Purinergic receptor P2Y12 involves in full-length Tau oligomers-induced microglial chemotaxis, phagocytosis and endocytic trafficking via filopodia-associated actin remodeling

by

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AcSIR Registration No. 10BB17A26053

A thesis submitted to the
Academy of Scientific & Innovative Research
for the award of the degree of
DOCTOR OF PHILOSOPHY
in
Science

Under the supervision of
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January, 2023

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Acknowledgments

I would like to express my heartfelt gratitude to the people who helped me during my Ph.D. journey. Firstly, I would like to thank my research advisor **Dr. Subashchandrabose Chinnathambi** for his constant guidance, support, scientific discussion and making critical suggestions in my research. His valuable comments and suggestions helped me to follow the direction of my research work and to obtain fruitful results.

I am highly thankful to **Director, CSIR-NCL** for providing me with the opportunity to work in this prestigious laboratory and the necessary infrastructure for carrying out my research work. I express my gratitude to the **University Grant commission (UGC)** for the fellowship award which provided financial support in the course of my Ph.D. I am extremely thankful to the **Academy of Scientific and Innovative research (AcSIR)** for their academic support. I sincerely thank **Chair, Biochemical Science Division**, for extending support and providing institutional facilities. I owe special thanks to my Doctoral Advisory Committee (DAC) members – **Dr. Manjusha Shelke, Dr. Dhanasekaran Shanmugam** and **Dr. Mahesh Dharne** for their critical comments and valuable suggestions which helped a lot in my research. I would like to thank **Dr. HV Thulasiram, Dr. Suresh Kumar Ramasamy** and **Dr. Anuya Nishal** for permitting me to use their lab facilities for my research work.

I am immensely thankful to the Central facilities for CD spectroscopy, TEM and HR-TEM facilities, ATC lab, FACS facilities at CSIR-NCL. In this regard, I am also greatly thankful to **Pankaj, Venkatesh and Tushar** for their help in acquiring the electron microscopy samples and helpful suggestions. I thank **Dr. Prebhanjan Giram** for providing Clopidogrel as a kind gift. I would like to extend my sincere thanks to the administrative facilities at CSIR-NCL office (Biochemical Sciences Division), Student Academic Office, AcSIR Office at CSIR-NCL, Digital Information Resource Centre (DIRC), Library, Stores and Purchase, Finance, Dispatch, housekeeping and Engineering sections for their help in the processing of official works.

I take this opportunity to express my sincere gratitude to my lab-mates – **Dr. Nalini, Dr. Shweta, Dr. Abhishek, Dr. Tushar, Smita, Hariharakrishnan, Tazeen, Mahima, Ankita and Aashima** for their constant support, care and maintaining a nice lab and learning environment. I especially thank **Shweta** and **Abhishek** for their support, matured thoughts and life-learning suggestions. I am thankful to the trainees and project assistants **– Lisni, Tanuja, Navya, Shivkanya and Drone** who worked with me and provided me an opportunity to learn from them. For my collaborative work, I would like to thank **Nalini** for Small molecule project, **Abhishek** for the Melatonin project, **Hari and Smita** for their *in silico* studies and GPCR project. Special thanks to **Smita** and **Hari** for proof-reading the thesis and generous helps.

Apart from my labmates, I would like to extend my sincere gratitude to my friends and colleagues in Biochemical Sciences Division, CSIR-NCL during my Ph.D. I am immensely thankful to the members of Dr. Suresh Kumar's Lab – **Dr. Deepanjan, Dr. Vijay, Dr. Yashpal and Dr. Debjyoti**, members of Dr. Mahesh Kulkarni's Lab –

Dr. Rajeshwari, Dr. Shakuntala, Dr. Prachi, Shabda, Arvind, Shiva and Babasaheb, members of Dr. Dhanasekaran's Lab – **Dr. Anurag, Dr. Meenakshi, Dr. Rupali, Sindhuri, Dr. Rahul, Dr. Parag, Shweta and Ajinkya**, members of Dr. Kiran Kulkarni's Lab – **Dr. Zenia, Debopriya and Aishwarya,** members of Dr. Thulasiram's Lab – **Ashish, Dr. Shrikant and Bhagyashree,** members of Dr. Koteshwara Rao Lab – **Amol and Vishwambhar** and members of Plant Molecular Biology Lab – **Dr. Santosh, Dr. Shamala, Dr. Ashwini, Ranjit, Gayatri and Bhakti**. I highly appreciate and express my thankfulness to my friends at NCL – **Sanjukta, Amrita, Mira, Sagar, Abhijit, Gargi, Siru, Aditya, Soham and** others from Bengali group. I would also like to mention my friends back at Kolkata – **Apurba, Ananya, Abhijit, Joy, Raj, Debayan Da, Priyanka di, Anwesha di, Monica, Rajanya, Jyoti, Priyanka, Debraj, Pramita,** My friends from Pune- **Anwesha, Swati di, Ashok, Ramaraju, Sunil, Arpan and Bhavna** who extended their kind support and were always there in times of need.

Lastly, I am thankful to my **Maa- Aparna Das, Baba- Tamal Das, Mr. Soumya Bhattacharya** and **my extended family** for their constant love, support and encouragement. I am extremely thankful to my husband **Dr. Yashvant Bansode** for his extreme patience, constant tolerance, scientific discussion, good wishes and life-long support. I dedicate my work to all those persons who believed in me, giving me the strength and courage to walk fearlessly in the path of life.

Thank you.

Contents

List of Abbreviations

5x FAD	Familiar Alzheimer's Disease- transgenic mice bearing 5 mutations
AD	Alzheimer's disease
ADP	Adenosine diphosphate
Akt	Akt serine/threonine kinase or Protein kinase B
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid-β
BBB	Blood brain barrier
Ca^{2+}	Calcium ion (signaling)
CBD	Corticobasal degeneration
CCR	CC chemokine receptor
CD11b	Cluster of differentiation integrin receptor (αMβ2, Mac-1, and CR3)
CD11c	Cluster of differentiation integrin receptor (CR4)
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
CR	Complement receptor
CSF1R	Colony stimulating factor-1 receptor
Cst7	Cystatin F
CX3CR1	CX3C motif chemokine receptor 1
DAMs	Disease-associated microglia
DAP12	TYRO protein tyrosine kinase-binding protein
DLB	Dementia with Lewy bodies
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FTDP-17	Fronto-temporal dementia with Parkinsonism-linked to Chromosome-17
FTLD	Frontotemporal Lobar Degeneration

GABA	γ-amino butyric acid
Gal-3	Galectin-3
GPCRs	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase-3β
HD	Huntington disease
HMGB1	High mobility group box protein 1
HMW	High molecular weight
HSP	Heat-shock proein
HSPG	Heparan sulfate proteoglycan
Iba1	Ionized Ca^{2+} binding adaptor protein 1
IF	Immunofluorescence
IL	Interleukins
LMW	Low molecular weight
LPS	Lipopolysaccharides
mAB	Monoclonal antibody
MCI	Mild cognitive impairment
MHC II	Major histocompatibility complex II (HLA-DR)
MTOC	Microtubule organizing center
MTs	Microtubules
NFAT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFTs	Neurofibrillary tangles
NMDA	N-methyl –D-Aspartic acid
P2X	Inotropic purinergic receptors
P2Y	Metabotropic purinergic receptor
P38-MAPK	p38 mitogen-activated protein kinases
P53	Tumor protein P53
PD	Parkinson’s disease

PHFs	Paired helical filaments
PI3K	Phosphatidylinositol 3-kinase
PIP2/PIP3	Phosphatidylinositol 2/3-phosphate
PKA/PKC	Protein kinase A/ C
PLC	Phospholipase C
PNS	Peripheral nervous system
PSP	Progressive supranuclear palsy
p-Tau	Phosphorylated Tau
PTMs	Post-translational modifications
Rabs	Ras-associated binding (Rab) small GTPases proteins
RIPK	Receptor-interacting serine/threonine-protein kinase 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEC	Size-exclusion chromatography
STAT	signal transducer and activator of transcription
Syk	Tyrosine-protein kinase SYK
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor-β
TLR	Toll-like receptor
TNFα	Tumor necrosis factorα
TREM2	Triggering receptor expressed on myeloid cells 2
UPS	Ubiquitin proteasomal system
WB	Western blot
ZnF UBP	Zinc finger ubiquitin binding protein
α-Syn	α-synuclein protein

