A novel role for SHARPIN in Aβ- mediated macrophage function in Alzheimer's disease

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Highlights

- SHARPIN regulates Aβ-phagocytic receptor expression by macrophages
- SHARPIN controls Aβ-mediated NLRP3 expression and macrophage polarization
- Aβ-induced SHARPIN mediates inflammatory damage, resulting in neuronal apoptosis
- Aβ-induced oxidative stress stimulates SHARPIN expression in macrophages
- NF-κB- mediated signalling alter SHARPIN protein in Aβ-treated macrophages

Research in Context

1. **Systematic review:** The authors reviewed the literature using PubMed and Google scholar to identify studies describing mechanisms underlying ineffective Aβ phagocytosis, and inflammation in the pathophysiology of Alzheimer's disease. The authors found that studies focussing on the role of SHARPIN in progression of AD is lacking.
2. **Interpretation:** Our *in vitro* and *ex vivo* findings demonstrate a novel role for SHARPIN in AD. Specifically, SHARPIN was found to mediate Aβ-phagocytosis and inflammation in peripheral macrophages exposed to Aβ. Further, SHARPIN expression was correlated with AD progression in patient blood-derived macrophages.
3. **Future directions:** The data in the present study show evidence for association between SHARPIN expression and progression of AD. Hence, a direct correlation with SHARPIN and the pathogenesis of AD needs to be explored in knockout mice models of AD and in AD patient-derived brain samples.

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-beta; MCI, Mild Cognitive Impairment; SHARPIN, Shank-associated RH domain-interacting protein; NLRP3, nucleotide-binding domain (NOD)-like receptor protein 3; LUBAC, linear ubiquitination assembly complex; iNOS, induced Nitric Oxide Synthase; IL-1β, Interleukin-1beta; TGF-β, Transforming Growth Factor-1beta; TNF-α, Tumor Necrosis Factor-alpha; PBMC, Peripheral Blood Mononuclear cells; CRP, C-Reactive Protein.

Abstract

INTRODUCTION: 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 remain elusive.

METHODS: Differentiated THP-1 cells treated with Aβ were used as *in-vitro* model. The role of SHARPIN was analysed using siRNA-mediated knockdown followed by immunoblotting, ELISA, real-time PCR, immunoprecipitation and flow cytometry. Differentiated SHSY5Y cells were used to study inflammation-mediated apoptosis.

RESULTS: SHARPIN was found to regulate Aβ- phagocytosis and NLRP3 expression in THP-1 derived macrophages. Further, it was found to promote macrophage polarization to an M1 (pro-inflammatory) phenotype resulting in enhanced inflammation and associated neuronal death, demonstrated using *in-vitro* culture systems and AD patient-derived macrophages.

DISCUSSION: The novel protein, SHARPIN has been shown to play critical roles in regulation of Aβ-phagocytosis and inflammation in AD and the mechanism by which SHARPIN is activated by Aβ in macrophages has been elucidated.

Keywords: SHARPIN, amyloid-beta, Alzheimer's disease, mild cognitive impairment, macrophage, NLRP3, inflammation, phagocytosis, oxidative stress.

1. Introduction

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 plaques and neurofibrillary tangles formed by the accumulation of amyloid beta (Aβ) proteins and hyperphosphorylated tau proteins [1]. During normal physiology, Aβ level in the brain is maintained through a homeostasis in production and degradation [2]. This is effectuated mainly by immune cells, namely microglia in the brain and macrophages and monocytes in the peripheral system [3,4]. 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 [5,6]. 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 [7,8]. 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 [9]. Although NLRP3 has been linked to the progression of AD [10,11], 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 [12]. Nevertheless, its role in AD has not been studied yet.

In the present study, we sought to address the link between NLRP3 inflammasome and 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 THP1 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 SHARPIN silencing protects neurons from Aβ-induced inflammatory damage.

2. Methods

2.1. Inclusion of study subjects

AD and MCI patients were recruited from the Memory & Neurobehavioral Clinic (MNC) at Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), 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 to avoid the possibility of peripheral infection or inflammation- mediated alteration of protein expression patterns and cell function. The diagnostic criteria of NINCDS–ADRDA [13] were used to confirm AD and MCI pathology. The severity of AD was determined using the

Clinical Dementia Rating Scale [14]. 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) [15]. The study population comprised of 65 individuals in three groups of 31 Alzheimer's disease, 13 Mild Cognitively Impaired 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.

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 anti-coagulated blood. Anti-coagulated blood was layered on Ficoll-Paque medium and centrifuged at 2000 rpm for 20 min. The PBMCs, collected from the interface between the plasma and the density medium, were washed twice in 1X PBS and seeded in RPMI medium supplemented with 10% autologous serum. Autologous serum was isolated by centrifugation of coagulated blood at 2500 rpm for 15 min, complement inactivated by heating at 56°C for 30 min and filtered through 0.22 µm filter.

2.3. Cell culture and differentiation

THP-1 acute monocytic leukemia cell line (obtained from National Centre for Cell Sciences, Pune) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and the cells were differentiated into macrophages by incubating with 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. All the experiments were carried out in PMA-differentiated THP-1 cell line.

SHSY5Y neuroblastoma 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 at 50% confluency 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. Amyloid-beta (Aβ) preparation

Lyophilized Aβ (1-42) and FITC- Aβ (1-42) was purchased from Abcam (Cambridge, UK) and Anaspec (Fremont, California, USA) respectively. The lyophilized powder was reconstituted in 1% NH_4OH to 2mg/ml concentration and was further reconstituted in 1X PBS to 1mg/ml concentration. The working stock (10mM) was prepared by reconstituting the stock in 1X PBS. Aβ thus prepared was analysed using western blotting and confirmed that majority of the protein prepared was in the cytotoxic oligomeric, tetrameric and trimeric forms (soluble Aβ).

2.5. siRNA transfection

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

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. The intracellular Reactive Oxygen Species (ROS) levels were quantified after incubating the cells with 40 μM Aβ and 10 mM N-acetyl cysteine (NAC), an ROS scavenger, for 12 h. The cells were then trypsinized and incubated with 10 μM H_2DCFDA for 1 h at 37°C, washed and subjected to FACS analysis.

2.7. Immunoprecipitation

Differentiated THP-1 cells were pre-treated with the NF-κB inhibitor, Bay-117082 for 1 h and then with Aβ for 12 h and 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 were washed and incubated with 3X Laemelli buffer at 70°C for 5 min and then exposed to magnetic field to pull down the coated protein.

2.8. Immunoblot analysis

Differentiated THP-1 cells were treated with 10 μM Aβ for 6 h to analyse the expression of SHARPIN and NLRP3 and for 12 h to analyse the expression of macrophage polarization markers and phagocytic receptors. Cells were pre-treated with NAC or Bay-117082 for 1 h and then with Aβ for 6 and 12 h respectively to analyse protein expression patterns. After incubation, total protein was isolated from the cells using 1X RIPA buffer containing 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°C. 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.

2.9. mRNA expression analysis

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

2.10. Analysis of cytokine release

The release of inflammatory cytokines, namely IL-1β, TNF-α, IL-10 and TGF-β, 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 (G-Biosciences, St.Louis, Missouri, 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 450 nm and the relative absorbance was calculated.

2.11. 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 1 μg/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 Lysotracker Red (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. Mean Fluorescent Intensity over three different fields per sample were subjected to analysis and the MFI were calculated by taking the ratio of ital. fluorescent intensity to the total number of cells in each field.

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 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 h and the expression of apoptotic markers were analysed.

2.13. Statistical analyses

One way ANOVA with Dunnett's multiple comparisons test 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 age-matched control subjects. Results were represented as mean ± SEM and a p value<0.05 was considered as statistically significant.

