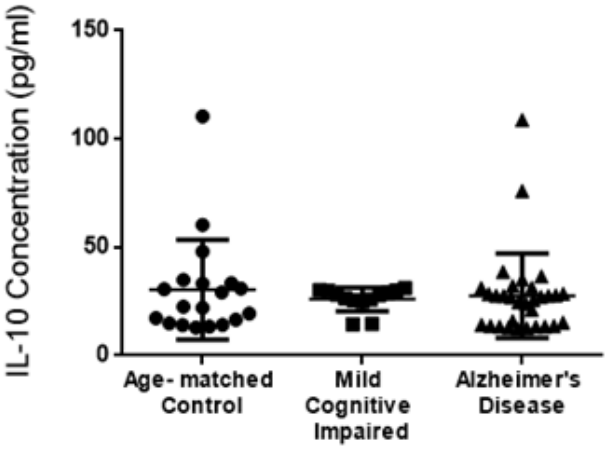


Figure IV. 47: Scatter plot showing the concentration of IL-10 in the plasma of study subjects

Plasma samples were isolated from the blood of the study subjects and were subjected to ELISA for analysis of the concentration of IL-10 in the peripheral circulation, quantified and represented graphically. The MCI and AD subjects were showing a slight lower concentration compared to control subjects.

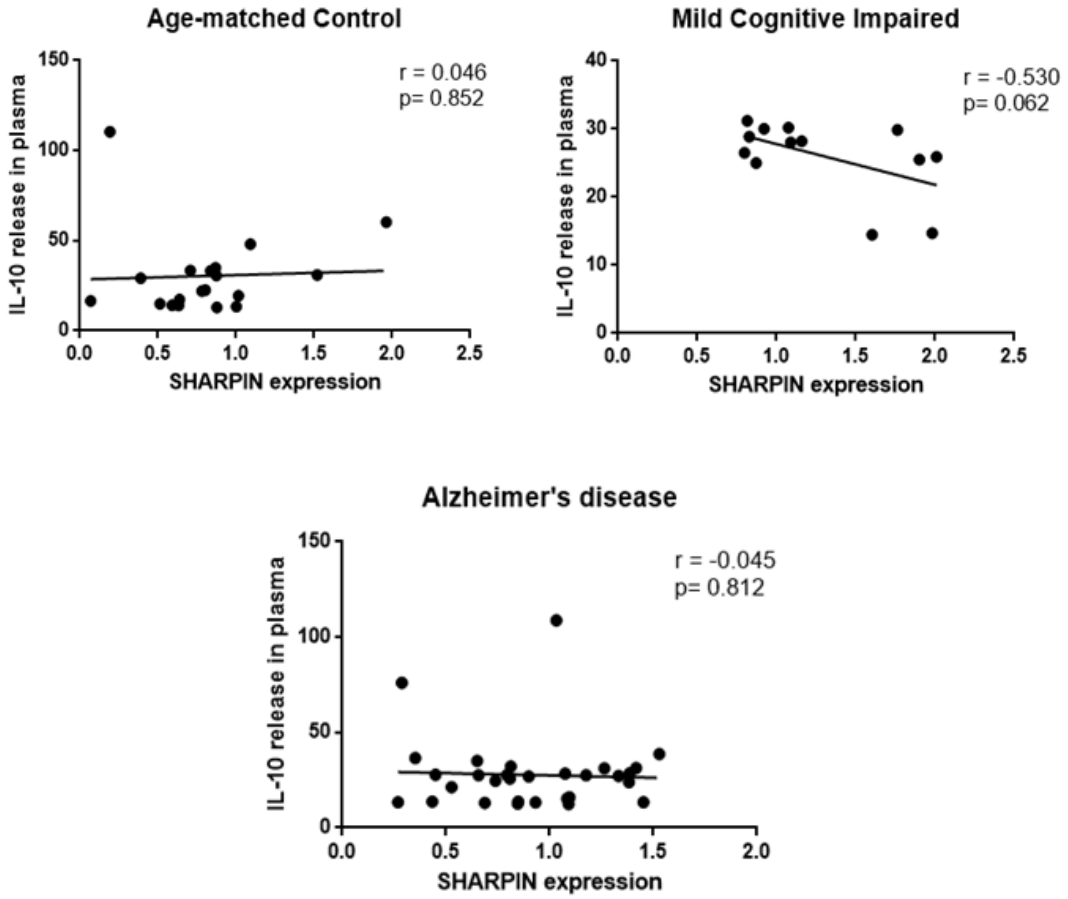


Figure IV. 48: Scatter plot showing correlation of SHARPIN expression in macrophages with IL-10 concentration in the plasma of control, MCI and AD subjects

SHARPIN expression by peripheral blood-derived macrophages was analyzed using western blotting (Figure IV.36) and the concentration of IL-10 was analyzed using ELISA (Figure IV.47) as described in the methods section in each study subject. The two factors were correlated in each study subjects and are represented as control MCI and AD groups separately. SHARPIN showed a very mild positive trend in the expression patterns with IL-10release in the plasma of control, but a mild to moderate negative correlation in the MCI and AD subjects.

IV. 10.7.4. Correlation of SHARPIN expression in macrophages with TGF-β1 concentration in the plasma of study subjects

The anti-inflammatory cytokine, TGF-β1 release in the plasma of the study subjects were analyzed using ELISA and were then correlated with SHARPIN expression by the peripheral blood- derived macrophages in the study subjects. The release of the cytokine was found to be significantly higher in control subjects compared to MCI and AD subjects. A correlation analysis of SHARPIN expression with the cytokine release was found to be showing a very mild positive correlation among control group and a mild negative correlation in all the study groups.

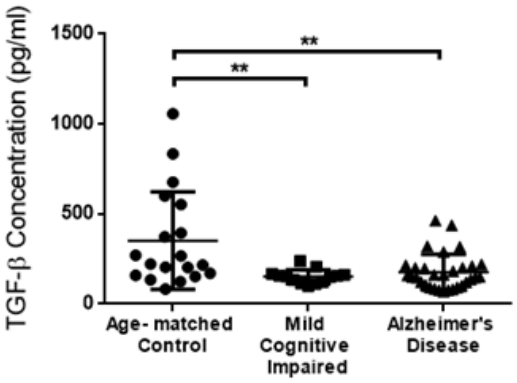


Figure IV. 49: Scatter plot showing the concentration of TGF-β1in the plasma of study subjects

*Plasma samples were isolated from the blood of the study subjects and were subjected to ELISA for analysis of the concentration of TGF-β1 in the peripheral circulation, quantified and represented graphically. The control subjects were showing significant higher concentration compared to MCI and AD subjects.(*p>0.05, **p>0.01, ***p>0.001)*

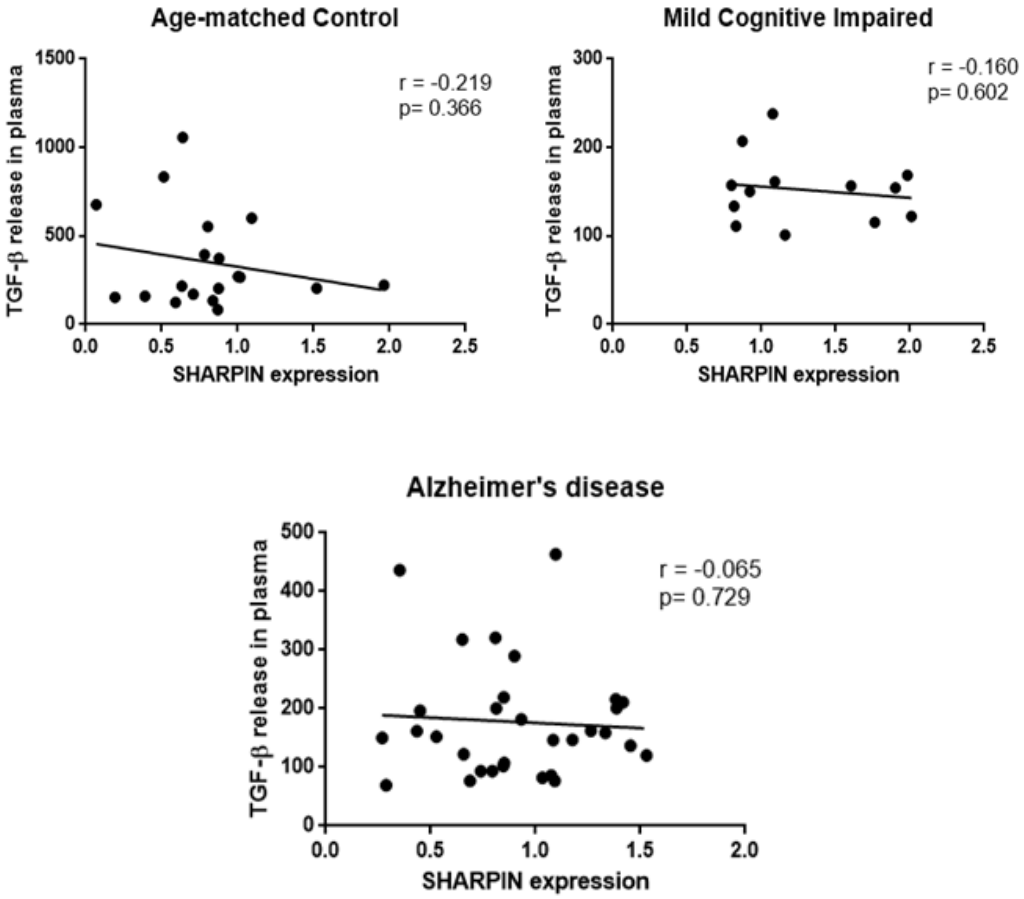


Figure IV. 50: Scatter plot showing correlation of SHARPIN expression in macrophages with TGF-β1 concentration in the plasma of control, MCI and AD subjects

SHARPIN expression by peripheral blood-derived macrophages was analyzed using western blotting (Figure IV.36) and the concentration of TGF-β1 was analyzed using ELISA (Figure IV.49) as described in the methods section in each study subject. The two factors were correlated in each study subjects and are represented as control MCI and AD groups separately. SHARPIN showed a mild negative correlation in the expression patterns with IL-10release in the plasma of all the study subjects.

V. Discussion

Alzheimer's disease (AD), the most devastating neurodegenerative disease known, is pathologically characterized by the accumulation of amyloid-beta (Aβ) and hyperphosphorylated tau proteins (de Paula *et al.*, 2009). According to the well accepted amyloid-cascade hypothesis, the mismetabolism of amyloid precursor protein (APP) sequentially by β- and γ- secretase instead of α- and γ- secretase leads to the overproduction of Aβ, which is highly hydrophobic in nature, accumulates and form amyloid plaques, causing neurodegeneration and brain atrophy (Hardy and Higgins, 1992). However, apart from the overproduction of Aβ due to genetic mutations in APP and APP processing enzymes in familial early- onset AD, Aβ is normally produced within a human brain and is essential for the maintenance of normal physiological functioning of the brain (Lazarevic *et al.*, 2017; Brothers *et al.*, 2018). During normal physiology, an adequate concentration of Aβ in the brain is maintained through homeostasis in production and degradation (Baranello *et al.*, 2015). This is effectuated mainly by three mechanisms: degradation by Aβ-degrading enzymes, clearance into the systemic circulation by shuttling through the blood-brain barrier (BBB), and clearance by immune cells, namely microglia in the brain and macrophages and monocytes in the peripheral system through Aβ phagocytosis and degradation (Wang *et al.*, 2006; Zhao *et al.*, 2009; Baranello *et al.*, 2015). However, under certain conditions mostly associated with aging, the immune cells fail to phagocytose Aβ, leading to a dysregulation in the balance between Aβ production and degradation (Mawuenyega *et al.*, 2010; Wildsmith *et al.*, 2013). In the long term, excessive Aβ accumulation accompanied by reduced degradation leads to chronic inflammatory activation of the immune cells causing neuronal death resulting in the progression of AD, mainly in the sporadic late- onset form (Rogers *et al.*, 2007; Van Eldik *et al.*, 2016). Thus, defective immune cell-mediated clearance of Aβ, is a major contributor of Aβ accumulation in the brain, leading to AD pathogenesis (Fiala *et al.*, 2005; Jairani *et al.*, 2019b). Further, Aβ accumulation-associated inflammatory activation of microglia in the brain leads to the recruitment of blood- derived macrophages to the brain from peripheral circulation, which plays a major role in causing neuronal apoptosis, leading to amplified rate of progression of AD (Sweeney *et al.*, 2018). Even though there are studies that correlate Aβ phagocytosis efficiency and inflammatory activation by the immune cells in AD (Aderem, 2003; Lund *et al.*,

2014), mechanisms that underlie inefficient Aβ phagocytosis and enhanced inflammation by macrophages remain insufficiently addressed.

The NLRP3 (NOD-like receptor protein 3) inflammasome, a protein complex composed of NLRP3, the adaptor protein Apoptosis-associated Speck-like protein containing a CARD (ASC) and the inflammatory caspase-1, is responsible for the cleavage and maturation of inflammatory cytokines like IL-1β and IL-18 (Mangan *et al.*, 2018). Although NLRP3 has been linked to the progression of AD (Heneka *et al.*, 2013; Yin *et al.*, 2018), studies focussing on the regulatory mechanism of the protein in AD have not been reported. SHARPIN (SHANK-associated RH domain-interacting protein), a part of the LUBAC (linear ubiquitination assembly complex) has been proven to be controlling the expression of NLRP3 through NF-κB activation (Gurung *et al.,* 2015). Nevertheless, its functional role in AD has not been studied yet.

V. 1. A functional role for SHARPIN in mediating macrophage response to Aβ in Alzheimer's disease: in vitro findings

SHARPIN has been recognised as an upstream activator of NF-κB, and an important mediator of inflammatory mechanism (Ikeda *et al.*, 2011b; Tokunaga *et al.*, 2011; Aksentijevich and Zhou, 2017).A recent study by YuyaAsanomi et.al identified a rare functional variant of SHARPIN as a genetic risk factor for late-onset AD (LOAD) proving the role of the protein in the progression of AD (Asanomi *et al.*, 2019).While the study identified genetic mutations in SHARPIN as a risk factor for LOAD, the molecular basis of SHARPIN in AD pathogenesis remains unclear.

In the present study, we focused on the role of SHARPIN in the regulation of macrophage function and its contribution to the progression of AD. Using differentiated THP-1 macrophages exposed to Aβ as an *in-vitro* model, we demonstrated a significant increase in the expression of SHARPINin the presence of Aβ, indicating a link between Aβ exposure and SHARPIN expression in macrophages.

SHARPIN has been implicated inthe regulation of inflammatory mechanisms (Wang *et al.*, 2012a; Kasirer-Friede *et al.*, 2019). Since macrophage-mediated inflammatory mechanisms in response to Aβ are dependent on phagocytic receptor interaction with Aβ (Aderem, 2003; Fiala, Cribbs, *et al.*, 2007), we analysed whether SHARPIN expression in macrophages could play a role in phagocytosis of Aβ phagocytosis. Our study showed for the first time the involvement of SHARPIN in Aβ phagocytosis by macrophages. Hence, SHARPIN might play a significant role in AD progression since Aβ phagocytosis by macrophages has been reported to be impaired in AD (Fiala *et al.*, 2005; Fiala, Liu, *et al.*, 2007; Masoumi *et al.*, 2009).

Aβ phagocytosis by macrophages is initiated by binding with Aβ phagocytic receptors, the most prominent among them being SCARA-1, CD36, type I and type II scavenger receptors, RAGE-1 and LRP-1 (Lai and McLaurin, 2012). Since SHARPIN knockdown significantly reduced Aβ phagocytosis, we explored whether SHARPIN mediates Aβ phagocytosis through regulating the expression of Aβ phagocytic receptors. Previous studies have shown reported a reduction in the expression levels of these receptors in the brain of AD subjects that lead to impaired Aβ clearance, contributing to increased Aβ load in the brain, eventually leading to neurodegeneration. Additionally, knockdown of these receptors in AD transgenic mice models promoted Aβ accumulation, supporting the role of these receptors in mediating the phagocytic clearance of Aβ (Fiala *et al.*, 2005; Mawuenyega *et al.*, 2010; Frenkel *et al.*, 2013; Wildsmith *et al.*, 2013). In the present study, we found that SHARPIN regulates Aβ through modulating the expression of receptors involved in Aβ uptake, namely SCARA-1, CD36, LRP-1 and RAGE-1, thus affecting the overall cellular intake of Aβ. This was evidenced by a significant reduction in FITC-Aβ fluorescence in SHARPIN-silenced THP-1 macrophages compared to the control.

A phagocytic stimulus in macrophages is followed by an inflammatory response. However, mechanisms underlying the regulation of this response in Aβ -stimulated macrophages have been relatively less explored. A study conducted in mice carrying mutant SHARPIN (*Sharpin*^{cpdm}) that caused loss of SHARPIN function identified that SHARPIN is required for NLRP3 activation (Gurung *et al.*, 2015). NLRP3, a

central regulator of pro-inflammatory response has been widely explored in AD and has been identified as one of the primary mediators of inflammatory damage in AD (Heneka *et al.*, 2013; Yin *et al.*, 2018). On the basis of these findings we explored the link between SHARPIN and NLRP3 and their regulatory role in mediating the inflammatory response in macrophages exposed to Aβ. We have found a significant reduction in NLRP3 expression in SHARPIN-knockdown macrophages exposed to Aβ demonstrating, a novel link between the two inflammatory mediators- SHARPIN and NLRP3 in Aβ stimulated macrophages.

Since SHARPIN regulates NLRP3 which is an activator of pro-inflammatory signalling (Abderrazak *et al.*, 2015; Shimizu *et al.*, 2019), we analysed the expression of inflammatory markers in SHARPIN-silenced macrophages exposed to Aβ. It is known that Aβ induces macrophage and microglia into a pro-inflammatory M1 phenotype, thus stimulating these cells to release inflammatory cytokines, chemokines and ROS in response to Aβ (Lund *et al.*, 2014; Famenini *et al.*, 2017). We found that while Aβ polarizes THP-1 macrophages to the M1 pro-inflammatory phenotype, knockdown of SHARPIN in the presence of Aβ prevented macrophage M1 polarization. On the contrary, knockdown of SHARPIN polarized THP-1 macrophages to an anti-inflammatory M2 phenotype as evidenced by an increase in the expression of anti-inflammatory markers TGF-β and IL-10 in SHARPIN-silenced macrophages exposed to Aβ. Together, these findings point to a role for SHARPIN-dependent NLRP3 in the regulation of macrophage polarization to a pro-inflammatory phenotype, in response to Aβ. This strongly suggests the SHARPIN-NLRP3- mediated inflammatory activation of macrophages in response to Aβ which further confirms the role of SHARPIN in the progression of Alzheimer's disease.

It has been reported that brain-resident and peripheral circulation-derived immune cells, while attempting to eliminate aggregated Aβ deposits in the brain, promotes inflammatory response through the secretion of pro-inflammatory cytokines and reactive oxygen species, leading to neuronal damage in AD (Van Eldik *et al.*, 2016). Since SHARPIN regulates inflammation, we sought to analyse the role of SHARPIN in mediating neuronal cell apoptosis. Using differentiated SHSY5Y neurons, we have observed significant reduction in the apoptosis of neurons treated with

conditioned media derived from SHARPIN-knockdown macrophages compared to the cells treated with conditioned media from macrophages incubated with Aβ. We found that while Aβ functioned through SHARPIN-mediated mechanisms to promote inflammatory cytokine release by THP-1 macrophages, knockdown of SHARPIN prevented the release of pro-inflammatory cytokines, leading to increased viability of differentiated SHSY5Y neurons in culture. The results clearly indicate that Aβ-induced neuronal apoptosis is primarily mediated through Aβ- mediated inflammatory mechanisms that involve SHARPIN. Thus our study demonstrates SHARPIN as a critical protein that acts as a double-edged sword regulating phagocytosis and inflammation, where its downregulation reduces inflammation and protects inflammatory neuronal damage, but at the same time, affecting macrophage-mediated Aβ phagocytosis.

Since SHARPIN was found to regulate critical macrophage functions involving phagocytosis and macrophage polarization, the study explored the factors that could be involved in the regulation of Aβ -stimulated SHARPIN expression. Aβ is well known to induce oxidative stress through ROS production (Butterfield *et al.*, 2013; Cheignon *et al.*, 2017). Further, SHARPIN is an upstream regulator of the redox sensitive transcription factor NF-κB, which points to a possible role for oxidative stress in stimulating SHARPIN expression (Tokunaga *et al.*, 2011). Since Aβ induced the expression of SHARPIN, we explored the role of ROS in mediating Aβ -induced SHARPIN. Treatment of macrophages with NAC, a potent ROS scavenger, prevented Aβ -induced SHARPIN expression, demonstrating ROS to play an important role in mediating SHARPIN expression in response to Aβ.

Since NF-κB is the redox-sensitive transcription factor (Kabe *et al.*, 2005), the role of NF-κB activation in regulating SHARPIN expression was explored and we found an increase in the molecular weight of SHARPIN in THP-1 cells treated with NF-κB inhibitor. This clearly states that the protein is getting modified in the absence of NF-κB signalling. SHARPIN is an ubiquitinylating protein that forms LUBAC by interacting with HOIL-1 L and HOIP and the functioning of SHARPIN as a part of the LUBAC complex is transfer of ubiquitin molecules to the NF-κB essential modulator (NEMO). Hence, inhibition of NF-κB signalling could be acting as an

inhibitor of SHARPIN activity, which leads us to the finding that ubiquitination is the post-translational modification in SHARPIN. Thus inhibition of NF-κB signalling pathway either promoted ubiquitination of SHARPIN or inhibited the transfer of ubiquitin from SHARPIN to NEMO thereby hindering SHARPIN function. The functional implications and regulatory mechanisms of SHARPIN need to be explored in detail in both on an immunological basis and a therapeutic basis. The role of SHARPIN has been explored in many disease conditions, especially in cancer stressing its multiple roles in LUBAC- independent regulatory mechanisms (Tamiya *et al.*, 2018; Zhou *et al.*, 2019), suggesting that this modification that we have identified, if not targeted for a proteosomal degradation, might be inhibiting only the LUBAC- dependent SHARPIN function, promoting its other cellular functions, which is beyond the scope of this study.

V. 2. SHARPIN expression by patient- derived macrophages: ex-vivo analysis

Analysis of SHARPIN in AD and MCI patient-derived macrophages pointed to a significant increase in SHARPIN expression in MCI patient-derived macrophages compared to AD and control subjects. MCI is regarded as the preclinical stage of AD. Consistent with our *in vitro* findings on the stimulatory effect of $A\beta_{42}$ on SHARPIN expression, the relative levels of SHARPIN was found to be significantly higher in the MCI patient-derived macrophages. However, the relative levels of SHARPIN expression in the AD patient-derived macrophages were significantly less compared to the MCI group (Figure IV.33). It has been widely reported that there is a gradual reduction in the concentration of $A\beta_{42}$ in the plasma of AD subjects with progression of the disease, resulting from decreased Aβ clearance through the BBB and its accumulation in the brain with the progression of disease from MCI to AD (Seppälä *et al.*, 2010; Toledo *et al.*, 2013; Janelidze *et al.*, 2016; Hanon *et al.*, 2018; Chen *et al.*, 2019; Zetterberg and Burnham, 2019). Hence, owing to a higher concentration of $A\beta_{42}$ in the peripheral circulation of MCI subjects with respect to AD, the relative levels of SHARPIN was found to be higher in the MCI group

compared to the control or AD. It has been reported that with the progression of AD, more of Aβ gets accumulated in the brain with reduced clearance to the peripheral system, resulting in a reduced concentration of Aβ in the plasma. In support of this, the expression of SHARPIN showed a positive correlation with $A\beta_{42/40}$ concentrationin the plasma of the respective study subjects (Figure IV.34).

Due to the heterogeneity of the study population, we further classified the subjects on the basis of age and diabetes. Our *in vitro* data suggests that Aβ-induced oxidative stress and oxidative stress in the absence of Aβ could induce the expression of SHARPIN. These data point out that age-induced oxidative stress could also play an important role in SHARPIN expression. Using patient-derived macrophages *ex-vivo*, we found that SHARPIN expression was higher in a higher age group and that those with diabetes were also showing an increased SHARPIN expression pointing out the diversity of factors that could modulate SHARPIN expression and inflammatory condition *in-vivo*.

In line with the *in vitro* observation on the regulatory role of SHARPIN in promoting macrophage polarization to a pro-inflammatory phenotype, the expression of SHARPIN was found to be positively correlated with the pro-inflammatory marker IL-1β and negatively correlated with the anti-inflammatory marker TGF-β in blood plasma of study subjects. However, SHARPIN expression did not corelate with other markers of pro-inflammation or anti-inflammation such as TNF-α and IL-10 respectively, in the blood plasma of study subjects. Further, the expression of SHARPIN did not corelate with phagocytic efficiency of Aβ by peripheral blood-derived macrophages. This indicates the presence of complex mechanisms regulating inflammation and phagocytosis and the interference of several exogenous factors like diet, drug interventions, life style factors, genetic composition, presence of other communicable and non-communicable diseases, etc. under *in-vivo* conditions contrary to the controlled *in-vitro* setting. An elaborate study on a larger cohort in a lesser heterogenic population needs to be conducted to explore the role of SHARPIN in the peripheral system *in-vivo*.

In summary, our study demonstrates, for the first time, a novel role for SHARPIN in the regulation of macrophage function in response to Aβ in a setting of AD. Our *in vitro* observations point to a role for SHARPIN in regulating Aβ phagocytosis and Aβ-stimulated inflammatory mechanisms in macrophages, where SHARPIN promoted the polarization of macrophages to a pro-inflammatory M1 phenotype. Further, we demonstrated a role for SHARPIN in mediating Aβ-induced neuronal cell death, demonstrating its role in promoting neurodegeneration in AD. Importantly, SHARPIN expression positively correlated with the levels of $A\beta_{42/40}$ in patient-derived macrophages demonstrating a link between SHARPIN expression and AD progression.

V. 3. Significance of the study

Enhanced inflammation and altered Aβ phagocytosis are hallmarks of AD pathogenesis. The present study identified a novel role for SHARPIN in mediating Aβ phagocytosis and macrophage polarization to an M1 pro-inflammatory phenotype and in a setting of AD. This data, highlighting the importance of SHARPIN in the regulation of inflammation and Aβ phagocytosis that are key determinant of AD pathogenesis, identify SHARPIN as a critical factor that promotes AD progression.