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Chapter 1
Introduction

1.1 Pathophysiology of Alzheimer's disease: Amyloid-β cascade, Tauopathy and elevated neuroinflammation

Alzheimer's disease (AD) is the leading cause of age-related dementia which is associated with memory deficit, cognitive decline, impaired communication skills, daily-to-daily activity, behavioral/ social withdrawal, etc. [1, 2]. There is more than 50 million people have been affected by AD-related neurodegenerative diseases world-wide which will follow the exponential increase in the recent future globally. The early onset of AD starts at 45 years of age while; the moderate to severe symptoms develop at the age of 60-65 years [3, 4]. The main pathophysiology of AD-related neurodegenerative disease includes; the accumulation of two proteins in the brain- extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) of Tau protein. The microglia and astrocytes mediate the neuroinflammation in the central nervous system (CNS) that lead to synaptic loss and cognitive impairment. Moreover, glial population exacerbate the massive oxidative damage, propagation of seed species, cytokine response, and peripheral immune infiltration in brain [5-7]. In normal conditions, the Amyloid precursor protein (APP) undergoes proteolytic cleavage by α- and β-secretase enzymes, which was subsequently cleared from the CNS milieu. But, in pathological conditions, the APP is cleaved by β- and γ- secretase enzymes, which lead to the formation of altered Aβ cleaved peptides- Aβ1-40 and Aβ1-42 [8]. These two Aβ species have higher aggregation propensity and are deposited as extracellular Aβ oligomers and plaques. Secondly, the microtubule-associated protein Tau regularly functions in microtubule-treadmilling, tubulin network stabilization and axonal transport in neurons. Now, Tau undergoes various post-translational modification (PTMs) such as- acetylation, glycation, ubiquitination, methylation, glycosylation and most common hyperphosphorylation in disease condition [9]. The hyperphosphorylated Tau detaches from the microtubule and self-aggregated in the neuronal axons, which further missorted in the somatodendritic region. Tau protein consists of six isoforms, which are generated by alternative splicing of exon 2, 3 and 10. The various Tau isoforms are expressed differentially in the peripheral and central nervous system which is also associated with developmental stages. The full-length human Tau (hTau40) is abundantly expressed in CNS and consists of multiple domains. The flexible N-terminal domain is involved in protein solubilisation, molecular spacing and interaction with other proteins. The repeat domain is associated with microtubule-binding and also plays a role in self-oligomerization, followed by aggregation. The internal proline-rich domain of Tau contains SP/TP motif which acts as docking site for many cellular kinases and regulates phosphorylation at serine-threonine residues [10]. The accumulated Tau aggregates blocked the release of neurotransmitters from neuronal synapses and eventually become secreted from the neurons *via* various mechanisms such as- exosomes, neurotransmitters, membrane leakage and cell-to-cell connections, etc. The oligomerized/ hyperphosphorylated aggregated Tau spread through the synaptically connected neuronal circuits by prion-like propagation and transmitting the 'seed' component for further aggregation within healthy neurons [7]. The spreading of Tau started from frontotemporal cortex to the hippocampus and other brain regions which directly correlate with the progressive rate of cognitive decline. Braak and Braak first correlated the development of brain pathology and the clinical symptoms during the progression of AD; which consists of six stages. Stages I and II are associated with the formation/ accumulation of NFTs in the brain region without any cognitive impairment. While Stages III and IV relate the progression of pathological destruction of the cerebral cortex and disease propagation from the neocortex to hippocampus, which begins the cognitive decline. Lastly, Stages V and VI are associated with severe damage of the neocortex and other brain regions with complete cognitive decline and clinical diagnostic changes [11].

Tauopathies are a class of neurodegenerative diseases, which include Alzheimer's disease (AD), Primary age-related Tauopathy, Fronto-temporal dementia and Parkinsonism-linked to chromosome-17 (FTDP-17), Progressive supranuclear palsy (PSP), Cortico-basal degeneration, Ganglioglioma, etc. In Tauopathies, the Tau protein is abnormally

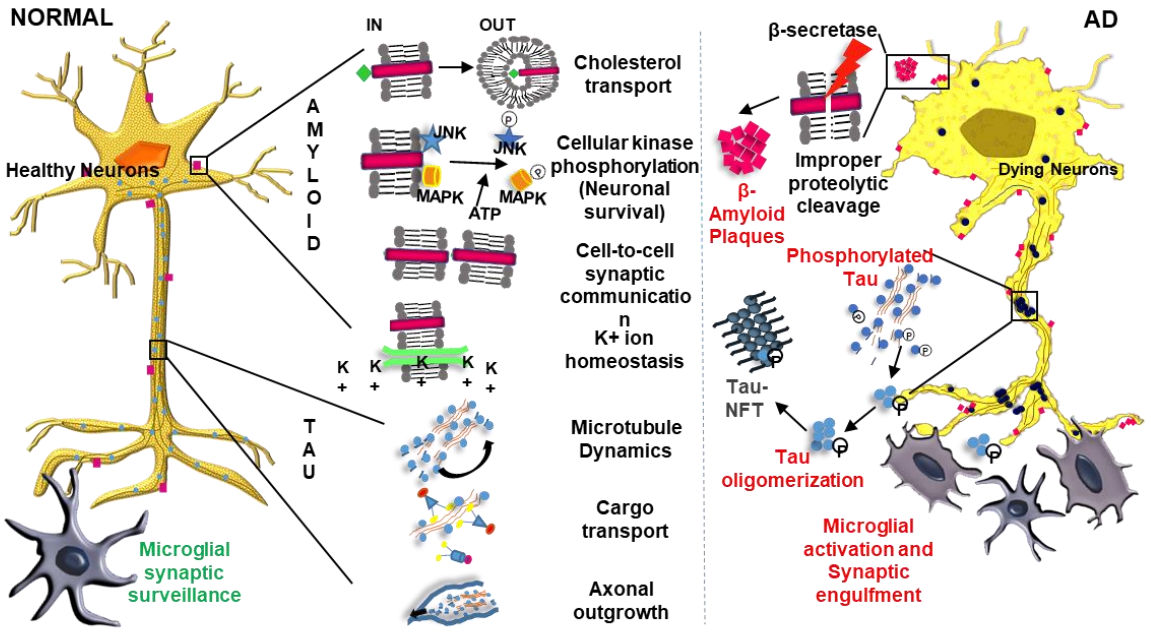

Figure 1. Proteopathic accumulation of Amyloid-β and Tau in neurons and microglial activation in AD. Alzheimer's disease (AD) is associated with the accumulation of Amyloid-β (Aβ) as plaques and Tau as neurofibrillary tangles (NFTs) in brain. In normal conditions, Aβ protein is involved in cholesterol transport, cell-to-cell communication and K^+ ion homeostasis in the neuronal membrane. Whereas, microtubule-associated protein Tau mediated microtubule dynamics, cargo transport and axonal outgrowth. In AD, APP is cleaved by the β-secretase enzyme and aggregated as senile plaques. Tau undergoes various PTMs such as hyperphosphorylation which lead to oligomerization and intracellular aggregation/ NFTs formation. The extracellular presence of Aβ plaques and released Tau proteins in the synaptic junction are recognized by surveilling microglia which resulted in activation and synaptic engulfment, and neurodegeneration. (*Adopted from Das and Chinnathambi, 2019, Cellular and Molecular Life Sciences*)