3. Results

3.1. SHARPIN regulates Aβ Receptor Expression and Phagocytosis by Macrophages

A strong correlation exists between inflammation, accumulation of Aβ and progression of AD. SHARPIN is an important positive regulator of pro-inflammatory signaling [16]. However, the role of SHARPIN in pathogenesis of AD has not been well studied. Here, we show that Aβ enhanced the expression of SHARPIN by approximately two-fold in THP-1 derived macrophages (Fig.1B). Further, we observed that knockdown of SHARPIN in macrophages significantly reduced the Aβ phagocytic efficiency as observed using FITC- Aβ phagocytic assay and flow cytometry, demonstrating a critical role for SHARPIN in modulating macrophage function in a context of AD (Fig. 2).

As Aβ phagocytosis is mediated by phagocytosis receptors, the expression of receptors involved in Aβ phagocytosis and uptake were analyzed. 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 (Fig.2).

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

The NLRP3 inflammasome is considered as a master switch in activating the pro-inflammatory cascade. Activation of NLRP3 inflammasome has also been reported to promote progression of AD. A previous study had reported SHARPIN in the regulation of NLRP3 inflammasome components in Chronic proliferative dermatitis (cpdm) [12]. However, 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 was abolished by silencing SHARPIN in macrophages (Fig.3A), pointing to the possibility that SHARPIN and NLRP3 act in tandem to promote pro-inflammatory signaling.

3.3. SHARPIN regulates Aβ- induced Macrophage Polarization

NLRP3 inflammasome controls the maturation and release of pro-inflammatory cytokines like IL-1β and IL-18 in macrophages [17]. 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 results in a decrease in the pro-inflammatory markers induced nitic oxide synthase (iNOS) (Fig. 3B), IL-1β and TNF-α release (Fig.3C), and mRNA expression of TLR2, CX3CR1 and CD68 (Fig.3D) and increase in the anti-

inflammatory markers TGF-β expression (Fig. 3B) and release, IL-10 release (Fig.3C) and mRNA expression of TLR1, CCR2 and CD163 (Fig.3D), suggesting that Aβ-induced SHARPIN expression polarizes the macrophages to pro-inflammatory M1 phenotype and SHARPIN knockdown reverses the polarization to M2 anti-inflammatory phenotype.

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

The effect of SHARPIN-mediated inflammation on neuronal apoptosis was analyzed using conditioned media experiments. For deriving a neuronal cell culture model, SHSY5Y cells were differentiated into mature neurons by treatment with 10 μM Retinoic acid (RA) for 4 days. The differentiated neuronal cells showed increased size and synaptic connections together with decreased expression of the neuronal stem cell marker Nestin (Fig.4A).

Differentiated SHSY5Y neuronal cell culture 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 differentiated SHSY5Y neuronal cell culture with conditioned media derived from SHARPIN-silenced THP-1 macrophages was found to significantly reduce the expression of pro-apoptotic markers cleaved caspase 3 and cleaved PARP (Fig.4B).

3.5. Aβ-induced oxidative stress affects SHARPIN expression

Aβ is known to enhance Reactive Oxygen Species (ROS) generation and oxidative stress [18] 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. Addition of the ROS scavenger N-acetyl cysteine (NAC) in the presence of Aβ reduced ROS levels confirming the role of Aβ in stimulating ROS production in macrophages (Fig.5A). To check the role of ROS in stimulating SHARPIN expression in macrophages exposed to Aβ,

the role of NAC on SHARPIN expression was analyzed. NAC treatment attenuated Aβ-stimulated ROS, demonstrating the role of ROS in mediating Aβ- stimulated SHARPIN expression (Fig.5B).

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

Since NFkB is a redox-sensitive transcription factor, its regulatory role on SHAPIN was investigated. Pharmacological inhibition of NF-κB using Bay-117082 was found to cause a shift in the mobility of SHARPIN protein migration in the gel. This was evident from an increase in the molecular weight of SHARPIN in Aβ + Bay-117082-treated group compared to the control and Aβ groups (Fig.5C). Ubiquitination is an important post-translation modification and also targets proteins for proteosomal degradation. Since addition of ubiquitin groups could alter the molecular weight of SHARPIN, immunoprecipitation assay for SHARPIN was performed (Fig.5D). Immunoprecipitation of SHARPIN and probing with anti-ubiquitin antibody in the Aβ + Bay-117082 treated group showed a significant increase in ubiquitinylation (Fig.5E), suggesting that the transcription factor NF-κB may function to ubiquitinate the protein SHARPIN.

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

Study subjects were categorized into 31 Alzheimer's disease, 13 mild cognitively impaired and 19 age-matched control subjects on the basis of clinical and psychological analysis (MMSE and ACE scores) (Fig.6A). We have observed that SHARPIN expression by macrophages was showing a similar pattern as the Aβ42/40 concentration in the plasma, with an increased expression in MCI subjects and AD compared to control subjects (Fig. 6B). To check the correlation between SHARPIN expression and Aβ42/40 levels, macrophages isolated from AD, MCI and age-matched control subjects were cultured in the autologous serum samples that were derived from the respective groups of study subjects. Importantly,

SHARPIN expression showed a mild correlation with Aβ42/40 in control and AD subjects and a moderate correlation in MCI subjects (Fig.6C) which corresponds to the fact that SHARPIN expression by macrophages, in the absence of peripheral infection, might be stimulated by the concentration of Aβ42/40 in the blood plasma.

4. Discussion

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 microglia in the brain and the macrophages entering the brain from peripheral circulation through the leaky blood-brain barrier in AD plays a major role in causing neuronal cell death, leading to enhanced neurodegeneration and amplified rate of progression of AD [19]. Although several studies have shown a correlation between inflammatory mediators and phagocytic receptor expression by immune cells [20], the underlying mechanisms that affect Aβ phagocytosis and promote pro-inflammatory conditions in the AD brain remain elusive.

The protein SHARPIN has been recognised as an upstream activator of NF-κB thus acting as an important mediator of inflammatory activation [21–23]. A recent study by Yuya Asanomi et.al identified a rare functional variant of SHARPIN as a genetic risk factor for late-onset Alzheimer's disease (LOAD) proving the role of the protein in the progression of AD [24]. 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 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. Further, 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.

SHARPIN and NLRP3 are widely regarded as the two principal mediators of inflammation. A study conducted in mice carrying mutant SHARPIN (*Sharpin*^{cpdm}) that caused loss of SHARPIN function found a reduction in NLRP3 activation demonstrating for the first time, a novel link between the two inflammatory mediators in a context of AD [12]. Since SHARPIN regulates NLRP3 which is an activator of pro-inflammatory signalling, we analysed the expression of inflammatory markers in SHARPIN-silenced macrophages exposed to Aβ. We found that while Aβ polarizes macrophages to a pro-inflammatory M1 phenotype, knockdown of SHARPIN in the presence of Aβ prevented M1 polarization. In fact, Aβ treated macrophages were found to polarize toward the pro-inflammatory M1 phenotype, thus demonstrating the role of SHARPIN in the regulation of macrophage polarization to a proinflammatory M1 phenotype in response to Aβ.

It has been reported that brain-resident and peripheral circulation-derived immune cells eliminate aggregated Aβ protein deposits in the brain, causing inflammatory response through the secretion of inflammatory cytokines and reactive oxygen species, leading to neuronal damage in AD. Since SHARPIN regulates inflammation that causes neuronal apoptosis, we sought to analyse the role macrophage SHARPIN in mediating neuronal cell apoptosis. Using differentiated SHSY5Y neurons, we have observed significantly reduced apoptosis of neurons in cells treated with conditioned media derived from SHARPIN-knockdown macrophages compared to the cells treated with conditioned media from macrophages incubated with Aβ. While Aβ functioned through SHARPIN-mediated mechanisms to promote inflammatory cytokine release by the macrophages in the media that

induced neuronal cell death, knockdown of SHARPIN in macrophages prevented the release of pro-inflammatory cytokines, leading to the survival of neurons in culture. The results clearly indicate that Aβ-induced neuronal apoptosis is primarily mediated through Aβ-induced pro-inflammatory mechanisms that involve SHARPIN. Thus 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.

