श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेंद्रम, तिरुवनन्तपुरम - 695 011, केरल, भारत
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
THIRUVANANTHAPURAM - 695 011, KERALA, INDIA
(एक राष्ट्रीय महत्व का संस्थान, विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार)
(An Institution of National Importance, Department of Science and Technology, Government of India)
टेलीफोन नं./Telephone No.: 0471-2443152 फैक्स/Fax: 0471-2446433, 2550728
ई-मेल/E-mail: sct@sctimst.ac.in वेबसाइट/Website: www.sctimst.ac.in

PROJECT COMPLETION REPORT

1. **Project Number** : 5301

2. **Title of the Project** : In vitro beta amyloid uptake by peripheral blood macrophages: predictor for progression of Mild cognitive impairment (MCI) to Alzheimer's disease (AD)

3. **Funding Agency Name** : ICMR

4. **Project Reference Number provided by the Funding Agency**: No. 53/02/2011-CMB/BMS

5. **Principal Investigator (Name & Address) :** Dr Srinivas Gopala
Scientist D
Department of Biochemistry

6. **Co-Investigators (Name & Address):** Dr. Ramsekhar N Menon, Associate

Professor, Department of Neurology, SCTIMST

7. **Implementing Institution : ** Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvanthapuram, Kerala

8. **Collaborating Institutions** : Nil

9. **Date of Commencement** : 01 March 2015

10. **Duration** : 3 YEARS

11. **Date of Completion** : November 30, 2018

12. **Objectives as approved:**

(i) Determination of de novo Aβ levels in plasma as well as in macrophages from AD patients, MCI subjects and age-matched controls.

(ii) To elucidate the role of macrophages in altering the rate of brain amyloidosis, by

an amyloid uptake assay using macrophages from AD patients, MCI subjects and age-matched controls.

(iii) Analysis of macrophage proteome for altered proteins by 2D gel electrophoresis.

(iv) ApoE genotyping in the study population.

13. Deviation made from original objectives if any, while implementing the project and reasons thereof :

i. To check whether SHARPIN regulates Aβ-mediated NLRP3 expression and polarization in macrophages

ii. To check whether SHARPIN – mediated inflammation induces neuronal apoptosis

iii. To check whether SHARPIN is involved in the regulation of Aβ receptor expression and phagocytosis

iv. To analyse whether Aβ- mediated oxidative stress regulates SHARPIN expression and whether the transcription factor NF-κB modulates SHARPIN expression

v. To analyze SHARPIN expression patterns in Alzheimer's Disease, MCI and control subjects and correlate with FITC-Aβ phagocytosis and inflammatory cytokine release in plasma of study subjects

14. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs :
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, 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. Subjects with other neurological disorders and infectious diseases which may alter the peripheral immune function were excluded from the study. All the recruited subjects were tested for hypertension, hyperlipidaemia, hypercholesterolemia, Vitamin B12 deficiency, thyroid dysfunction, diabetes, cardiopathy or any history of cranial trauma. Subjects with high plasma CRP level were excluded from the study to avoid the possibility of peripheral infection or inflammation- mediated alteration of protein expression patterns and cell function. 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). Preclinical AD cases were classified as MCI, on the basis of their MMSE (Mini Mental State Examination) scores and

performance on the Addenbrook's Cognitive Examination (ACE) (Mathuranath et al., 2000). The study population comprised of 34 individuals in four groups of 12 early- stage AD (eAD), 4 moderate- stage AD (mAD), 11 MCI and 6 cognitively unimpaired age-matched controls. The blood specimens (20 ml) were obtained from all subjects by venipuncture; and collected in heparin-lysed vacutainers for isolation of monocytes, DNA, plasma and serum.

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 anti-coagulated blood. Anti-coagulated blood was layered on top of equal volume of Ficoll-Paque medium and centrifuged at 2000rpm for 20 minutes. The PBMCs, collected from the interface between the plasma and the density medium, were washed twice in 1X PBS and seeded in culture dishes in RPMI medium supplemented with 10% autologous serum. Autologous serum was isolated by centrifugation of coagulated blood at 2500rpm for 15 minutes, complement inactivated by heating at 56^{o}C for 30 minutes and filtered through 0.22micron filter. Subjects with high plasma CRP levels were excluded from the study.

Cell culture and differentiation

THP-1 acute monocytic leukemia cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and the cells were differentiated into macrophages by incubating with 100nM phorbol 12-myristate 13-acetate (PMA) for 48 hours. All the treatments were carried out in PMA- induced differentiated THP-1 cell lines.

SHSY5Y neuroblastoma cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS. The cells at 50% confluency were treated with 10μM retinoic acid (RA) in 1% FBS for 3-4 days for differentiation of SHSY5Y neuroblastoma cells into mature neurons. Neuronal differentiation was confirmed by analysing the expression of neuronal stem cell marker Nestin and mature neuronal marker β-III tubulin.

siRNA transfection

Differentiated THP-1 cells were transfected with siRNA (Cell Signaling Technology, USA) with jetPrime PolyPlus transfection reagent (Thermofischer Scientific) as per the protocol. Transfection efficiency was confirmed by analysing protein expression using western blotting technique.

Assessment of Reactive Oxygen Species production

H_2DCFDA (dichlorofluorescin diacetate) assay was used to detect Aβ- induced oxidative stress in differentiated THP-1 cells. The intracellular Reactive Oxygen Species (ROS) levels were quantified after incubating the cells with 40μM Aβ and 10mM N-acetyl cysteine (NAC), an ROS scavenger, for 12 hours. The cells were then trypsinized and incubated with 10μM H_2DCFDA for 1 hour at 37^{o}C, washed and subjected to FACS analysis.

Immunoprecipitation

Differentiated THP-1 cells were pre-treated with Bay-117082 for 1 hour and then with Aβ for 12 hours and the total protein were isolated using low-ionic isolation buffer. The protein isolated was incubated with primary antibody and then with Protein-A coated magnetic beads and pulled down by applying magnetic field. The protein bound with magnetic beads are washed and incubated with 3X Laemelli buffer at 70_oC for 5 minutes and then exposed to magnetic field to pull down the coated protein.

Immunoblot analysis

Differentiated THP-1 cells were treated with 10μM Aβ for 6 hours to analyse the expression of SHARPIN and NLRP3 and for 12 hours to analyse the expression of macrophage polarization markers and phagocytic receptors. Cells were pre-treated with NAC or Bay-117082 for 1 hour and then with Aβ for 6 and 12 hours respectively to analyse protein expression patterns. After incubation, total protein was isolated from the cells using 1X RIPA buffer with protease and phosphatase inhibitors and quantified using BCA protein quantification assay. Total protein was denatured in Laemmeli buffer and loaded on SDS-PAGE gels for separation. The separated protein were transferred to PVDF membrane, blocked with 5% skimmed milk and incubated overnight with the respective antibodies in 3% bovine serum albumin (BSA) at 4^oC. After overnight incubation, the washed blots were incubated with HRP- labelled secondary antibody and developed using enhanced chemiluminescence. The relative protein expression was quantified densitometrically using ImageJ software and normalized with β-actin expression.

mRNA expression analysis

Total RNA was isolated from differentiated THP-1 cells using the kit protocol (Invitrogen) and cDNA was synthesised from the isolated RNA. The mRNA expression was analysed using TaqMan Primers with human tubulin as internal control.