accumulated in the various locations of brain, especially in the peri-nuclear region of both neurons and glia (microglia, oligodendrocytes and astrocytes), leading to neuronal loss, inflammation and dementia [12, 13]. Primary age-associated Tauopathy subjects showed a slower rate of memory decline, social behavior, language and visuospatial performance with lower ApoE4 allele frequency than AD patients during the progressive disease stage [14]. It has been previously postulated that the early onset of Tau pathology dictates Aβ plaque formation and targets neuronal glutamate projections in the aged brain cortex of human and non-human primates. In a mechanistic way, abnormally phosphorylated Tau regulates the trapping of APP^+ endosomes; initiating a vicious cycle of Aβ production and calcium dysregulation along with genetic risk factors accelerating the disease pathology [15]. Similarly, acetylated Tau at Lys residue resulted in the blockage of long-term potentiation (LTP) of hippocampus synapses, impaired recruitment of AMPA-type glutamate receptor and postsynaptic localization of KIBRA protein related to memory and synaptic plasticity in AD-associated dementia [16]. Different amyloidogenic proteins such as; Aβ, Tau and Transactive response DNA binding protein (TDP43) exhibit differential patterns of brain atrophy in the hippocampus and temporal neocortex. Moreover, the accumulation of amyloid proteins is directly correlated with cognitive decline and the progression of AD and primary age-related Tauopathies [17, 18]. For eg., High TDP43 and NFT stages are proportional to hippocampal atrophy and faster disease acceleration but, slower death progression. While lower TDP43 stages were associated with a slower early progression rate and later disease acceleration that is linked to high NFT Braak's stages [17]. Therefore, the continuous accumulation of Aβ plaques and hyperphosphorylated Tau-NFTs directly connect the progression of AD associated-Tauopathy with distinctive cognitive decline which requires full-time medical attention.

There are three classes of glia in CNS- microglia, astrocytes and oligodendrocytes, that perform neuronal surveillance, tissue repair and homeostasis maintenance. Astrocytes mainly function as an integral component of the blood-brain-barrier (BBB) and neurotransmitter/ metabolites maintenance, while; microglia mediates the pre/post-natal synaptic pruning, adult synaptic surveillance and neuronal repair upon brain injury [19, 20]. The extracellular Aβ plaques and Tau oligomers can be sensed by surveilling microglia, which leads to the phagocytosis of patho-proteins and amyloid-bearing synapses in CNS [21]. The P301S Tau mice model showed hippocampal synaptic loss, axonal spheroids formation and activated microglial phenotype before the onset of Tau tangle formation in the brain [22]. Experimentally, the extracellular addition of Tau facilitates the gradual loss of live neurons by the phagocytosis of proliferative microglia through the exposure of neuronal surface phosphatidylserine. But in this scenario, the neurons did not undergo necrosis or apoptosis as observed in the neuron-glia ex-vivo co-culture system [23]. But during the progression of disease, Disease-associated microglia (DAMs) transform into a tolerance state of inflammation and phagocytosis in 5xFAD mice. Moreover the Interferon-γ (IFN-γ)-mediated metabolic reprogramming reverses the glycolytic metabolism and inflammation [24]. Another report emphasizes the microglial activation through NLRP3 inflammasome assembly by the Tau hyperphosphorylation and aggregation. Moreover, the intracerebral injection of Aβ-containing brain homogenates has induced Tauopathy *via* NLRP3 inflammasome which is downstream of microglial activation and neurodegeneration [25]. Further advancements also prove that the involvement of adaptive immune response in the due course of neurodegenerative diseases can be indicated by the infiltration of vascular and peripheral T-cells in AD patient's brain [26]. Henceforth, it is evident that the interconnected signaling cascade of Aβ pathway, Tauopathy and glia-mediated innate and adaptive inflammation are aggravating the progressive pathological stages of AD-related neurodegenerative diseases (Fig. 1).

1.2 Implications of soluble oligomers in AD

The protein misfolding and the protein aggregation is a complex multistep process that includes the conversion of monomers into mature fibrils through the formation of many intermediate oligomeric species. Various factors contribute in protein oligomerization, such as- surface charges, PTMs, hydrophobicity and propensity which regulate the formation of ordered periodic structures. The kinetics of the Aβ and Tau protein aggregation was well-studied which depicts an initial slow nucleation phase, then the accumulation of intermediate oligomeric species, followed by the exponential increase in fibrillar aggregation. Oligomers are short-lived, unstable but reactive species, which can accelerate the aggregation through interaction with either monomers or other pre-fibrillar species. The ON-pathway oligomers are converted into larger aggregates, but the OFF-pathway oligomers can be released from affected neurons and propagate in healthy cells [27, 28]. Oligomers act as a conformational template for normal proteins for further aggregation and can interact with other proteins non-specifically and exacerbate cell signaling [29, 30]. In past decades, various pieces of literature reported different methodologies for either the preparation or the isolation of soluble/insoluble and non-filamentous Tau oligomers in the scenario of AD [31]. A variety of factors such as- solvent composition, methods and timing of protein/peptide preparation and type of inducer used to initiate aggregation can be used for the aggregation. *In vitro* Tau aggregation can be induced *via* various anionic mediators such as- heparin, arachidonic acid, RNA, etc. Previous reports elucidated the *in vitro* preparation and biophysical characterization of truncated Tau oligomers by sucrose-gradient centrifugation or changing buffer pH composition [32, 33]. Another group reported the formation of various forms of Tau oligomers by using single molecular fluorescence techniques and thermodynamic stability assay. These are- electrostatically interacted short-lived species, OFF-pathway cytotoxic oligomers, ON-pathway oligomers for growing filament and transient oligomers species with internal cross-β structures [34]. Many studies introduced the use of thiol-blocker agents to

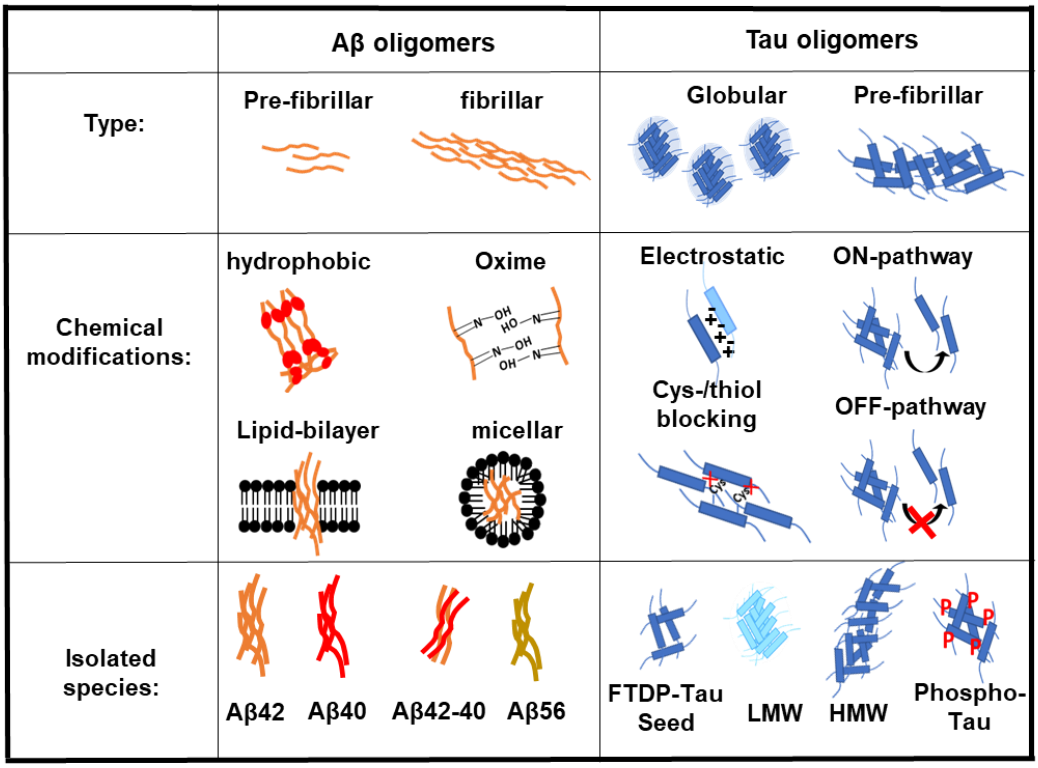

Figure 2. Isolated and in vitro chemically prepared oligomers and their implications in AD. Aβ forms different oligomers such as pre-fibrillar and fibrillar oligomers in AD. Aβ peptide was used to prepare in vitro oligomers *via* various chemical modifications. Tau was found to form globular and pre-fibrillar oligomers where the electrostatic interaction or Cys-thiol blockage were used to induce Tau oligomerization in vitro. The AD brain-derived Aβ oligomers contain homo/ hetero-oligomeric species. The isolated Tau oligomers from AD brain comprise of p-Tau, HMW, LMW and FTDP-17 mutant oligomers. *(Adopted from Balmik and Das et al., 2022, Royal Society of Chemistry)*