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 [18,25]. 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, the role of NFkB activation in regulating SHARPIN expression was explored. Further exploration into the regulatory mechanisms on SHARPIN lead us to the finding that NF-κB promoted ubiquitination of SHARPIN. However, the functional role of this ubiquitinylation in altering SHARPIN stability or function was not analysed.

The functional implications and regulatory mechanisms of SHARPIN need to be explored in detail in both on an immunological basis and therapeutic basis. SHARPIN has been highly explored in many disease conditions, especially in cancer stressing out its multiple roles in many independent regulatory mechanisms other than LUBAC [26,27], suggesting that this modification that we have identified, if not targeted for a proteosomal degradation, might be

inhibiting only the LUBAC- dependent SHARPIN function and might be promoting its other cellular functions, which is out of scope for this study.

Using peripheral blood-derived macrophages and plasma obtained from AD patient samples, the correlation between SHARPIN expression and Aβ42/40 levels was analysed. Analysis of SHARPIN in AD and MCI patient-derived macrophages pointed out a significant increase in SHARPIN expression in MCI-derived macrophages compared to AD and age-matched control subjects. MCI is regarded as the preclinical stage of AD. It has been reported that with 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 blood plasma of AD subjects compared to the MCI subjects. The SHARPIN expression patterns in our study subjects can be reflected as a result of the stimuli induced by varied concentration of Aβ in the peripheral circulation, where a higher concentration of Aβ in the circulation in the MCI subjects induced the enhanced expression of SHARPIN by MCI macrophages and the lower concentration of Aβ in AD and control subjects contributes to a lower stimuli for SHARPIN expression. Hence, the reduced rate of Aβ clearance from the AD brain coupled with decreased Aβ concentration in peripheral circulation of AD patients may explain the decrease in SHARPIN expression in AD-patient-derived macrophages compared to MCI. A correlation study of SHARPIN expression with Aβ42/40 in the plasma of the same study subjects shows a mild to moderate positive correlation suggesting the same. Further, although we tried to correlate SHARPIN expression in macrophages derived from the study subjects with the phagocytic efficiency of macrophages and inflammatory markers in the plasma of in the respective study subjects, a significant correlation was not found (Supplementary Fig.6S). This indicates the presence of complex mechanisms regulating inflammation and phagocytosis under *in-vivo* conditions contrary to the *in-vitro* conditions. Further, in account of the small sample size, we could not consider other factors that may alter the inflammatory

conditions in the peripheral system, such as diabetes, ApoE and other genetic risk factors. An elaborate study on a larger cohort with a similar genetic and non-genetic profile 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 response to Aβ in a setting of AD. SHARPIN was found to regulate Aβ phagocytosis and Aβ-stimulated inflammatory mechanisms in macrophages, highlighting a novel role for SHARPIN in regulating macrophage function in a context of AD. 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. Oxidative stress, with its calamitous role in most age-associated diseases, was found to be regulating the protein expression, which was further modulated by NF-κB-mediated signaling mechanism. Importantly, SHARPIN expression correlated with the levels of Aβ42/40 in MCI subject-derived macrophages demonstrating a link between SHARPIN expression and progression of AD. 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. Further, the functional role of ubiquitination in altering SHARPIN function and its implications to 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 since these are the cells that respond primarily to Aβ accumulation in AD brain.

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Figure legends:

Fig.1A: Western blot image showing that amyloid-beta prepared was in the cytotoxic soluble form (oligomeric, tetrameric and trimeric). **C.** Differentiated THP-1 macrophages were treated with 10μM Aβ for 6h and SHARPIN expression was analysed using western blotting and represented graphically.

Fig.2: Differentiated THP-1 macrophages were treated with 10μM Aβ or FITC-Aβ for 12 h. **A.** Western blot data showing siRNA transfection efficiency for SHARPIN. **B.** Fluorescent microscopic image and graphical representation of mean fluorescence intensity (MFI) showing decreased phagocytosis of FITC-labelled Aβ by SHARPIN- knockdown macrophages siRNA compared to control. **C.** Flow cytometry data and graphical representation of MFI and the number of FITC-Aβ phagocytosed cells. **D.** Western blot and graphical representation of major Aβ-phagocytic receptors (SCARA1 and CD-36) and receptors involved in receptor- mediated uptake of Aβ (RAGE-1 and LRP-1) showing increased expression when stimulated with Aβ and decreased when SHARPIN expression was silenced in Aβ-treated macrophages. Statistical analysis- One-way NOVA with *p>0.05, **p>0.01, ***p>0.001.

Fig.3: Differentiated THP-1 macrophages were transfected with SHARPIN siRNA and then treated with 10μM Aβ for 6h. **A.** Western blot data showing siRNA validation for SHARPIN in the presence of Aβ. **B.** Western blot data and graphical representation showing an increase in the expression of NLRP3 by macrophages in the presence of Aβ which was significantly downregulated in SHARPIN knockdown cells. **C.** Differentiated THP-1 macrophages were transfected with SHARPIN siRNA and then treated with 10μM Aβ for 12h. Western blot data and graphical representation of M1 macrophage markers showing protein expression of iNOS, ELISA data showing the release of pro-inflammatory cytokines (IL-1β and TNF-α)

and Real-time PCR data showing mRNA expression of TLR-2, CX3CR1 and CD68 with tubulin as endogenous control. **D.** Western blot data and graphical representation of M2 macrophage markers showing protein expression of TGF-β, ELISA data showing the release of anti-inflammatory cytokines (IL-10 and TGF-β) and Real-time PCR data showing mRNA expression of TLR-1, CCR2 and CD163 with tubulin as endogenous control (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.

Fig.4: A. Immunocytochemistry image showing decreased expression of Nestin, a marker of stem cell lineage cells, in the differentiated neurons and phase contrast image showing undifferentiated neuroblastoma cell lines and the differentiated mature neurons with increased size and synaptic connections. **B.** Differentiated neurons were treated with conditioned media obtained from macrophages transfected with SHARPIN siRNA and then treated with 10μM Aβ, for 24h. Western blot data and graphical representation showing expression of cleaved caspase-3, caspase-3, PARP and cleaved PARP. Statistical analysis- One-way NOVA with *p>0.05, **p>0.01, ***p>0.001.

Fig.5: A. Differentiated THP-1 macrophages were pre-incubated with 10mM NAC for 1h and the with 40μM Aβ for 12h. Then the cells were incubated with H2DCFDA for 1h and subjected to flow cytometry. Flow cytometry data and graphical representation show 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 expression of SHARPIN in the presence of Aβ and NAC. **C.** Differentiated THP-1 macrophages were pre-incubated with NF-κB inhibitor, Bay 11-7082 for 1h and then treated with 10μM Aβ for 12h. Western blot

data showing increase in the molecular weight of SHARPIN when the cells are subjected to Bay 11-7082. **D.** SHARPIN protein was immunoprecipitated for further analysis and the purity of the immunoprecipitated protein was shown with western blot data. **E.** Western blot data and graphical representation showing increased ubiquitination of SHARPIN in the Bay 11-7082 treated cells compared to the control.

Fig.6: A. Study subject demographics. **B.** Scatter plot showing SHARPIN expression by macrophages cultured in media supplemented with autologous serum which contains Aβ in varied concentration. **C.** Scatter plot showing Aβ42/40 ratio in the blood plasma of the study subjects. The SHARPIN expression by macrophages in the study subjects were correlated with Aβ42/40 ratio in the blood plasma. Pearson correlation coefficient, r = 0.222 (p=0.36, ns) for age-matched control, r = 0.552 (p=0.05, ns) for mild-cognitive impaired and r = 0.183 (p= 0.32, ns) for Alzheimer's disease subjects (ns- non significant).