Analysis of Cytokine Release

The release of inflammatory cytokines and the amount of Aβ40 and Aβ42 in plasma samples and THP-1 cell conditioned media were analysed using Enzyme-Linked Immuno-Sorbent Assay. The samples were pre-treated and diluted as per the assay kit protocol (ImmunoTag, USA) and incubated in specific antibody pre-coated wells. The wells were washed and incubated with primary antibody, HRP-conjugated secondary antibody and TMB substrate sequentially and the absorbance was read at 450nm and the relative absorbance was calculated.

Macrophage Aβ internalization assay

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 1μg/ml HiLyte Flour 488-labeled Amyloid-β 1-42 (FITC-Aβ), washed with 1X PBS and examined by fluorescence microscopy for

analysing Aβ uptake. Lysosomal marker LysotrackerRed (Life Technologies, Carlsbad, CA, USA) was used as the counter stain to analyse the extent of intra-lysosomal localization of phagocytosed Aβ. Image analysis was performed using ImageJ software. MFI over six different fields per sample were subjected to analysis.

THP-1 cell lines: Differentiated cells were incubated overnight with FITC-Aβ, washed twice with 1X PBS and examined by fluorescent microscopy. The images obtained were quantified for Mean Fluorescent Intensity using ImageJ software.

Conditioned media Experiments

Conditioned media collected from differentiated THP-1 treated with Aβ with/without siRNA were centrifuged and removed cellular debris. Differentiated SHSY5Y cells were treated with the conditioned media for 24-48 hours and the expression of apoptotic markers were analysed.

Statistical Analyses

One way ANOVA with Dunnett's multiple comparisons test was used to compare control parameters with treatment groups. Pearson 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.

Data collected with figures:

a. SHARPIN is required for Aβ- induced NLRP3 expression in macrophages

The role of SHARPIN in regulating the expression of the inflammasome components, especially the NLRP3 protein through NF-κB signaling pathway is studied in Chronic proliferative dermatitis (cpdm) (1), however, the regulatory pathways involved in NLRP3 expression in Alzheimer's Disease has not been well documented. To analyze the role of SHARPIN in the regulation of Aβ- mediated NLRP3 expression, we silenced SHARPIN expression using siRNA- mediated gene knockdown in differentiated THP-1 macrophages. We have observed that Aβ could induce the expression of SHARPIN in the *in-vitro* system and the NLRP3 protein expression induced by Aβ is almost completely inhibited by silencing the expression of SHARPIN in macrophages. This study proves the novel role of the protein SHARPIN in Alzheimer's disease pathogenesis.

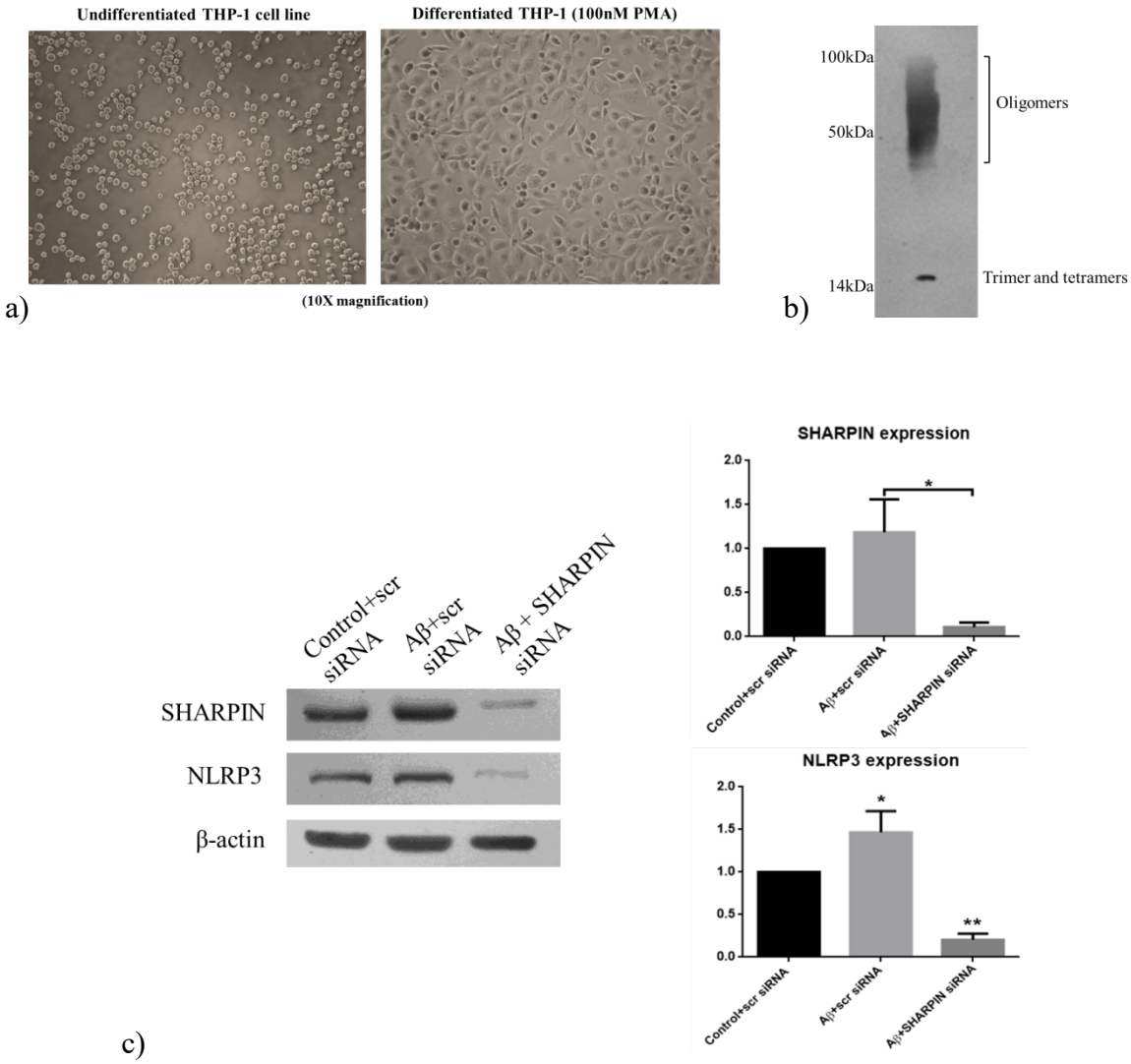
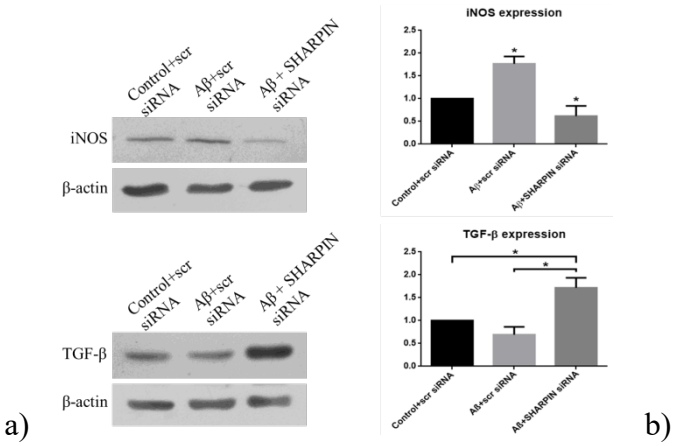