prepare Tau oligomers by preventing an accelerated aggregation process [35, 36]. Similarly, various studies emphasized the isolation and stabilization of Tau oligomers which can act as seed species in the course of Tauopathy. Frontotemporal Lobar Dementia (FTLD) mutants Tau protein was found to form soluble smaller oligomers as compared to wild-type control brain [37, 38]. Hyperphosphorylated high molecular weight (HMW) oligomers are more abundant than low molecular weight (LMW) oligomers in severe AD patient's brain. While; $TOC1^{+}$ Tau oligomers were evident in CTE brain with a direct correlation with cognitive impairment [39]. The cis p-Tau and its oligomers arise during traumatic brain injury and AD, while; targeting these cis p-Tau oligomers by monoclonal antibody (mAb) reduced Tauopathy and restore cognitive function [40]. Recent evidence showed that α-synuclein (α-syn) can also form co-oligomers more efficiently with Aβ and Tau, which showed a clear impact on the neurotoxicity and progression of Tauopathy and synucleopathy [41]. Similarly, the interaction between TDP43 and Tau oligomers potentiate the cross-seeding properties in P301L Tau-inducible cell model and in AD, FTLD, and amyotrophic lateral sclerosis (ALS) patient's brain. This speculates the converging role of TDP43 and Tau in Tauopathy-related neurodegenerative disease progression [42]. Tau oligomers are also found to interact with p53, a cell cycle transcriptional regulator which is also oligomerized in AD brain and P301L mice model. Moreover, p53-Tau co-oligomers were mislocalized into the neuronal cytosol, leading to DNA damage and cell death in AD brain [43]. Moreover, Aβ oligomers induce p-Tau and its incorporation as co-aggregates in hippocampal neurons of diabetic 3XTg-AD mice model. Therefore, the trapping and targeting of these toxic oligomers is the most

challenging and promising therapeutic target to combat the progression of neurodegenerative disease like AD.

Oligomers can be transmitted through synapses in neuro-anatomical circuits where it acts as a template for endogenous Tau aggregation and its subsequent release into extracellular space [44]. The soluble Aβ oligomers form membrane pore, increased cytosolic Ca^{2+} level, mitochondrial dysfunction and neuronal death [45, 46]. Similarly, Tau oligomers were also found to be accumulated in response to mitochondrial oxidative stress and impaired respiratory metabolism in cortical neurons and trans-mitochondrial "cybrid'' model of transgenic Tau mice [47]. Various forms of Tau oligomers such as- hyperphosphorylated, soluble, and prefibrillar oligomers mediate synaptic loss, miss-sorting in axonal compartments, seeding Tau pathology and Aβ toxicity [48, 49]. Other groups have shown that the propagated Tau oligomers lead to reduced long-term potentiation and increased short-term depression in cortical neurons, which was partially blocked by the administration of oligomer-specific antibody [50, 51]. The insoluble p-Tau (CP3 and AT8) oligomers and the soluble Aβ oligomers were found to be present in AD patient's brain in correlation with intellectual impairment [52]. A clinical decrease or absence of Tau oligomers at neuronal synapses correlates with synaptic functional integrity, neurogenesis and cognitive intactness in non-demented with Alzheimer's neuropathology (NDAN) individuals which emphasizes the primary role of oligomeric Tau in the synaptic and cognitive decline of sporadic AD cases [53], Similarly, Tau oligomers were detected in neuronal somatic extension and the serum of AD patients and healthy aged individuals but non-related to healthy-Mild cognitive impairment (MCI) *vs*. AD-MCI subjects. This signifies the peripheral clearance of Tau oligomers from interstitium to blood occurred early to the accumulation of Aβ pathology and Tau aggregate deposition in brain [54, 55]. Therefore the increasing evidence of Tau oligomer level in cerebrospinal fluid and serum of AD patients can be considered as a biomarker in the diagnosis and therapeutics target (Fig. 2).

Amyloidosis and Tauopathy resulted in excitatory NMDA activation and Ca^{2+} release, excitotoxic neurodegeneration and most importantly glia-mediated inflammation. Tau oligomers isolated from Traumatic brain injury (TBI) and aging subjects lead to *in vitro* Tau aggregation and cognitive dysfunction in the hTau mice model. Recent reports strongly correlated glia-mediated neuronal loss with the accumulation of toxic Tau oligomers in the pre-clinical AD model [56]. Astrocytic overexpression of risk factor ApoE4 allele has significantly increased Tau hyperphosphorylation, neuronal accumulation of Tau oligomers in neuron-astrocyte co-culture and transgenic Tau mice model [57]. Moreover, Tau oligomers are found to be associated with Aβ deposition near to the astrocytes in cerebral amyloid angiopathy (CAA) mice brain [58]. A recent study showed the presence of $Iba1^{low}$, HLA-DR^{high}, $CD74^{high}$, L-$ferritin^{high}$ microglial populations which effectively correlated with the Tau pathology but not the Aβ load in middle temporal gyrus of the human AD brain [59]. However, $Iba1^{+}$ NOS^{+} areas in the brain are associated with plaque deposition which concludes the accumulation of activated microglia [60]. Pharmacological inhibition of colony-stimulating factor-1 receptor (CSF1R) signaling resulted in a significant reduction of pre-fibrillar soluble oligomers, intraneural amyloids and neuritic plaques by ablating microglial population which showed a visible benefit in cognitive improvement in 5xFAD mice [61]. Moreover, the Tau oligomer-specific TOMA antibody injection significantly restores cognitive and motor function and also reduces the Tau oligomer level in A53T synucleopathy transgenic mouse model [62]. Therefore, the accumulating evidences emphasized the immense importance of Tau oligomers in the progressive scenario of AD where the oligomers-targeted immunotherapy could be a potential strategy to treat many neurodegenerative diseases.

1.3 Microglial functions in physiology and AD pathology

The central nervous system (CNS) mainly comprises of two different types of cells- Neurons and glia (astrocytes, microglia and oligodendrocytes). Neurons are the building blocks of the

central and peripheral nervous system, which mediate various functions such as- the generation and storage of memory, sensory to motor functions and inter-neuronal communication through the neurotransmitter. Astrocytes are the brain-resident glia that surveil the BBB along with the regulation of CNS metabolism, homeostasis and neurotransmitter recycling. Oligodendrocytes maintain myelination, axon potential and neuronal metabolism. Lastly, microglia is the prime-most immune cells in CNS, consisting of 5-20% of total glia, which continuously surveils the invasion of pathogen, synaptic health surveillance and maintains tissue homeostasis. Microglia originate from the myeloid-progenitor cells in the embryonic yolk sac during early developmental stages and migrated to the brain axis, having self-renewal and proliferation properties [20]. Microglia as the CNS-resident immune effector cells, contribute in various physiology and pathological functions. For eg., synaptic pruning, circuitry development, adult neuronal rewiring, phagocytosis of pathogen and cellular debris, inflammatory regulation etc. But the improper functionality of senescent, aged or DAMs microglia leads to the development of neurological disorders such as Nasu-hakola disease and age-related neurodegenerative diseases Alzheimer's disease, ALS, Multiple sclerosis (MS) and Parkinson's disease (PD) etc [63, 64]. The pharmacological replacement of CSF1R in senescent microglial population and its repopulation exerted an improvement of spatial memory and microglial tissue density morphology without altering inflammation-related genes expression in 24 months aged mice. Furthermore, the replaced microglia induced actin remodeling, synapse formation, dendritic spine density, improved long-term potentiation and neurogenesis in hippocampus [65]. But on contrary, the depletion of tissue-resident microglia and increased pool of peripheral macrophages didn't alter the accumulation of p-Tau and Tau oligomers in the aged hTau mice model. Therefore, this study significantly questions the direct involvement of microglia in the progression of AD [66].