Suppl. Fig.6S: A. Scatter plot of MFI showing phagocytosis efficiency of FITC- Aβ after overnight incubation by macrophages isolated from study subjects. SHARPIN expression by macrophages in the study subjects were correlated with MFI. Pearson correlation coefficient, r = -0.256 (p=0.28, ns) for age-matched control, r = -0.334 (p=0.26, ns) for mild-cognitive impaired and r = -0.337 (p= 0.06, ns) for Alzheimer's disease subjects. **B.** Scatter plot showing release of pro-inflammatory cytokine IL-1β in the blood plasma of the study subjects. SHARPIN expression by macrophages in the study subjects were correlated with IL-1β release in the blood plasma. Pearson correlation coefficient, r = 0.430 (p=0.06, ns) for age-matched control, r = -0.472 (p=0.10, ns) for mild-cognitive impaired and r = -0.005 (p=0.97, ns) for Alzheimer's disease subjects. **C.** Scatter plot showing release of pro-inflammatory cytokine TNF-α in the blood plasma of the study subjects. SHARPIN expression by macrophages in the study subjects were correlated with TNF-α release in the blood plasma. Pearson correlation coefficient, r = -0.135 (p=0.57, ns) for age-matched control, r = 0.183 (p=0.54, ns) for mild-

cognitive impaired and r = -0.062 (p=0.73, ns) for Alzheimer's disease subjects. **D.** Scatter plot showing release of anti-inflammatory cytokine IL-10 in the blood plasma of the study subjects. SHARPIN expression by macrophages in the study subjects were correlated with IL-10 release in the blood plasma. Pearson correlation coefficient, r = 0.078 (p=0.75, ns) for age-matched control, r = -0.539 (p=0.05, ns) for mild-cognitive impaired and r = -0.040 (p=0.82, ns) for Alzheimer's disease subjects. **E.** Scatter plot showing release of anti-inflammatory cytokine TGF-β in the blood plasma of the study subjects. SHARPIN expression by macrophages in the study subjects were correlated with TGF-β release in the blood plasma. Pearson correlation coefficient, r = -0.153 (p=0.53, ns) for age-matched control, r = -0.181 (p=0.55, ns) for mild-cognitive impaired and r = -0.020 (p= 0.91, ns) for Alzheimer's disease subjects (ns- non significant).

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Compliance with Ethical Standards

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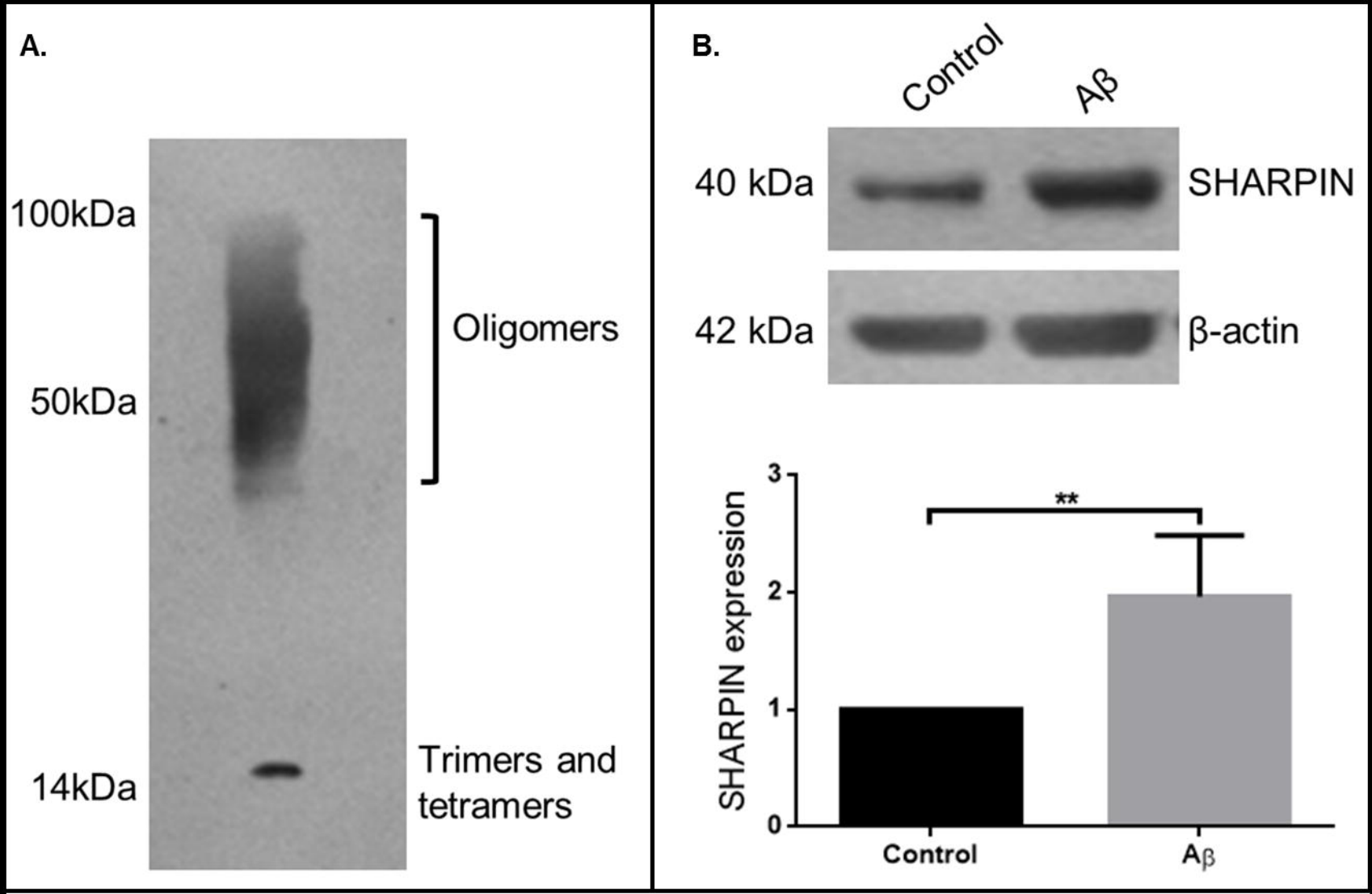
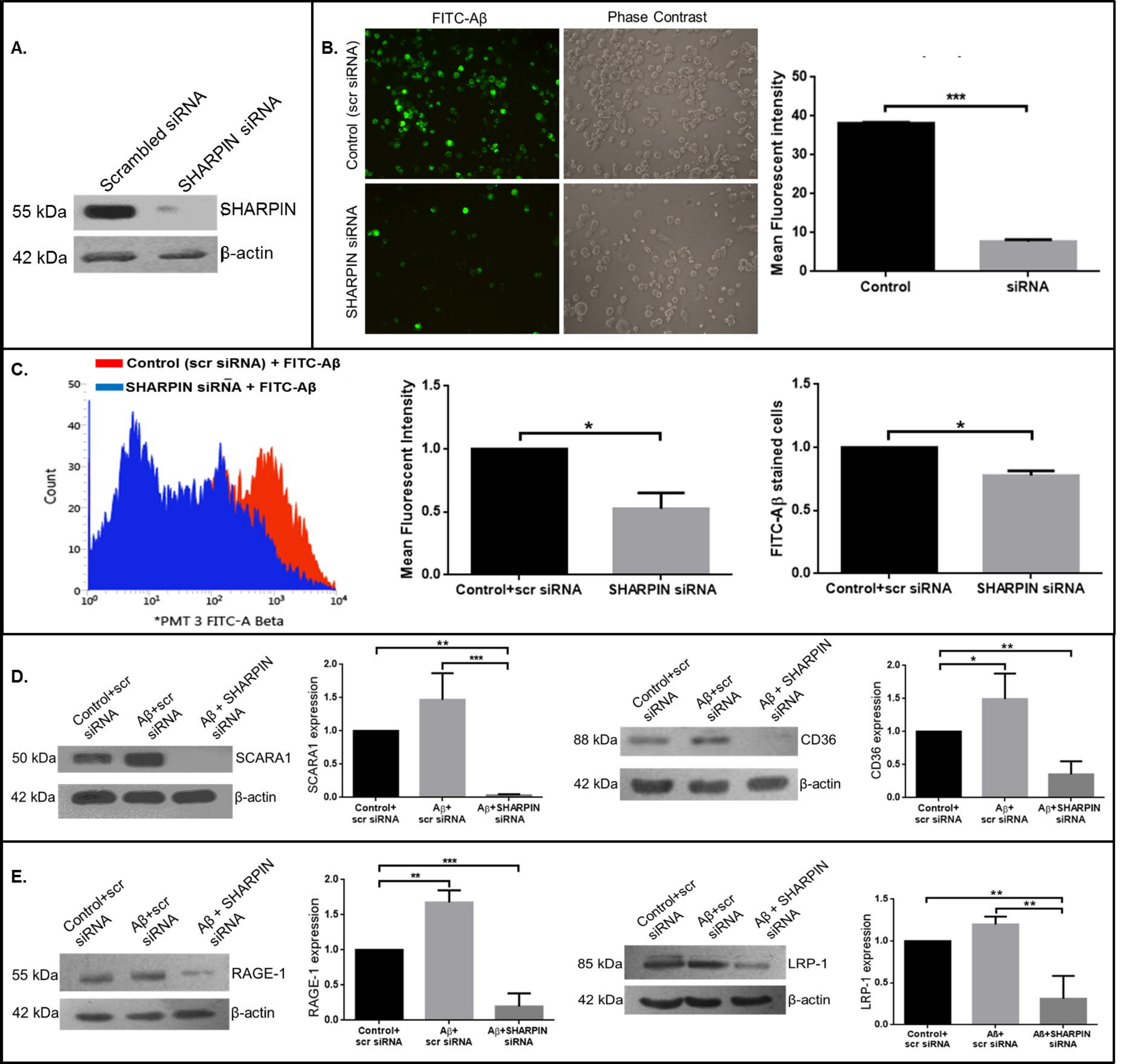
Conflict of Interest: All authors (DK, RNM, PSM & SG) declare no conflict of interest.