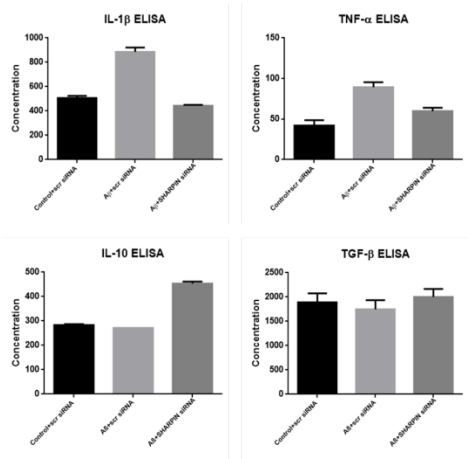
Fig.1: a) Phase contrast microscopic image of human monocytic leukemia cell lines and the differentiated macrophages after treatment with PMA. b) Western blot image showing that amyloid-beta prepared was in the toxic forms (oligomeric, tetrameric and trimeric). c) Western blot data and graphical representation showing increased expression of SHARPIN and NLRP3 in the presence of Aβ and that SHARPIN knockdown has downregulated theexpression of NLRP3 proving that SHARPIN controls NLRP3 expression.

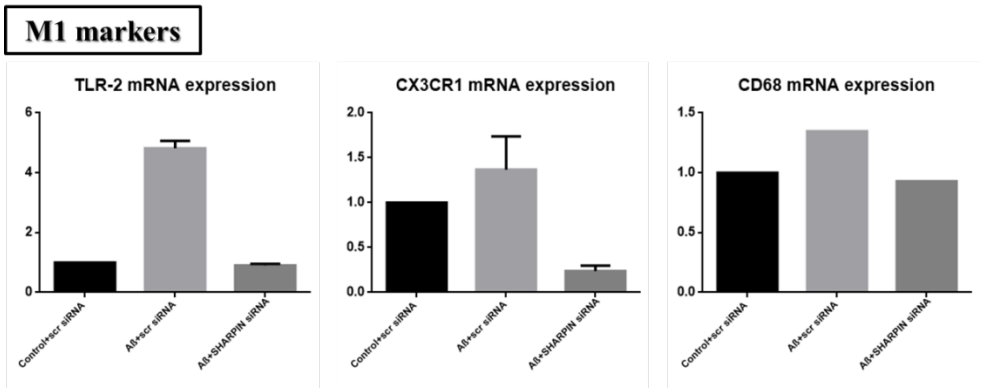
b. SHARPIN regulates Aβ- induced Macrophage Polarization

NLRP3 inflammasome can be regarded as a master regulator of inflammatory mechanisms in macrophages since the protein complex controls the maturation and release of the major pro-inflammatory cytokines like IL-1β and IL-18 (ref). Since we found that NLRP3 itself is regulated by SHARPIN, we analyzed if SHARPIN protein could control the fate of macrophages to become pro-inflammatory (M1 phenotype) or anti-inflammatory (M2 phenotype). It has been well known that Aβ and all the other toxic proteins in the AD brain

stimulate the macrophages to polarize to the M1 phenotype, which contributes to bystander damage in the AD brain neurons (ref). We observed that SHARPIN knockdown could polarize the macrophages to the anti-inflammatory phenotype even in the presence of Aβ.







c)

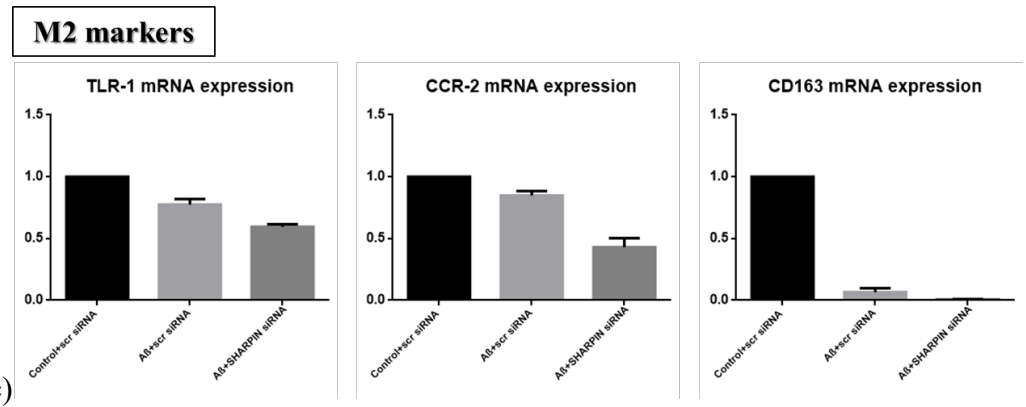
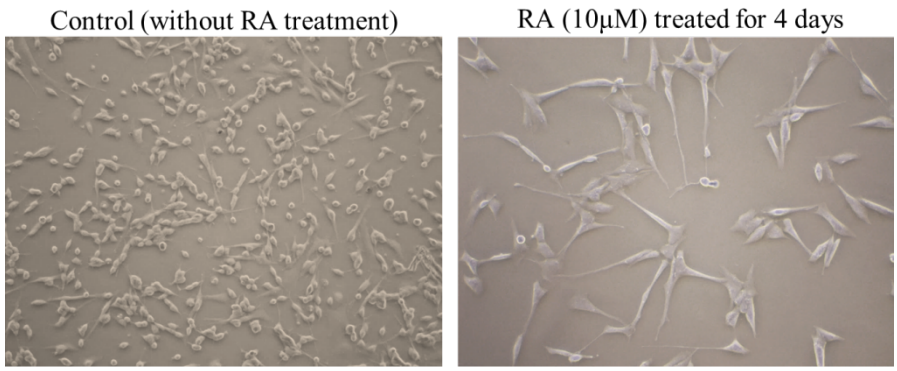