Initially microglial was thought to be supporting cells in the CNS but recent studies in the last few decades prove the importance and complexities of this glia as a major innate immune player in brain which is directly involved in AD or other age-related dementia [67]. Microglia display a degenerated phenotype with fragmented dystrophic processes, reduced cellular and surveilling area, impaired immune protection and neuronal survival in AD. Moreover, the soluble hippocampal fractions containing p-Tau (AT8, AT100) from AD and THY-T22 mice brain were found to be cytotoxic to cultured microglia with impaired phagocytic activity [68]. Similarly, Tau hyperphosphorylation and aggregation occur early in hTau CX3CR1 $^{(-/-)}$ mice model. While; the inoculation of purified microglia from hTau CX3CR1 $^{(-/-)}$ mice brain induces Tauopathy in control recipient mice brain [69]. Phenotypically altered DAMs were found to degrade plaques and associated peri-neural nets in 5xFAD and 3XTg-AD mice brain, similar to LPS-activated microglia [70]. Recent understanding showed the involvement of TREM-2 along with genetic variants of ApoE regulates various microglial functions [71, 72]. Ionized calcium-binding adaptor protein-1 (Iba-1) was considered as an identification/activation marker of microglia/macrophages. But, recent studies emphasized Iba1 as an actin cytoskeleton crosslinking protein that modulates various functions in microglia and macrophages, like- migration, proliferation, membrane ruffling, phagocytosis and immune surveillance. [73]. Iba1 deficient mice$^{(-/-)}$ showed a distinct microglial phenotype with reduced ramification, motility, and performed altered synaptic pruning with less number of hippocampal excitatory synapses in early and postnatal development that lead to cognitive decline [74]. Moreover, Iba1 and Pax5 interaction mediate cell proliferation, differentiation, immune activation and neurogenesis via actin remodeling in brain [75]. The IFN-γ, Interleukin-1β (IL-1β) and tissue injury were reported to increase the Iba1 levels related to cellular proliferation and migration [76].

Microglia were found to perform dual but extremely opposite functions in the onset and progression of AD and associated neurodegenerative diseases. The beneficial function includes the phagocytic and complement-mediated clearance of Aβ and Tau deposits in brain parenchyma. On contrary, microglial activation, neurotoxic inflammatory cytokine and chemokine production, improper synaptic engulfment and spreading of the seed component

can be targeted against the glial population in AD treatment [77, 78]. Complement protein C5a/C5aR1 signaling induces the activation of $CD11c^+$ microglial population, A1 astrocyte migration and inflammatory signaling relating disease progression in AD mice model [79]. Tumor necrosis factor-α (TNF-α) mediates neuronal death *via* Aβ-induced microglial activation by secreting excessive glial glutamate with an upregulated level of glutaminase and connexin 34 hemi-channel in gap junction. Therefore, the glutaminase inhibitor or hemi-channel blockers can be therapeutically targeted in glial inflammatory diseases such as AD, PD, ALS etc [80, 81]. The polarization of microglia from M1 to M2 in CA3 region mitigates the neuropathic pain and attenuates the AD-associated cognitive behavior in the doxycycline-induced AD mice model [82]. Therefore, it is extremely necessary to interfere the appropriate functions of pathology-associated microglia and sets of targeting molecules in the diseased population to obtain a successful strategy against AD-like neuroinflammatory condition.

1.4 Actin remodeling and microstructures formation in immune cell function

Actin is a cytoskeleton protein network, like other two cytoskeleton networks- microtubule and intermediate filaments in cells that perform various physiological functions [83]. Actin monomer is a 42 KDa protein, which is the building block of the double-stranded helical actin filament, having a diameter of 7 nm and a size of 10-100 μm. The dimerization/ trimerization of monomeric actin (globular actin: G-actin) is the thermodynamically limited step that requires the involvement of ATP-dependent actin nucleation complex (Arp2/3 complex) association. Actin network helps to maintain various functions in cells such as- structural integrity, shape-changing to a specific environment, migration, movement through a narrow passage, cell division, endo/ exocytosis, extracellular matrix (ECM) adhesion and cell-to-cell communication etc. The membrane-associated actin polymerization from G-actin to F-actin generates mechanical tensile forces on the cytoplasmic membrane and mediates the directional migration. The organization of actin polymers can be of different types- branched, cross-linked networks, parallel and anti-parallel bundles, cortical actin sheets and stress fibers etc. The branched and cross-linked actin network at the cell front makes a two-dimensional sheet-like structure called lamellipodia which mainly helps in forward movement, phagocytosis and endo/ exocytosis of the cells. Similarly, bundled actin network is present within the finger-like projection of cells called filopodia, which is associated with mechanosensing, object trapping and migration [84, 85]. A thin layer of cross-linked actin network makes up the cell cortex at the substratum layer, which is involved in cell structure maintenance, matrix adhesion and even invasion. While inside the cells, the three-dimensional cross-linked actin network along with contractile actin-myosin complex and integrin-associated stress fibers maintain the actin gliding, global contraction and signaling transduction at the focal adhesion sites [86]. A specialized membrane-associated actin structure called podosome contains a cross-linked actin core network, surround by actin-binding proteins- vinculin, talin and membrane adhesive proteins- integrins. Podosome (similar to invadopodia in cancer cells) is short-lived protrusive structures at the ventral cell surface, size of 0.5-2 μm in diameter in lamellipodia. The core of the podosome is associated with various actin-associated proteins such as- Wiskott–Aldrich Syndrome protein (WASP), tyrosine kinase substrate with 4/5 SH3 domains (TKS4/5) etc., important for membrane attachment of actin core and extracellular signal transduction [87, 88]. Podosome has directly involved in many physiological functions such as- cellular migration, matrix adhesion, tissue invasion, wound healing, inflammation and ECM degradation. The Arp2/3 complex and formins are involved in the initiation, cross-linking and elongation of actin polymer. Then, various actin cross-linking proteins maintain the structural accuracy of the actin filaments (F-actin) networks. For eg., Fascin maintains the parallel actin bundles, α-actinin, profilin, cofilin and Arp2/3 complex withhold the branched and cross-linked actin network in lamellipodia. A complete tandem of assembly and disassembly machinery of actin cytoskeleton network eventuate in the migratory immune cells to mediate a series of physiological functions, which got affected in pathological conditions like- AD(Fig. 3A).