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.


A.
100kDa
50kDa
14kDa
Oligomers
Trimers and tetramers
B.
Control
Aβ
40 kDa
SHARPIN
42 kDa
β-actin
SHARPIN expression
3
2
1
0
**
Control
Aβ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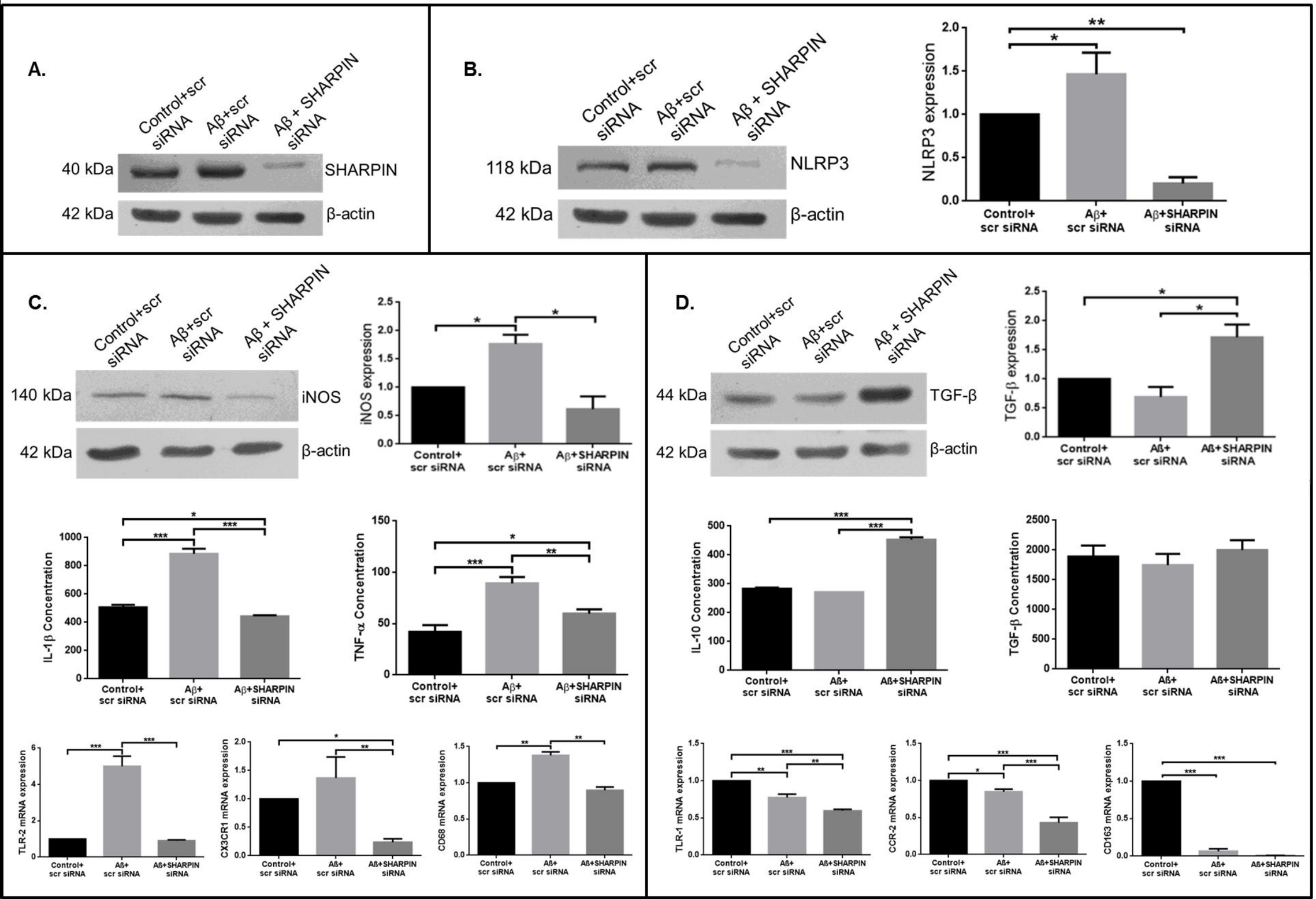
Scrambled siRNA
SHARPIN siRNA
55 kDa
SHARPIN
42 kDa
β-actin
B.
FITC-Aβ
Phase Contrast
Control (scr siRNA)
SHARPIN siRNA
Mean Fluorescent intensity
***
Control
siRNA
C.
Control (scr siRNA) + FITC-Aβ
SHARPIN siRNA + FITC-Aβ
Count
*PMT 3 FITC-A Beta
Mean Fluorescent Intensity
FITC-Aβ stained cells
*
Control+scr siRNA
SHARPIN siRNA
D.
Control+scr siRNA
Aβ+scr siRNA
Aβ + SHARPIN siRNA
50 kDa
SCARA1
42 kDa
β-actin
SCARA1 expression
**
***
Control+ scr siRNA
Aβ+ scr siRNA
Aβ+SHARPIN siRNA
88 kDa
CD36
CD36 expression
*
E.
55 kDa
RAGE-1
RAGE-1 expression
85 kDa
LRP-1
LRP-1 expression
Aß+ scr siRNA
Aß+SHARPIN siRNA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Control+scr siRNA
Aβ+scr siRNA
Aβ + SHARPIN siRNA
40 kDa
SHARPIN
42 kDa
β-actin
B.
118 kDa
NLRP3
NLRP3 expression
Control+ scr siRNA
Aβ+ scr siRNA
Aβ+SHARPIN siRNA
C.
140 kDa
iNOS
iNOS expression
IL-1β Concentration
TNF-α Concentration
TLR-2 mRNA expression
CX3CR1 mRNA expression
CD68 mRNA expression
D.
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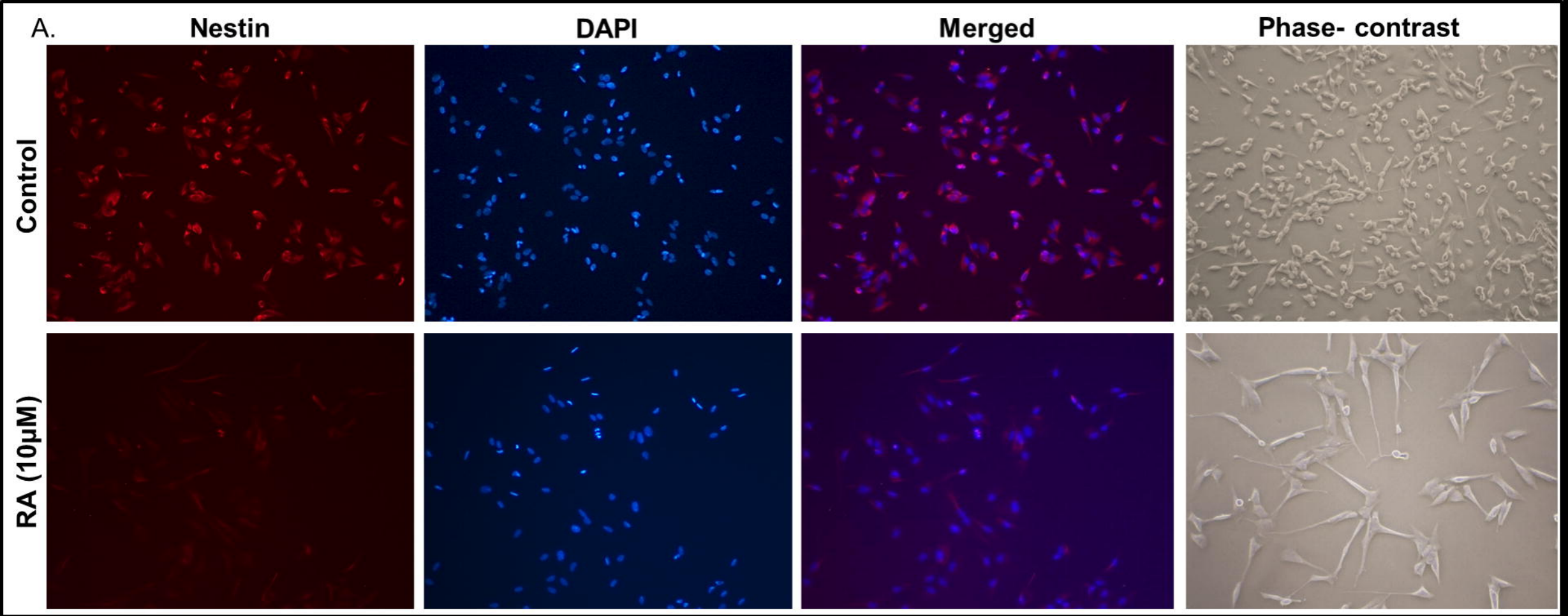
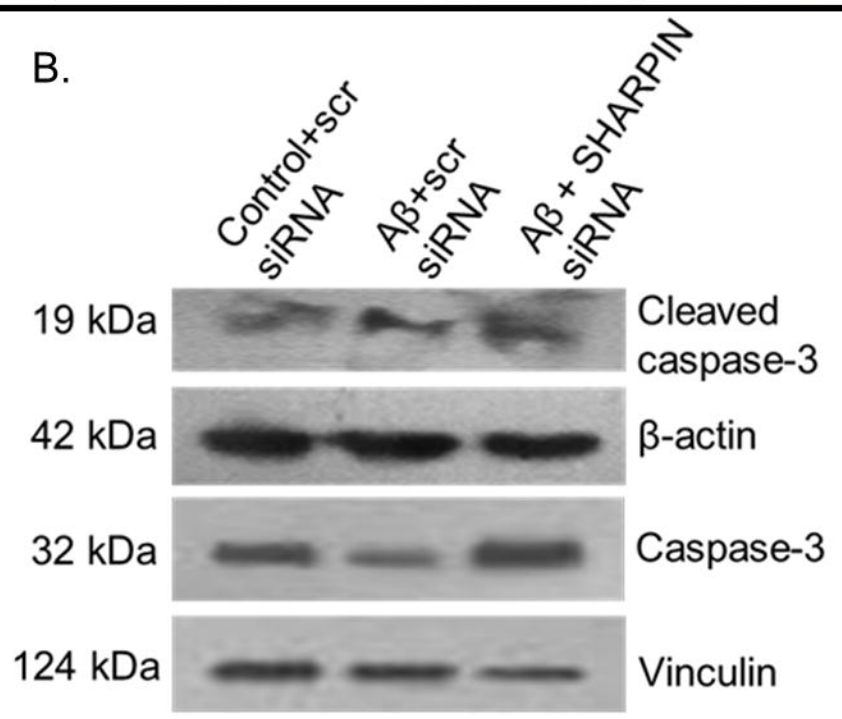
TLR-1 mRNA expression
CCR-2 mRNA expression
CD163 mRNA expression

A.


Nestin
DAPI
Merged
Phase- contrast
Control
RA (10μM)