Fig.2: a) Western blot data and graphical representation showing increased expression of iNOS (M1 marker) and decreased expression of TGF-β (M2 marker) in the presence of Aβ proving that Aβ polarizes macrophages to the M1 phenotype and the expression levels got reversed when SHARPIN was knocked down. b) ELISA data showing decreased release of anti-inflammatory cytokines (IL-10 and TGF-β) and increased release of pro-inflammatory cytokines (IL-1β and TNF-α) in the presence of Aβ and that also got reversed once SHARPIN is knocked down. c) Real-time PCR data showing increased mRNA expression of M1 markers and decreased expression of M2 markers in the presence of Aβ. The M1 marker expression got reduced in the presence of SHARPIN siRNA 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.

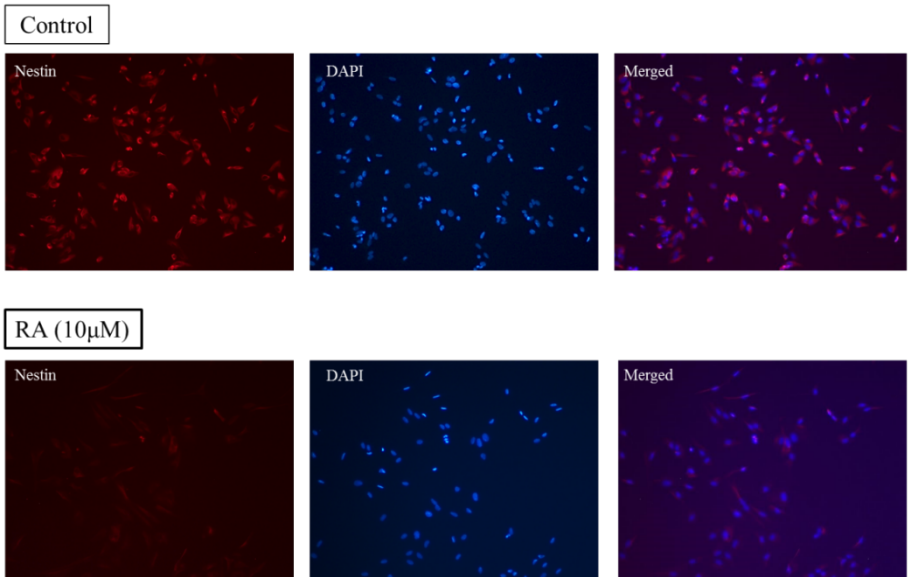
c. SHARPIN down- regulation prevents inflammation-mediated neuronal cell death

Aβ-mediated inflammation has been regarded as one of the major cause for neuronal apoptosis in AD brain. The effect of SHARPIN-mediated inflammatory mechanisms on neuronal apoptosis was analyzed using the conditioned media from SHARPIN-silenced THP-1 macrophages in RA- differentiated SHSY5Y cells. SHSY5Y cells were differentiated into mature neurons by treatment with 10μM retinoic acid for 4 days and the cells were showing decreased expression of stem cell marker Nestin. Differentiated neurons treated with conditioned media obtained from macrophages incubated with Aβ showed increased expression of apoptotic markers in which was found to be reduced when SHARPIN was knockdown confirming that Aβ-induced SHARPIN-mediated inflammation contributes to major part of neuronal apoptosis in AD brain.



(10X magnification)

a)



b)

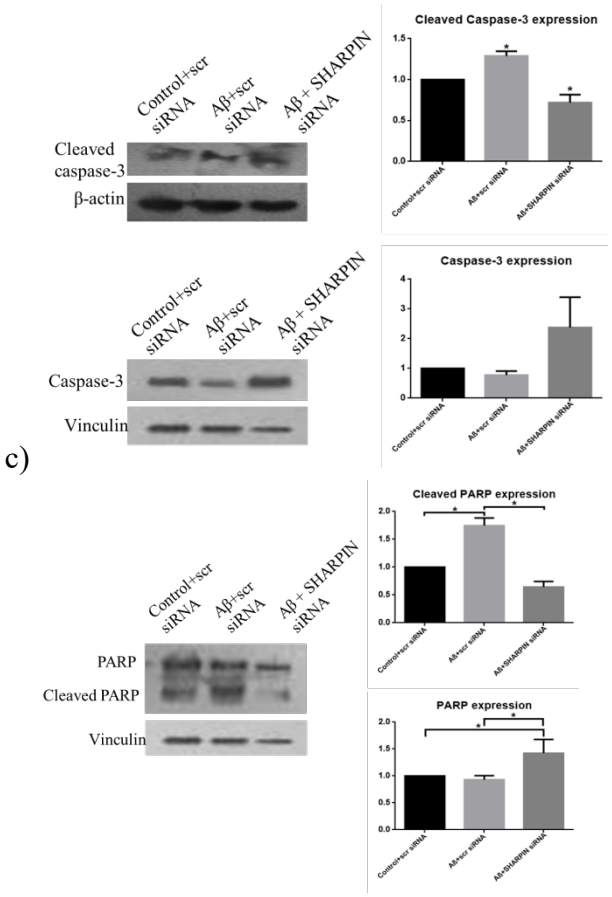


Fig.3: a) Phase contrast image showing undifferentiated neuroblastoma cell lines and the differentiated mature neurons with increased size and synaptic connections. b) Immunocytochemistry image showing decreased expression of Nestin, a marker of stem cell lineage cells, in the differentiated neurons. c) Western blot data and graphical representation showing increased expression of cleaved caspase-3 and cleaved PARP in the presence of Aβ-containing conditioned media, suggesting increased apoptosis in neuronal cells which got decreased when treated with conditioned media from SHARPIN-knockdown cells suggesting that Aβ-induced SHARPIN mediated inflammatory mechanisms are causing the major fraction of neuronal death.

d. SHARPIN regulates Aβ Receptor Expression and Phagocytosis in Macrophages

According to the immune hypothesis, brain-resident microglia and peripheral macrophages show inefficiency in Aβ- phagocytosis due to some unknown mechanisms. Although several studies have shown a correlation between inflammatory mediators and phagocytic receptor expression by immune cells, the underlying mechanisms that promote pro-inflammatory conditions in the AD brain remain elusive. To demonstrate whether SHARPIN, which has been observed as a regulator of inflammation could also play a role in Aβ phagocytosis, we analyzed the phagocytosis efficiency of SHARPIN silenced macrophages by incubating the cells with FITC-labeled Aβ. The SHARPIN knockdown cells show significant decrease in Aβ uptake, which has been demonstrated using fluorescent imaging and flow cytometry

analysis. The expression of receptors involved in Aβ phagocytosis and receptor-mediated uptake were also analyzed which were shown to increase the expression in the presence of Aβ and the expression is brought down by silencing SHARPIN in the presence of Aβ. These results prove the role of SHARPIN in macrophage function, namely phagocytosis and inflammation in Alzheimer's disease.