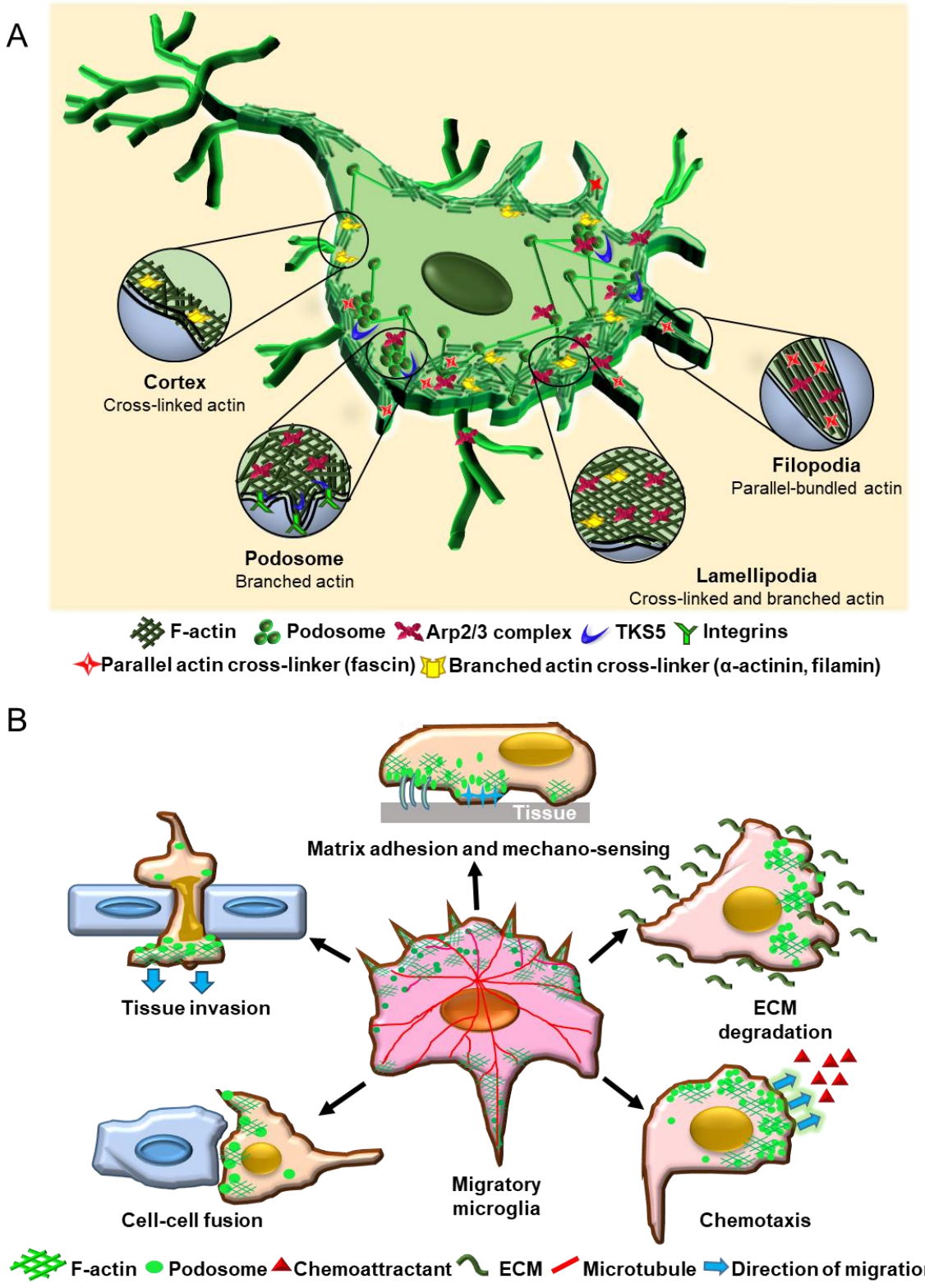

Figure 3. Structure and function of various actin microstructures in AD-associated microglia. A. Actin remodeling consists of various microstructures which are different from others based on their actin organization, cellular location, functions and particular molecular signature. Migratory immune cells such as microglia contain various actin structures such as lamellipodia, filopodia, podosome, uropod and actin cortex. The actin remodeling involves the functioning of various other proteins such as Arp2/3 complex, TKS5/4 adaptor, various actin cross-linkers and cell receptors- Integrins. **B.** The migratory microglia remodels the cytoskeleton actin and tubulin network to mediate various

physiological and effector functions. The actin-rich podosome formation regulates ECM sensing, degradation and invasion by remodeling tissue degradation and homeostasis.

Microglia display various distinct morphologies such as- 'ramified' (homeostatic), 'amoeboid' (activated), dystrophic (fragmented and branches) and stress-primed phenotype in different physiological and pathological events [89, 90]. The morphological changes of activated microglia include the retraction of cellular processes, the increase of soma and the expression of different myeloid and phagocytic receptors [91]. But, microglial activation leads to rapid migration, secretion of cytotoxic factors, such as- pro-inflammatory cytokine, ROS, NO, resulting in neurodegeneration [92]. It has been previously reported that actin remodeling and microglial migration become impaired due to aging, which probably relates the development of AD [93, 94]. Microglial calcium-binding proteins: calmodulin along with Iba1 regulates the reorganization of the cortex actin network and also influences cell viability, proliferation and fluid-phase phagocytosis, in activated rat microglia [95]. But, with increasing time, the phagocytosis of microglia increases with the loss of directed process extension [96]. The α-linolenic acid, a class of omega-3 fatty acids induces the formation of membrane ruffling and phagocytic cup, colocalized with Arp2/3 complex in lamellipodia for microglial movement and Tau internalization [97]. On the other hand, Phosphatidylinositol (PI) signaling plays important roles in Aβ- and Tau-induced microglial migration, actin remodeling, phagocytic cup formation and endosomal trafficking, by depending on the differential inositol ring phosphorylation and actin binding protein association [98]. Similarly, the disruption of actin dynamics resulted in reduced secretion of NO, TNF-α, IL-6 and induced release of IL-1β and IL-18 from LPS stimulated microglia. Furthermore, the impaired actin cytoskeleton affects the IGF1 secretion, p-STAT6 nuclear translocation and behavioural alteration in IL-4 stimulated microglia and attenuates M2 polarization [99]. Therefore, the impaired actin dynamics can strongly regulate the cellular senescence, microglial activation, migration, proliferation, phagocytosis and improper effector function leading to pathological neurodegeneration (Fig. 3B).

1.5 Microglial and astrocyte receptors in neurodegenerative diseases,

In AD, the abundance of extracellular protein aggregates/ oligomers and damaged neurons can activate the surveilling microglia, which resulted in increased migration, oxidative stress, phagocytosis of protein depositions, antigen presentation and adaptive inflammation. But, the improper elimination of live neurons or damaged synapses by triggered microglia results in synaptic loss, gradual cognitive decline and neurodegeneration [100]. Activated microglia can alter the expression of various surface receptors as a 'death signal' response, which include Toll-like receptors (TLR2, TLR4), Receptor for advanced glycated end product (RAGE), Integrins such as- α6-β1 integrin, CD14, CD36, Metabotropic and inotropic purinergic receptors (P2Y6, P2Y4, P2Y12, P2X7, P2X4), antigen-presenting MHCII and MAC-L, etc. The phagocytic receptors which are involved in patho-proteins clearance include Triggering receptor expressed in myeloid cells 2 (TREM2), Fcγ receptor, Complement receptor (C1q, C5aR), Mannose receptor, Scavenging receptors (SCARA1, MARCO, SCARB1) and Lipoprotein receptor-related protein (LRP) etc [101-103]. Aged microglia showed a decreased expression of Aβ phagocytic receptors such as- SRA, CD36, RAGE with increased production of pro-inflammatory cytokines- IL1β and TNFα which correlate further Aβ accumulation and microglial activation in AD progression [104]. The microglia-specific TREM2-DAP12 signaling and neuro-glial communicative CX_3CL_1-CX_3CR_1 axis play a central role in microglial survival, proliferation, maturation and phagocytosis in CNS aging, AD and other neurodegenerative diseases [105]. A recent single-cell Transcriptomics and the functional study revealed that TREM2 knock out resulted in reduced phagocytosis of ApoE and Aβ plaques, cell survival and diminished CXC_4-mediated chemotaxis in the AD-associated DAMs population [106]. Furthermore, a risk variants of TREM2 exhibit Aβ accumulation, NFTs formation, TNF-α induced glutamatergic excitatory transmission and reduced inhibitory GABA-ergic signaling, contributing to elevated neuronal dysfunction and neurodegeneration in sporadic AD and other dementia [107, 108]. Recently,

various GPCRs and TREM2 receptor were reviewed for their direct role in microglial phagocytosis, inflammation, Aβ and pathogenic Tau production and in therapeutically important neuroprotection through PI3K-AKT and small GTPase signaling in AD [109] (Fig. 4A).