B.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Aβ+scr siRNA
Aβ + SHARPIN siRNA
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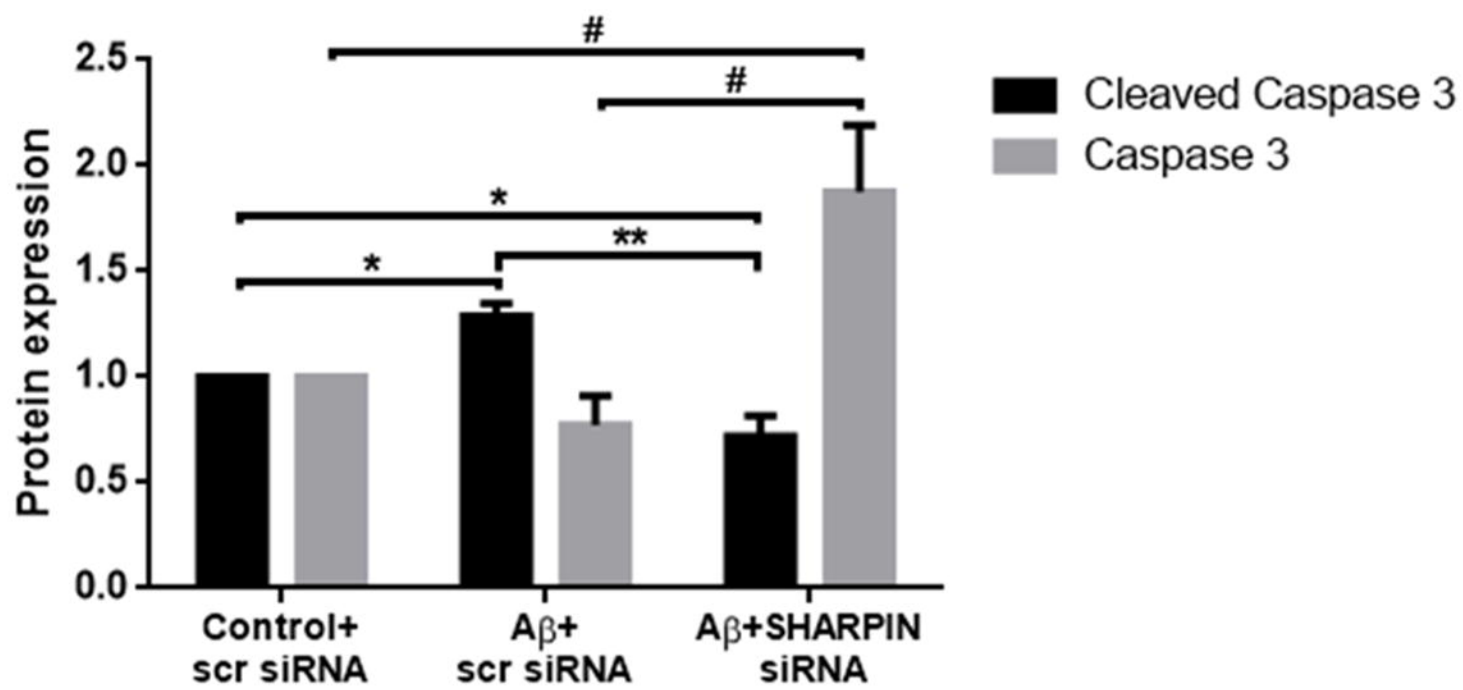