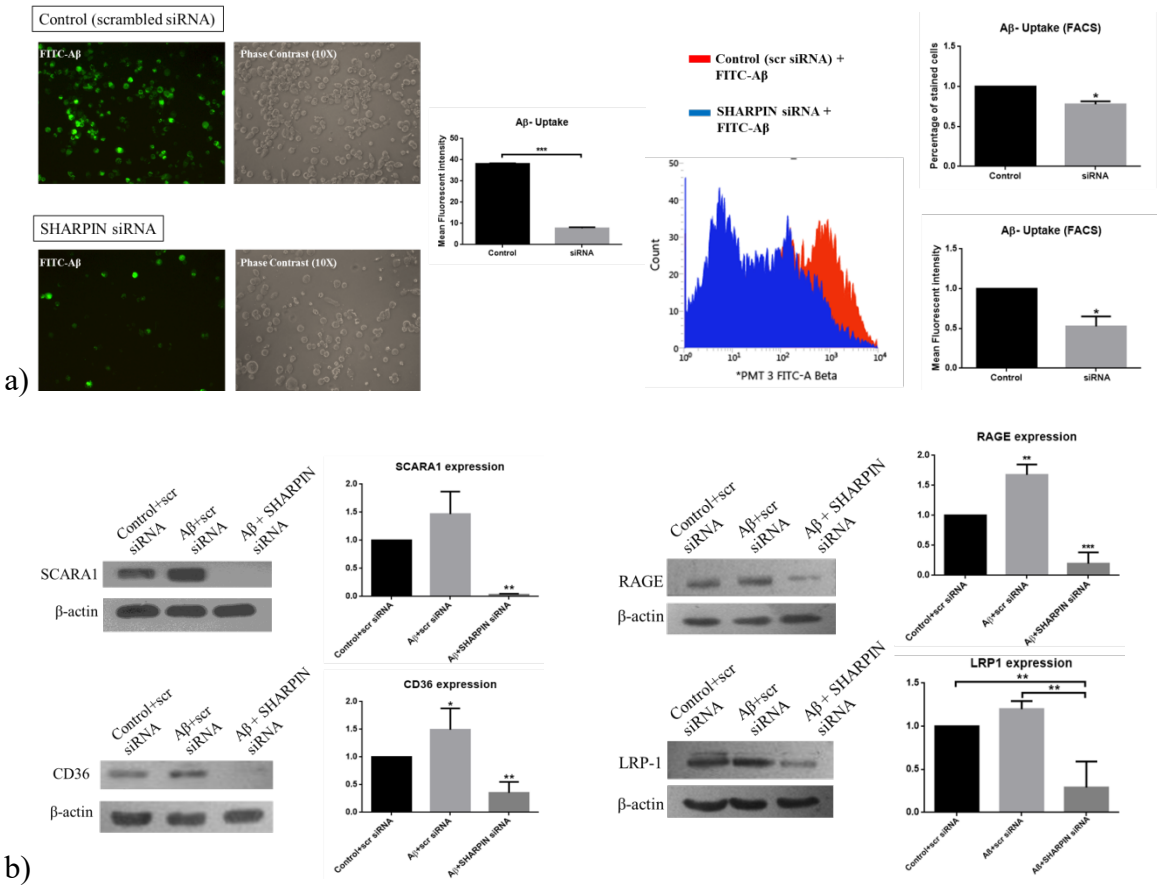


Fig.4: a) Microscopic image and flow cytometry data showing decreased phagocytosis of FITC-labelled Aβ in the presence of SHARPIN siRNA compared with the untreated cells. b) Western blot and graphical representation showing decreased expression of major Aβ-phagocytic receptors (SCARA1 and CD-36) and receptors involved in receptor mediated uptake of Aβ (RAGE-1 and LRP-1) when SHARPIN expression was silenced in Aβ-treated macrophages.

e. Aβ-induced oxidative stress affects SHARPIN expression

Aβ has been well known to induce reactive oxygen species (ROS) generation and oxidative stress (ref) in AD brain and culture systems. Our study has also demonstrated the generation of ROS, using H2- DCFDA assay, which was confirmed by using the ROS scavenger N-acetyl cysteine in the presence of Aβ. SHARPIN has been identified as an upstream signaling molecule regulating the activation of NF-κB transcription factor (ref). Since NF-κB is highly sensitive to oxidative and inflammatory stimuli and SHARPIN is an upstream regulator, we analyzed and found that SHARPIN expression is mediated by Aβ-induced

oxidative stress, where the Aβ- induced SHARPIN expression was brought down by the ROS scavenger NAC.

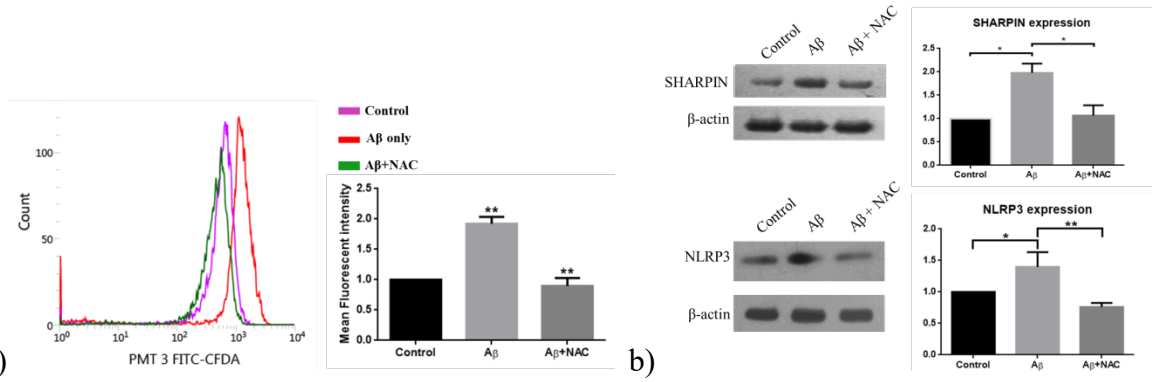


Fig.5: a) DCFDA-flow cytometry data and graphical representation showing increased fluorescence as a result of increased ROS production in Aβ treated cells compared to control and the fluorescence is decreased in the presence of the ROS scavenger N-acetyl cysteine. b) Western blot and graphical data showing increased expression of SHARPIN and NLRP3 in the presence of Aβ which is brought down by treating with NAC suggesting that Aβ-induced oxidative stress is promoting SHARPIN and thereby NLRP3 expression.