V. 4. Limitations of the study

Although the present study demonstrates a role for SHARPIN in Alzheimer's disease and its functional implications with respect to promoting Aβ phagocytosis and inflammation, neuro-inflammatory mechanisms contributing as a leading factor for neurodegeneration in AD, future studies in brain resident immune cells in AD transgenic mice models are required to explore the detailed mechanistic role of SHARPIN in AD. Further, owing to a small sample size, SHARPIN expression did not significantly correlate with macrophage phagocytosis efficiency of Aβ and inflammation. Hence, these data need to be analysed in a larger cohort of study

subjects. Since age plays an important role in the functioning and alteration of immune cells in the peripheral system, the study subjects might be showing a high variability in inflammatory mechanisms which further changes with life style factors, drug interventions, presence of undiagnosed diseases, genetic factors including ApoEpolymorphism, etc. which will also reflect in the study results in contrast to a well-controlled *in vitro* culture system.

V. 5. Future directions

Future studies need to address the role of SHARPIN in AD using a larger cohort of study subjects in order to establish the role of this protein in AD pathogenesis. Importantly, exploring microglial- and astrocyte- mediated SHARPIN expression and its role in phagocytosis and inflammatory pathways are relevant in this field since these are the cells that respond primarily to Aβ accumulation in AD brain. Further, the protein might be a potential biomarker for AD pathogenesis, which needs to be explored on a genetic and molecular basis.

VI. Summary & Conclusions

Accumulation of A-beta in the brain as a consequence of impaired immune cell-mediated A-beta clearance, in the long term, leads to LOAD. . Although brain-resident microglia play a major role in the clearance of A-beta clearance in AD, the phagocytic machinery of these cells gradually become ineffective and hence recruit peripheral blood-derived monocytes/macrophages to aid in A-beta phagocytosis and clearance. . Although these cells aid in phagocytic clearance of A-beta, activation of A-beta-induced inflammatory response in PBMCs cause neuronal cell death, leading to neurodegeneration. Hence, defective immune cell-mediated clearance of Aβ and Aβ-associated inflammatory activation of immune cells are key contributors of Aβ accumulation and neurodegeneration in AD, however, the underlying mechanisms contributing to these remain elusive.

SHARPIN is a protein that has been reported to promote inflammatory response via promoting expression and activation of the inflammasome. Although inflammation is a primary mediator in promoting AD pathogenesis, the role of SHARPIN – a key regulator of inflammation remained unexplored, until recently wherein two studies reported the genetic involvement of SHARPIN in AD, identifying a novel functional variant of the protein as a genetic risk factor for LOAD. Although these studies show a causative link between SHARPIN dysfunction and AD progression, reasons for However, the mechanisms by which functional implications of SHARPIN in AD remain to be explored.

In this study, differentiated THP-1 cells treated with Aβ were used as *in-vitro* model to analyse the role of SHARPIN in AD. SHARPIN was silenced in differentiated THP-1 cells and the functional role of the protein in regulating phagocytosis and inflammatory mechanisms in macrophages exposed to Aβ was analysed using immunoblotting, ELISA, real-time PCR, immunoprecipitation and flow cytometry. The effect of SHARPIN-mediated inflammatory mechanisms in neurons was analysed using differentiated SHSY5Y cells. Further, the expression patterns of the protein in different stages of AD were analysed in macrophages derived from AD, MCI and control subject blood samples.

The findings in the present study point to a role for SHARPIN in the pathogenesis of AD. The major conclusions of the study can be summarized as given below:

- Aβ upregulates SHARPIN expression in macrophages
- SHARPIN enhances Aβ uptake via regulating the expression of Aβ phagocytic receptors in macrophages
- SHARPIN upregulates Aβ-mediated NLRP3 expression by macrophages
- SHARPIN polarizes macrophages to the M1 pro-inflammatory phenotype in response to Aβ
- SHARPIN-mediated macrophage polarization induces neuronal apoptosis in response to Aβ
- SHARPIN expression is regulated by Aβ- induced oxidative stress
- SHARPIN ubiquitination is mediated via an NF-κB- mediated signalling mechanism
- SHARPIN expression in patient-derived macrophages positively correlates with $A\beta_{42/40}$ concentration in the plasma of study subjects
- SHARPIN expression increases with age and the presence of diabetes in the study subjects
- SHARPIN expression in macrophages positively correlates with pro-inflammatory cytokine IL-1β and negatively correlates with anti-inflammatory cytokine TGF-β in the plasma of the study subjects.
- No correlation was observed between SHARPIN expression in macrophages and Aβ- phagocytosis efficiency of the macrphages in study subjects.

In short, the present study demonstrates a novel role for SHARPIN in the regulation of Aβ-phagocytosis and inflammation in Alzheimer's disease.

VII. References

Abbas N, Bednar I, Mix E, Marie S, Paterson D, Ljungberg A, Morris C, Winblad B, Nordberg A, and Zhu J (2002) Up-regulation of the inflammatory cytokines IFN-gamma and IL-12 and down-regulation of IL-4 in cerebral cortex regions of APP(SWE) transgenic mice. *J Neuroimmunol* **126**:50–57.

Abderrazak A, Syrovets T, Couchie D, El Hadri K, Friguet B, Simmet T, and Rouis M (2015) NLRP3 inflammasome: From a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. *Redox Biol* **4**:296–307.

Aderem A (2003) Phagocytosis and the Inflammatory Response. *J Infect Dis* **187**:S340-5.

Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, and Tschopp J (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* **20**:319–325.

Akiyama H., Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, and Wyss-Coray T (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging* **21**:383–421.

Akiyama Haruhiko, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WST, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata–Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, and Wyss–Coray

T (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging* **21**:383–421.

Aksentijevich I, and Zhou Q (2017) NF-κB Pathway in Autoinflammatory Diseases: Dysregulation of Protein Modifications by Ubiquitin Defines a New Category of Autoinflammatory Diseases. *Front Immunol* **8**.

Algotsson A, and Winblad B (2007) The integrity of the blood-brain barrier in Alzheimer's disease. *Acta Neurol Scand* **115**:403–408.

Alvarez JI, Dodelet-Devillers A, Kebir H, Ifergan I, Fabre PJ, Terouz S, Sabbagh M, Wosik K, Bourbonnière L, Bernard M, van Horssen J, de Vries HE, Charron F, and Prat A (2011) The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science* **334**:1727–1731.

Asanomi Y, Shigemizu D, Miyashita A, Mitsumori R, Mori T, Hara N, Ito K, Niida S, Ikeuchi T, and Ozaki K (2019) A rare functional variant of SHARPIN attenuates the inflammatory response and associates with increased risk of late-onset Alzheimer's disease. *Mol Med* **25**.

Atri C, Guerfali FZ, and Laouini D (2018) Role of Human Macrophage Polarization in Inflammation during Infectious Diseases. *Int J Mol Sci* **19**:1801.

Baatar D, Patel K, and Taub DD (2011) The effects of ghrelin on inflammation and the immune system. *Mol Cell Endocrinol* **340**:44–58.

Bales KR, Du Y, Dodel RC, Yan GM, Hamilton-Byrd E, and Paul SM (1998) The NF-kappaB/Rel family of proteins mediates Abeta-induced neurotoxicity and glial activation. *Brain Res Mol Brain Res* **57**:63–72.

Ballenger JF (2006) Progress in the history of Alzheimer's disease: the importance of context. *J Alzheimers Dis JAD* **9**:5–13.

Baranello RJ, Bharani KL, Padmaraju V, Chopra N, Lahiri DK, Greig NH, Pappolla MA, and Sambamurti K (2015) Amyloid-Beta Protein Clearance and Degradation (ABCD) Pathways and their Role in Alzheimer's Disease. *Curr Alzheimer Res* **12**:32–46.

Bartus RT, Dean RL, Beer B, and Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**:408–414.

Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, and Latz E (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol Baltim Md 1950* **183**:787–791.

Beech JS, Wheeler DW, Reckless J, Grant AJ, Price J, Mastroeni P, Grainger DJ, and Menon DK (2007) The MHP36 line of murine neural stem cells expresses functional CXCR1 chemokine receptors that initiate chemotaxis in vitro. *J Neuroimmunol* **184**:198–208.

Bejanin A, Schonhaut DR, La Joie R, Kramer JH, Baker SL, Sosa N, Ayakta N, Cantwell A, Janabi M, Lauriola M, O'Neil JP, Gorno-Tempini ML, Miller ZA, Rosen HJ, Miller BL, Jagust WJ, and Rabinovici GD (2017) Tau pathology and neurodegeneration contribute to cognitive impairment in Alzheimer's disease. *Brain J Neurol* **140**:3286–3300.

Bergsbaken T, Fink SL, and Cookson BT (2009) Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* **7**:99–109.

Bird TD (2008) Genetic Aspects of Alzheimer Disease. *Genet Med Off J Am Coll Med Genet* **10**:231–239.

Blennow K, Mattsson N, Schöll M, Hansson O, and Zetterberg H (2015) Amyloid biomarkers in Alzheimer's disease. *Trends Pharmacol Sci* **36**:297–309.

Bolmont T, Haiss F, Eicke D, Radde R, Mathis CA, Klunk WE, Kohsaka S, Jucker M, and Calhoun ME (2008) Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. *J Neurosci Off J Soc Neurosci* **28**:4283–4292.

Bornemann KD, Wiederhold KH, Pauli C, Ermini F, Stalder M, Schnell L, Sommer B, Jucker M, and Staufenbiel M (2001) Abeta-induced inflammatory processes in microglia cells of APP23 transgenic mice. *Am J Pathol* **158**:63–73.

Bossù P, Ciaramella A, Salani F, Vanni D, Palladino I, Caltagirone C, and Scapigliati G (2010) Interleukin-18, from neuroinflammation to Alzheimer's disease. *Curr Pharm Des* **16**:4213–4224.

Brothers HM, Gosztyla ML, and Robinson SR (2018) The Physiological Roles of Amyloid-β Peptide Hint at New Ways to Treat Alzheimer's Disease. *Front Aging Neurosci* **10**.

Butovsky O, Kunis G, Koronyo-Hamaoui M, and Schwartz M (2007) Selective ablation of bone marrow-derived dendritic cells increases amyloid plaques in a mouse Alzheimer's disease model. *Eur J Neurosci* **26**:413–416.

Butterfield DA, Swomley AM, and Sultana R (2013) Amyloid β-Peptide (1–42)-Induced Oxidative Stress in Alzheimer Disease: Importance in Disease Pathogenesis and Progression. *Antioxid Redox Signal* **19**:823–835.

Cartier L, Hartley O, Dubois-Dauphin M, and Krause K-H (2005) Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain Res Brain Res Rev* **48**:16–42.

Cerf E, Sarroukh R, Tamamizu-Kato S, Breydo L, Derclaye S, Dufrêne YF, Narayanaswami V, Goormaghtigh E, Ruysschaert J-M, and Raussens V (2009) Antiparallel beta-sheet: a signature structure of the oligomeric amyloid beta-peptide. *Biochem J* **421**:415–423.

Cheignon C, Tomas M, Bonnefont-Rousselot D, Faller P, Hureau C, and Collin F (2017) Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox Biol* **14**:450–464.

Chen T-B, Lee Y-J, Lin S-Y, Chen J-P, Hu C-J, Wang P-N, and Cheng IH (2019) Plasma Aβ42 and Total Tau Predict Cognitive Decline in Amnestic Mild Cognitive Impairment. *Sci Rep* **9**:1–10.

Chitre N, Ghumatkar P, and Sathaye DrS (2016) The Role of M1/M2 Transition of the Brain Macrophages in Alzheimer's Disease. *IOSR J Pharm Biol Sci* **11**:72–78.

Cho HJ, Jin SM, Youn HD, Huh K, and Mook-Jung I (2008) Disrupted intracellular calcium regulates BACE1 gene expression via nuclear factor of activated T cells 1 (NFAT 1) signaling. *Aging Cell* **7**:137–147.

Chung H, Brazil MI, Soe TT, and Maxfield FR (1999) Uptake, Degradation, and Release of Fibrillar and Soluble Forms of Alzheimer's Amyloid β-Peptide by Microglial Cells. *J Biol Chem* **274**:32301–32308.

Citron BA, Dennis JS, Zeitlin RS, and Echeverria V (2008) Transcription factor Sp1 dysregulation in Alzheimer's disease. *J Neurosci Res* **86**:2499–2504.

Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, and Ashe KH (2005) Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. *Nat Neurosci* **8**:79–84.

Conductier G, Blondeau N, Guyon A, Nahon J-L, and Rovère C (2010) The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases. *J Neuroimmunol* **224**:93–100.

Crowther RA, and Wischik CM (1985) Image reconstruction of the Alzheimer paired helical filament. *EMBO J* **4**:3661–3665.

Cudaback E, Li X, Montine KS, Montine TJ, and Keene CD (2011) Apolipoprotein E isoform-dependent microglia migration. *FASEB J* **25**:2082–2091.

de Paula V de JR, Guimarães FM, Diniz BS, and Forlenza OV (2009) Neurobiological pathways to Alzheimer's disease: Amyloid-beta, TAU protein or both? *Dement Neuropsychol* **3**:188–194.

Deane R, Bell RD, Sagare A, and Zlokovic BV (2009) Clearance of amyloid-beta peptide across the blood-brain barrier: implication for therapies in Alzheimer's disease. *CNS Neurol Disord Drug Targets* **8**:16–30.

Dementia (n.d.).

Dementia statistics | Alzheimer's Disease International (n.d.).

Dheen ST, Kaur C, and Ling E-A (2007) Microglial activation and its implications in the brain diseases. *Curr Med Chem* **14**:1189–1197.

Doens D, and Fernández PL (2014) Microglia receptors and their implications in the response to amyloid β for Alzheimer's disease pathogenesis. *J Neuroinflammation* **11**:48.

Duan R-S, Yang X, Chen Z-G, Lu M-O, Morris C, Winblad B, and Zhu J (2008) Decreased fractalkine and increased IP-10 expression in aged brain of APP(swe) transgenic mice. *Neurochem Res* **33**:1085–1089.

Duara R, Lopez-Alberola RF, Barker WW, Loewenstein DA, Zatinsky M, Eisdorfer CE, and Weinberg GB (1993) A comparison of familial and sporadic Alzheimer's disease. *Neurology* **43**:1377–1384.

Dyrks T, Weidemann A, Multhaup G, Salbaum JM, Lemaire HG, Kang J, Müller-Hill B, Masters CL, and Beyreuther K (1988) Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. *EMBO J* **7**:949–957.

El Khoury J, Hickman SE, Thomas CA, Cao L, Silverstein SC, and Loike JD (1996) Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. *Nature* **382**:716–719.

El Khoury J, and Luster AD (2008) Mechanisms of microglia accumulation in Alzheimer's disease: therapeutic implications. *Trends Pharmacol Sci* **29**:626–632.

Famenini S, Rigali EA, Olivera-Perez HM, Dang J, Chang MT, Halder R, Rao RV, Pellegrini M, Porter V, Bredesen D, and Fiala M (2017) Increased intermediate M1-M2 macrophage polarization and improved cognition in mild cognitive impairment patients on ω-3 supplementation. *FASEB J* **31**:148–160.

Fani Maleki A, and Rivest S (2019) Innate Immune Cells: Monocytes, Monocyte-Derived Macrophages and Microglia as Therapeutic Targets for Alzheimer's Disease and Multiple Sclerosis. *Front Cell Neurosci* **13**.

Fiala M, Cribbs DH, Rosenthal M, and Bernard G (2007) Phagocytosis of amyloid-beta and inflammation: two faces of innate immunity in Alzheimer's disease. *J Alzheimers Dis JAD* **11**:457–463.

Fiala M, Lin J, Ringman J, Kermani-Arab V, Tsao G, Patel A, Lossinsky AS, Graves MC, Gustavson A, Sayre J, Sofroni E, Suarez T, Chiappelli F, and Bernard G (2005) Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. *J Alzheimers Dis JAD* **7**:221–232; discussion 255-262.

Fiala M, Liu PT, Espinosa-Jeffrey A, Rosenthal MJ, Bernard G, Ringman JM, Sayre J, Zhang L, Zaghi J, Dejbakhsh S, Chiang B, Hui J, Mahanian M, Baghaee A, Hong P, and Cashman J (2007) Innate immunity and transcription of MGAT-III and Toll-like receptors in Alzheimer's disease patients are improved by bisdemethoxycurcumin. *Proc Natl Acad Sci* **104**:12849–12854.

Francis PT, Palmer AM, Snape M, and Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry* **66**:137–147.

Frenkel D, Wilkinson K, Zhao L, Hickman SE, Means TK, Puckett L, Farfara D, Kingery ND, Weiner HL, and El Khoury J (2013) Scara1 deficiency impairs clearance of soluble Amyloid-β by mononuclear phagocytes and accelerates Alzheimer's-like disease progression. *Nat Commun* **4**:2030.

Glenner GG, and Wong CW (1984) Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Commun* **122**:1131–1135.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, and James L (1991) Segregation of a missense mutation

in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**:704–706.

Goedert M, Spillantini MG, Jakes R, Rutherford D, and Crowther RA (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* **3**:519–526.

Gosselin D, and Rivest S (2007) Role of IL-1 and TNF in the brain: twenty years of progress on a Dr. Jekyll/Mr. Hyde duality of the innate immune system. *Brain Behav Immun* **21**:281–289.

Granic I, Dolga AM, Nijholt IM, van Dijk G, and Eisel ULM (2009) Inflammation and NF-kappaB in Alzheimer's disease and diabetes. *J Alzheimers Dis JAD* **16**:809–821.

Grathwohl SA, Kälin RE, Bolmont T, Prokop S, Winkelmann G, Kaeser SA, Odenthal J, Radde R, Eldh T, Gandy S, Aguzzi A, Staufenbiel M, Mathews PM, Wolburg H, Heppner FL, and Jucker M (2009) Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat Neurosci* **12**:1361–1363.

Guerreiro RJ, Gustafson DR, and Hardy J (2012) The genetic architecture of Alzheimer's disease: beyond APP, PSENs and APOE. *Neurobiol Aging* **33**:437–456.

Guo H, Callaway JB, and Ting JP-Y (2015) Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med* **21**:677–687.

Gurung P, and Kanneganti T-D (2015) SHARPIN, a novel regulator of the NLRP3 inflammasome (INM6P.334). *J Immunol* **194**:193.8-193.8.

Gurung P, Lamkanfi M, and Kanneganti T-D (2015) SHANK-associated RH domain interacting protein (SHARPIN) is required for optimal NLRP3 inflammasome activation. *J Immunol Baltim Md 1950* **194**:2064–2067.

Hanon O, Vidal J-S, Lehmann S, Bombois S, Allinquant B, Tréluyer J-M, Gelé P, Delmaire C, Blanc Frederic, Mangin J-F, Buée L, Touchon J, Hugon J, Vellas B, Galbrun E, Benetos A, Berrut G, Paillaud E, Wallon D, Castelnovo G, Volpe-Gillot L, Paccalin M, Robert P-H, Godefroy O, Dantoine T, Camus V, Belmin J, Vandel P, Novella J-L, Duron E, Rigaud A-S, Schraen-Maschke S, Gabelle A, Hanon O, Blanc Frédéric, Boudali Y, Gabelle A, Seux M, Lenoir H, Bayle C, Bombois S, Delbeuck X, Moulin F, Duron E, Latour F, Plichart M, Pichierri S, Orvoën G, Galbrun E, Castelnovo G, Volpe–Gillot L, Labourée F, Cassagnaud P, Paquet C, Lala F, Dumurgier J, Rigaud A, Perret–Guillaume C, Alonso E, du Boisgueheneuc F, Hugonot–Diener L, Rollin–Sillaire A, Martinaud O, Boully C, Spivac Y, Devendeville A, Belmin J, Robert P, Dantoine T, Caillard L, Wallon D, Hannequin D, Sastre N, Haffen S, Kearney–Schwartz A, Novella J, Deramecourt V, Chauvire V, Abitbol G, Schwald N, Hommet C, Sellal F, Cariot M, Abdellaoui M, Benisty S, Gherabli S, Anthony P, Bloch F, Charasz N, Chauvelier S, Gaubert J, Sacco G, Guerin O, Boddaert J, Paccalin M, Mackowiak M, Rabus M, Gissot V, et al. (2018) Plasma amyloid levels within the Alzheimer's process and correlations with central biomarkers. *Alzheimers Dement* **14**:858–868.

Hardy J, and Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci* **12**:383–388.

Hardy JA, and Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256**:184–185.

Hawkes CA, and McLaurin J (2009) Selective targeting of perivascular macrophages for clearance of beta-amyloid in cerebral amyloid angiopathy. *Proc Natl Acad Sci U S A* **106**:1261–1266.

Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Saecker A, Griep A, Axt D, Remus A, Tzeng T-C, Gelpi E, Halle A, Korte M, Latz E, and Golenbock D (2013) NLRP3 is activated in Alzheimer´s disease and contributes to pathology in APP/PS1 mice. *Nature* **493**:674–678.

Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A, Axt D, Remus A, Tzeng T-C, Gelpi E, Halle A, Korte M, Latz E, and Golenbock DT (2013) NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* **493**:674–678.

Hickman SE, Allison EK, and El Khoury J (2008) Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J Neurosci Off J Soc Neurosci* **28**:8354–8360.

Hippius H, and Neundörfer G (2003) The discovery of Alzheimer's disease. *Dialogues Clin Neurosci* **5**:101–108.

Hohsfield LA, and Humpel C (2015) Migration of blood cells to β-amyloid plaques in Alzheimer's disease. *Exp Gerontol* **65**:8–15.

Hsieh CL, Koike M, Spusta SC, Niemi EC, Yenari M, Nakamura MC, and Seaman WE (2009) A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem* **109**:1144–1156.

Hughes CP, Berg L, Danziger WL, Coben LA, and Martin RL (1982) A new clinical scale for the staging of dementia. *Br J Psychiatry J Ment Sci* **140**:566–572.

Ikeda F, Deribe YL, Skånland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, van Wijk SJL, Goswami P, Nagy V, Terzic J, Tokunaga F, Androulidaki A, Nakagawa T, Pasparakis M, Iwai K, Sundberg JP, Schaefer L, Rittinger K, Macek B, and Dikic I (2011a) SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. *Nature* **471**:637–641.

Ikeda F, Deribe YL, Skånland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, van Wijk SJL, Goswami P, Nagy V, Terzic J, Tokunaga F, Androulidaki A, Nakagawa T, Pasparakis M, Iwai K, Sundberg JP, Schaefer L, Rittinger K, Macek B, and Dikic I (2011b) SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. *Nature* **471**:637–641.

Iqbal K, and Grundke-Iqbal I (2008) Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention. *J Cell Mol Med* **12**:38–55.

Iram T, and Frenkel D (2012) Targeting the Role of Astrocytes in the Progression of Alzheimers Disease. *Curr Signal Transduct Ther* **7**:20–27.

Irwin DJ (2016) Tauopathies as Clinicopathological Entities. *Parkinsonism Relat Disord* **22**:S29–S33.

Jairani PS, Aswathy PM, Krishnan D, Menon RN, Verghese J, Mathuranath PS, and Gopala S (2019a) Apolipoprotein E Polymorphism and Oxidative Stress in Peripheral Blood-Derived Macrophage-Mediated Amyloid-Beta Phagocytosis in Alzheimer's Disease Patients. *Cell Mol Neurobiol* **39**:355–369.

Jairani PS, Aswathy PM, Krishnan D, Menon RN, Verghese J, Mathuranath PS, and Gopala S (2019b) Apolipoprotein E Polymorphism and Oxidative Stress in Peripheral Blood-Derived Macrophage-Mediated Amyloid-Beta Phagocytosis

in Alzheimer's Disease Patients. *Cell Mol Neurobiol*, doi: 10.1007/s10571-019-00651-1.

Janelidze S, Stomrud E, Palmqvist S, Zetterberg H, van Westen D, Jeromin A, Song L, Hanlon D, Tan Hehir CA, Baker D, Blennow K, and Hansson O (2016) Plasma β-amyloid in Alzheimer's disease and vascular disease. *Sci Rep* **6**.

Jarosz-Griffiths HH, Noble E, Rushworth JV, and Hooper NM (2016) Amyloid-β Receptors: The Good, the Bad, and the Prion Protein. *J Biol Chem* **291**:3174–3183.

Jiang Q, Heneka M, and Landreth GE (2008) The role of peroxisome proliferator-activated receptor-gamma (PPARgamma) in Alzheimer's disease: therapeutic implications. *CNS Drugs* **22**:1–14.

Jofre-Monseny L, Loboda A, Wagner AE, Huebbe P, Boesch-Saadatmandi C, Jozkowicz A, Minihane A-M, Dulak J, and Rimbach G (2007) Effects of apoE genotype on macrophage inflammation and heme oxygenase-1 expression. *Biochem Biophys Res Commun* **357**:319–324.

Kabe Y, Ando K, Hirao S, Yoshida M, and Handa H (2005) Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal* **7**:395–403.

Kanekiyo T, and Bu G (2014) The low-density lipoprotein receptor-related protein 1 and amyloid-β clearance in Alzheimer's disease. *Front Aging Neurosci* **6**.

Kasirer-Friede A, Tjahjono W, Eto K, and Shattil SJ (2019) SHARPIN at the nexus of integrin, immune, and inflammatory signaling in human platelets. *Proc Natl Acad Sci* **116**:4983–4988.

Lai AY, and McLaurin J (2012) Clearance of amyloid-β peptides by microglia and macrophages: the issue of what, when and where. *Future Neurol* **7**:165–176.

Lancour D, Naj A, Mayeux R, Haines JL, Pericak-Vance MA, Schellenberg GD, Crovella M, Farrer LA, and Kasif S (2018) One for all and all for One: Improving replication of genetic studies through network diffusion. *PLOS Genet* **14**:e1007306.

Landreth GE, and Reed-Geaghan EG (2009) Toll-like receptors in Alzheimer's disease. *Curr Top Microbiol Immunol* **336**:137–153.

Lazarevic V, Fieńko S, Andres-Alonso M, Anni D, Ivanova D, Montenegro-Venegas C, Gundelfinger ED, Cousin MA, and Fejtova A (2017) Physiological Concentrations of Amyloid Beta Regulate Recycling of Synaptic Vesicles via Alpha7 Acetylcholine Receptor and CDK5/Calcineurin Signaling. *Front Mol Neurosci* **10**:221.