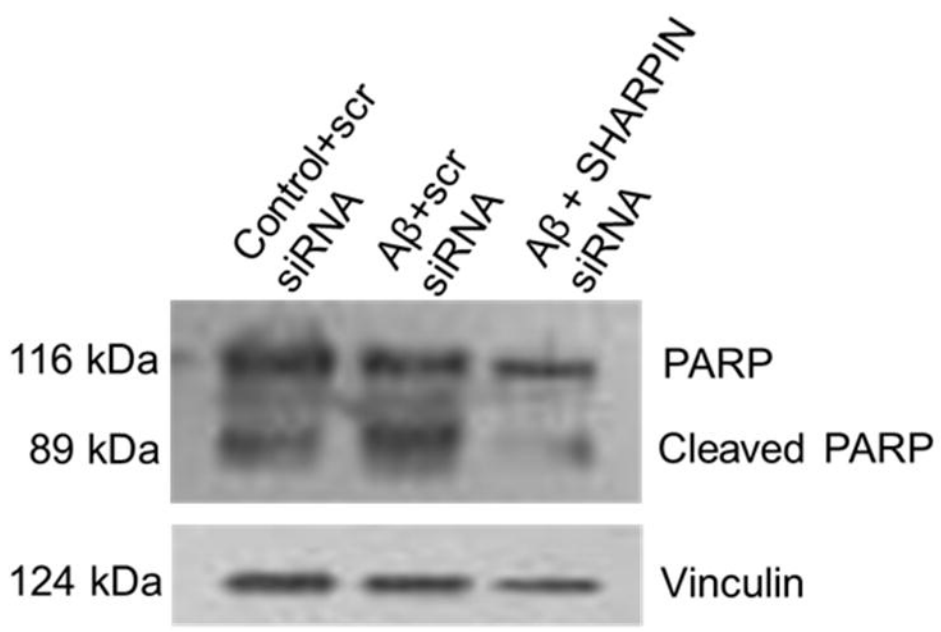
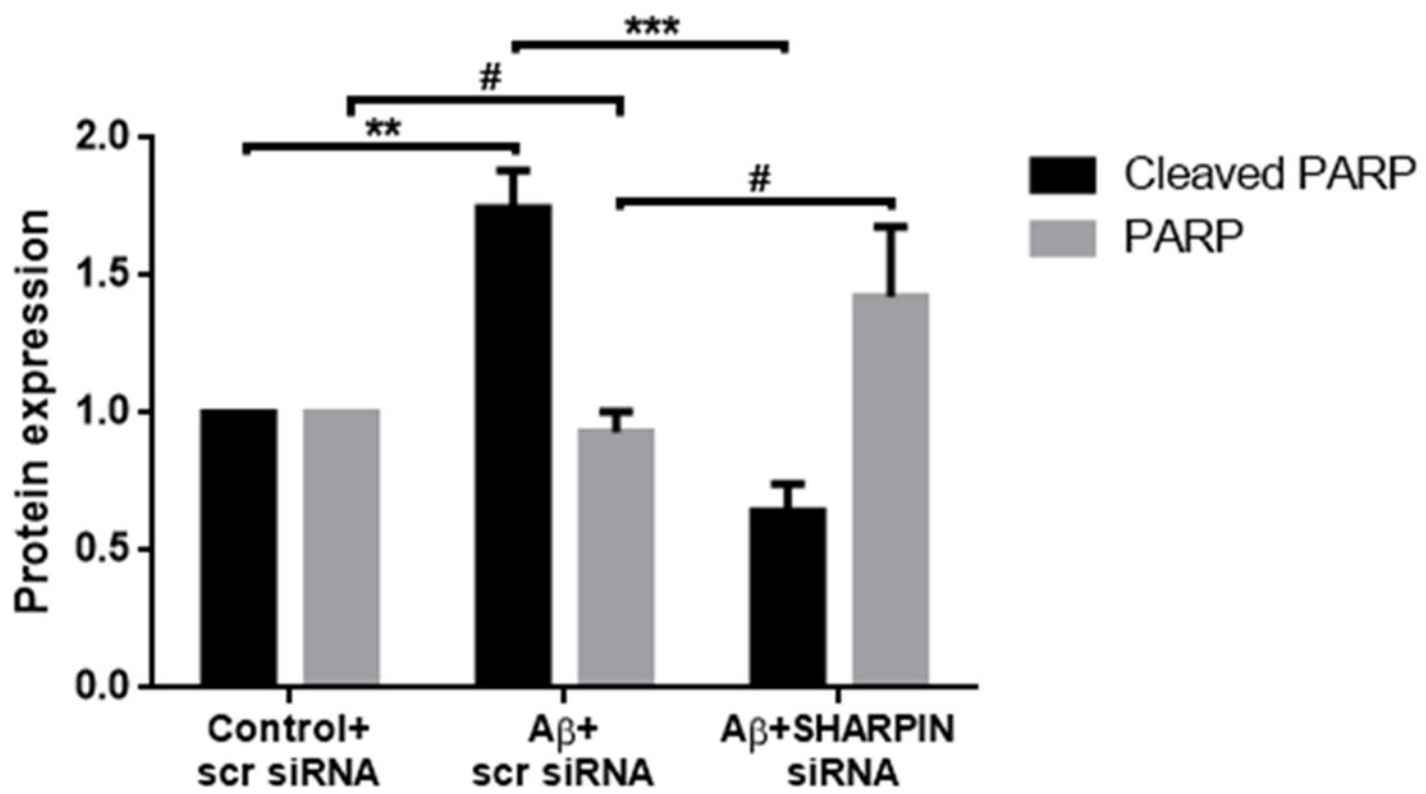
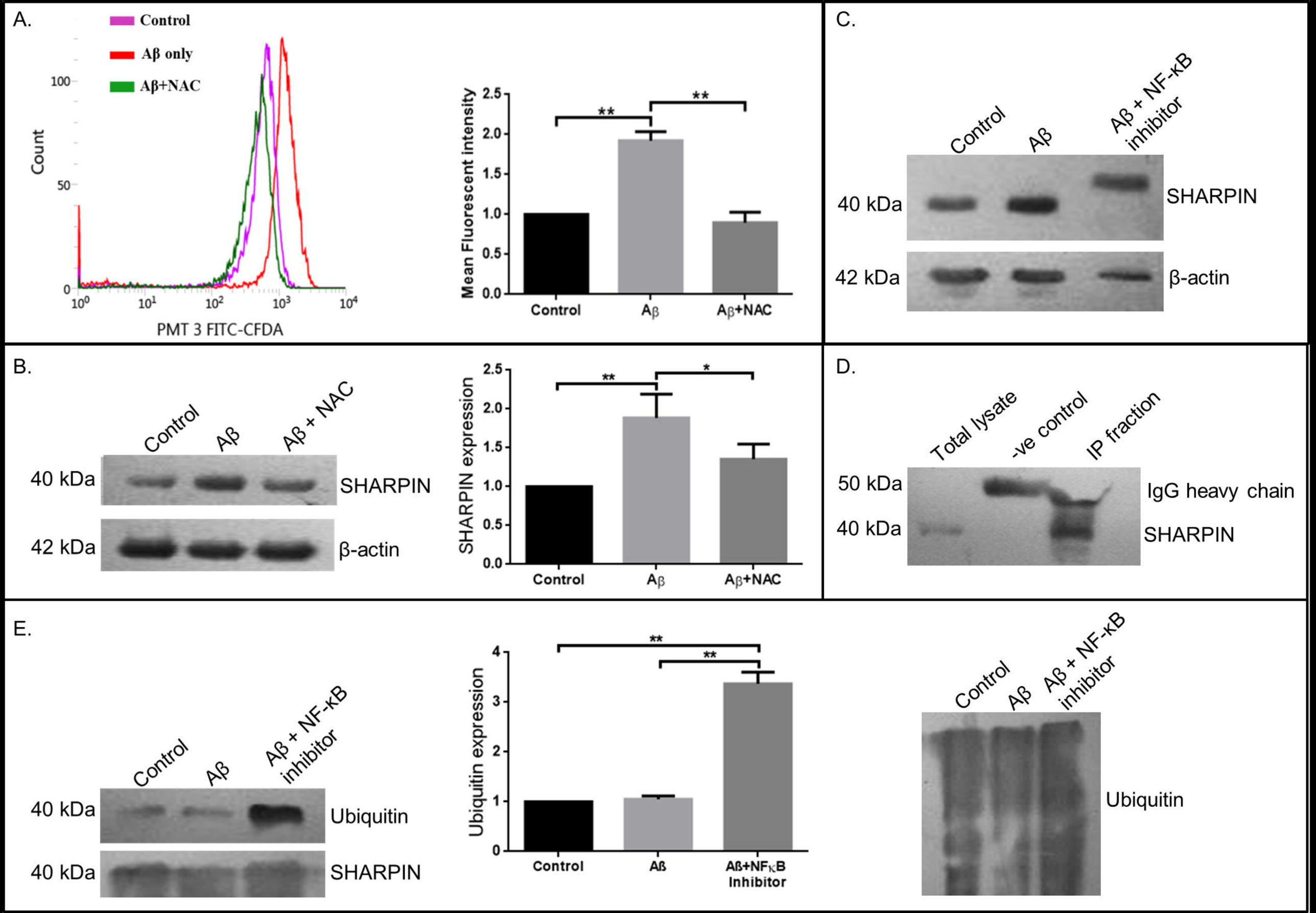
Cleaved caspase-3
42 kDa
β-actin
32 kDa
Caspase-3
124 kDa
Vinculin