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

We found SHARPIN as an important mediator of Aβ- induced inflammation and phagocytosis by macrophages. However, what regulates SHARPIN need to be elucidated. Since NF-κB is a highly sensitive transcription factor to oxidative stress and inflammation, there is a possibility that NF-κB plays a role in SHARPIN regulation. So we looked at the expression of SHARPIN after inhibiting NF-κB using Bay-117082 and found an increase in the molecular weight of SHARPIN suggesting that SHARPIN is controlled by NF-κB mediated indirect signaling mechanism. This has to be confirmed using mass spectrometry analysis.

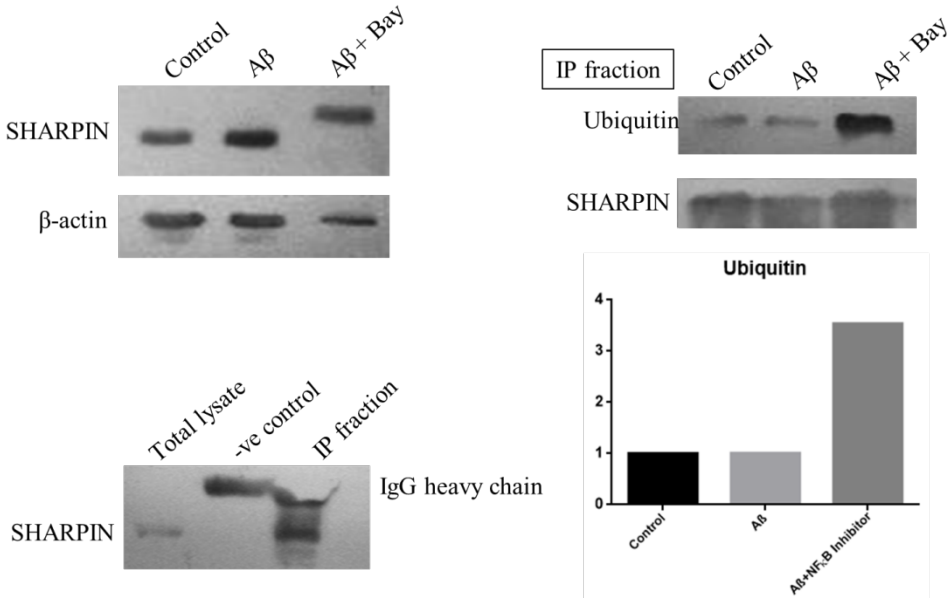


Fig.6: a) Western blot data showing increase in the molecular weight of SHARPIN when the cells are subjected to NF-κB inhibitor. The SHARPIN protein was immunoprecipitated for further analysis and the purity of the immunoprecipitated protein was shown with western blot data. b) Western blot data and graphical representation showing increased ubiquitination of SHARPIN in the Bay- treated cells compared to the untreated.

g. Patient characteristics

Study subjects were categorized into four groups: i) age-matched control subjects are healthy volunteers without any symptoms of memory or cognitive decline, ii) Mild cognitive impaired subjects, which is considered as a transition stage between healthy subjects and AD, iii) early stage AD and iv) moderate stage AD. The sujects showing normal CRP level in blood plasma was selected for the study to exclude the possibility of peripheral infection or inflammation-mediated SHARPIn expression and inflammatory mechanisms. As expected, the MCI group show a higher mean value of Aβ42:40 in the plasma compared to the control which got decreased further with the progression of AD.

Study Subjects	Total number with normal CRP	MMSE scores	ACE scores	Age	Aβ 42:40 ratio
Control	6	-	-	65.8 ± 7.4	0.28 ± 0.07
MCI	11	27 ± 3	82 ± 10	70.6 ± 5.7	0.38 ± 0.04
Early stage AD	12	24 ± 3	70 ± 3	68.7 ± 8.5	0.28 ± 0.15
Moderate stage AD	4	20 ± 2	67 ± 6	64.5 ± 5.3	0.25 ± 0.17

Fig.7: Subject demographics

h. SHARPIN expression is altered in Alzheimer's disease patient macrophages

SHARPIN expression was analyzed in macrophages isolated from the blood samples of the study subjects. All the study subjects show varied expression of SHARPIN however, the mean value correlates with the mean value of the Aβ content in the blood plasma. This pattern could explain that SHARPIN expression by the circulating monocytes and the macrophages cultured in the autologous serum collected from the same subjects was affected by the concentration of Aβ present in the blood plasma.

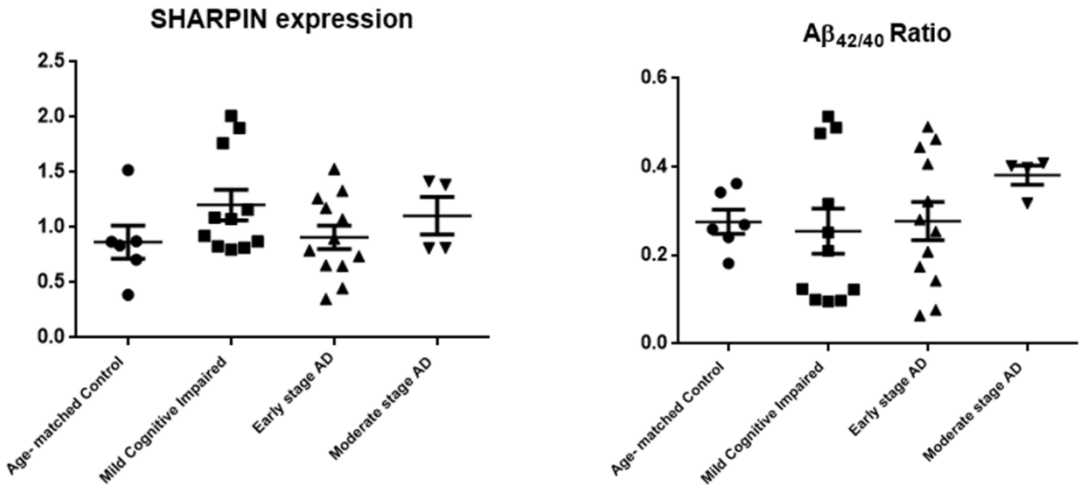


Fig.8: Scatter plot showing SHARPN expression by macrophages cultured in media supplemented with autologous serum which contains Aβ in varied concentration.

i. Correlation of SHARPIN expression with Aβ phagocytosis and inflammatory cytokine release by Alzheimer's disease macrophages

Phagocytic efficiency of isolated macrophages from the study subjects were analyzed using FITC labeled-Aβ and quantified using imageJ software. The release of the pro-inflammatory and anti-inflammatory cytokines in the plasma was also quantified using ELISA and represented graphically. However, these factors does not show significant correlation with SHARPIN expression by the macrophages eventhough there is a positive trend in the patterns (data not shown). Analysis in more number of samples needs to be done for conclusive evidence in the role of the protein *in-vivo*.