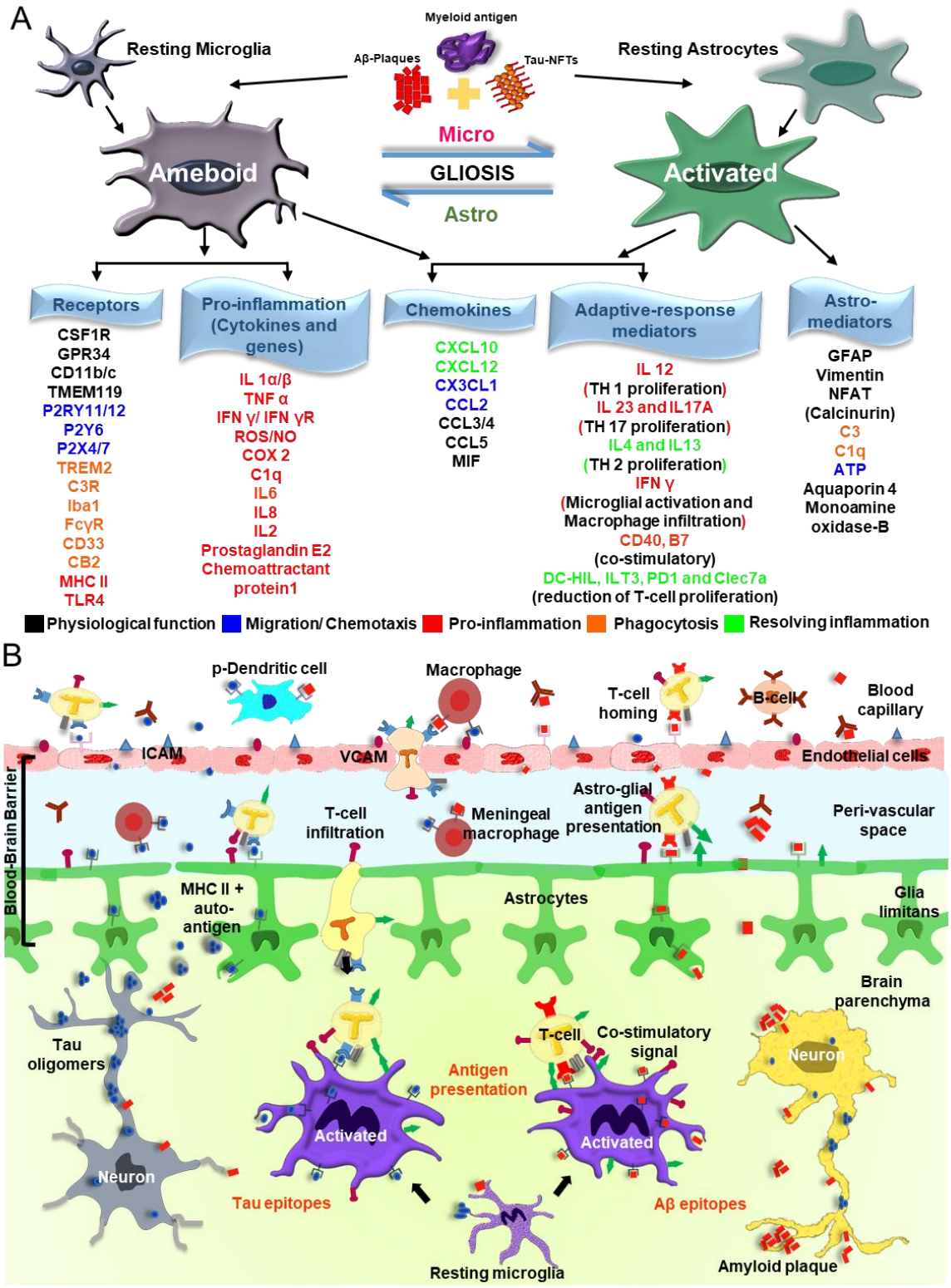

Figure 4. Micro-astroglial receptors and microglia-mediated antigen presentation of Tau for immune infiltration in CNS. A. Activated microglia and astrocytes express various surface receptors which regulate cellular proliferation, migration/ chemotaxis, inflammation and phagocytosis. But, at resolving stages, microglia and astrocytes perform neuroprotection and anti-inflammation. **B.** The lymphatic antigen drainage (soluble Tau and Aβ oligomers) attracted the dendritic cells and

meningeal macrophages for CNS infiltration and antigen presentation to the adaptive immune cells. The extravasation of T-cells in CNS, mediates the microglial antigen presentation. *(Adopted from Das and Chinnathambi, 2019, Cellular and Molecular Life Sciences)*

DAMs can exhibit both pro and anti-inflammatory phenotypes as observed in 5XFAD mice model as well as AD patient brains. Pro-inflammatory phenotype is associated with the activation of TLR2, PTGS2, IL12b, IL1β, surface CD44, Kv1.3 channel and transcriptional regulators NF-kB, STAT1, Rel1 which relate the causal effects of neuropathy, inflammation and cognitive decline. While; the anti-inflammatory subtypes expressed phagocytic genes- IGF1, ApoE, $CXCR_4$, and regulator LxR-α/β, ATF1 that mediate neuromodulatory functions and Aβ-Tau clearance in mice model and AD brain [110-112]. Galectin-3 (Gal-3/ MAC2) mediates the age-dependant Aβ oligomerization, plaque accumulation, and Iba1-GFAP expression in plaque-associated regions in AD mice brain. Gal-3 was also found to colocalize with microglial Iba1 and interact with TREM2 for oligomeric Aβ-mediated glial activation in FTLD-AD patients [113]. Moreover, Gal-3 involves in microglial morphological transformation, phagocytosis through F-actin-rich lamellipodia and filopodia formation *via* cofillin activation and actin-myosin contraction in MS and traumatic axonal injury model [114]. An immune checkpoint regulator PD-1/PDL1 in microglia-astrocyte pair mediates Aβ phagocytosis from plaque deposits, CD36 receptor expression and inflammatory regulation in APP/PS1 mice and AD brain [115]. Various chemotactic signaling such as chemerin/CMKLR1 and CCR2 axis regulates the microglial migration and its recruitments to Aβ plaque area through the formation of actin-rich protrusions, microtubule polarization and golgi reorientation *via* p38-MAPK in AD mice model [116, 117]. An elevated expression of CSF1/CSF1R and diminished mRNA level of IL34 were observed in mild to severe pathology of AD brain which subsequently links the inflammatory activation and reduced level of Aβ removal by lysosomal degradation in aged microglia [118]. Recent preclinical studies with non-human primates showed that the long-term use of class-B CpG ODN 2006- a TLR9 agonist has improved clearance of Aβ and Tau without sustained inflammation in the aged squirrel monkey with improved cerebral amyloid angiopathy and cognitive improvement [119]. A study emphasized that the TREM2 activation leads to the accumulation of IFN-responsive MHC-II expressing microglia with increased Aβ uptake in 5xFAD mice brain [120]. The engulfed plaques are degraded by activated microglia after which, the MHCII-bound peptides can be presented to the infiltrated T-cells, leading to the adaptive immune response in CNS [103]. Therefore, targeting a single/combinatorial receptor signaling pathway in disease-associated microglia-astrocytes would be of immense importance to identify and implement a promising approach for CNS-degenerative diseases by controlling plaque clearance, neuroprotection and balanced inflammation (Fig. 4B).