Leal MC, Dorfman VB, Gamba AF, Frangione B, Wisniewski T, Castaño EM, Sigurdsson EM, and Morelli L (2006) Plaque-associated overexpression of insulin-degrading enzyme in the cerebral cortex of aged transgenic tg2576 mice with Alzheimer pathology. *J Neuropathol Exp Neurol* **65**:976–987.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, and Wang K (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* **269**:973–977.

Ley K (2017) M1 Means Kill; M2 Means Heal. *J Immunol* **199**:2191–2193.

Li J, Lai Y, Cao Y, Du T, Zeng L, Wang G, Chen X, Chen J, Yu Y, Zhang S, Zhang Y, Huang H, and Guo Z (2015) SHARPIN overexpression induces tumorigenesis in human prostate cancer LNCaP, DU145 and PC-3 cells via

NF-κB/ERK/Akt signaling pathway. *Med Oncol Northwood Lond Engl* **32**:444–444.

Li R, Strohmeyer R, Liang Z, Lue L-F, and Rogers J (2004) CCAAT/enhancer binding protein delta (C/EBPdelta) expression and elevation in Alzheimer's disease. *Neurobiol Aging* **25**:991–999.

Lim S, Sala C, Yoon J, Park S, Kuroda S, Sheng M, and Kim E (2001) Sharpin, a novel postsynaptic density protein that directly interacts with the shank family of proteins. *Mol Cell Neurosci* **17**:385–397.

Liu B, Zhu Y, Zhou J, Wei Y, Long C, Chen M, Ling Y, Ge J, and Zhuo Y (2014) Endoplasmic reticulum stress promotes amyloid-beta peptides production in RGC-5 cells. *Cell Stress Chaperones* **19**:827–835.

Liu C-C, Kanekiyo T, Xu H, and Bu G (2013) Apolipoprotein E and Alzheimer disease: risk, mechanisms, and therapy. *Nat Rev Neurol* **9**:106–118.

Liu M, Bian C, Zhang J, and Wen F (2014) Apolipoprotein E gene polymorphism and Alzheimer's disease in Chinese population: a meta-analysis. *Sci Rep* **4**:4383.

Luber-Narod J, and Rogers J (1988) Immune system associated antigens expressed by cells of the human central nervous system. *Neurosci Lett* **94**:17–22.

Lue LF, Rydel R, Brigham EF, Yang LB, Hampel H, Murphy GM, Brachova L, Yan SD, Walker DG, Shen Y, and Rogers J (2001) Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia in vitro. *Glia* **35**:72–79.

Lue L-F, Walker DG, Jacobson S, and Sabbagh M (2009) Receptor for advanced glycation end products. *Future Neurol* **4**:167–177.

Lund H, Zhang X, and Harris R (2014) Regulation of amyloid beta oligomer phagocytosis by M1 and M2 macrophage polarization. *J Neuroimmunol* **275**:87–88.

Maccioni RB, Farías G, Morales I, and Navarrete L (2010) The Revitalized Tau Hypothesis on Alzheimer's Disease. *Arch Med Res* **41**:226–231.

Majumdar A, Chung H, Dolios G, Wang R, Asamoah N, Lobel P, and Maxfield FR (2008) Degradation of fibrillar forms of Alzheimer's amyloid beta-peptide by macrophages. *Neurobiol Aging* **29**:707–715.

Malm TM, Koistinaho M, Pärepalo M, Vatanen T, Ooka A, Karlsson S, and Koistinaho J (2005) Bone-marrow-derived cells contribute to the recruitment of microglial cells in response to beta-amyloid deposition in APP/PS1 double transgenic Alzheimer mice. *Neurobiol Dis* **18**:134–142.

Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, and Latz E (2018) Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov* **17**:588–606.

Masoumi A, Goldenson B, Ghirmai S, Avagyan H, Zaghi J, Abel K, Zheng X, Espinosa-Jeffrey A, Mahanian M, Liu PT, Hewison M, Mizwickie M, Cashman J, and Fiala M (2009) 1alpha,25-dihydroxyvitamin D3 interacts with curcuminoids to stimulate amyloid-beta clearance by macrophages of Alzheimer's disease patients. *J Alzheimers Dis JAD* **17**:703–717.

Mathuranath PS, Cherian JP, Mathew R, George A, Alexander A, and Sarma SP (2007) Mini mental state examination and the Addenbrooke's cognitive examination: effect of education and norms for a multicultural population. *Neurol India* **55**:106–110.

Mathuranath PS, Hodges JR, Mathew R, Cherian PJ, George A, and Bak TH (2004) Adaptation of the ACE for a Malayalam speaking population in southern India. *Int J Geriatr Psychiatry* **19**:1188–1194.

Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, and Bateman RJ (2010) Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science* **330**:1774.

McGeer PL, Itagaki S, and McGeer EG (1988) Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. *Acta Neuropathol (Berl)* **76**:550–557.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, and Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**:939–944.

Melo JD, and Tang D (2015) Elevation of SIPL1 (SHARPIN) Increases Breast Cancer Risk. *PLOS ONE* **10**:e0127546.

Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, de Calignon A, Rozkalne A, Koenigsknecht-Talboo J, Holtzman DM, Bacskai BJ, and Hyman BT (2008) Rapid appearance and local toxicity of amyloid-beta plaques in a mouse model of Alzheimer's disease. *Nature* **451**:720–724.

Michell-Robinson MA, Touil H, Healy LM, Owen DR, Durafourt BA, Bar-Or A, Antel JP, and Moore CS (2015) Roles of microglia in brain development, tissue maintenance and repair. *Brain J Neurol* **138**:1138–1159.

Mott RT, Ait-Ghezala G, Town T, Mori T, Vendrame M, Zeng J, Ehrhart J, Mullan M, and Tan J (2004) Neuronal expression of CD22: novel mechanism for inhibiting microglial proinflammatory cytokine production. *Glia* **46**:369–379.

Mouton-Liger F, Paquet C, Dumurgier J, Bouras C, Pradier L, Gray F, and Hugon J (2012) Oxidative stress increases BACE1 protein levels through activation of the PKR-eIF2α pathway. *Biochim Biophys Acta BBA - Mol Basis Dis* **1822**:885–896.

Moynagh PN (2005) The interleukin-1 signalling pathway in astrocytes: a key contributor to inflammation in the brain. *J Anat* **207**:265–269.

MUDr. Dana Maňasková (n.d.).

Naert G, and Rivest S (2011) CC chemokine receptor 2 deficiency aggravates cognitive impairments and amyloid pathology in a transgenic mouse model of Alzheimer's disease. *J Neurosci Off J Soc Neurosci* **31**:6208–6220.

O'Brien RJ, and Wong PC (2011) Amyloid Precursor Protein Processing and Alzheimer's Disease. *Annu Rev Neurosci* **34**:185–204.

Okamura N, and Yanai K (2017) Brain imaging: Applications of tau PET imaging. *Nat Rev Neurol* **13**:197–198.

Pan X-D, Zhu Y-G, Lin N, Zhang J, Ye Q-Y, Huang H-P, and Chen X-C (2011) Microglial phagocytosis induced by fibrillar β-amyloid is attenuated by oligomeric β-amyloid: implications for Alzheimer's disease. *Mol Neurodegener* **6**:45.

Parajuli B, Sonobe Y, Horiuchi H, Takeuchi H, Mizuno T, and Suzumura A (2013) Oligomeric amyloid β induces IL-1β processing via production of ROS: implication in Alzheimer's disease. *Cell Death Dis* **4**:e975.

Paresce DM, Ghosh RN, and Maxfield FR (1996) Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron* **17**:553–565.

Perlmutter LS (1994) Microvascular pathology and vascular basement membrane components in Alzheimer's disease. *Mol Neurobiol* **9**:33–40.

Perry VH, and Teeling J (2013) Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Semin Immunopathol* **35**:601–612.

Petersen RC (2009) Early Diagnosis of Alzheimer's Disease: Is MCI Too Late? *Curr Alzheimer Res* **6**:324–330.

Petersen RC (2004) Mild cognitive impairment as a diagnostic entity. *J Intern Med* **256**:183–194.

Priller C, Bauer T, Mitteregger G, Krebs B, Kretzschmar HA, and Herms J (2006) Synapse formation and function is modulated by the amyloid precursor protein. *J Neurosci Off J Soc Neurosci* **26**:7212–7221.

Pryor NE, Moss MA, and Hestekin CN (2012) Unraveling the Early Events of Amyloid-β Protein (Aβ) Aggregation: Techniques for the Determination of Aβ Aggregate Size. *Int J Mol Sci* **13**:3038–3072.

Rantala JK, Pouwels J, Pellinen T, Veltel S, Laasola P, Mattila E, Potter CS, Duffy T, Sundberg JP, Kallioniemi O, Askari JA, Humphries MJ, Parsons M, Salmi M, and Ivaska J (2011) SHARPIN is an endogenous inhibitor of β1-integrin activation. *Nat Cell Biol* **13**:1315–1324.

Reed-Geaghan EG, Savage JC, Hise AG, and Landreth GE (2009) CD14 and toll-like receptors 2 and 4 are required for fibrillar A{beta}-stimulated microglial activation. *J Neurosci Off J Soc Neurosci* **29**:11982–11992.

Ries M, and Sastre M (2016) Mechanisms of Aβ Clearance and Degradation by Glial Cells. *Front Aging Neurosci* **8**.

Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, and Peterson PK (2004) Role of microglia in central nervous system infections. *Clin Microbiol Rev* **17**:942–964, table of contents.

Rodríguez JJ, Olabarria M, Chvatal A, and Verkhratsky A (2009) Astroglia in dementia and Alzheimer's disease. *Cell Death Differ* **16**:378–385.

Rogers J, Luber-Narod J, Styren SD, and Civin WH (1988) Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol Aging* **9**:339–349.

Rogers J, Mastroeni D, Leonard B, Joyce J, and Grover A (2007) Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? *Int Rev Neurobiol* **82**:235–246.

Rossor MN, Fox NC, Freeborough PA, and Harvey RJ (1996) Clinical features of sporadic and familial Alzheimer's disease. *Neurodegener J Neurodegener Disord Neuroprotection Neuroregeneration* **5**:393–397.

Saido T, and Leissring MA (2012) Proteolytic Degradation of Amyloid β-Protein. *Cold Spring Harb Perspect Med* **2**.

Saresella M, La Rosa F, Piancone F, Zoppis M, Marventano I, Calabrese E, Rainone V, Nemni R, Mancuso R, and Clerici M (2016) The NLRP3 and NLRP1 inflammasomes are activated in Alzheimer's disease. *Mol Neurodegener* **11**.

Saresella M, Marventano I, Calabrese E, Piancone F, Rainone V, Gatti A, Alberoni M, Nemni R, and Clerici M (2014) A complex proinflammatory role for peripheral monocytes in Alzheimer's disease. *J Alzheimers Dis JAD* **38**:403–413.

Sastre M, Richardson JC, Gentleman SM, and Brooks DJ (2011) Inflammatory risk factors and pathologies associated with Alzheimer's disease. *Curr Alzheimer Res* **8**:132–141.

Schachter AS, and Davis KL (2000) Alzheimer's disease. *Dialogues Clin Neurosci* **2**:91–100.

Schmitz A, Tikkanen R, Kirfel G, and Herzog V (2002) The biological role of the Alzheimer amyloid precursor protein in epithelial cells. *Histochem Cell Biol* **117**:171–180.

Schubert D, Jin LW, Saitoh T, and Cole G (1989) The regulation of amyloid beta protein precursor secretion and its modulatory role in cell adhesion. *Neuron* **3**:689–694.

Seppälä TT, Herukka S-K, Hänninen T, Tervo S, Hallikainen M, Soininen H, and Pirttilä T (2010) Plasma Aβ42 and Aβ40 as markers of cognitive change in follow-up: a prospective, longitudinal, population-based cohort study. *J Neurol Neurosurg Psychiatry* **81**:1123–1127.

Sharma D, and Kanneganti T-D (2016) The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *J Cell Biol* **213**:617–629.

Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, Becker CE, Ediriweera HN, Mullick AE, Golenbock DT, Stuart LM, Latz E, Fitzgerald KA, and Moore KJ (2013) CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat Immunol* **14**:812–820.

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HA, Haines JL, Perkicak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, and St George-Hyslop PH (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* **375**:754–760.

Shimizu H, Sakimoto T, and Yamagami S (2019) Pro-inflammatory role of NLRP3 inflammasome in experimental sterile corneal inflammation. *Sci Rep* **9**:1–11.

Simard AR, Soulet D, Gowing G, Julien J-P, and Rivest S (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron* **49**:489–502.

Singhrao SK, Neal JW, Rushmere NK, Morgan BP, and Gasque P (1999) Differential expression of individual complement regulators in the brain and choroid plexus. *Lab Investig J Tech Methods Pathol* **79**:1247–1259.

Sofroniew MV, and Vinters HV (2010) Astrocytes: biology and pathology. *Acta Neuropathol (Berl)* **119**:7–35.

Soheili-Nezhad S, Jahanshad N, Guelfi S, Khosrowabadi R, Saykin AJ, Thompson PM, Beckmann CF, Sprooten E, Zarei M, and Initiative for the ADN (2019) A Non-Synonymous SHARPIN Variant is Associated with Limbic Degeneration and Family History of Alzheimer's Disease. *bioRxiv* 196410.

Song L, Pei L, Yao S, Wu Y, and Shang Y (2017) NLRP3 Inflammasome in Neurological Diseases, from Functions to Therapies. *Front Cell Neurosci* **11**.

Stieglitz B, Haire LF, Dikic I, and Rittinger K (2012) Structural analysis of SHARPIN, a subunit of a large multi-protein E3 ubiquitin ligase, reveals a novel dimerization function for the pleckstrin homology superfold. *J Biol Chem* **287**:20823–20829.

Sweeney MD, Sagare AP, and Zlokovic BV (2018) Blood–brain barrier breakdown in Alzheimer's disease and other neurodegenerative disorders. *Nat Rev Neurol* **14**:133–150.

Tabaton M, Zhu X, Perry G, Smith MA, and Giliberto L (2010) Signaling Effect of Amyloid-β42 on the Processing of AβPP. *Exp Neurol* **221**:18–25.

Tachibana M, Holm M-L, Liu C-C, Shinohara M, Aikawa T, Oue H, Yamazaki Y, Martens YA, Murray ME, Sullivan PM, Weyer K, Glerup S, Dickson DW, Bu G, and Kanekiyo T (2019) APOE4-mediated amyloid-β pathology depends on its neuronal receptor LRP1. *J Clin Invest* **129**:1272–1277.

Tai LM, Mehra S, Shete V, Estus S, Rebeck GW, Bu G, and LaDu MJ (2014) Soluble apoE/Aβ complex: mechanism and therapeutic target for APOE4-induced AD risk. *Mol Neurodegener* **9**:2.

Tamiya H, Kim Hyungsoo, Klymenko O, Kim Heejung, Feng Y, Zhang T, Han JY, Murao A, Snipas SJ, Jilaveanu L, Brown K, Kluger H, Zhang H, Iwai K, and Ronai ZA (2018) SHARPIN-mediated regulation of protein arginine methyltransferase 5 controls melanoma growth. *J Clin Invest* **128**:517–530.

Tan M-S, Yu J-T, Jiang T, Zhu X-C, Wang H-F, Zhang W, Wang Y-L, Jiang W, and Tan L (2013) NLRP3 polymorphisms are associated with late-onset Alzheimer's disease in Han Chinese. *J Neuroimmunol* **265**:91–95.

Tanaka Y, Tateishi K, Nakatsuka T, Kudo Y, Takahashi R, Miyabayashi K, Yamamoto K, Asaoka Y, Ijichi H, Tateishi R, Shibahara J, Fukayama M, Ishizawa T, Hasegawa K, Kokudo N, and Koike K (2016) Sharpin promotes hepatocellular carcinoma progression via transactivation of Versican expression. *Oncogenesis* **5**:e277–e277.

Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler TD, Fieremans E, Axel L, Rusinek H, Nicholson C, Zlokovic BV, Frangione B, Blennow K, Ménard J, Zetterberg H, Wisniewski T, and Leon MJ de (2015) Clearance systems in the brain—implications for Alzheimer diseaser. *Nat Rev Neurol* **12**:248–248.

Thériault P, ElAli A, and Rivest S (2015) The dynamics of monocytes and microglia in Alzheimer's disease. *Alzheimers Res Ther* **7**:41.

Tian L, Zhang K, Tian Z-Y, Wang T, Shang D-S, Li B, Liu D-X, Fang W-G, Wang Z-Y, and Chen Y-H (2014) Decreased expression of cathepsin D in monocytes is related to the defective degradation of amyloid-β in Alzheimer's disease. *J Alzheimers Dis JAD* **42**:511–520.

Tiribuzi R, Crispoltoni L, Porcellati S, Di Lullo M, Florenzano F, Pirro M, Bagaglia F, Kawarai T, Zampolini M, Orlacchio Aldo, and Orlacchio Antonio (2014)

miR128 up-regulation correlates with impaired amyloid β(1-42) degradation in monocytes from patients with sporadic Alzheimer's disease. *Neurobiol Aging* **35**:345–356.

Tokunaga F, and Iwai K (2012) LUBAC, a novel ubiquitin ligase for linear ubiquitination, is crucial for inflammation and immune responses. *Microbes Infect* **14**:563–572.

Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, Sakata S, Tanaka K, Nakano H, and Iwai K (2011) SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. *Nature* **471**:633–636.

Toledo JB, Shaw LM, and Trojanowski JQ (2013) Plasma amyloid beta measurements - a desired but elusive Alzheimer's disease biomarker. *Alzheimers Res Ther* **5**:8.

Turner PR, O'Connor K, Tate WP, and Abraham WC (2003) Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog Neurobiol* **70**:1–32.

Van Eldik LJ, Carrillo MC, Cole PE, Feuerbach D, Greenberg BD, Hendrix JA, Kennedy M, Kozauer N, Margolin RA, Molinuevo JL, Mueller R, Ransohoff RM, Wilcock DM, Bain L, and Bales K (2016) The roles of inflammation and immune mechanisms in Alzheimer's disease. *Alzheimers Dement Transl Res Clin Interv* **2**:99–109.

Von Bernhardi R, Eugenin-von Bernhardi L, and Eugenin J (2015) Microglial cell dysregulation in Brain Aging and Neurodegeneration. *Front Aging Neurosci* **7**.

Walker DG, Dalsing-Hernandez JE, Campbell NA, and Lue L-F (2009) Decreased expression of CD200 and CD200 receptor in Alzheimer's disease: a potential mechanism leading to chronic inflammation. *Exp Neurol* **215**:5–19.

Walker DG, and Lue L-F (2005) Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases. *J Neurosci Res* **81**:412–425.

Wang D-S, Dickson DW, and Malter JS (2006) β-Amyloid Degradation and Alzheimer's Disease. *J Biomed Biotechnol* **2006**.

Wang Y, Zhou TH, Zhi Z, Barakat A, Hlatky L, and Querfurth H (2013) Multiple effects of β-amyloid on single excitatory synaptic connections in the PFC. *Front Cell Neurosci* **7**:129.

Wang Z, Potter CS, Sundberg JP, and Hogenesch H (2012a) SHARPIN is a key regulator of immune and inflammatory responses. *J Cell Mol Med* **16**:2271–2279.

Wang Z, Potter CS, Sundberg JP, and Hogenesch H (2012b) SHARPIN is a key regulator of immune and inflammatory responses. *J Cell Mol Med* **16**:2271–2279.

Wegiel J, Wang KC, Imaki H, Rubenstein R, Wronska A, Osuchowski M, Lipinski WJ, Walker LC, and LeVine H (2001) The role of microglial cells and astrocytes in fibrillar plaque evolution in transgenic APP(SW) mice. *Neurobiol Aging* **22**:49–61.

What are the Three Stages of Alzheimer's Disease | SeniorDirectory.com (n.d.).

White JA, Manelli AM, Holmberg KH, Van Eldik LJ, and Ladu MJ (2005) Differential effects of oligomeric and fibrillar amyloid-beta 1-42 on astrocyte-mediated inflammation. *Neurobiol Dis* **18**:459–465.

Wildsmith KR, Holley M, Savage JC, Skerrett R, and Landreth GE (2013) Evidence for impaired amyloid β clearance in Alzheimer's disease. *Alzheimers Res Ther* **5**:33.

Wilkinson K, and El Khoury J (2012) Microglial scavenger receptors and their roles in the pathogenesis of Alzheimer's disease. *Int J Alzheimers Dis* **2012**:489456.

Wu M, Nissen JC, Chen EI, and Tsirka SE (2012) Tuftsin Promotes an Anti-Inflammatory Switch and Attenuates Symptoms in Experimental Autoimmune Encephalomyelitis. *PLoS ONE* **7**.

Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, and Husemann J (2003) Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. *Nat Med* **9**:453–457.

Wyss-Coray T, and Mucke L (2002) Inflammation in neurodegenerative disease--a double-edged sword. *Neuron* **35**:419–432.

Yan SS, Chen D, Yan S, Guo L, and Chen JX (2012) RAGE is a key cellular target for Aβ-induced perturbation in Alzheimer's disease. *Front Biosci Sch Ed* **4**:240–250.

Yang H, Yu S, Wang W, Li X, Hou Y, Liu Z, Shi Y, Mu K, Niu G, Xu J, Wang H, Zhu J, and Zhuang T (2017) SHARPIN Facilitates p53 Degradation in Breast Cancer Cells. *Neoplasia N Y N* **19**:84–92.

Yang LB, Li R, Meri S, Rogers J, and Shen Y (2000) Deficiency of complement defense protein CD59 may contribute to neurodegeneration in Alzheimer's disease. *J Neurosci Off J Soc Neurosci* **20**:7505–7509.

Yin J, Zhao F, Chojnacki JE, Fulp J, Klein WL, Zhang S, and Zhu X (2018) NLRP3 Inflammasome Inhibitor Ameliorates Amyloid Pathology in a Mouse Model of Alzheimer's Disease. *Mol Neurobiol* **55**:1977–1987.

Yin K-J, Cirrito JR, Yan P, Hu X, Xiao Q, Pan X, Bateman R, Song H, Hsu F-F, Turk J, Xu J, Hsu CY, Mills JC, Holtzman DM, and Lee J-M (2006) Matrix Metalloproteinases Expressed by Astrocytes Mediate Extracellular Amyloid-β Peptide Catabolism. *J Neurosci* **26**:10939–10948.

Zetterberg H, and Burnham SC (2019) Blood-based molecular biomarkers for Alzheimer's disease. *Mol Brain* **12**:26.

Zhang X, Li Y, Xu H, and Zhang Y (2014) The γ-secretase complex: from structure to function. *Front Cell Neurosci* **8**.

Zhao L, Lin S, Bales KR, Gelfanova V, Koger D, DeLong C, Hale J, Liu F, Hunter JM, and Paul SM (2009) Macrophage-Mediated Degradation of β-Amyloid via an Apolipoprotein E Isoform-Dependent Mechanism. *J Neurosci* **29**:3603–3612.

Zhou S, Liang Y, Zhang X, Liao L, Yang Y, Ouyang W, and Xu H (2019) SHARPIN Promotes Melanoma Progression via Rap1 Signaling Pathway. *J Invest Dermatol*, doi: 10.1016/j.jid.2019.07.696.

Zlokovic BV (2011) Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat Rev Neurosci* **12**:723–738.

Zuroff L, Daley D, Black KL, and Koronyo-Hamaoui M (2017) Clearance of cerebral Aβ in Alzheimer's disease: reassessing the role of microglia and monocytes. *Cell Mol Life Sci* **74**:2167–2201.

VIII. Annexures

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A novel role for SHARPIN in amyloid-β phagocytosis and inflammation by peripheral blood-derived macrophages in Alzheimer's disease

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ABSTRACT

Defective immune cell-mediated clearance of amyloid-beta (Aβ) and Aβ-associated inflammatory activation of immune cells are key contributors in pathogenesis of Alzheimer's disease (AD). However, the underlying mechanisms remain elusive. Shank-associated RH domain-interacting protein (SHARPIN) is a critical regulator of inflammatory response. Using in vitro cultures of THP-1-derived macrophages exposed to Aβ and AD patient–derived macrophages, we demonstrate the role of SHARPIN as an obligate regulator of Aβ phagocytosis and inflammation in macrophages. Specifically, Aβ-stimulated SHARPIN in THP-1 macrophages promoted Aβ phagocytosis and expression of proinflammatory markers. In addition, Aβ-stimulated SHARPIN in macrophages promoted neuronal cell-death in differentiated SHSY5Y neurons. Furthermore, we report a novel regulatory link between SHARPIN and the NLRP3 inflammasome in response to Aβ in THP-1 macrophages. In line with our in vitro observations, a strong positive association was demonstrated between levels of $A\beta_{42}$ in blood plasma of mild cognitive impairment and AD patients with SHARPIN expression in macrophages obtained from respective patient-derived peripheral blood mononuclear cells. Together, our findings show SHARPIN as a critical determinant in mediating macrophage response to Aβ and pathogenesis of AD.

1. Introduction

Alzheimer's disease (AD) is characterized by the accumulation of amyloid-beta (Aβ) and hyperphosphorylated tau proteins in the brain (de Paula et al., 2009). During normal physiology, a homeostatic balance is maintained between Aβ production and degradation (Baranello et al., 2015) and is effectuated mainly by immune cells, namely microglia in the brain and macrophages and monocytes in the peripheral circulation (Wang et al., 2006; Zhao et al., 2009). Under certain conditions, mostly associated with aging, these cells fail to phagocytose Aβ leading to a dysregulation in the balance between Aβ production and degradation (Mawuenyega et al., 2010; Wildsmith et al., 2013). In the long term, excessive Aβ accumulation accompanied by reduced degradation leads to chronic inflammatory activation of macrophages and microglia, causing neuronal death, resulting in the progression of AD (Rogers et al., 2007; Van Eldik et al., 2016). Importantly, although macrophage-mediated Aβ phagocytosis and inflammation constitute pathophysiological hallmarks that promote the progression of AD, mechanisms that contribute to the dysfunction of these key processes in macrophages in a setting of AD remain insufficiently addressed.

In this regard, SHARPIN (Shank-associated RH domain-interacting protein) is reported as a critical regulator of the inflammatory response. Prior studies have reported *Sharpin* mutations to result in chronic inflammation and demonstrated the role of SHARPIN in activation of the NF-κB signaling pathway (Gurung et al., 2015, p. 3; Tokunaga et al., 2011). A recent study identified a rare functional variant of SHARPIN as a genetic risk factor for late-onset Alzheimer's disease (LOAD) (Asanomi et al., 2019). However, although it is known that SHARPIN plays a pivotal role in the macrophage-driven inflammatory response, mechanisms that link SHARPIN in macrophages to the pathology of AD, a predominantly inflammatory-driven disease, have been underexplored.