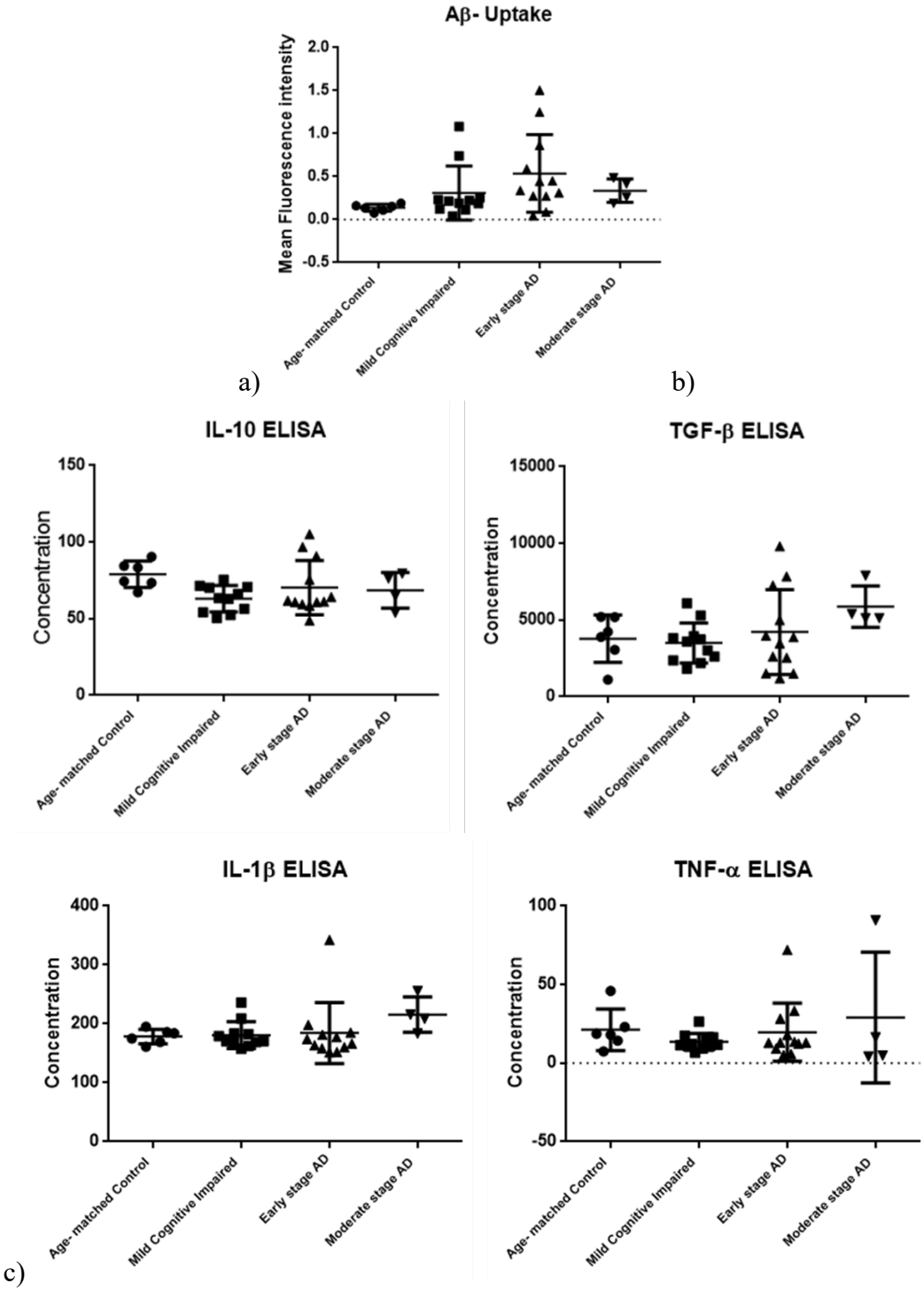


Fig.9: a) Scatter plot showing phagocytosis efficiency of Aβ by macrophages isolated from study subjects. b) Scatter plot showing release of anti-inflammatory cytokines in the blood plasma of the study subjects. c) Scatter plot showing release of pro-inflammatory cytokines in the blood plasma of the study subject

15. **Detailed anaylsis of results :** Defective immune cell-mediated clearance of amyloid-beta (Aβ), is a major contributor of Aβ accumulation in the brain, leading to the pathogenesis of Alzheimer's disease (AD). Aβ accumulation-associated inflammatory activation of macrophages in the brain and the peripheral circulation plays a major role in causing neuronal cell death, leading to the pathogenesis of AD. Although several studies have shown a correlation between inflammatory mediators and phagocytic receptor expression by immune cells, the underlying mechanisms that promote pro-inflammatory conditions in the AD brain remain elusive.

Using differentiated THP-1 macrophages as an in-vitro model, we have identified an increase in the expression of SHARPIN, a ubiquitinating protein that finally leads to the activation of the redox-sensitive transcription factor NF-κB, by Aβ- induced oxidative stress. Aβ- induced SHARPIN expression has been found to regulate the expression of NLRP3, a protein that forms a complex with ASC (Apoptosis-associated speck-like protein containing a CARD) and Caspase-1 to form the NLRP3 inflammasome complex, which has been identified as a prominent mediator of pro-inflammatory cytokine release like IL-1β and IL-18 and thus inflammatory damage in AD scenario. SHARPIN has also been found to regulate polarization of macrophages to the pro-inflammatory M1 phenotype in response to Aβ and siRNA-mediated knockdown of SHARPIN leads to macrophage polarization to the anti-inflammatory phenotype which could protect neurons from inflammatory damage. Using differentiated SHSY%Y neurons, we have also observed a reduced apoptosis of neurons when the cells were treated with conditioned media from SHARPIN-knockdown macrophages in presence of Aβ, suggesting the Aβ-mediated neuronal apoptosis is primarily through damages induced by Aβ-mediated inflammatory mechanisms.

Our study has also shown the role of SHARPIN in regulating Aβ phagocytosis by directly regulating the expression of receptors involved in Aβ-phagocytosis and receptor-mediated uptake thus affecting the overall intake of Aβ. Thus our study proves SHARPIN as a double-edged sword regulating phagocytosis and inflammation, where its downregulation protects inflammatory neuronal damage, but affects immune-mediated phagocytosis and clearance of Aβ. Further, to see the regulatory mechanisms of SHARPIN, we looked at the role of NF-κB, the highly sensitive transcription factor to oxidative stress, inflammatory and immune stimuli, in controlling SHARPIN through some feedback mechanism. Through immunoprecipitation and western blotting, we found that NF-κB mediated signaling mechanism is either inhibiting the transfer of ubiquitin molecules from SHARPIN to NEMO or is ubiquitinilating the protein targeting the protein for degradation or other specific functions. The result needs to be further validated using mass spectrometry analysis.