Protein expression
2.5
2.0
1.5
1.0
0.5
0.0
#
#
*
**
*
Control+ scr siRNA
Aβ+ scr siRNA
Aβ+SHARPIN siRNA
Cleaved Caspase 3
Caspase 3


Control+scr siRNA
Aβ+scr siRNA
Aβ + SHARPIN siRNA
116 kDa
PARP
89 kDa
Cleaved PARP
124 kDa
Vinculin

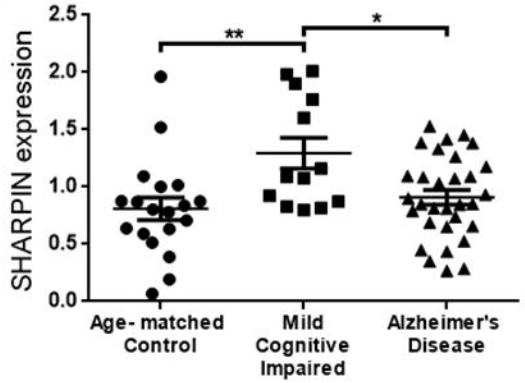
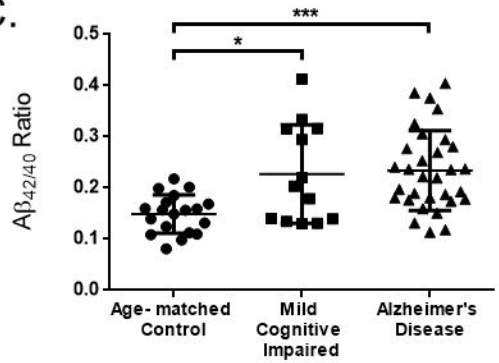
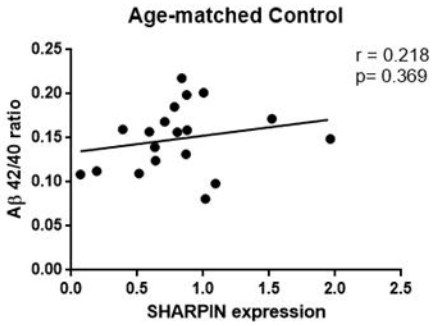
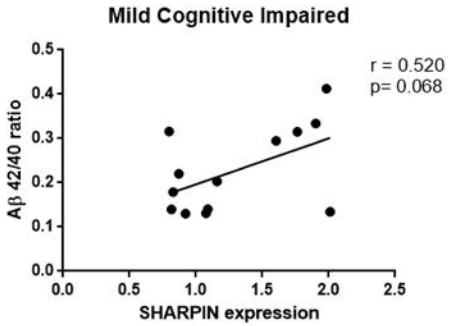
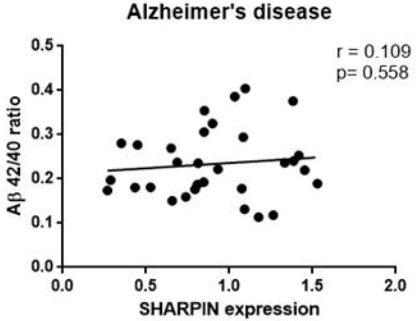

Protein expression
2.0
1.5
1.0
0.5
0.0
***
#
**
#
Control+ scr siRNA
Aβ+ scr siRNA
Aβ+SHARPIN siRNA
Cleaved PARP
PARP


A.
Control
Aβ only
Aβ+NAC
Count
PMT 3 FITC-CFDA
Mean Fluorescent intensity
Control
Aβ
Aβ+NAC
C.
Control
Aβ
Aβ + NF-κB inhibitor
40 kDa
SHARPIN
42 kDa
β-actin
B.
Control
Aβ
Aβ + NAC
40 kDa
SHARPIN
42 kDa
β-actin
SHARPIN expression
Control
Aβ
Aβ+NAC
D.
Total lysate
-ve control
IP fraction
50 kDa
IgG heavy chain
40 kDa
SHARPIN
E.
Control
Aβ
Aβ + NF-κB inhibitor
40 kDa
Ubiquitin
40 kDa
SHARPIN
Ubiquitin expression
Control
Aß
Aß+NFκB Inhibitor
Control
Aβ
Aβ + NF-κB inhibitor
Ubiquitin

A.

Study Subjects	Total number	MMSE scores	ACE scores	Age	Average Aβ 42:40 ratio
Control	19	-	-	54.7 ± 10	0.149
MCI	14	27 ± 3	82 ± 10	71.3 ± 5.5	0.227
AD	31	22 ± 5	67 ± 6	69.5 ± 8.6	0.234

B.



C.