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In the present study, we sought to address the role of SHARPIN and its impact on macrophage function in AD. Specifically, we hypothesized a role for SHARPIN in mediating Aβ phagocytosis and consequent activation of proinflammatory signaling through promoting polarization of macrophages to an M1 phenotype. To this end, using a combination of in vitro studies and patient-derived samples, we present evidence of an obligate regulatory role for SHARPIN in macrophage-mediated Aβ phagocytosis and transition of macrophages to a proinflammatory phenotype. Together, the observation that SHARPIN-mediated actions regulate fundamental macrophage responses to Aβ stimuli conclusively point to a novel role for SHARPIN in macrophages as a critical determinant in the pathogenesis of AD.

2. Materials and methods

2.1. Inclusion of study subjects

AD and mild cognitive impairment (MCI) patients were recruited from the Memory & Neurobehavioral Clinic at the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Kerala, after obtaining institutional ethical clearance. All the recruited subjects were tested for hypertension, hyperlipidemia, hypercholesterolemia, vitamin B12 deficiency, thyroid dysfunction, diabetes, cardiopathy, cranial trauma, or other neurological disorders. Subjects with high plasma CRP level were excluded to avoid systemic inflammation-mediated alterations in protein expression. The diagnostic criteria of NINCDS–ADRDA (McKhann et al., 1984) were used to confirm AD and MCI pathology. The severity of AD was determined using the Clinical Dementia Rating Scale (Hughes et al., 1982). Using Peterson's criteria, preclinical cases of AD were classified as MCI (Petersen, 2004) if they scored between 1.5 and 2.0 standard deviations below the corresponding age group's population mean score on the memory tests (Mathuranath et al., 2007) on the Addenbrook's Cognitive Examination (Mathuranath et al., 2004) administered in their native language of Malayalam. The study population comprised a total of 63 individuals with 31 AD, 13 MCI, and 19 cognitively unimpaired control subjects. For isolation of monocytes, plasma and serum, blood samples (20 mL) were collected from all subjects by venepuncture with informed consent.

2.2. Isolation of monocytes from blood samples

Peripheral blood mononuclear cells (PBMCs) were isolated using the density gradient Ficoll-Paque (Sigma-Aldrich, St. Louis, MO, USA) medium from anticoagulated blood. Anticoagulated blood was layered on Ficoll-Paque medium and centrifuged at 2000 rpm for 20 minutes. The PBMCs, collected from the interface between the plasma and the density medium, were washed twice in 1X PBS and seeded and cultured for 14 days in RPMI-1640 medium supplemented with 10% autologous serum until differentiation into macrophages. Autologous serum was isolated by centrifugation of coagulated blood at 2500 rpm for 15 minutes, complement inactivated and filtered through 0.22 μm filter.

2.3. Cell culture and differentiation

THP-1 acute monocytic leukemia cells (obtained from National Centre for Cell Sciences, Pune) were cultured in RPMI-1640 medium supplemented with 10% FBS and were differentiated into macrophages by incubating with 100 nM phorbol 12-myristate 13-acetate for 48 hours. Macrophage differentiation was ascertained through characterization of monocyte and macrophage marker CD14 and CD68, respectively (Supplemental Fig. 1S).

SHSY5Y neuroblastoma cells (obtained from CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum) were cultured in RPMI-1640 medium supplemented with 10% FBS. Treatment with retinoic acid (RA) induces neuronal differentiation in SHSY5Y cells (Cheung et al., 2009; Korecka et al., 2013). At a confluency of 50%, the cells were treated with 10 μM retinoic acid (RA) in 1% FBS for 3–4 days for differentiation into mature neurons. Neuronal differentiation was confirmed by analysing the morphology and the decreased expression of neuronal stem cell marker, Nestin.

2.4. Aβ preparation

Lyophilized $A\beta_{1-42}$ and HiLyte Flour 488-labeled $A\beta_{1-42}$ (FITC-Aβ) (Abcam, Cambridge, UK and Anaspec Fremont, California, USA respectively) were reconstituted in 1% NH_4OH to achieve a stock concentration of 2 mg/mL. From the stock, a working concentration of 1 mg/mL was obtained through dilution in 1X PBS. Aβ thus prepared was analyzed using western blotting (Cerf et al., 2009; Pryor et al., 2012) and confirmed that Aβ prepared was in the cytotoxic soluble oligomeric form (Fig. 1A).

2.5. siRNA transfection

Differentiated THP-1 cells were transfected with SHARPIN siRNA or scrambled (scr) control siRNA (Cell Signaling Technology, Danvers, Massachusetts, USA) using jetPrime PolyPlus transfection reagent (Thermofischer Scientific, Waltham, Massachusetts, USA) as per the protocol. Silencing efficiency of the siRNA used was checked by probing for protein expression by western blot analysis.

2.6. Assessment of reactive oxygen species production

H_2DCFDA (dichlorofluorescin diacetate) assay was used to detect Aβ-induced oxidative stress in differentiated THP-1 cells. Intracellular reactive oxygen species (ROS) levels were quantified after preincubation with 10 mM N-acetyl cysteine (NAC) for 1 hour followed by treatment with Aβ (40 μM) for 12 hours. The cells were trypsinized and incubated with 10 μM H_2DCFDA for 1 hour at 37°C, washed, and subjected to FACS analysis.

2.7. Immunoprecipitation

Differentiated THP-1 cells were pretreated with NF-κB inhibitor—BAY 11-7082 for 1 hour followed by treatment with Aβ for 12 hours. The total protein was isolated using low-ionic isolation buffer. The protein fraction was incubated with primary antibody and protein-A–coated magnetic beads and pulled down by using magnetic field. The protein-bound magnetic beads were washed, incubated with 3X Laemmli buffer at 70 °C for 5 minutes and the purified protein was pulled down applying magnetic field.

2.8. Immunoblot analysis

Total protein was isolated using 1X RIPA buffer containing protease and phosphatase inhibitors and quantified using BCA assay. Denatured protein was loaded on SDS-PAGE gels for separation and transferred onto PVDF membranes, blocked with 5% skimmed milk and incubated with the respective antibodies in 3% bovine serum albumin at 4 °C. After overnight incubation, the blots were incubated with HRP-labeled secondary antibody and developed using enhanced chemiluminescence. The relative protein expression was quantified densitometrically using ImageJ software and normalized with β-actin or vinculin as loading control.

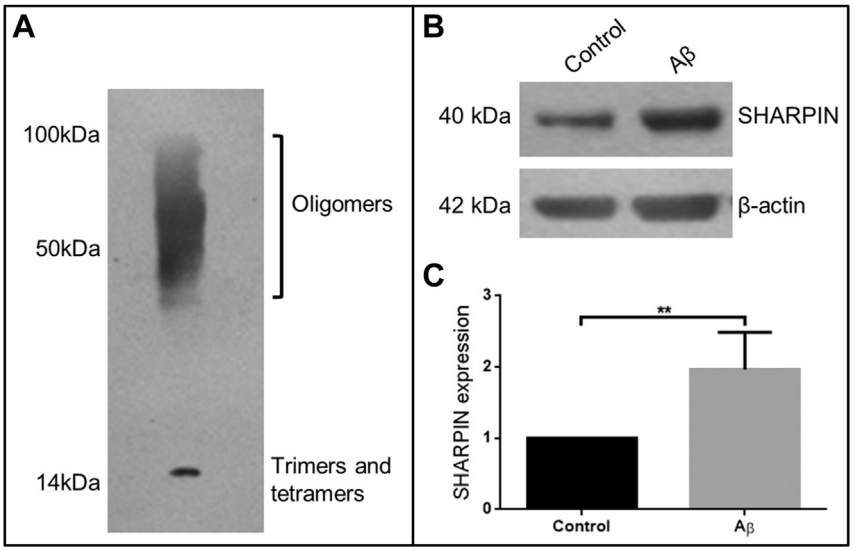

Fig. 1. Aβ enhances SHARPIN expression. Lyophilized $A\beta_{1-42}$ was dissolved in 1% NH_4OH to 2 mg/mL and then reconstituted in 1X PBS to 1 mg/mL. The prepared Aβ was analyzed by western blotting to confirm that the Aβ prepared was in the cytotoxic soluble (oligomeric, tetrameric, and trimeric) form (A). Differentiated THP-1 macrophages in RPMI-1640 medium supplemented with 1% FBS were treated with 10 μM Aβ for 6h and SHARPIN expression was analyzed by using western blotting (B), quantified, normalized with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium supplemented with 1% FBS), and represented graphically (fold change in expression: control vs. Aβ is 1: 1.97, n = 4) (C). β-actin was used as endogenous control. Statistical analysis—Student's *t-test* with $^{**}p > 0.01$.

2.9. mRNA expression analysis

Total RNA was isolated from differentiated THP-1 cells using the specified manufacturer's protocol (Invitrogen, Carlsbad, California, USA) and cDNA was synthesized from the isolated RNA. The mRNA expression was analyzed using TaqMan primers with human tubulin as internal control. The relative fold change in the mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

2.10. Analysis of cytokine release

The release of inflammatory cytokines and $A\beta_{40}$, $A\beta_{42}$ in plasma samples, and THP-1 cell conditioned media were analyzed using enzyme-linked immunosorbent assay (ELISA). The samples were pretreated as per the manufacturer's protocol (G-Biosciences, St. Louis, MO, USA) and incubated in specific antibody precoated wells. The wells were washed and incubated with primary antibody, HRP-conjugated secondary antibody and TMB substrate sequentially. The absorbance was read at 450 nm followed by estimation of the concentration from the standard curve.

2.11. Macrophage Aβ internalization assay

2.11.1. Primary cells

Monocytes isolated were cultured in RPMI 1640 medium supplemented with 10% autologous serum for 14 days until complete differentiation. Differentiated macrophages were incubated overnight with 1 μg/mL HiLyte Fluor 488-labeled Amyloid-β 1–42 (FITC-Aβ), washed with 1X PBS, and examined by fluorescence microscopy for analyzing Aβ uptake. Lysosomal marker LysoTracker Red (Life Technologies, Carlsbad, CA, USA) was used as the counter stain to analyze the extent of intralysosomal localization of phagocytosed Aβ. Image analysis was performed using ImageJ software. Mean fluorescent intensity (MFI) over 3 different fields per sample were subjected to analysis and the MFI were calculated by taking the ratio of total fluorescent intensity to the total number of cells in each field.

2.11.2. THP-1 cells

Differentiated cells were incubated overnight with FITC-Aβ, washed twice with 1X PBS and examined by fluorescent microscopy. The images obtained were quantified for MFI using ImageJ software.

2.12. Conditioned media experiments

Conditioned media collected from THP-1-derived macrophages were centrifuged and removed cellular debris. Differentiated SHSY5Y cells were treated with the conditioned media derived from differentiated THP-1 cells that were exposed to Aβ treatment with or without SHARPIN siRNA for 48 hours. The expression of apoptotic markers cleaved caspase-3 and cleaved PARP were analyzed using western blotting in these cells.

2.13. Statistical analyses

For experiments involving 2 groups, Student's *t*-test was used. For experiments involving more than 2 groups, one-way ANOVA was used to compare control parameters with the treatment groups. Pearson's correlation coefficient was used to correlate each parameter with SHARPIN expression in AD, MCI, and control subjects. Results are represented as mean ± SEM and p value <0.05 was considered as statistically significant.

3. Results

3.1. SHARPIN regulates Aβ receptor expression and phagocytosis by macrophages

The effect of varying concentrations of Aβ (1–10 μM) on SHARPIN expression was checked initially (Supplemental Fig. 1S. B). Aβ at a concentration of 10 μM significantly enhanced the expression of SHARPIN by approximately two-fold in THP-1 derived macrophages (Fig. 1B and C). Furthermore, we observed that knockdown of SHARPIN in macrophages significantly reduced the Aβ phagocytic efficiency using FITC-Aβ internalization assay, demonstrating a role

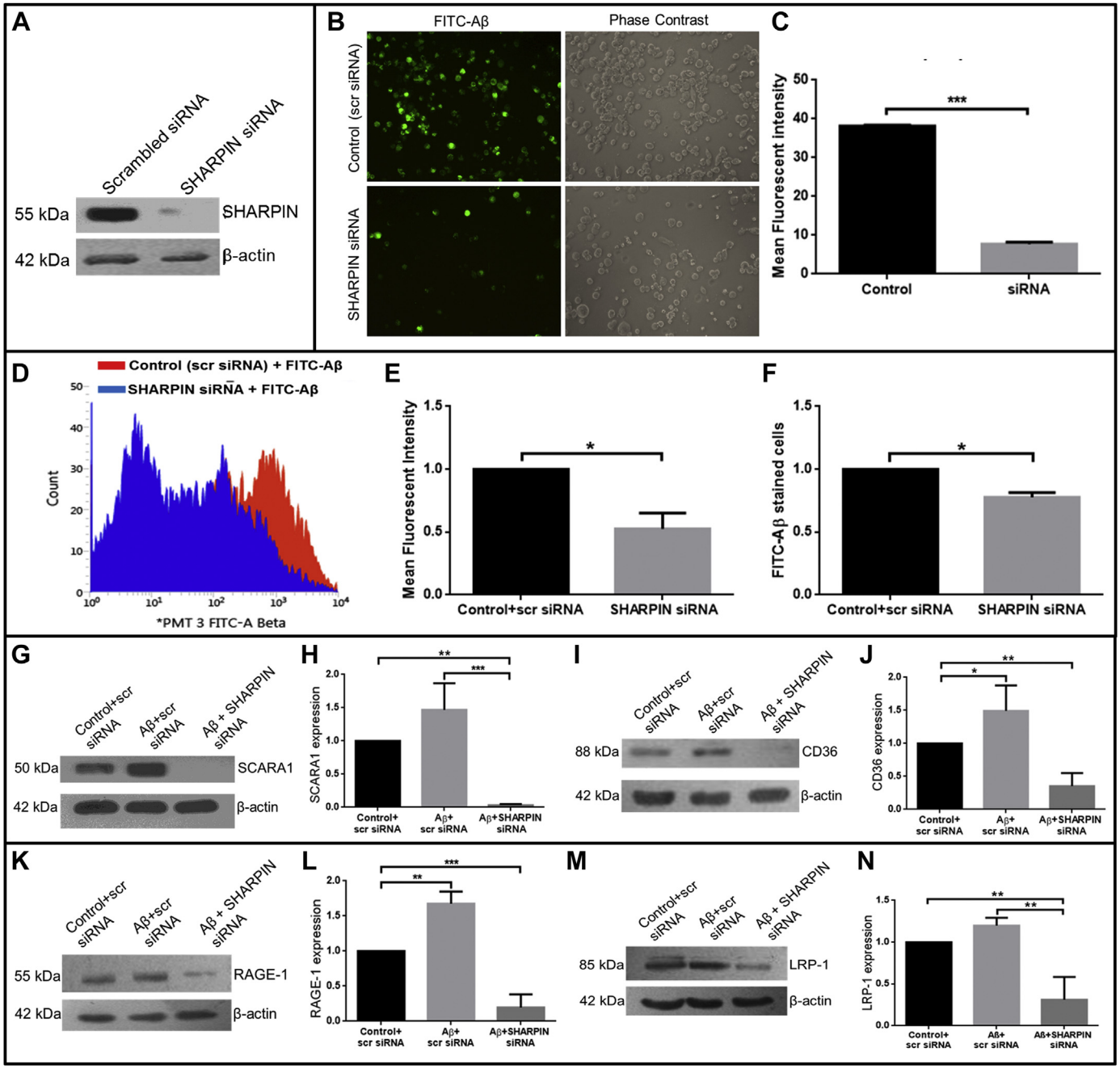

Fig. 2. Aβ-induced SHARPIN expression regulates Aβ phagocytosis. Differentiated THP-1 macrophages were transfected with SHARPIN or scrambled (scr) siRNA and the transfection efficiency was analyzed by western blotting (A). The efficiency of Aβ phagocytosis was analyzed after incubating the transfected cells with FITC-Aβ for 12 hours. Aβ uptake was analyzed using fluorescent imaging (B) and the mean fluorescent intensity (MFI) was quantified and represented graphically (fold change in expression: control+scr siRNA vs. SHARPIN siRNA is 1: 1.38, n = 3) (C). The cells were subjected to flow cytometry analysis (D), MFI of FITC-Aβ was quantified with respect to the control (scr siRNA) (fold change in expression: control vs. SHARPIN siRNA is 1: 1.90, n = 3) (E), and the number of cells positive for FITC-Aβ was quantified after normalization with control and represented graphically (fold change in expression: control+scr siRNA vs. SHARPIN siRNA is 1: 1.29, n = 3) (F). Differentiated THP-1 macrophages were transfected with SHARPIN siRNA or scr siRNA. After stimulation with Aβ for 12h, the SCARA1, CD-36, RAGE-1, and LRP-1 (G–N) were analyzed by western blotting. β-actin was used as loading control (fold change in expression: control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.47: 0.033, n = 4 for SCARA1; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.49: 0.35, n = 5 for CD-36; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.68: 0.197, n = 3 for RAGE-1; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.20: 0.31, n = 3 for LRP-1). Statistical analysis—one-way ANOVA with $*p > 0.05$, $**p > 0.01$, $***p > 0.001$.

for SHARPIN in modulating macrophage function in a setting of AD (Fig. 2B–D, Supplemental Fig. 2S. A).

Aβ phagocytosis in macrophages is mediated by phagocytic receptors (Baranello et al., 2015; Lai and McLaurin, 2012). Because SHARPIN silencing was found to decrease Aβ phagocytosis, the possibility that SHARPIN affects Aβ-phagocytic receptor expression to modulate Aβ phagocytosis was examined. SHARPIN knockdown attenuated Aβ-induced expression of the phagocytic receptors—scavenger receptor class A1 (SCARA1), CD36, receptor for advanced glycation end products (RAGE-1), and low-density lipoprotein receptor-related protein 1 (LRP-1) (Fig. 2), demonstrating that SHARPIN mediates Aβ phagocytosis via its regulatory control over

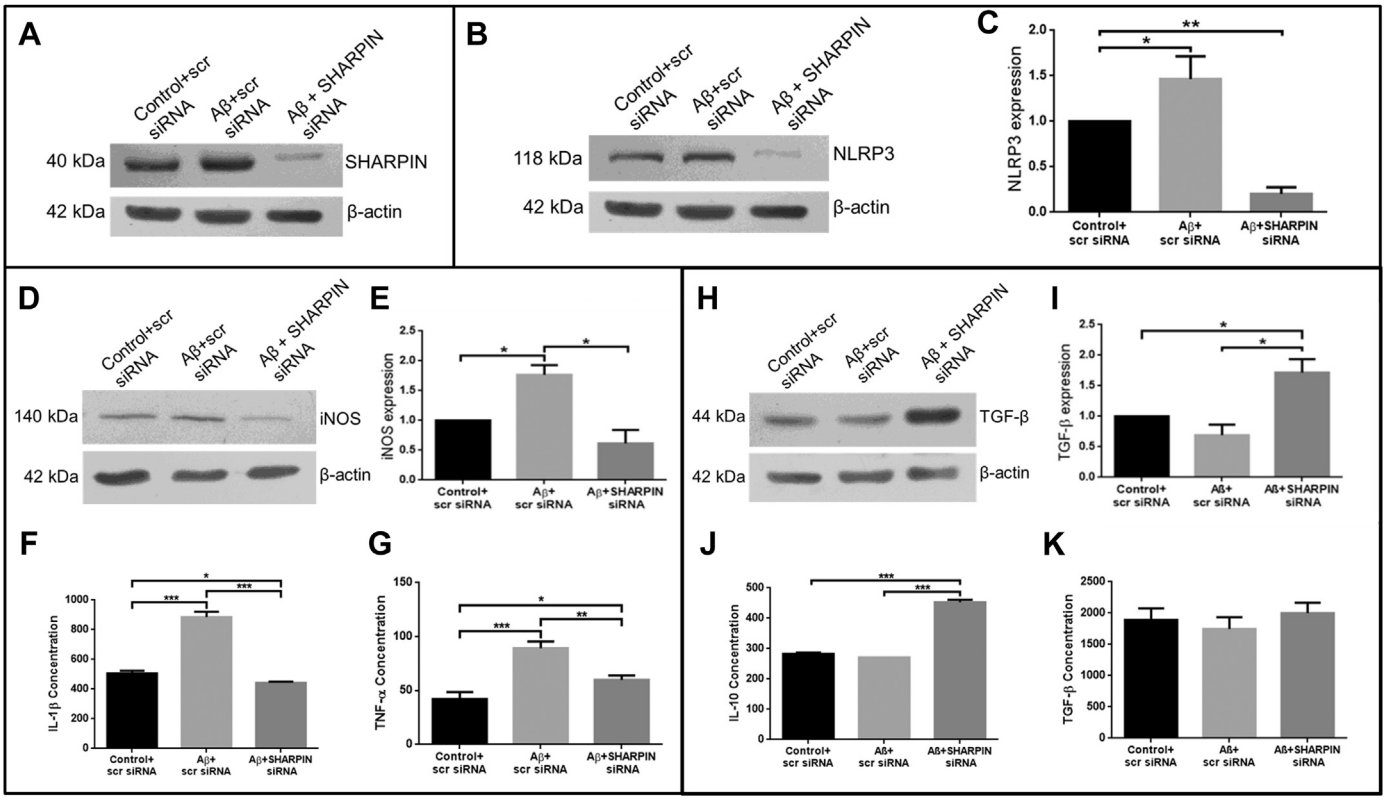

Fig. 3. SHARPIN knockdown inhibits Aβ-induced M1 polarization of macrophages. Differentiated THP-1 macrophages were transfected with SHARPIN siRNA or scr siRNA and the transfection efficiency was analyzed by western blotting (A). After transfection with SHARPIN siRNA or scr siRNA, the cells were treated with 10 μM Aβ for 6 hours and NLRP3 expression was analyzed by western blotting (B), normalized with control (scr siRNA) and represented graphically (fold change in expression: control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.47: 0.20, n = 3). β-Actin was used as loading control (C). After transfection with SHARPIN siRNA or scr siRNA, the cells were treated with 10 μM Aβ for 12 hours. The expression of M1 marker iNOS (induced nitric oxide synthase) (D, E) and the release of proinflammatory cytokines (IL-1β and TNF-α) in the conditioned media were quantified using ELISA and represented graphically (fold change in expression: control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.77: 0.62, n = 3 for iNOS; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.75: 0.87, n = 3 for IL-1β; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 2.12: 1.42, n = 4 for TNF-α) (F, G). The expression of M2 marker TGF-β (H, I) and the release of anti-inflammatory cytokines (IL-10 and TGF-β) in the conditioned media were quantified using ELISA and represented graphically (fold change in expression: control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 0.70: 1.72, n = 3 for TGF-β; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 0.92: 1.06, n = 3 for IL-10; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 0.96: 1.6, n = 4 for TGF-β release) (J, K). Statistical analysis—one-way ANOVA with $^{*}p > 0.05$, $^{**}p > 0.01$, $^{***}p > 0.001$.

the expression of Aβ phagocytosis receptors. SHARPIN knockdown reduced basal expression of Aβ receptors in macrophages (Supplemental Fig. 2S. B).

We also looked at the phagocytosis efficiency of Aβ after chronic stimulation with lipopolysaccharide (LPS) or Aβ for 6 hours. We found that prior stimulation or priming with LPS or Aβ reduced the phagocytosis efficiency of Aβ by the macrophages (Supplemental Fig. 4S. G and H) (Figs. 1 and 2).

3.2. SHARPIN regulates Aβ-induced macrophage polarization

Macrophage polarization to a proinflammatory M1 phenotype and resultant upregulation of proinflammatory cytokines contribute to neuroinflammation, a major hallmark of AD (Akiyama et al., 2000; Rogers et al., 2007). NLRP3 inflammasome is reported to mediate macrophage polarization to an M1 phenotype. In addition to NLRP3, prior studies report SHARPIN as a key regulator of inflammatory response. Here, SHARPIN knockdown attenuated Aβ-induced NLRP3 expression in macrophages (Fig. 3B), pointing to the possibility that SHARPIN and NLRP3 act in tandem to promote a proinflammatory signaling cascade in response to Aβ stimulation. Furthermore, we observed that SHARPIN knockdown resulted in a decrease in markers of the M1 phenotype (Fig. 3, Supplemental Fig. 3S) and increase in M2 phenotypic markers (Fig. 3, Supplemental Fig. 3S). Together, the data show that Aβ-induced SHARPIN expression predominantly polarizes macrophages to a proinflammatory M1 phenotype and enhances the expression of proinflammatory cytokines in Aβ-stimulated THP-1 macrophages. Using LPS, a well-known activator of macrophage M1 polarization, we checked whether the expression of SHARPIN was specific to stimulation with Aβ. We found that stimulation of macrophages with LPS enhanced the expression of SHARPIN, NLRP3, and iNOS and decreased the expression of TGF-β (Supplemental Fig. 4S). Furthermore, priming of THP-1-derived macrophages with LPS for 1 hour significantly enhanced Aβ-mediated SHARPIN, NLRP3, and iNOS expression and reduced TGF-β expression (Supplemental Fig. 4S) (Fig. 3).

3.3. SHARPIN knockdown prevents inflammation-mediated neuronal cell death

The effect of SHARPIN-mediated inflammation on neuronal apoptosis was analyzed using conditioned media experiments. Differentiated neurons exhibit decreased expression of the stem cell marker Nestin and are also identified by morphological analysis. Differentiation of SHSY5Y cells to neurons post-RA treatment was confirmed by morphological analysis and immunocytochemistry that showed an increase in the cell size and number of synaptic connections together with decreased expression of Nestin (Fig. 4A).