Analysis of SHARPIN expression in patient samples concluded a trend of expression that depends on the level of Aβ in the plasma, which might be acting as a trigger for SHARPIN expression. Corelation of SHARPIN with phagocytosis efficiency and inflammatory cytokine release in the plasma was not able to find a significant correlation (data not shown). However, analysis in more number of sample are required to conclude the role of the protein in in-vivo.

The findings implicate SHARPIN in regulating Aβ-mediated macrophage polarization to pro-inflammatory M1-phenotype and also in Aβ-phagocytic receptor expression in macrophages thus affecting phagocytosis. In summary, our study demonstrates, for the first time, a novel role for SHARPIN in the regulation of macrophage response to Aβ in the setting of AD.

16. Conclusions summarising the achievements and indication of scope for future work :

Alzheimer's disease 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 plaques and neurofibrillary tangles formed by the accumulation of amyloid beta (Aβ) proteins and hyperphosphorylated tau proteins. Excessive Aβ accumulation along with reduced degradation leads to neuronal death and inflammation leading to the progression of AD. In normal physiology, Aβ is a protein required by the brain in picomolar quantities. However, when secreted in excessive amounts, it will be eliminated through degradation. Immune cells, namely microglia in the brain and macrophages and monocytes in the peripheral system play an important role in Aβ degradation through phagocytosis. Once stimulated, microglia phagocytose Aβ and stimulate the monocytes from blood to migrate into the brain through the secretion of chemokines. But, in AD, it has been well proven that these microglia and macrophages fail to phagocytose Aβ through a downregulated expression of Aβ receptors, leading to increased Aβ accumulation and in that process; the immune cells remain continuously activated by Aβ resulting in an inflammatory milieu in the brain. This will cause bystander damage to neurons apart from the toxicity caused by increased Aβ and p-tau accumulation.

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. Among the family of NOD-like receptors (NLRs), NLRP3 is the most versatile one activated by diverse stimuli other than PAMPs and DAMPs like cellular stress including oxidative stress. The role of NLRP3 inflammasome has been well established in AD however, its regulatory mechanisms in AD have been seldom investigated. SHARPIN (SHANK-associated RH domain-interacting protein), a part of the LUBAC (linear ubiquitin chain assembly complex) has been proven to be controlling the expression of NLRP3 through ubiquitinilating NEMO (NF-kappa B Essential Modulator) and activating the transcription factor NF-κB. Nevertheless, its role in AD has not been studied yet.

In this study, we investigated the role of SHARPIN in Alzheimer's disease through its regulatory role in the macrophage function using differentiated THP-1 cells treated with Aβ as an in-vitro model. SHARPIN has not only been found to be controlling NLRP3 expression in AD macrophages, it was also found to regulate macrophage polarization to M1 (pro-inflammatory) phenotype and Aβ phagocytosis by regulating the expression of Aβ-phagocytic receptors. Thereby, we established the novel role of SHARPIN in Aβ- mediated

macrophage polarization and phagocytosis in Alzheimer's disease. Further, Aβ- induced oxidative stress has been identified as the stimulating factor of SHARPIN expression, whose activation is again controlled by NF-κB signalling mediated inhibition of the ubiquitin transferring ability of SHARPIN protein. The signalling mechanisms regulating SHARPIN expression need to be further explored for identifying the mechanisms controlling macrophage function, thereby promoting the identification of possible targets to control inflammatory damage in neurodegerenative diseases as well as in inflammatory disorders.

17. Science and Technology benefits accrued :

a. List of research publications with complete details :

1. P M Aswathy, P S Jairani, Joe Verghese, Srinivas Gopala, P S Mathuranath. *MAPT* genetic variations are uncommon cause of frontotemporal dementia in south India. ***Neurobiology of Aging*** 35: 443.e23–443.e24, 2014.
2. P S Jairani, P M Aswathy, Srinivas Gopala, Joe Verghese, P S Mathuranath, Interaction with MAPT H1/H1 haplotype increases dementia risk in APOE ε4 carriers in a population of southern India. ***Dementia and Geriatric Cognitive Disorders***, 42: 255-264, 2016.
3. P M Aswathy, P S Jairani, Joe Verghese, Sheelakumari R, Srinivas Gopala, Priya Srinivas, P S Mathuranath. Progranulin mutation analysis: Identification of one novel mutation in exon 12 associated with frontotemporal dementia. **Neurobiology of Aging** 39: 218e1-218.e3, 2016.
4. Jairani PS, Aswathy PM, Dhanya Krishnan, Ramsekhar N Menon, Joe Varghese, Mathuranath PS, Srinivas Gopala. Apolipoprotein E Polymorphism and Oxidative Stress in Peripheral Blood- Derived Macrophage-Mediated Amyloid-Beta Phagocytosis in Alzheimer's Disease Patients. ***Cellular & Molecular Neurobiology***, 39: 355-369, 2019. **IF: 3.895**
5. Dhanya Krishnan, Ramsekhar Menon, Mathuranath PS, Srinivas Gopala. A novel role for SHARPIN in Aβ- phagocytosis and inflammation by peripheral blood derived macrophages in Alzheimer's disease. ***Neurobiology of Aging,*** 93: 131-141, 2020. **IF: 4.673**
6. Dhanya Krishnan, Ramsekhar Menon, Srinivas Gopala. SHARPIN: Role in Finding NEMO and in Amyloid Beta Clearance and Degradation (ABCD) Pathway in Alzheimer's Disease? ***Cellular and Molecular Neurobiology****, 42:* 1267-1281, 2022. **IF: 5.046.**

b. Manpower trained on the project :

i. Research Scientists or Research Fellows	**:**	Three JRFs
ii. No. of PhD's produced	**:**	Two
iii. Other Technical Personnel trained	**:**	Nil

c. Patents taken, if any : Nil

d. Products developed, if any : Nil

18. Procurement/Usage of Equipment:

1. Procurement/ usage of equipment:
   a.

Sl no	Name of Equipment	Make/Model	Cost (Rs)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
	MINI-PROTEAN TETRA CELL, MINI TRANS-BLOT TRANS BLOT SD SEMI DRY ELECTROPHORETIC APPARATTUS	Biorad	366510/-	08/11/2017	100%	Nil

Suggestions for disposal of equipment(s)

The equipment and the accessories are working conditions and currently being heavily utilized in the department by research students.

(Name and Signature of PI)