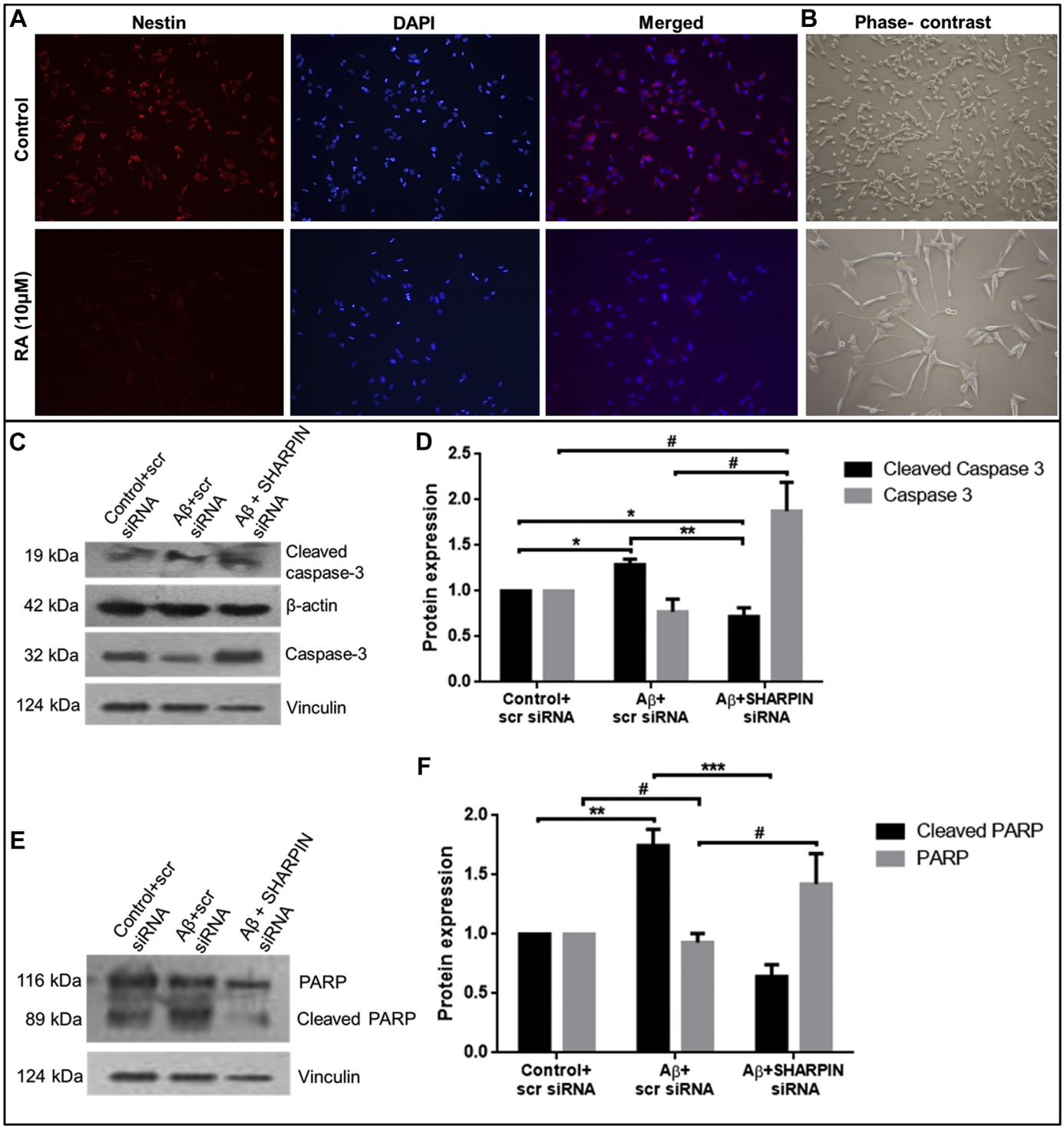

Fig. 4. SHARPIN knockdown in macrophages prevents Aβ-induced inflammation mediated neuronal damage. SHSY5Y neuroblastoma cell lines were differentiated into mature neurons by incubating the cells with 10 μM retinoic acid (RA) for 3–4 days in RPMI-1640 supplemented with 1% FBS. The differentiated cells were analyzed for the stem cell lineage marker Nestin using immunocytochemistry and analyzed using fluorescent imaging (A). The cells were further analyzed for cell size, morphology, and synaptic connections before and after differentiation using phase-contrast microscopy (B). Differentiated SHSY5Y cells were treated with conditioned media from SHARPIN-silenced THP-1 macrophages exposed to 10 μM Aβ for 48 hours. The expression of apoptotic markers cleaved caspase-3 (C) and cleaved PARP (E) were analyzed by western blotting, normalized with control and represented graphically. β-actin and vinculin were used as loading controls (fold change in expression: control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.29: 0.72, n = 3 for cleaved caspase-3 and 1: 0.77: 1.88, n = 3 for caspase-3; control+scr siRNA vs. Aβ+scr siRNA vs. Aβ+SHARPIN siRNA is 1: 1.75: 0.64, n = 3 for cleaved PARP and 1: 0.93: 1.42, n = 3 for PARP) (D, F). Statistical analysis- One-way ANOVA with $^{*/\#}p > 0.05$, $^{**}p > 0.01$.

Neuronal cells treated with conditioned media obtained from macrophages incubated with Aβ showed increased expression of apoptotic markers cleaved caspase-3 and cleaved PARP. Importantly, incubation of the neuronal cells with conditioned media derived from SHARPIN-silenced THP-1 macrophages was found to significantly reduce the expression of the proapoptotic markers (Fig. 4).

3.4. Aβ-induced oxidative stress affects SHARPIN expression

Aβ is known to enhance ROS generation and oxidative stress (Butterfield et al., 2013) in the AD brain and in vitro cell cultures. In line with these prior reports, we found enhanced ROS generation in Aβ-treated macrophages compared with the control. Furthermore, as shown by FACS analysis, addition of the ROS scavenger NAC reduced Aβ-stimulated ROS, confirming the role of Aβ in enhancing ROS levels in macrophages (Fig. 5A and B). To check the role of ROS in stimulating SHARPIN expression in macrophages exposed to Aβ, the cells were preincubated with NAC, followed by analysis of SHARPIN expression. NAC treatment downregulated SHARPIN expression, demonstrating the role of ROS in mediating Aβ-stimulated SHARPIN expression in macrophages (Fig. 5D and E).

3.5. SHARPIN is controlled by NF-κB-mediated feedback regulation

Because NF-κB is a redox-sensitive transcription factor, its regulatory role in SHARPIN was investigated. Pharmacological inhibition of NF-κB using BAY 11-7082 was found to cause an increase in the molecular weight of SHARPIN compared with the control and Aβ groups (Fig. 5C). Immunoprecipitation of SHARPIN (Fig. 5F) and probing with anti-ubiquitin antibody in the Aβ+BAY 11-7082–treated group showed a significant increase in ubiquitinylation

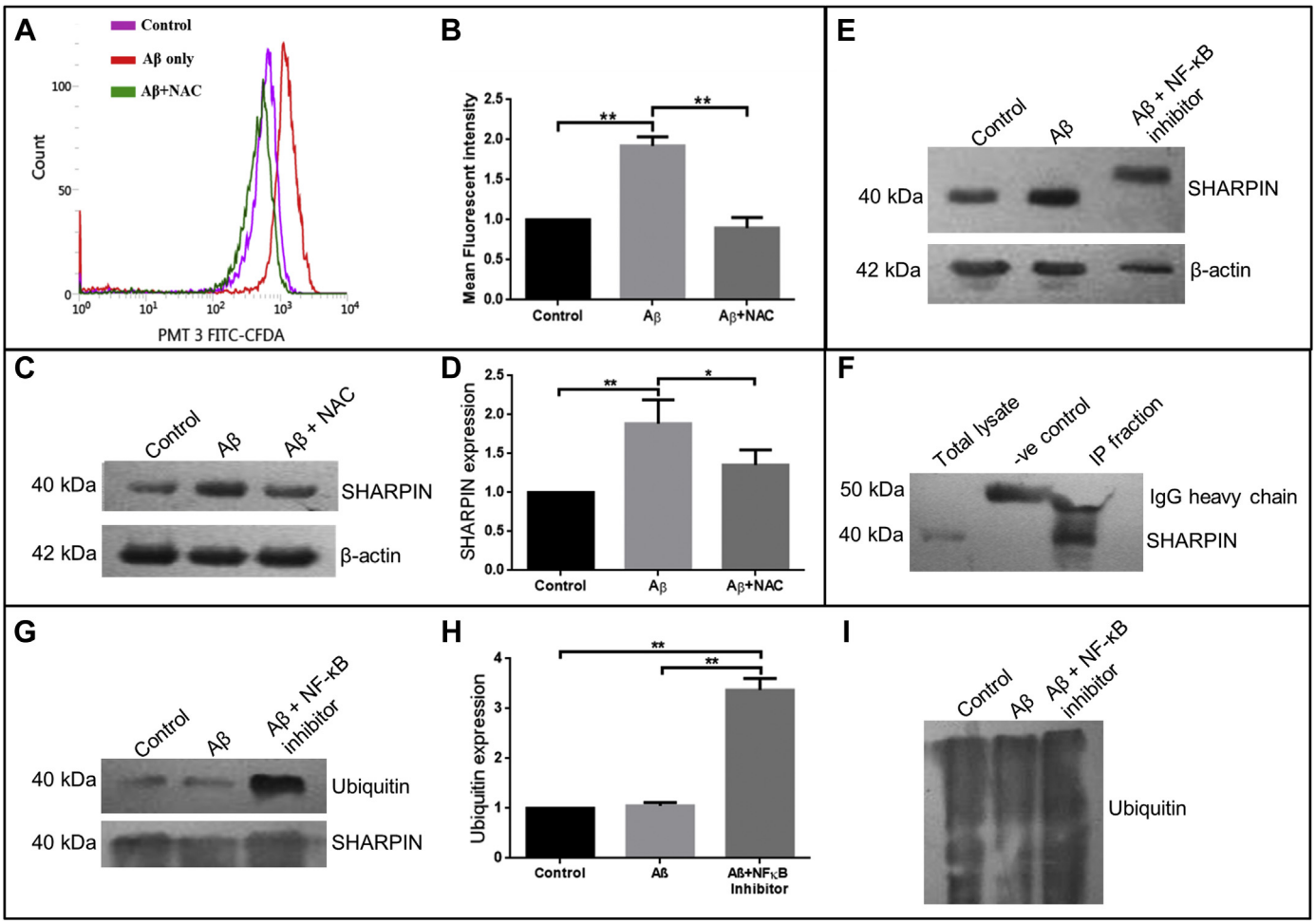

Fig. 5. SHARPIN expression is stimulated by Aβ-induced oxidative stress and is post-translationally modified by NF-κB-mediated signaling pathway. Differentiated THP-1 macrophages cultured in RPMI-1640 medium were preincubated with 10 mM NAC for 1h followed by treatment with 40 μM Aβ for 12 hours. Then the cells were incubated with H_2DCFDA for 1 hour and subjected to flow cytometry for analyzing intracellular reactive oxygen species (ROS) production induced by Aβ (A) the mean fluorescent intensity was quantified, normalized with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium without FBS), and represented graphically (fold change in expression: control vs. Aβ vs. Aβ+NAC is 1: 1.92: 0.89, n = 3) (B). Differentiated THP-1 macrophages were pre-incubated with 10 mM NAC for 1 hour followed by treatment with 10 μM Aβ for 6 hours. The expression of SHARPIN was analyzed by western blotting (C), normalized with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium without FBS) and was represented graphically (fold change in expression: control vs. Aβ vs. Aβ+NAC is 1: 1.89: 1.35, n = 3) (D). Differentiated THP-1 macrophages were preincubated with NF-κB inhibitor, BAY 11-7082 (10 μM) for 1h and then treated with BAY 11-7082 + 10 μM Aβ for 12 hours in RPMI-1640 medium supplemented with 10% FBS. SHARPIN expression was analyzed using western blotting and the increase in molecular weight of SHARPIN in BAY 11-7082 + 10 μM Aβ-treated cells compared with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium supplemented with 10% FBS) and Aβ was represented (E). SHARPIN protein was immunoprecipitated for further analysis and the purity of the immunoprecipitated protein was shown with western blot data (F). Immunoprecipitated SHARPIN protein was probed for ubiquitination using anti-ubiquitin antibody and the ubiquitination of SHARPIN protein (G) and total protein ubiquitination (I) was analyzed using western blotting, normalized with control and represented graphically (fold change in expression: control vs. Aβ vs. Aβ+ BAY 11-7082 is 1: 1.05: 3.37, n = 3) (H). Statistical analysis—one-way ANOVA with $^{*}p > 0.05$, $^{**}p > 0.01$.

A

Study Subjects	Total number	MMSE scores	ACE scores	Age	Average Aβ 42:40 ratio
Control	19	-	-	54.7 ± 10	0.149
MCI	13	27 ± 3	82 ± 10	71.3 ± 5.5	0.227
AD	31	22 ± 5	67 ± 6	69.5 ± 8.6	0.234

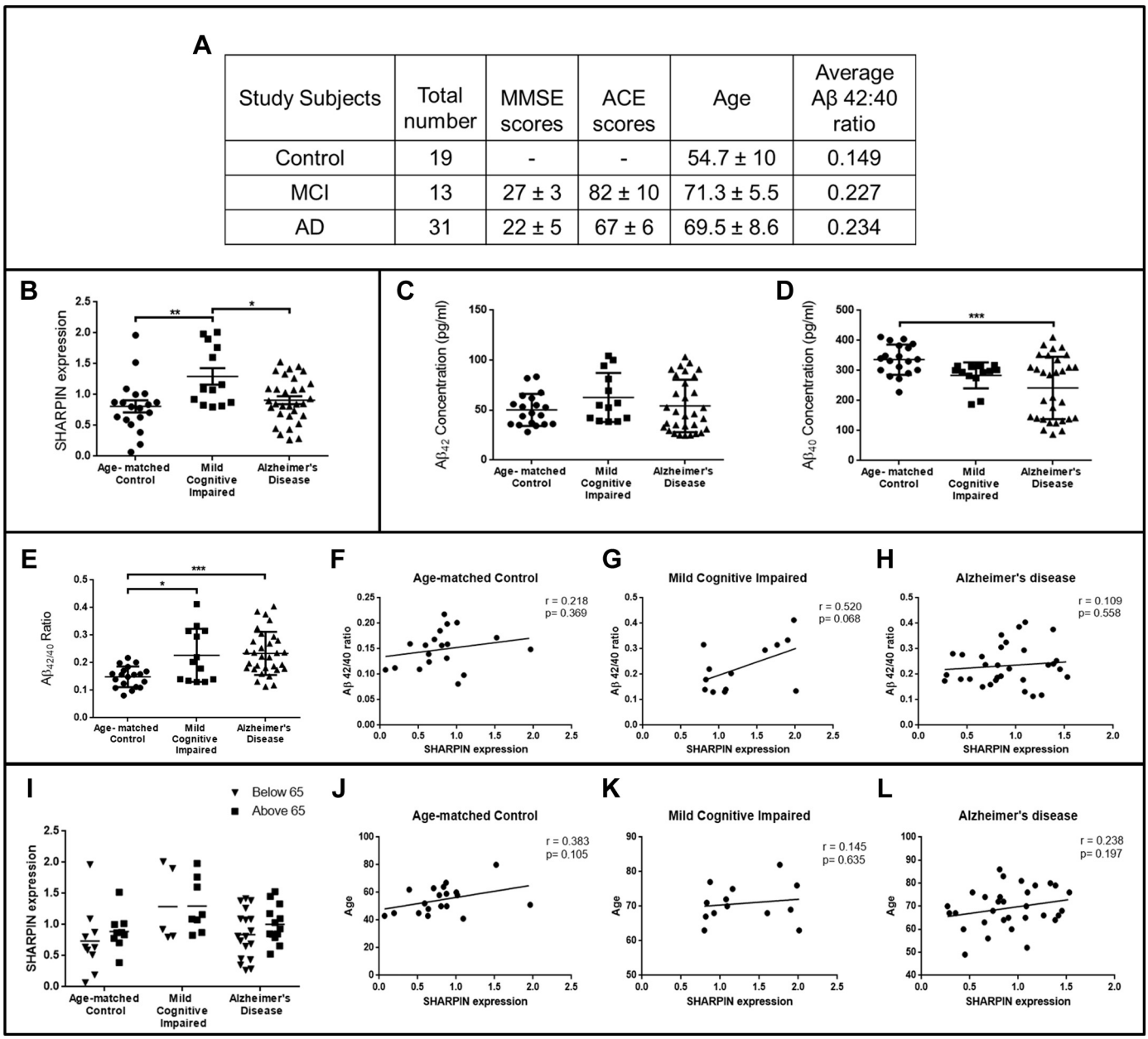

Fig. 6. SHARPIN expression in mild cognitively impaired (MCI), Alzheimer's disease (AD) and control subjects. Details of the study subjects were represented. Subjects with a high plasma CRP level were excluded from the study population (A). Peripheral blood mononuclear cells (PBMCs) were isolated from the study subjects' blood samples and cultured in RPMI-1640 supplemented with autologous serum were cultured for 14 days until complete differentiation into macrophages. Total protein was isolated from the mature macrophages and analyzed for SHARPIN expression in the study subjects and represented as scatter plot (B). $A\beta_{42}$ and $A\beta_{40}$ in the blood plasma isolated from the same study subjects were analyzed using ELISA (C, D) and the ratio of $A\beta_{42}$ to $A\beta_{40}$ was calculated and represented as scatter plot (E). SHARPIN expression in macrophages isolated from the study subjects (control, MCI, and AD) were correlated with $A\beta_{42/40}$ ratio in the blood plasma of control (F), MCI (G) and AD (H) subjects. The Pearson correlation coefficient, r = 0.218 ($p = 0.369$, ns) for age-matched control, r = 0.520 ($p = 0.068$, ns) for mild-cognitive impaired and r = 0.109 ($p = 0.558$, ns) for Alzheimer's disease subjects (ns, non significant). The study subjects were grouped into age groups below 65 and above 65 years and the expression of SHARPIN was analyzed and represented as scatter plot (I). SHARPIN expression in macrophages was correlated with age of the study subjects and represented graphically (J, K, L). The Pearson correlation coefficient, r = 0.383 ($p = 0.105$, ns) for age-matched control, r = 0.145 ($p = 0.635$, ns) for mild cognitive impaired and r = 0.238 ($p = 0.197$, ns) for Alzheimer's disease subjects. ns, non significant, $^*p > 0.05$, $^{**}p > 0.01$, $^{***}p > 0.001$.

(Fig. 5G), suggesting that the transcription factor NF-κB may function in the ubiquitinylation of SHARPIN.

3.6. SHARPIN expression is altered in AD patient–derived macrophages

Study subjects were categorized into 31 AD, 13 MCI, and 19 control subjects (Fig. 6A). In patient-derived macrophages isolated from these categories of subjects, the MCI patient samples displayed a prominent increase in expression of SHARPIN followed by AD and control subjects (Fig. 6B) similar to the concentration levels of $A\beta_{42}$ concentrations in the plasma (Fig. 6C) of the study subjects. Importantly, SHARPIN expression showed a positive correlation with respect to $A\beta_{42/40}$ concentrations (the concentration of $A\beta_{42}$ normalized to the total Aβ metabolism by taking the ratio of $A\beta_{42}$ to $A\beta_{40}$, the most abundant form of Aβ) in the plasma of control, MCI, and AD subjects (Fig. 6F–H), suggesting that SHARPIN expression in macrophages, in the absence of systemic inflammation, might be

stimulated by the concentration of $A\beta_{42}$, the toxic form of Aβ, in the blood plasma. Owing to the small sample size with a heterogeneous population, we segregated the subjects on the basis of age (Fig. 6I) and the presence of diabetes (Supplemental Fig. 7S) and found a correlation between increased SHARPIN expression, age, and diabetes in the study subjects.

4. Discussion

Defective immune cell–mediated clearance of Aβ is a major contributor of Aβ accumulation in the brain, leading to the pathogenesis of AD (Fiala et al., 2005; Jairani et al., 2019). Aβ accumulation–associated inflammatory activation of microglia in the brain and the macrophages entering the brain from peripheral circulation play a major role in promoting neuronal apoptosis, leading to progression of AD (Sweeney et al., 2018). Although several studies have shown a correlation between inflammatory mediators and phagocytic receptor expression by immune cells (Aderem, 2003), the underlying mechanisms that affect Aβ phagocytosis and promote proinflammatory conditions in the AD brain remain elusive.

SHARPIN has been recognized as an upstream activator of NF-κB thus acting as an important mediator of inflammatory signaling (Aksentijevich and Zhou, 2017; Ikeda et al., 2011; Tokunaga et al., 2011). A recent study by Asanomi et al. identified a rare functional variant of SHARPIN as a genetic risk factor for LOAD, proving the role of the protein in the progression of AD (Asanomi et al., 2019). Although the study identified genetic mutations in SHARPIN as a risk factor for LOAD, the molecular basis of SHARPIN in AD pathogenesis remains unclear.

In the present study, we focused on the role of SHARPIN in the regulation of macrophage function and its contribution to the progression of AD. Using differentiated THP-1 macrophages exposed to Aβ as an in vitro model, we have demonstrated a significant increase in the expression of SHARPIN in the presence of Aβ, indicating a link between Aβ exposure and SHARPIN expression in macrophages. Furthermore, our study has shown the role of SHARPIN in regulation of Aβ phagocytosis through modulating the expression of receptors involved in Aβ uptake thus, affecting the overall cellular intake of Aβ which was evident by the significant reduction in FITC-Aβ fluorescence in SHARPIN-silenced cells. We also checked whether the expression of SHARPIN and associated phagocytosis was exclusive to stimulation with Aβ. LPS is reported to promote inflammation in macrophages and alter expression of phagocytic receptors (Cai et al., 2012; Rosadini and Kagan, 2017). Here, increased expression of SHARPIN in response to LPS suggested that SHARPIN-mediated macrophage function is not specific to stimulation with Aβ. Chronic stimulus of THP-1-derived macrophages with LPS and then Aβ further enhanced SHARPIN expression and M1 polarization and decreased phagocytosis efficiency by the cells. Hence, while addressing the role of this protein in AD, the role of other inflammatory stimuli that alter SHARPIN expression should be taken into consideration.

SHARPIN and NLRP3 are widely regarded as the 2 principal mediators of inflammation. A study conducted in mice carrying mutant SHARPIN ($Sharpin^{cpdm}$) that caused loss of SHARPIN function identified that SHARPIN is required for NLRP3 activation (Gurung et al., 2015, p. 3). We found a reduction in NLRP3 expression in SHARPIN-knockdown macrophages exposed to Aβ demonstrating for the first time, a novel link between the 2 inflammatory mediators in the context of AD. Because SHARPIN regulates NLRP3, which is an activator of proinflammatory signaling, we analyzed the expression of inflammatory markers in SHARPIN-silenced macrophages exposed to Aβ. We found that while Aβ polarized macrophages to the M1 phenotype, knockdown of SHARPIN in the presence of Aβ prevented M1 polarization polarizing the cells to the neuroprotective M2 phenotype.

It has been reported that brain-resident and peripheral circulation–derived immune cells phagocytose Aβ in the brain and activate proinflammatory signaling leading to neuronal apoptosis and consequent progression of AD (Akiyama et al., 2000; Fiala et al., 2007; Rogers et al., 2007). Because we observed SHARPIN to promote proinflammatory cytokine expression, we analyzed its role in mediating neuronal cell apoptosis. A significant reduction in the apoptosis of neuronal cells treated with conditioned media derived from SHARPIN-knockdown macrophages compared with the control cells point to the role of SHARPIN in mediating Aβ-triggered inflammation-associated neuronal cell death. Specifically, because SHARPIN is a critical mediator of proinflammatory cytokine expression as shown by our data, the decrease in neuronal apoptosis can be attributed to a reduction in release of SHARPIN-mediated inflammatory cytokines in the conditioned-media of Aβ-stimulated macrophages transfected with SHARPIN-siRNA. Thus, our study identifies SHARPIN as a critical protein that acts as a double-edged sword in regulating phagocytosis and inflammation. While on the one hand SHARPIN promotes macrophage-mediated phagocytosis of Aβ, it also acts to promote proinflammatory signaling.

Because SHARPIN was found to regulate critical macrophage functions involving phagocytosis and macrophage polarization, the study explored the factors involved in the regulation of Aβ-stimulated SHARPIN expression in THP-1 macrophages. Aβ is reported to induce oxidative stress through ROS production (Butterfield et al., 2013; Cheignon et al., 2017). Because Aβ stimulated SHARPIN expression, we explored the role of ROS in mediating Aβ-induced SHARPIN. Treatment of macrophages with NAC, a potent ROS scavenger, prevented Aβ-induced SHARPIN expression, demonstrating ROS to play an important role in mediating SHARPIN expression in response to Aβ. Further analysis of downstream elements that could potentiate ROS signaling to influence SHARPIN expression leads to a serendipitous and important observation in the study. NF-κB is a redox-sensitive transcription factor that is reported to mediate ROS-dependent signaling and transcription of inflammatory genes (Liu et al., 2017; Morgan and Liu, 2011). In the present study, as opposed to our initial hypothesis on the role of NF-κB in the transcriptional regulation of Aβ-stimulated SHARPIN expression, we identified a novel role for NF-κB in mediating post-translational modifications in SHARPIN through mediating ubiquitination of SHARPIN. This is in line with the role of SHARPIN as a component of the linear ubiquitin chain assembly complex (LUBAC) which functions by transferring linear ubiquitin molecules to the NEMO protein (Tokunaga et al., 2011; Tokunaga and Iwai, 2012). However, the functional implication of NF-κB-dependent ubiquitination of SHARPIN needs to be explored further. SHARPIN has been highly explored in many disease conditions, especially in cancer stressing out its multiple roles in many LUBAC-independent regulatory mechanisms (Tamiya et al., 2018; Zhou et al., 2019), suggesting that this modification that we have identified, if not targeted for a proteasomal degradation, might be inhibiting only the LUBAC-dependent SHARPIN function, promoting its other cellular functions, which is out of scope for this study.

Analysis of SHARPIN in AD and MCI patient–derived macrophages demonstrated a significant increase in SHARPIN expression in MCI-derived macrophages compared with AD and control subjects. MCI is regarded as the preclinical stage of AD. Our findings show a higher degree of SHARPIN expression in MCI group compared with the control group. Previous studies have reported a reduction in concentration of Aβ levels in the blood plasma of AD patients compared with MCI due to reduced Aβ clearance into the peripheral circulation in AD patients compared with MCI (Fandos et al., 2017; Hanon et al., 2018; Song et al., 2011). Because Aβ

stimulates SHARPIN expression, reduced concentrations of $A\beta_{42}$ in peripheral circulation of AD patients might explain the decrease in SHARPIN expression in the AD group compared with MCI. Thus, the SHARPIN expression pattern in our study subjects can be reflected as a result of the stimuli induced by varied concentration of $A\beta_{42}$ in the peripheral circulation. A correlation comparing SHARPIN expression with $A\beta_{42/40}$ in the plasma of the same study subjects showed a positive correlation. Importantly, in line with our in vitro data demonstrating $A\beta_{1-42}$ in the stimulation of SHARPIN expression in macrophages, concentrations of the toxic $A\beta_{42}$ levels in the plasma showed a positive association with SHARPIN expression in macrophages obtained from the respective patient-derived PBMCs. Owing to the heterogeneity of the study population, we further classified the subjects on the basis of age (Fig. 6I) and diabetes (Supplemental Fig. 7S) and found that SHARPIN expression was higher in the age group above 65 years and in diabetic individuals pointing to the diversity of factors that could modulate SHARPIN expression and inflammatory conditions in vivo. Although SHARPIN was found to promote inflammation and Aβ phagocytosis in vitro, a significant correlation in SHARPIN expression in macrophages derived from the PBMCs of study subjects with the Aβ phagocytic efficiency and inflammatory markers in the plasma of the respective study subjects was not observed (Supplemental Fig. 6S). This indicates the presence of complex mechanisms regulating inflammation and phagocytosis under in vivo conditions contrary to the controlled in vitro setting. Hence, future studies need to examine a larger cohort with a lesser degree of population heterogeneity to meaningfully analyze the role of SHARPIN in macrophage phagocytosis of Aβ and inflammation in an in vivo setting.

In summary, our study demonstrates, for the first time, a novel role for SHARPIN in the regulation of macrophage function in response to Aβ in a setting of AD. SHARPIN was found to regulate Aβ phagocytosis and Aβ-stimulated inflammation in macrophages. Furthermore, we found that SHARPIN-mediated inflammation in macrophages exposed to Aβ promoted neuronal cell death, demonstrating its role in promoting neurodegeneration in AD. The data uncovered a role for ROS-dependent expression of SHARPIN in macrophages exposed to Aβ and a role for the ROS-sensitive transcription factor NF-κB in mediating the post-translational modification of SHARPIN through ubiquitination. Importantly, SHARPIN expression varied with the levels of $A\beta_{42}$, the toxic form of Aβ, in AD, MCI, and age-matched control subject–derived macrophages demonstrating a link between SHARPIN expression and AD progression. This correlates with our in vitro data where SHARPIN expression increases with increased concentrations of $A\beta_{1-42}$. Future studies need to address the role of SHARPIN in AD using a larger cohort of study subjects to establish the role of this protein in AD pathogenesis. Furthermore, the functional role of ubiquitination in altering SHARPIN function and its implications in macrophages needs to be addressed. Importantly, exploring microglial- and astrocyte-mediated SHARPIN expression and its role in phagocytosis and inflammatory pathways are relevant in this field because these are the cells that respond primarily to Aβ accumulation in AD brain.

Disclosure statement

All authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Dhanya Krishnan: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Ramsekhar N. Menon:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Pavagada Sivasankara Mathuranath:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Srinivas Gopala:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing.

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All procedures performed in the above study were in accordance with the ethical standards of the Institutional Human Ethical Committee (IEC/234/2009) and with The Code of Ethics of the World Medical Association (Declaration of Helsinki), 1964 and its later amendments or comparable standards.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2020.02.001.

References

Aderem, A., 2003. Phagocytosis and the inflammatory response. J. Infect Dis. 187, S340–S345.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L., Finch, C.E., Frautschy, S., Griffin, W.S.T., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I.R., McGeer, P.L., O'Banion, M.K., Pachter, J., Pasinetti, G., Plata–Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F.L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G., Wyss–Coray, T., 2000. Inflammation and Alzheimer's disease. Neurobiol. Aging 21, 383–421.

Aksentijevich, I., Zhou, Q., 2017. NF-κB pathway in autoinflammatory diseases: dysregulation of protein modifications by ubiquitin defines a new category of autoinflammatory diseases. Front. Immunol. 8, 399.

Asanomi, Y., Shigemizu, D., Miyashita, A., Mitsumori, R., Mori, T., Hara, N., Ito, K., Niida, S., Ikeuchi, T., Ozaki, K., 2019. A rare functional variant of SHARPIN attenuates the inflammatory response and associates with increased risk of late-onset Alzheimer's disease. Mol. Med. 25, 20.

Baranello, R.J., Bharani, K.L., Padmaraju, V., Chopra, N., Lahiri, D.K., Greig, N.H., Pappolla, M.A., Sambamurti, K., 2015. Amyloid-beta protein clearance and degradation (ABCD) pathways and their role in Alzheimer's disease. Curr. Alzheimer Res. 12, 32–46.

Butterfield, D.A., Swomley, A.M., Sultana, R., 2013. Amyloid β-peptide (1–42)-induced oxidative stress in alzheimer disease: importance in disease pathogenesis and progression. Antioxid. Redox Signal 19, 823–835.

Cai, L., Wang, Z., Meyer, J.M., Ji, A., van der Westhuyzen, D.R., 2012. Macrophage SR-BI regulates LPS-induced pro-inflammatory signaling in mice and isolated macrophages. J. Lipid Res. 53, 1472–1481.

Cerf, E., Sarroukh, R., Tamamizu-Kato, S., Breydo, L., Derclaye, S., Dufrêne, Y.F., Narayanaswami, V., Goormaghtigh, E., Ruysschaert, J.-M., Raussens, V., 2009. Antiparallel beta-sheet: a signature structure of the oligomeric amyloid beta-peptide. Biochem. J. 421, 415–423.

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C., Collin, F., 2017. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. Redox Biol. 14, 450–464.

Cheung, Y.-T., Lau, W.K.-W., Yu, M.-S., Lai, C.S.-W., Yeung, S.-C., So, K.-F., Chang, R.C.-C., 2009. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30, 127–135.

de Paula, V.J.R., Guimarães, F.M., Diniz, B.S., Forlenza, O.V., 2009. Neurobiological pathways to Alzheimer's disease: amyloid-beta, TAU protein or both? Dement. Neuropsychol. 3, 188–194.

Fandos, N., Pérez-Grijalba, V., Pesini, P., Olmos, S., Bossa, M., Villemagne, V.L., Doecke, J., Fowler, C., Masters, C.L., Sarasa, M., 2017. Plasma amyloid β 42/40 ratios as biomarkers for amyloid β cerebral deposition in cognitively normal individuals. Alzheimers Dement. 8, 179–187.

Fiala, M., Cribbs, D.H., Rosenthal, M., Bernard, G., 2007. Phagocytosis of amyloid-beta and inflammation: two faces of innate immunity in Alzheimer's disease. J. Alzheimers Dis. 11, 457–463.

Fiala, M., Lin, J., Ringman, J., Kermani-Arab, V., Tsao, G., Patel, A., Lossinsky, A.S., Graves, M.C., Gustavson, A., Sayre, J., Sofroni, E., Suarez, T., Chiappelli, F., Bernard, G., 2005. Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. J. Alzheimers Dis. 7, 221–232.

Gurung, P., Lamkanfi, M., Kanneganti, T.-D., 2015. SHANK-associated RH domain interacting protein (SHARPIN) is required for optimal NLRP3 inflammasome activation. J. Immunol. 194, 2064–2067.

Hanon, O., Vidal, J.-S., Lehmann, S., Bombois, S., Allinquant, B., Tréluyer, J.-M., Gelé, P., Delmaire, C., Blanc, F., Mangin, J.-F., Buée, L., Touchon, J., Hugon, Jacques, Vellas, B., Galbrun, E., Benetos, A., Berrut, G., Paillaud, E., Wallon, D., Castelnovo, G., Volpe-Gillot, L., Paccalin, M., Robert, P.-H., Godefroy, O., Dantoine, T., Camus, V., Belmin, J., Vandel, P., Novella, J.-L., Duron, E., Rigaud, A.-S., Schraen-Maschke, S., Gabelle, A., Hanon, O., Blanc, F., Boudali, Y., Gabelle, A., Seux, M., Lenoir, H., Bayle, C., Bombois, S., Delbeuck, X., Moulin, F., Duron, E., Latour, F., Plichart, M., Pichierri, S., Orvoën, G., Galbrun, E., Castelnovo, G., Volpe–Gillot, L., Labourée, F., Cassagnaud, P., Paquet, C., Lala, F., Dumurgier, J., Rigaud, A., Perret–Guillaume, C., Alonso, E., du Boisgueheneuc, F., Hugonot–Diener, L., Rollin–Sillaire, A., Martinaud, O., Boully, C., Spivac, Y., Devendeville, A., Belmin, J., Robert, P., Dantoine, T., Caillard, L., Wallon, D., Hannequin, D., Sastre, N., Haffen, S., Kearney–Schwartz, A., Novella, J., Deramecourt, V., Chauvire, V., Abitbol, G., Schwald, N., Hommet, C., Sellal, F., Cariot, M., Abdellaoui, M., Benisty, S., Gherabli, S., Anthony, P., Bloch, F., Charasz, N., Chauvelier, S., Gaubert, J., Sacco, G., Guerin, O., Boddaert, J., Paccalin, M., Mackowiak, M., Rabus, M., Gissot, V., Benetos, A., Picard, C., Guillemaud, C., Gervais, C., Hugon, J., Michel, J., David, J., Paulin, M., Ousset, P., Vandel, P., Pariel, S., Camus, V., Chawakilian, A., Joffredo, L., Troussiere, A., Adam, C., Dupuy, D., Paillaud, E., Briault, H., Saulnier, I., Mondon, K., Picat, M., Laurent, M., Godefroy, O., Daheb, R., Libercier, S., Krabchi, D., Chupin, M., Vidal, J., Chaussade, E., Lehmann, S., Schraen-Maschke, S., 2018. Plasma amyloid levels within the Alzheimer's process and correlations with central biomarkers. Alzheimers Dement. 14, 858–868.

Hughes, C.P., Berg, L., Danziger, W.L., Coben, L.A., Martin, R.L., 1982. A new clinical scale for the staging of dementia. Br. J. Psychiatry 140, 566–572.

Ikeda, F., Deribe, Y.L., Skånland, S.S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., van Wijk, S.J.L., Goswami, P., Nagy, V., Terzic, J., Tokunaga, F., Androulidaki, A., Nakagawa, T., Pasparakis, M., Iwai, K., Sundberg, J.P., Schaefer, L., Rittinger, K., Macek, B., Dikic, I., 2011. SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature 471, 637–641.

Jairani, P.S., Aswathy, P.M., Krishnan, D., Menon, R.N., Verghese, J., Mathuranath, P.S., Gopala, S., 2019. Apolipoprotein E polymorphism and oxidative stress in peripheral blood-derived macrophage-mediated amyloid-beta phagocytosis in Alzheimer's disease patients. Cell Mol. Neurobiol. 39, 355–369.

Korecka, J.A., van Kesteren, R.E., Blaas, E., Spitzer, S.O., Kamstra, J.H., Smit, A.B., Swaab, D.F., Verhaagen, J., Bossers, K., 2013. Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. PLoS One 8, e63862.

Lai, A.Y., McLaurin, J., 2012. Clearance of amyloid-β peptides by microglia and macrophages: the issue of what, when and where. Future Neurol. 7, 165–176.

Liu, T., Zhang, L., Joo, D., Sun, S.-C., 2017. NF-κB signaling in inflammation. Signal Transduct. Target. Ther. 2, 17023.

Mathuranath, P.S., Cherian, J.P., Mathew, R., George, A., Alexander, A., Sarma, S.P., 2007. Mini mental state examination and the Addenbrooke's cognitive examination: effect of education and norms for a multicultural population. Neurol. India 55, 106–110.

Mathuranath, P.S., Hodges, J.R., Mathew, R., Cherian, P.J., George, A., Bak, T.H., 2004. Adaptation of the ACE for a Malayalam speaking population in southern India. Int. J. Geriatr. Psychiatry 19, 1188–1194.

Mawuenyega, K.G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J.C., Yarasheski, K.E., Bateman, R.J., 2010. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science 330, 1774.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., Stadlan, E.M., 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of department of health and human services task force on Alzheimer's disease. Neurology 34, 939–944.

Morgan, M.J., Liu, Z., 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res 21, 103–115.

Petersen, R.C., 2004. Mild cognitive impairment as a diagnostic entity. J. Intern. Med. 256, 183–194.

Pryor, N.E., Moss, M.A., Hestekin, C.N., 2012. Unraveling the early events of amyloid-β protein (Aβ) aggregation: techniques for the determination of Aβ aggregate size. Int. J. Mol. Sci. 13, 3038–3072.

Rogers, J., Mastroeni, D., Leonard, B., Joyce, J., Grover, A., 2007. Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? Int. Rev. Neurobiol. 82, 235–246.

Rosadini, C.V., Kagan, J.C., 2017. Early innate immune responses to bacterial LPS. Curr. Opin. Immunol. 44, 14–19.

Song, F., Poljak, A., Valenzuela, M., Mayeux, R., Smythe, G.A., Sachdev, P.S., 2011. Meta-analysis of plasma amyloid-β levels in Alzheimer's disease. J. Alzheimers Dis. 26, 365–375.

Sweeney, M.D., Sagare, A.P., Zlokovic, B.V., 2018. Blood–brain barrier breakdown in Alzheimer's disease and other neurodegenerative disorders. Nat. Rev. Neurol. 14, 133–150.

Tamiya, H., Kim, H., Klymenko, O., Kim, H., Feng, Y., Zhang, T., Han, J.Y., Murao, A., Snipas, S.J., Jilaveanu, L., Brown, K., Kluger, H., Zhang, H., Iwai, K., Ronai, Z.A., 2018. SHARPIN-mediated regulation of protein arginine methyltransferase 5 controls melanoma growth. J. Clin. Invest 128, 517–530.

Tokunaga, F., Iwai, K., 2012. LUBAC, a novel ubiquitin ligase for linear ubiquitination, is crucial for inflammation and immune responses. Microbes Infect. 14, 563–572.

Tokunaga, F., Nakagawa, T., Nakahara, M., Saeki, Y., Taniguchi, M., Sakata, S., Tanaka, K., Nakano, H., Iwai, K., 2011. SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. Nature 471, 633–636.

Van Eldik, L.J., Carrillo, M.C., Cole, P.E., Feuerbach, D., Greenberg, B.D., Hendrix, J.A., Kennedy, M., Kozauer, N., Margolin, R.A., Molinuevo, J.L., Mueller, R., Ransohoff, R.M., Wilcock, D.M., Bain, L., Bales, K., 2016. The roles of inflammation and immune mechanisms in Alzheimer's disease. Alzheimers Dement. (N. Y.) 2, 99–109.

Wang, D.-S., Dickson, D.W., Malter, J.S., 2006. β-Amyloid degradation and Alzheimer's disease. J. Biomed. Biotechnol. 2006, 58406.

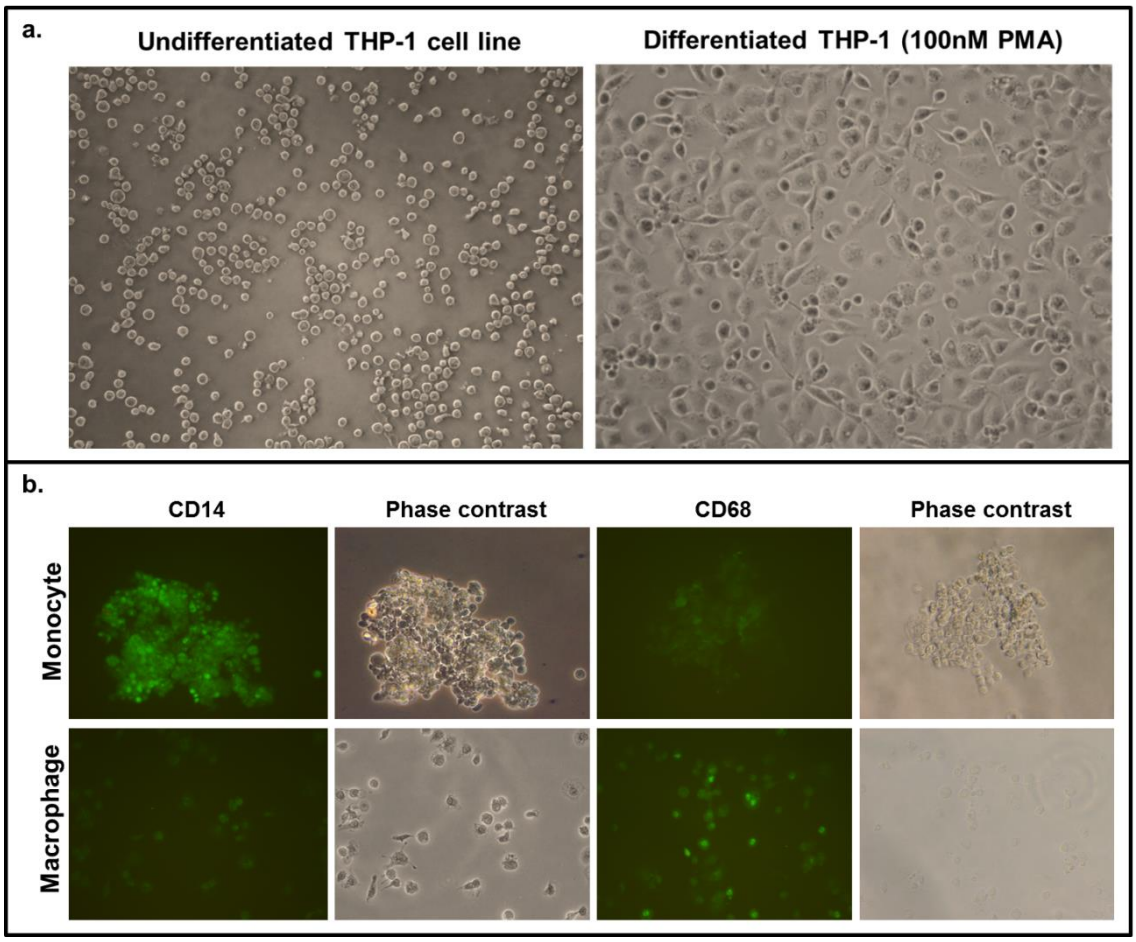
Wildsmith, K.R., Holley, M., Savage, J.C., Skerrett, R., Landreth, G.E., 2013. Evidence for impaired amyloid β clearance in Alzheimer's disease. Alzheimers Res. Ther. 5, 33.

Zhao, L., Lin, S., Bales, K.R., Gelfanova, V., Koger, D., DeLong, C., Hale, J., Liu, F., Hunter, J.M., Paul, S.M., 2009. Macrophage-mediated degradation of β-amyloid via an apolipoprotein E isoform-dependent mechanism. J. Neurosci. 29, 3603–3612.

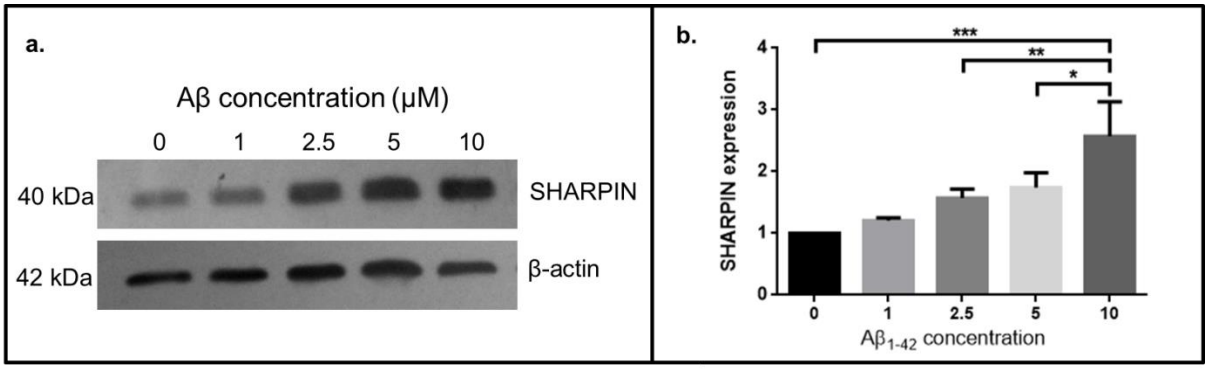
Zhou, S., Liang, Y., Zhang, X., Liao, L., Yang, Y., Ouyang, W., Xu, H., 2019. SHARPIN promotes melanoma progression via Rap1 signaling pathway. J. Invest. Dermatol. 140, 395–403.e6.

Supplementary Figures

A.



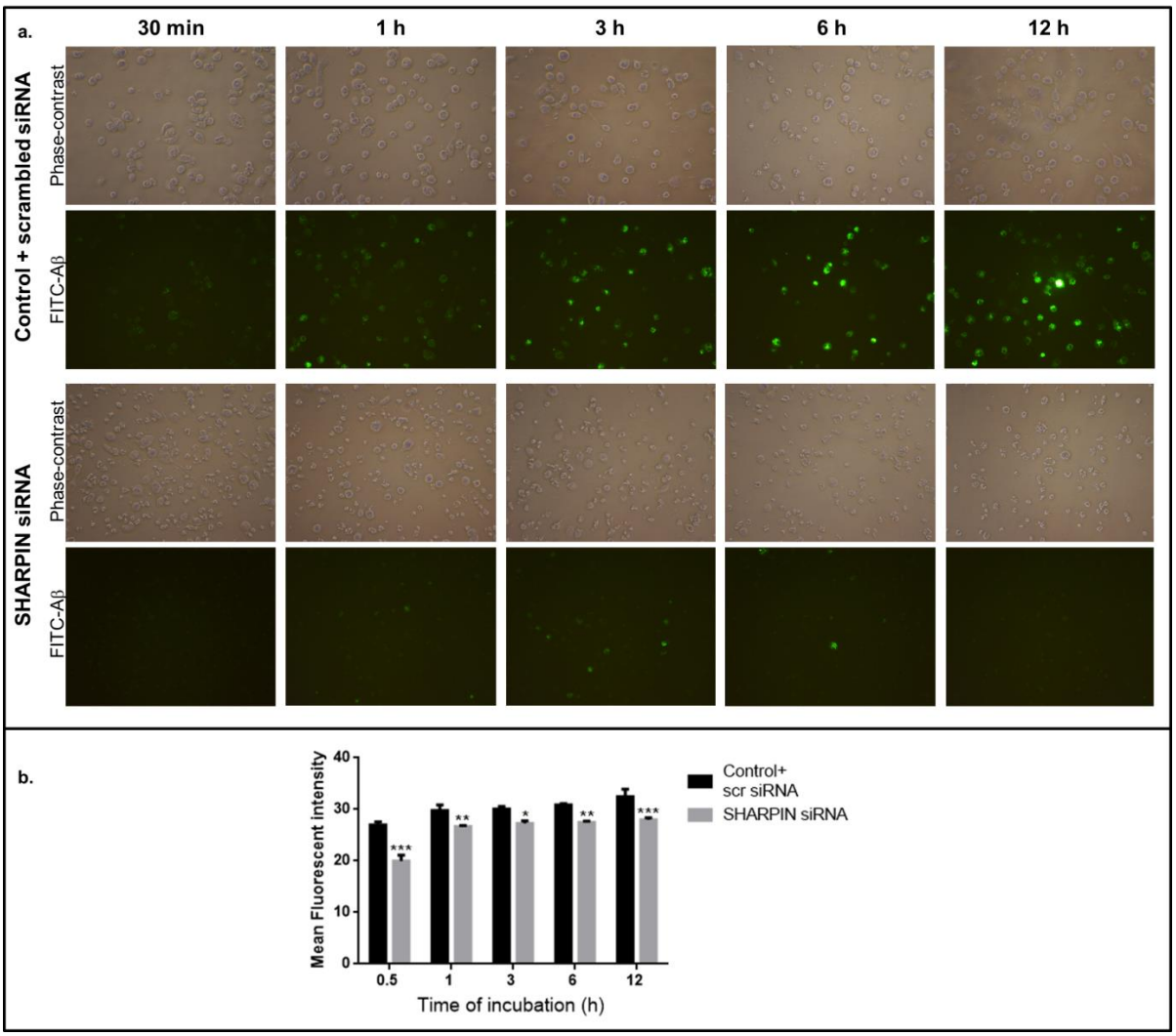
B.



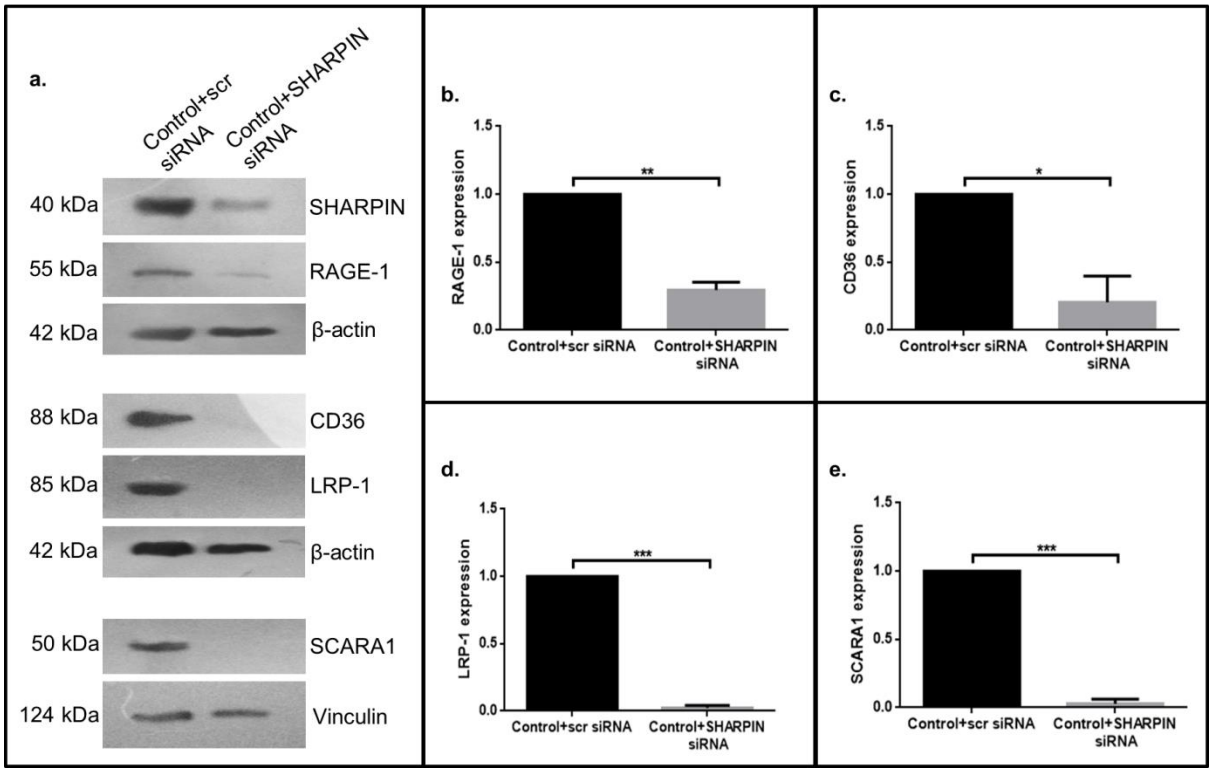
Supplemental Fig. 1S: A. Differentiation of THP-1 monocytes into macrophages. THP-1 acute monocyte leukaemia cells were treated with 100nM phorbol 12-myristate 13-acetate

(PMA) for 48 h in RPMI-1640 supplemented with 10% FBS for differentiation of monocytes into mature macrophages and the phase contrast image of cells before and after differentiation was analysed (**a**). THP-1 cells before and after PMA- induced differentiation were analysed for monocyte and macrophage markers CD14 and CD68 respectively using immunocytochemistry and analysed using fluorescent imaging (**b**).**B. Dose-response effect of Aβ on SHARPIN expression.** Differentiated THP-1 macrophages were treated withdifferent concentrations ofAβ (1 μM, 2.5 μM, 5 μM, 10 μM)in RPMI-1640 medium supplemented with 1% FBS for 6h and SHARPIN expression was analysed using western blotting (**a**), quantified, normalised with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium supplemented with 1% FBS) and represented graphically (**b**). β- actin was used as endogenous control. Statistical analysis- student's *t-test* with *p>0.05, **p>0.01, ***p>0.001.

A.

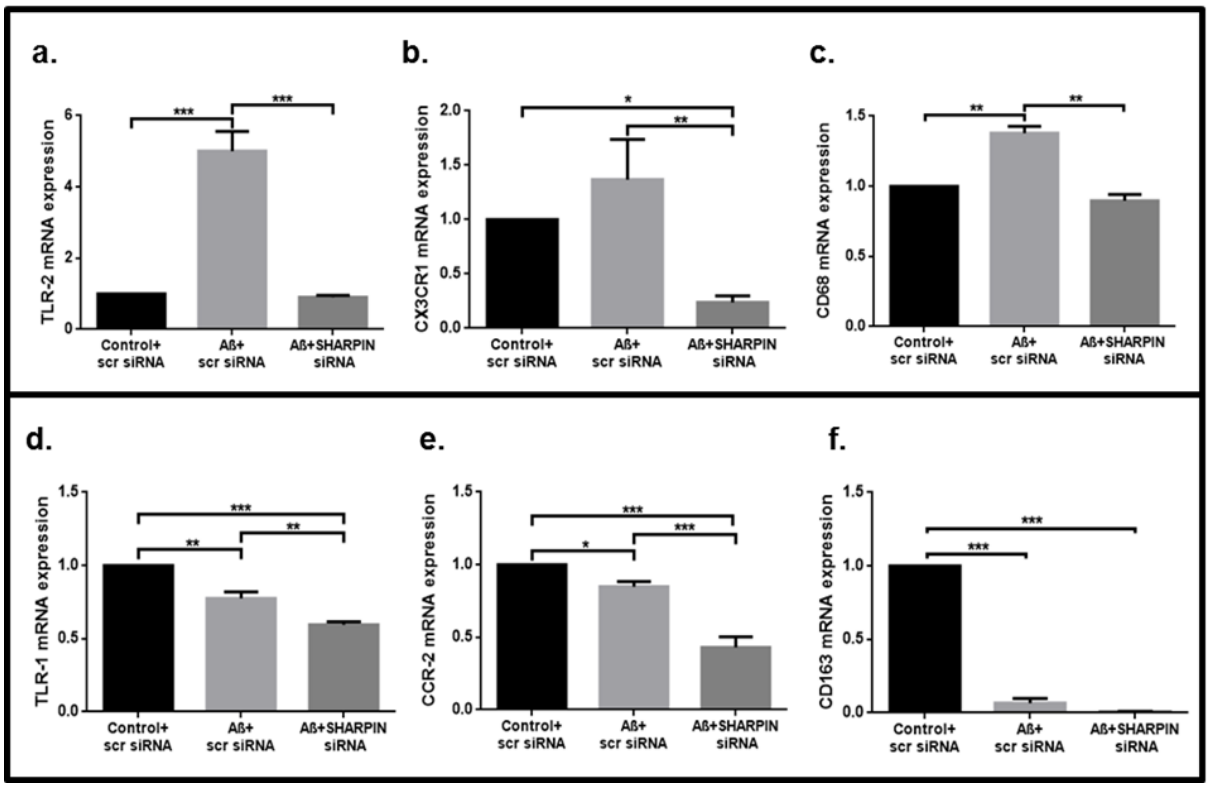

a.
30 min
1 h
3 h
6 h
12 h
Control + scrambled siRNA
Phase-contrast
FITC-Aβ
SHARPIN siRNA
Phase-contrast
FITC-Aβ
b.
Mean Fluorescent intensity
40
30
20
10
0
0.5
1
3
6
12
Time of incubation (h)
***
**
*
**
***
Control+
scr siRNA
SHARPIN siRNA

B.

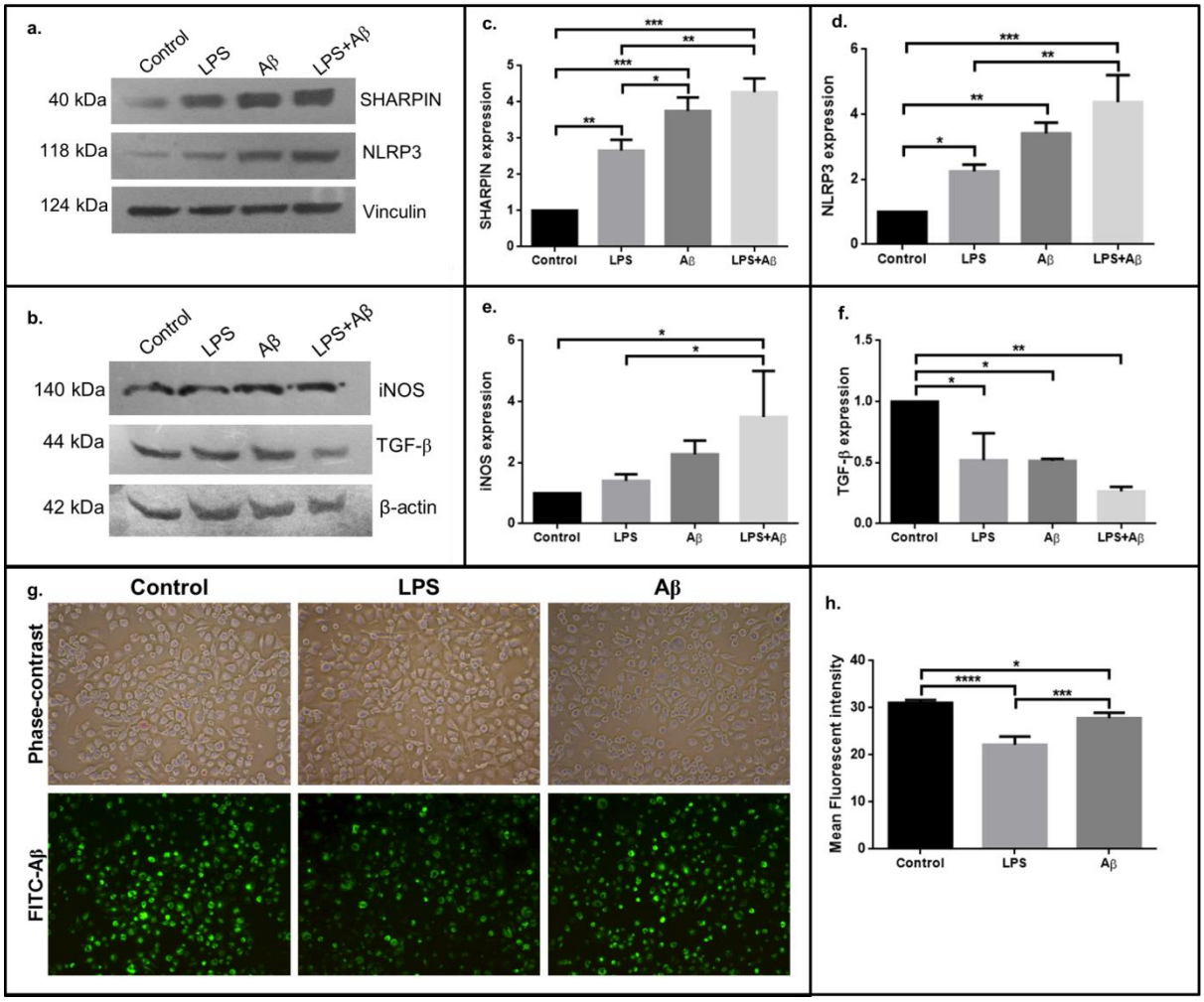


Supplemental Fig. 2S: A. SHARPIN knockdown inhibits Aβ uptake by macrophages irrespective of time of incubation. Differentiated THP-1 cells transfected with SHARPIN/scrambled (scr) siRNA were treated with 10μM FITC-Aβ for different time points (30 min, 1 h, 3 h, 6 h, 12 h) and the phagocytosis efficiency was analysed using fluorescent imaging (**a**) and the mean fluorescent intensity (MFI) was quantified and represented graphically (**b).**Statistical analysis- One-way ANOVA with *p>0.05, **p>0.01, ***p>0.001.

B. SHARPIN knockdown downregulates Aβ receptor expressions without Aβ stimulation. Differentiated THP-1 cells were transfected with SHARPIN or scr siRNA and the expression of Aβ-phagocytic receptors, RAGE-1, CD-36, LRP-1 and SCARA1 (**a**) were analysed using western blotting and represented graphically after normalising with control(**b, c, d and e**). Statistical analysis- student's *t-test* with *p>0.05, **p>0.01, ***p>0.001.

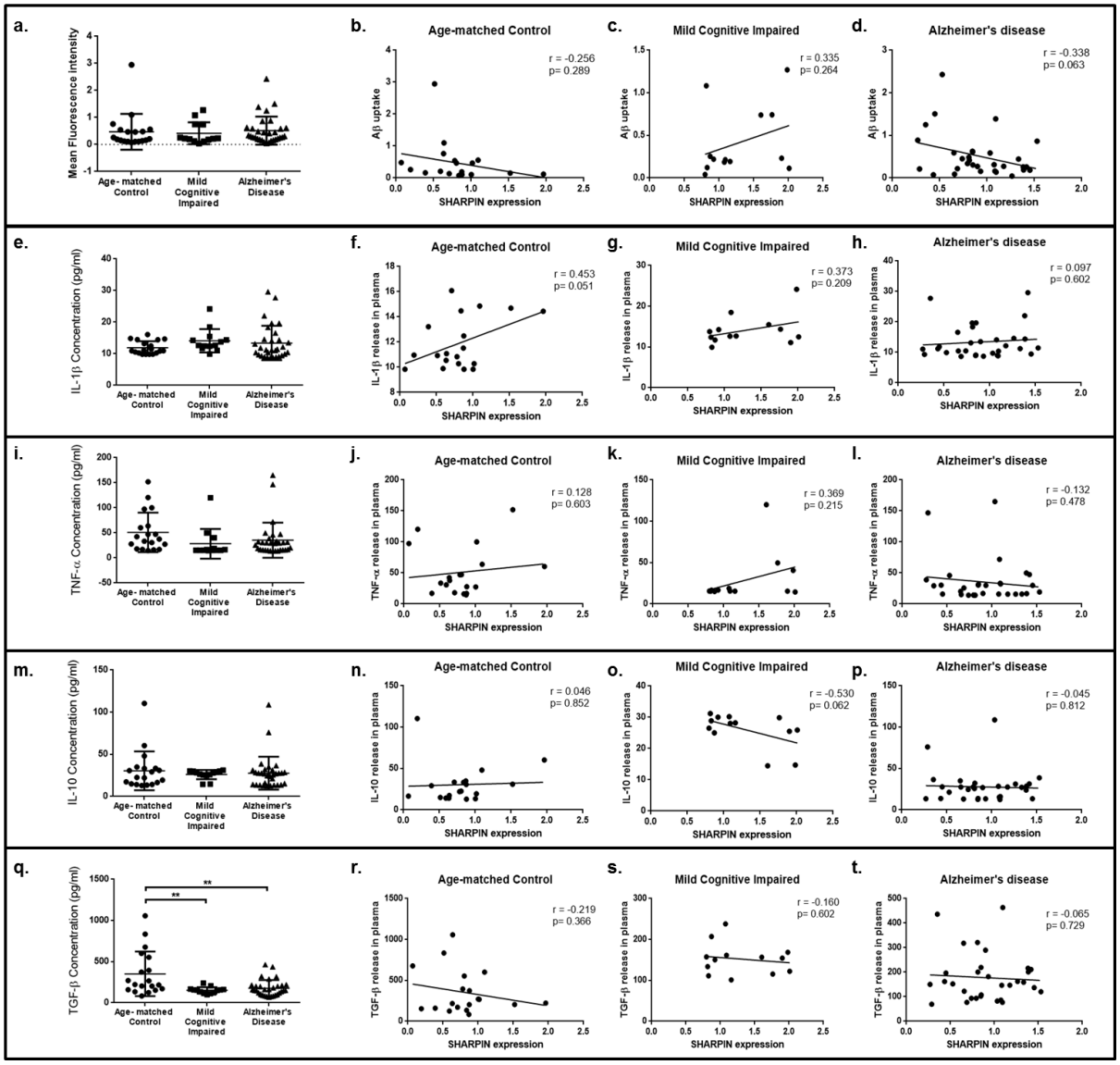


Supplemental Fig. 3S:SHARPIN knockdown inhibits Aβ-mediated M1 polarization of macrophages. Differentiated THP-1 cells transfected with SHARPIN/scrambled (scr) siRNA were treated with 10μM Aβ for 12 h and the mRNA expression of M1 markers; TLR-2 (**a**), CX3CR1 (**b**) and CD68 (**c**) and M2 markers; TLR-1 (**d**), CCR2 (**e**) and CD163 (**f**) were analysed using Real-time PCR with tubulin as endogenous control and represented graphically after normalising with control. However, the mRNA expression of M2 markers also got decreased possibly because SHARPIN mediated signaling mechanisms are activating NF-κB and inhibition of SHARPIN thus downregulates the transcription of genes controlled by NF-κB which includes the M2 markers.Statistical analysis- One-way NOVA with *p>0.05, **p>0.01, ***p>0.001.



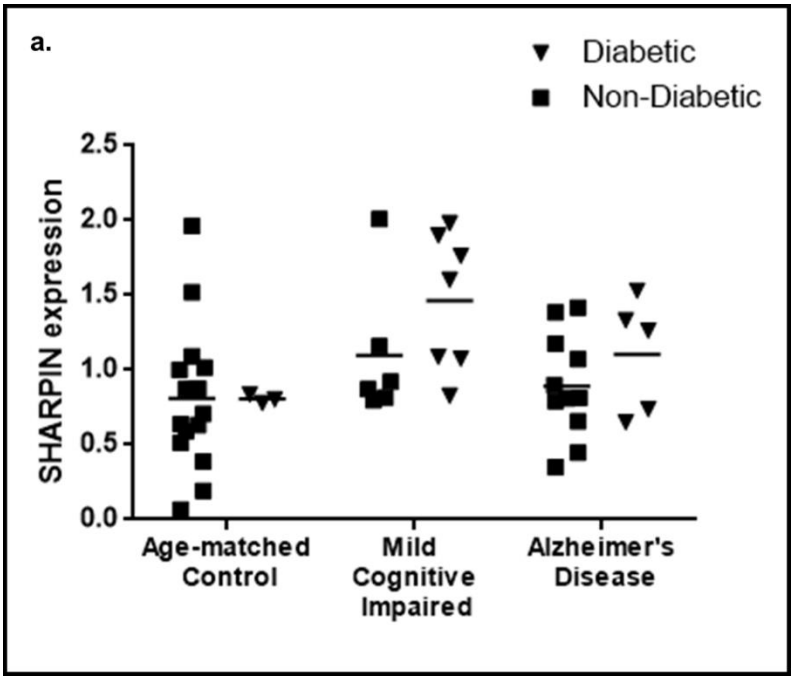
Supplemental. Fig.4S: Chronic activation of macrophages with LPS prior to Aβ stimuli enhanced SHARPIN expression and decreased Aβ phagocytosis. Differentiated THP-1 macrophages were treated with10μM Aβ or 1μM LPS in RPMI-1640 medium supplemented with 1% FBS for 6h with or without prior stimulation with 1μM LPS for 1 h and the expression of SHARPIN (**a**), NLRP3 (**a**), iNOS (**b**) and TGF-β(**b**)were analysed using western blotting, quantified, normalised with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium supplemented with 1% FBS) and represented graphically (**c, d, e and f**). β- actin was used as endogenous control. Differentiated THP-1 macrophages were treated with10μM Aβ or 1μM LPS in RPMI-1640 medium supplemented with 10% FBS for 6h and the efficiency of Aβ phagocytosis was analysed after incubating the cells with FITC-Aβ for 12 h. Aβ uptake was analysed using fluorescent imaging (**g**) and the mean

fluorescent intensity (MFI) was quantified, normalised with control (differentiated THP-1 macrophages cultured in RPMI-1640 medium supplemented with 10% FBS) and represented graphically (**h**). Statistical analysis- One-way NOVA with *p>0.05, **p>0.01, ***p>0.001.



Supplemental. Fig.6S: Correlation of SHARPIN expression by macrophages with phagocytosis and inflammation in study subjects. Peripheral blood mononuclear cells (PBMCs) were isolated from the study subjects' blood samples and were cultured in RPMI-1640 supplemented with autologous serum were cultured for 14 days until complete differentiation into macrophages. Aβ phagocytic efficiency of the macrophages were analysed at the 14th day by incubating the cells with FITC-labelled Aβ overnight and the

mean fluorescent intensity per cell (MFI) was calculated over three different fields per sample and represented as scatter plot (**a**). MFI of each study sample was correlated with SHARPIN expression by the macrophages of the same subjects. Pearson correlation coefficient, r = -0.256 (p=0.289, ns) for age-matched control (**b**), r = -0.335 (p=0.264, ns) for mild-cognitive impaired (**c**) and r = -0.338 (p= 0.063, ns) for Alzheimer's disease (**d**) subjects. The release of pro-inflammatory cytokines IL-1β (**e**), TNF-α (**i**) and anti-inflammatory cytokines IL-10 (**m**), TGF-β (**q**) in the blood plasma of the study subjects were analysed using ELISA and represented graphically. SHARPIN expression by macrophages in the study subjects were correlated with cytokine release in the blood plasma. Pearson correlation coefficient for IL-1β, r = 0.453(p=0.051, ns) for age-matched control (**f**), r = 0.373 (p=0.209, ns) for mild-cognitive impaired (**g**) and r = 0.097 (p=0.602, ns) for Alzheimer's disease (**h**) subjects. Pearson correlation coefficient for TNF-α, r = 0.128 (p=0.603, ns) for age-matched control (**j**), r = 0.369 (p=0.215, ns) for mild-cognitive impaired (**k**) and r = -0.132 (p=0.478, ns) for Alzheimer's disease (**l**) subjects. Pearson correlation coefficient for IL-10, r = 0.046 (p=0.852, ns) for age-matched control (**n**), r = -0.530 (p=0.062, ns) for mild-cognitive impaired (**o**) and r = -0.045 (p=0.812, ns) for Alzheimer's disease (**p**) subjects. Pearson correlation coefficientfor TGF-β, r = -0.219 (p=0.366, ns) for age-matched control (**r**) , r = -0.160 (p=0.602, ns) for mild-cognitive impaired (**s**) and r = -0.065 (p= 0.729, ns) for Alzheimer's disease (**t**) subjects (ns- non significant). *ns – non significant.



Supplemental Fig. 7S: SHARPIN expression in study subjects categorized as diabetic and non-diabetic subjects. The study subjects were categorised on the basis of presence of diabetes and the expression of SHARPIN by the macrophages of the subjects were analysed using western blotting and represented graphically (**a**).

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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

P. S. Jairani and P. M. Aswathy are equal first authors.

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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.

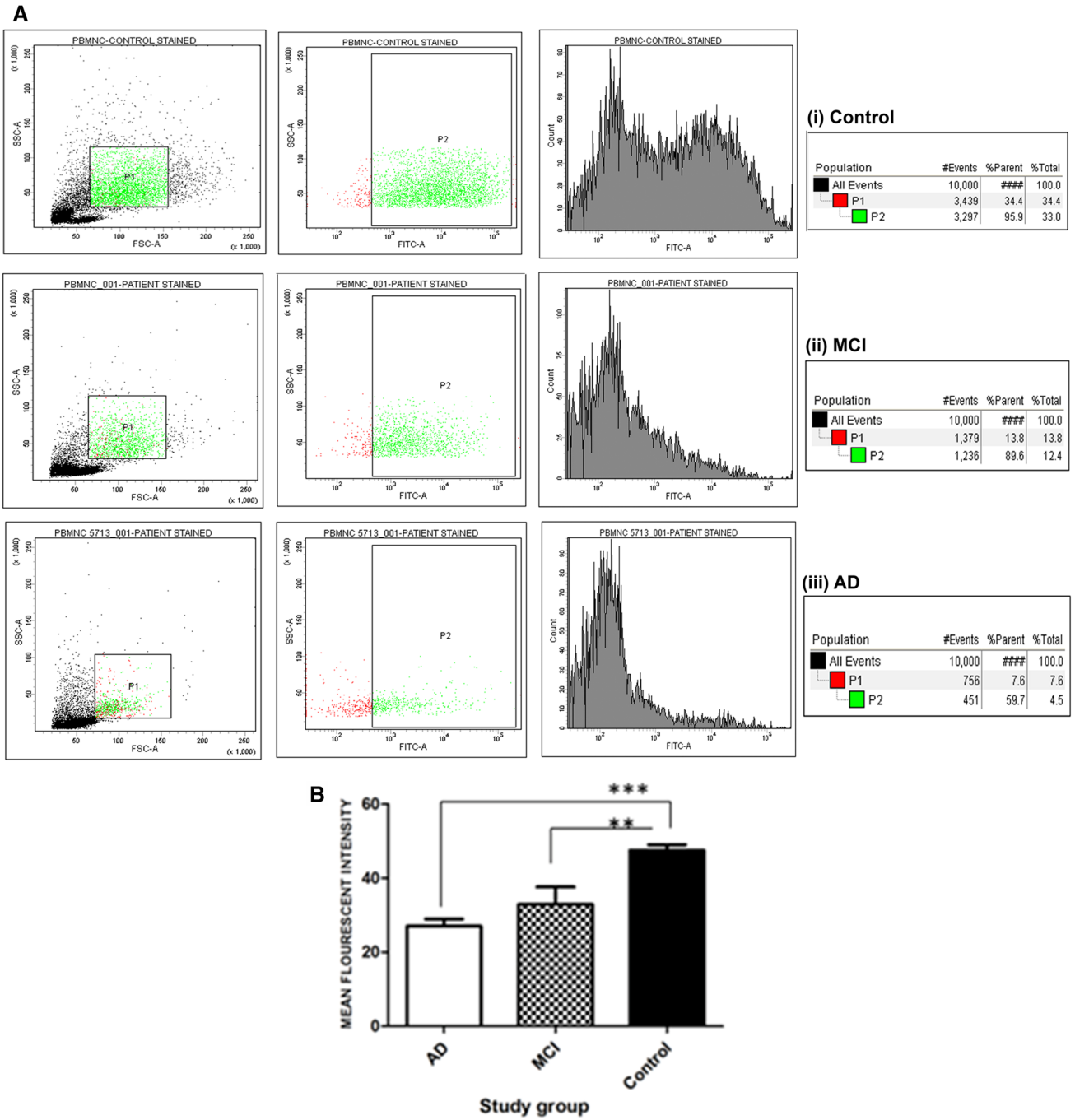

Fig. 1 **a** Representative flow cytometry histograms of cognitively normal control, MCI and AD patient (Histogram shown is from ungated population). Phagocytosis of Aβ = Mean fluorescence intensity (MFI) of FITC Aβ in upper right corner times % cells upper right corner. Top panel (i) (Control) showing 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).

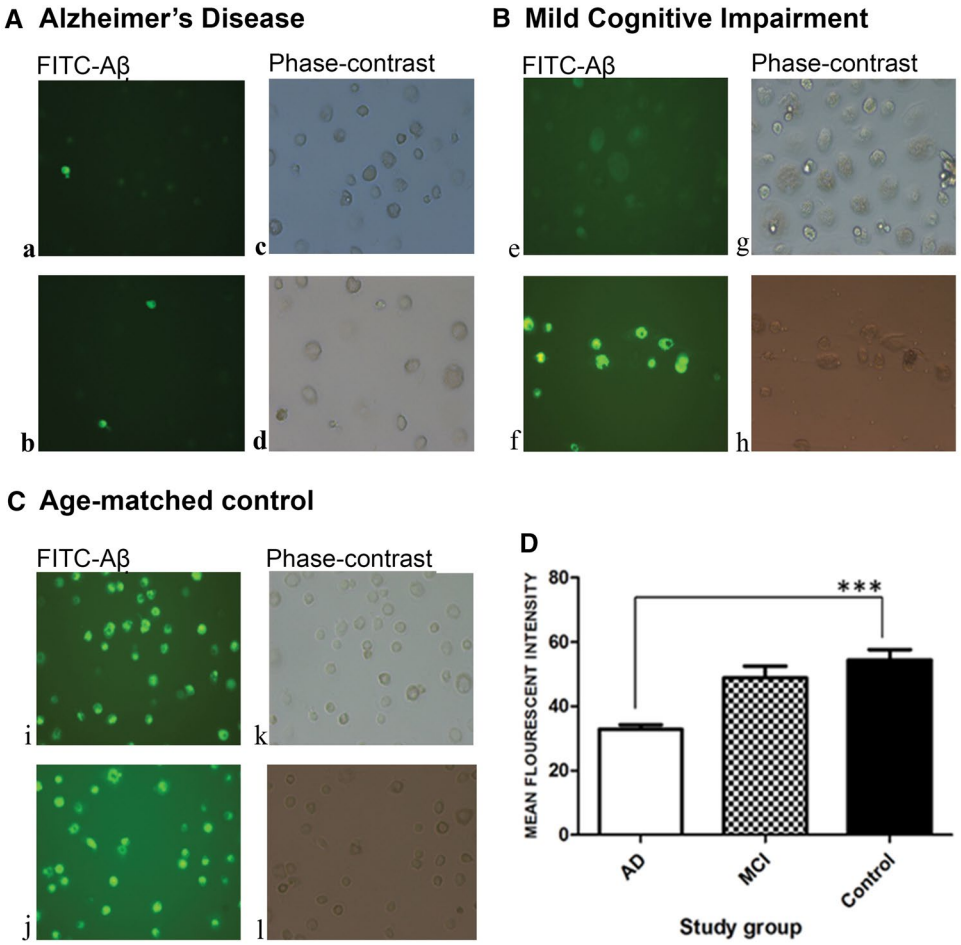

Fig. 2 MFI for macrophage amyloid β internalisation assay. Fluorescent microscopic images: mature macrophages, after overnight exposure to FITC-Aβ, were observed by fluorescent microscope. Representative images at ×40 magnification on fluorescent microscope are given along with respective phase contrast 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. ***$p < 0.0001$

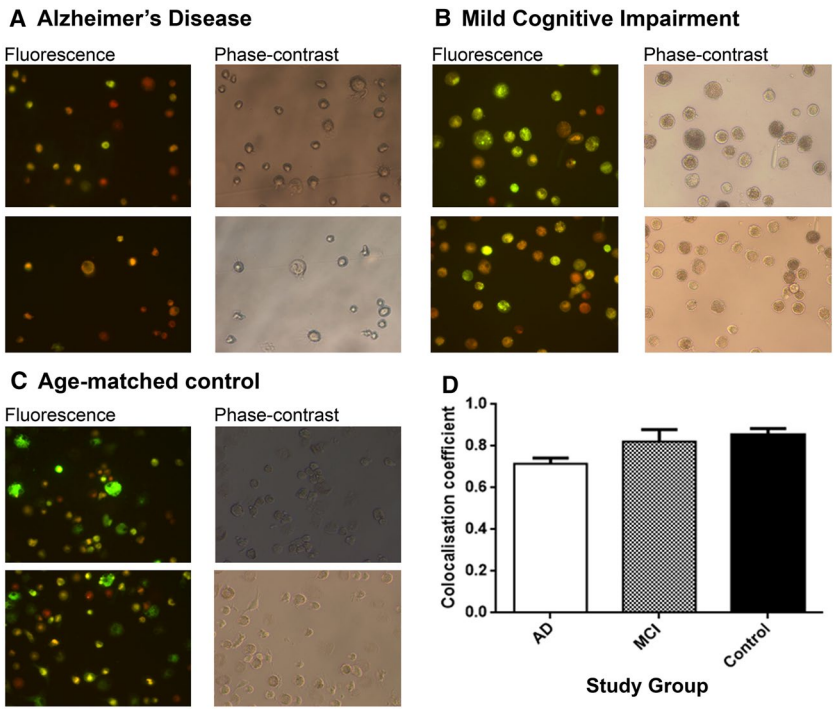

Fig. 3 Differentiated macrophages of an AD patient (**a**), MCI subject (**b**) and a control subject (**c**) were undergone overnight exposure to Aβ (1 µg/mL) and stained by Lysotracker red, examined by fluorescent microscopy (×40 magnification). **d** Degree of colocalization was determined using image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

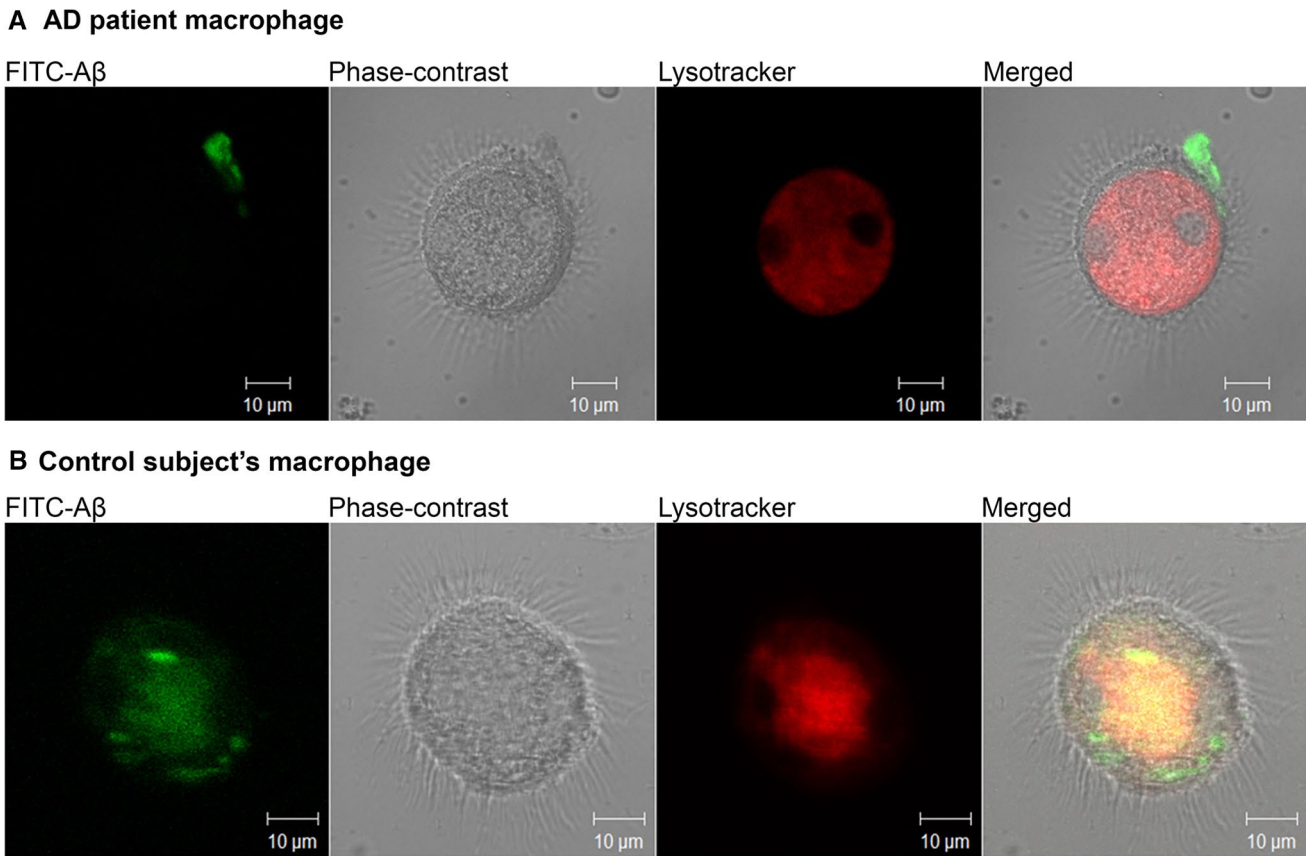

Fig. 4 Confocal images of AD and control macrophages after overnight exposure to fluorescent tagged Aβ. **a** Activated AD macrophage (with processes) being unsuccessful in internalizing Aβ, magnified image (×40) split XY into three channels and merged view showing only surface binding. **b** Control 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

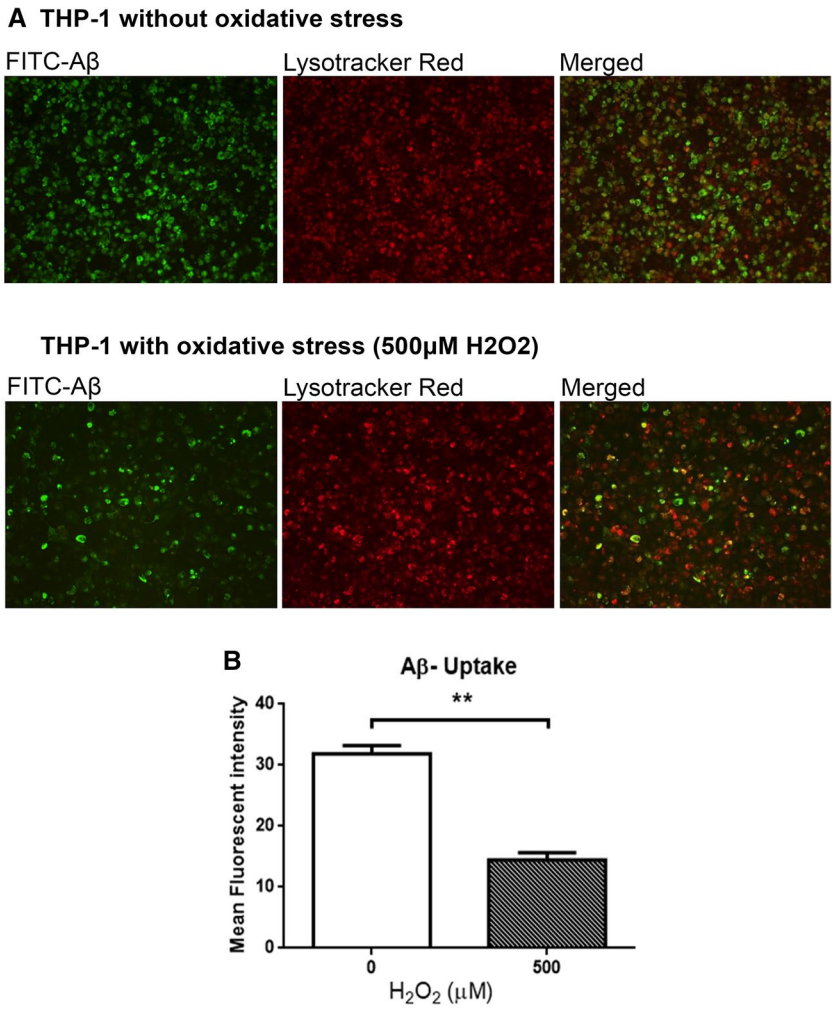

Fig. 5 **a** After pretreatment with 500 µM H_2O_2 for 30 min, the cells were incubated with FITC-Aβ overnight. Total fluorescence was analyzed using fluorescent microscope (×10 magnification). The cells were stained with lysosomal marker Lysotracker Red to visualize lysosomal colocalization of phagocytozed 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

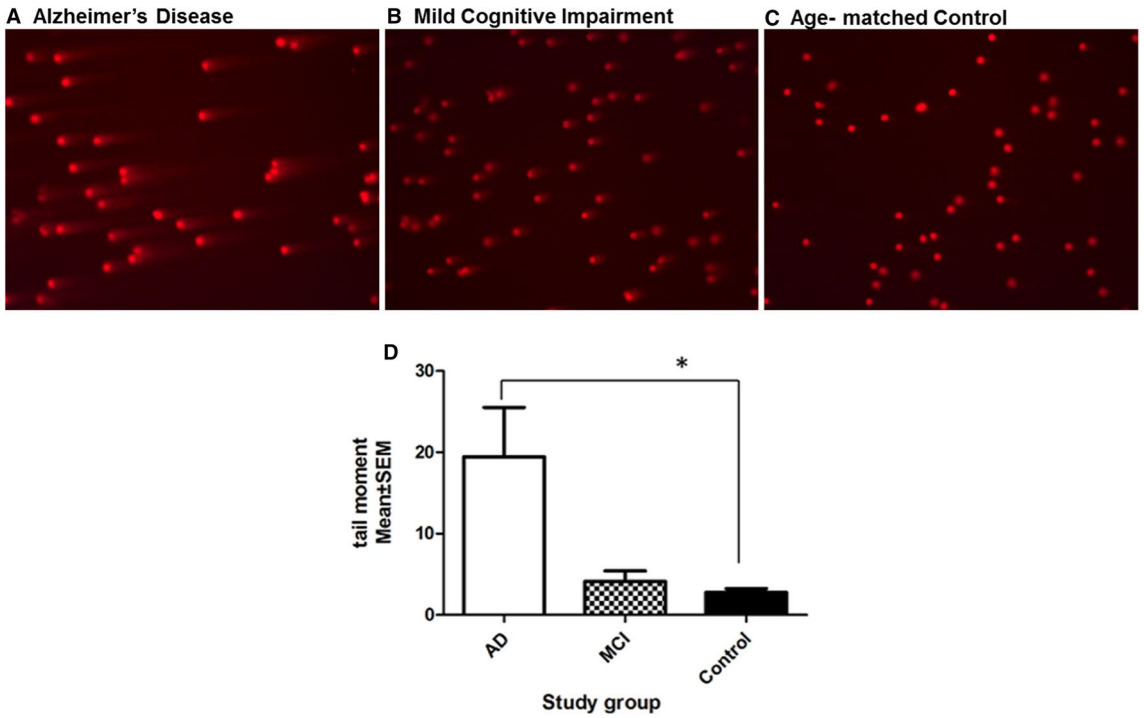

Fig. 6 Representative images of comets on peripheral blood cells of AD patient (**a**), MCI subject (**b**) and age-matched control (**c**) after electrophoresis under UV microscopic observation. **d** Graphical representation of mean tail moments of AD, MCI and controls on comet assay. *$p < 0.05$

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).

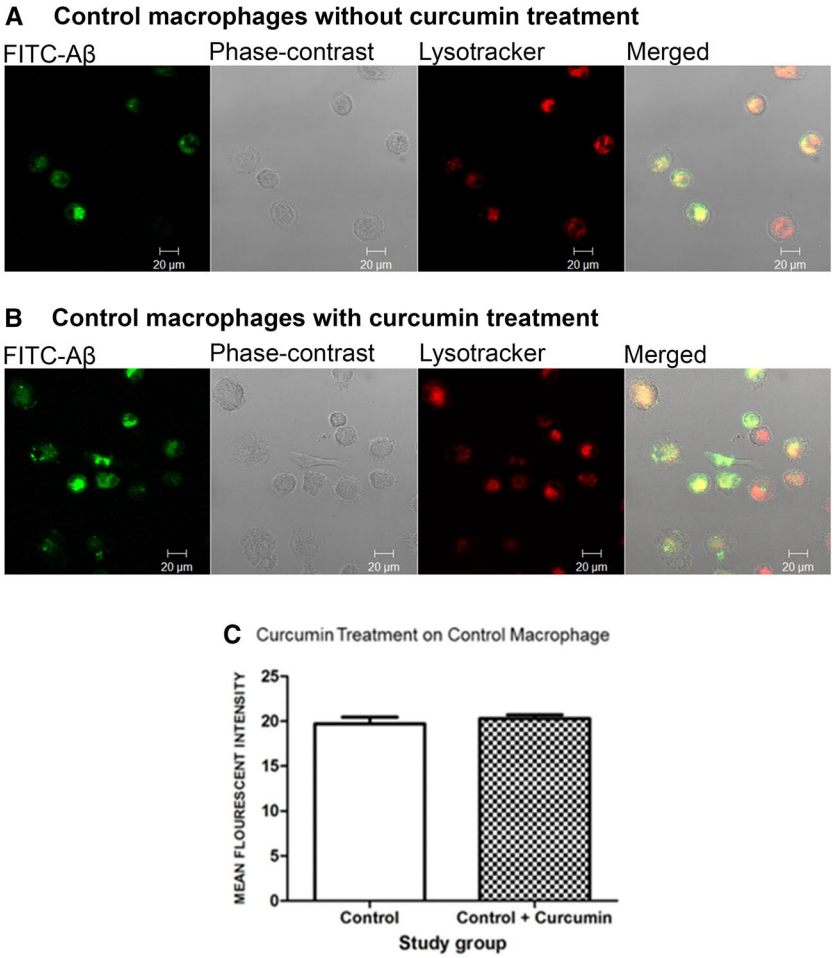

Fig. 7 **a** Control macrophages Showing Baseline Internalization of Fluorescent Aβ. **b** Confocal image of macrophages derived from control subjects showing no significant difference in the phagocytosis efficiency after curcumin treatment. **c** Graphical representation of **a** and **b**

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.

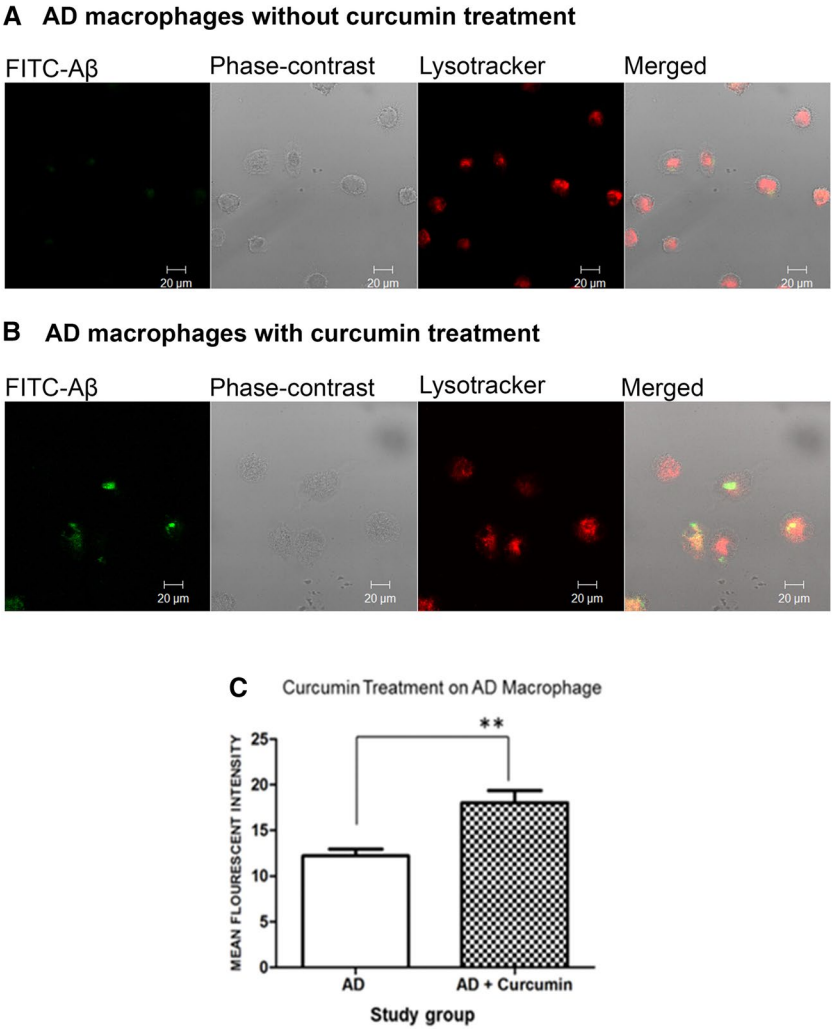

Fig. 8 **a** Confocal images showing significant reduction in phagocytosis efficiency of AD macrophages compared to macrophages derived from age-matched control. **b** Confocal image of AD macrophages showing enhancement of phagocytic efficiency after curcumin treatment when compared to untreated macrophages. **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.

References

Ak T, Gülçin I (2008) Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact 174(1):27–37

Butterfield DA, Reed T, Newman SF, Sultana R (2007) Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment. Free Radic Biol Med 43(5):658–677

Cash JG et al (2012) Apolipoprotein E4 impairs macrophage efferocytosis and potentiates apoptosis by accelerating endoplasmic reticulum stress. J Biol Chem 287(33):27876–27884

Castellano JM et al (2011) Human apoE isoforms differentially regulate brain amyloid-β peptide clearance. Sci Trans Med 3(89):89ra57

Coppedè F, Migliore L (2015) DNA damage in neurodegenerative diseases. Mutat Res 776:84–97

Cudaback E, Li X, Montine KS, Montine TJ, Keene CD (2011) Apolipoprotein E isoform-dependent microglia migration. FASEB J 25(6):2082–2091

Dementia statistics | Alzheimer's Disease International [Online]. https://www.alz.co.uk/research/statistics. Accessed: 09 Mar 2018

Doens A, Fernández PL (2014) Microglia receptors and their implications in the response to amyloid β for Alzheimer's disease pathogenesis. J Neuroinflamm 11:48

Fiala M et al (1998) Amyloid-beta induces chemokine secretion and monocyte migration across a human blood–brain barrier model. Mol Med 4(7):480–489

Fiala M et al (2007) Innate immunity and transcription of MGAT-III and Toll-like receptors in Alzheimer's disease patients are improved by bisdemethoxycurcumin. Proc Natl Acad Sci USA 104(31):12849–12854

Garcia-Alloza M, Borrelli LA, Rozkalne A, Hyman BT, Bacskai BJ (2007) Curcumin labels amyloid pathology in vivo, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. J Neurochem 102(4):1095–1104

Grainger DJ, Reckless J, McKilligin E (2004) Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. J Immunol 173(10):6366–6375

Hixson JE, Vernier DT (1990) Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. J Lipid Res 31(3):545–548

Hughes CP, Berg L, Danziger WL, Coben LA, Martin RL (1982) A new clinical scale for the staging of dementia. Br J Psychiatry J Ment Sci 140:566–572

Jairani PS, Aswathy PM, Gopala S, Verghese J, Mathuranath PS (2016) Interaction with the MAPT H1H1 genotype increases dementia risk in APOE ε4 carriers in a population of Southern India. Dement Geriatr Cogn Disord 42(5–6):255–264

Kirkham P (2007) Oxidative stress and macrophage function: a failure to resolve the inflammatory response. Biochem Soc Trans 35(Pt 2):284–287

Kline A (2012) Apolipoprotein E, amyloid-ß clearance and therapeutic opportunities in Alzheimer's disease. Alzheimers Res Ther 4(4):32

Lai AY, McLaurin J (2012) Clearance of amyloid-β peptides by microglia and macrophages: the issue of what, when and where. Fut Neurol 7(2):165–176

Lee CYD, Tse W, Smith JD, Landreth GE (2012) Apolipoprotein E promotes β-amyloid trafficking and degradation by modulating microglial cholesterol levels. J Biol Chem 287(3):2032–2044

Lim GP, Chu T, Yang F, Beech W, Frautschy SA, Cole GM (2001) The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J. Neurosci 21(21):8370–8377

Liu C-C, Kanekiyo T, Xu H, Bu G (2013) Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol 9(2):106–118

Mathuranath PS, Nestor PJ, Berrios GE, Rakowicz W, Hodges JR (2000) A brief cognitive test battery to differentiate Alzheimer's disease and frontotemporal dementia. Neurology 55(11):1613–1620

Mathuranath PS et al (2010) Dementia in Kerala, South India: prevalence and influence of age, education and gender. Int J Geriatr Psychiatry 25(3):290–297

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34(7):939–944

Mishra S, Palanivelu K (2008) The effect of curcumin (turmeric) on Alzheimer's disease: an overview. Ann Indian Acad Neurol 11(1):13–19

Mohandas E, Rajmohan V, Raghunath B (2009) Neurobiology of Alzheimer's disease. Indian J Psychiatry 51(1):55–61

Montagne A, Zhao Z, Zlokovic BV (2017) Alzheimer's disease: A matter of blood–brain barrier dysfunction? J Exp Med 214(11):3151–3169

Owen JB et al (2010) Oxidative modification to LDL receptor-related protein 1 in hippocampus from subjects with Alzheimer disease: implications for Aβ accumulation in AD brain. Free Radic Biol Med 49(11):1798–1803

Pantelidis P, Lambert-Hammill M, Wierzbicki AS (2003) Simple sequence-specific-primer-PCR method to identify the three main apolipoprotein E haplotypes. Clin Chem 49(11):1945–1948

Petersen RC, Smith GE, Waring SC, Ivnik RJ, Kokmen E, Tangelos EG (1997) Aging, memory, and mild cognitive impairment. Int Psychogeriatr 9(Suppl 1):65–69

Petrlova J et al (2011) A differential association of Apolipoprotein E isoforms with the amyloid-β oligomer in solution. Proteins 79(2):402–416

Raley MJ, Loegering DJ (1999) Role of an oxidative stress in the macrophage dysfunction caused by erythrophagocytosis. Free Radic Biol Med 27(11–12):1455–1464

Reddy PH et al (2016) Protective effects of a natural product, curcumin, against amyloid β induced mitochondrial and synaptic toxicities in Alzheimer's disease. J Investig Med 64(8):1220–1234

Reddy PH et al (2018) Protective effects of Indian spice curcumin against amyloid beta in Alzheimer's disease. J Alzheimers Dis JAD 61(3):843–866

Splettstoesser WD, Schuff-Werner P (2002) Oxidative stress in phagocytes—"the enemy within". Microsc Res Tech 57(6):441–455

Zhu Y et al (2012) APOE genotype alters glial activation and loss of synaptic markers in mice. Glia 60(4):559–569

Zuroff L, Daley D, Black KL, Koronyo-Hamaoui M (2017) Clearance of cerebral Aβ in Alzheimer's disease: reassessing the role of microglia and monocytes. Cell Mol Life Sci 74(12):2167–2201

Research Articles:

- **Dhanya Krishnan**, Ramsekhar N Menon, Mathuranath PS, Srinivas Gopala ―*A novel role for SHARPIN in amyloid-β phagocytosis and inflammation by peripheral blood- derived macrophages in Alzheimer's disease*, published in the journal of ***Neurobiology of Aging*** (PMID: 32165044) as first author. Impact factor: 4.398

- P. S. Jairani, P. M. Aswathy, **Dhanya Krishnan**, Ramsekhar N. Menon, Joe Verghese, P. S. Mathuranath, Srinivas Gopala ―*Apolipoprotein E Polymorphism and Oxidative Stress in Peripheral Blood- Derived Macrophage-Mediated Amyloid-Beta Phagocytosis in Alzheimer's Disease Patients* published in the journal of ***Cellular and Molecular Neurobiology*** (PMID: 30694418) as 2nd author. Impact factor: 3.811

Conferences:

- Best Paper Presentation Award (3rd) in International Conference on Advanced Degenerative Diseases and Molecular Interventions conducted at Trivandrum, 2017

- Best Paper Presentation Award (1st) in National Seminar on Omics Approaches in Biotechnology conducted by Department of Biotechnology, Government college, Karyavattom, 2019

- Best Poster Presentation Award (1st) in International Symposium on Metabolism and Ageing conducted at Trivandrum, 2019

- Best Paper Presentation Award (2nd) in Biochemistry Talks: Targeting Metabolism in Non Communicable Diseases conducted at SCTIMST, 2019

- Poster presented (virtually) at Keystone Symbosia: Neurodegenerative Diseases: New Insights and Therapeutic Opportunities conducted at Keystone, Colorado USA, 2019

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