1.6 Purinergic signaling in AD-associated neurodegenerative diseases

Damaged neurons are the source of released energy nucleotides (ATP, ADP, UDP, etc.) and extracellular protein aggregates. Purinergic receptors are of two types- adenosine receptors- P1 and nucleotide di/triphosphate receptors- P2. The P2 purinoceptors are further subdivided into two groups, such as- Ionotropic purinergic receptors (P2X1- P2X7) and Metabotropic purinergic receptors (P2Y1- P2Y14) [121]. The P2X receptors are two-transmembrane ligand-gated ion channels, where the extracellular N-terminal ligand-binding domain recognizes nucleotide triphosphates. The ligand binding imparts conformational changes in a transmembrane domain which allow the opening of ion channels, leading to the ion influx and intracellular Ca^{2+} signaling. The P2Y receptors are seven-transmembrane GPCRs that recognize nucleotide di/tri-phosphates by external ligand-binding pocket, leading to G-protein signal transduction or receptor internalization (co-endocytosis) [122]. Purinergic receptors are previously investigated and therapeutically intervened in cardio-vascular and hematological diseases. But the abundance of these purinoceptors in CNS showed its involvement in various physiological and pathological functions such as- memory-locomotor function, neuronal stem cell and microglial migration, synaptic transmission, cell proliferation, microglial activation, phagocytosis, regulated apoptosis, neuro-glial

communication, inflammation and neuropathic pain [123]. In past decades, an enormous amount of research focussed on purinergic receptors depicted their role singularly or synergistically in AD, PD, ALS, TBI and various other neurodegenerative and neuroinflammatory diseases, which makes them a potential candidate for therapeutically intervened. A previous report showed that extracellular ADP induces microglial chemotaxis and membrane ruffling through the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and β-arrestin signaling cascade, which leads to the formation of paxillin-containing lamellipodia structures [124]. Similarly, the extracellular adenosine is associated with the CD73-A2B receptor pathway which exhibits gamma radiation-induced glioblastoma cell migration and actin remodeling for acquiring resistant malignant phenotype *via* DNA damage response [125]. A neuron-specific overexpression of A2A receptor induces Tau hyperphosphorylation, memory deficit, loss of glutamatergic synapses and hippocampal C1q upregulation in the THY-Tau22 mice model, similar to FTLD brain [126]. Moreover, the depletion of A2A improved the GSK3β and PKA-dependent Tau phosphorylation, axonal injury, aquaporin-associated dendritic spine density and cognitive behavior in traumatic brain injury and Tauopathy mice model which can be targeted in therapeutics [127-129] (Fig. 5).

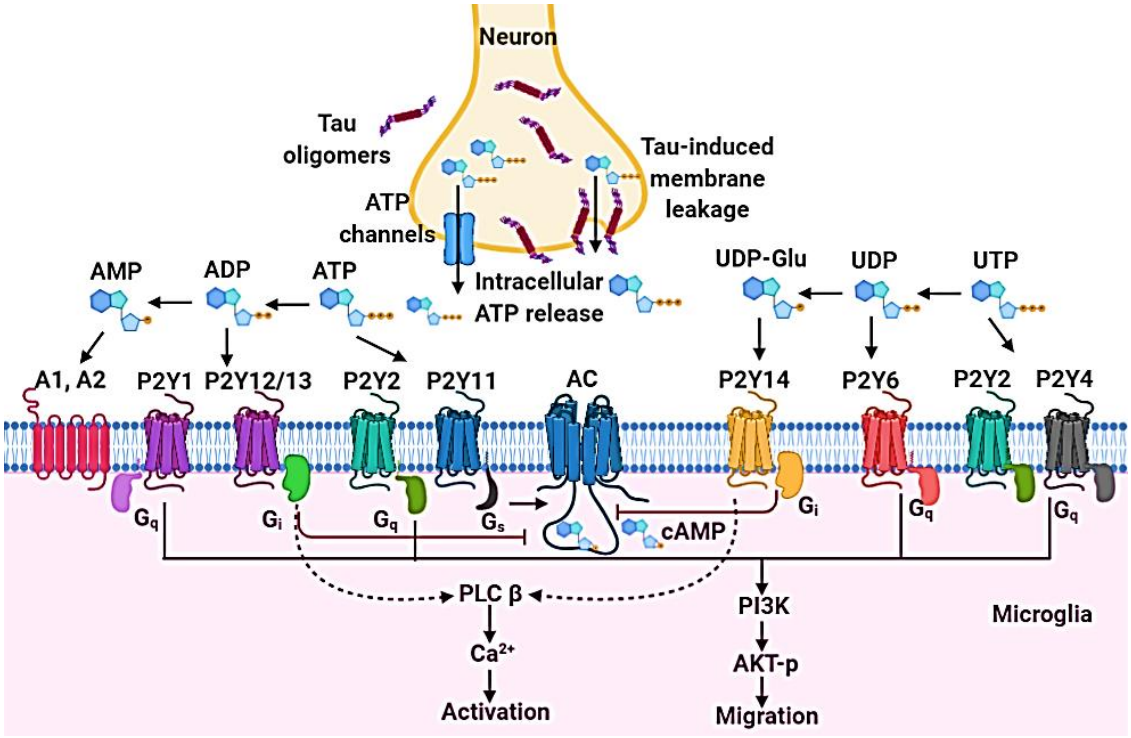

Figure 5. Purinergic receptors signaling in AD. Tau oligomers and NFTs bearing neurons are associated with membrane leakage which resulted in the secretion of intracellular nucleotides in CNS. The released nucleotides were captured by purinergic P2Y and P1 receptors that lead to target G-proteins activation. Further, the activation of PLCβ and PI3K-Akt signaling led to Ca^{2+} oscillation, microglial activation and migration.

Purinergic signaling plays a differential function in different cell types and disease pathologies. Purinergic P2X4 activates microglial motility *via* PI3K-AKT signaling pathway which leads to the inflammation-associated production of prostaglandin, TNF-α and BDNF during aging and stroke-related neuropathology condition [130]. Similarly, chemokine CCL_2 stimulation elevates the cell surface localization of P2X4 receptor by inducing lysosomal exocytosis and ATP-mediated Akt phosphorylation in primary microglia during nerve injury [131]. Moreover, the release of ATP through the pore of oligomeric-Aβ leads to the activation of microglia and astrocytes by the involvement of P2X receptors [132, 133]. However, the pharmacological inhibition of p38, ROS, P2X7 and CAMK-II significantly reduce the ATP-induced microglial apoptosis which is downstream to P2X7 or ROS signaling cascade [134]. The stimulation of P2X7 receptor resulted in the release of various neurotoxic factors such

as- proinflammatory cytokines (IL1β, IL6, TNFα), chemokines, ROS, NO and excitatory glutamate, ATP etc. Moreover, P2X7-mediated transmembrane flux of Na^+, K^+, Ca^{2+} influx and intracellular ion/metabolite depletion induce microglial apoptosis and neuronal death in neurodegenerative diseases- AD, PD, ALS and psychiatric disease [135-137]. Previously, it was reported that extracellular α-syn induces the P2X7-dependent intracellular mobilization within the neuritic extension, pannexin recruitment for ATP release and reduces extracellular ecto-ATPase release which regulates neuronal toxicity and death. Moreover, the cholesterol and ATP-dependent physical association and signaling crosstalk of P2X7 and pannexin-1 are important in receptor clustering and endocytosis in neurons [138, 139]. The pharmacological/ genetic ablation of P2X7 receptor leads to the reduction of microglial migration, proliferation, exosomal release, abnormal phagocytic function and GSK3β-mediated Tau phosphorylation ($MC1^+$ and ALZ50) in the hippocampus with improved motor and memory function in P301S mice model [140, 141]. An interesting computational model-based study showed that either a high concentration of ATP or co-activation of P2X4 and P2X7 receptor can trigger TNF-α production, *via* NFAT signaling and Ca^{2+} homeostasis in inflammatory microglia [142].

The microglial function is regulated by an array of purinergic receptors such as- P2X4, P2X7, P2Y4, P2Y6, P2Y12 and adenosine receptor A2 and A3 which plays a significant role in neurodegenerative diseases like- AD, PD, ALS, MS etc. Previous reports showed that P2Y1 is found to be colocalized with NFTs and Aβ plaques, regulates neuronal self-renewal, astrocyte proliferation and microglial migration by interacting with αv/β3/5 integrin and induces the IL1β secretion in AD mice and human brain [143-145]. Moreover, reactive astrocytes were found to surround the Aβ plaques and exhibit hyperactivity *via* P2Y1 receptor and connexion hemi channel [146]. The LPS-activated microglia express an increased level of P2Y6 and P2Y12 with a high level of Iba1, CD11b, DAP12 and Na^+/Ca^{2+} ion exchanger, for migration and phagocytic clearance of faulty neurons [147]. Another report showed that combined administration of LPS and P2Y agonist can significantly induce the microglial expression of Iba1, purinergic receptors (P2Y6, P2Y2 and P2Y12), NCX1 (Na^+/Ca^{2+} exchanger), Ca^{2+} channel, MARCKs related protein (MRP) which all are linked to microglial activation, migrations machinery component and CD11b-DAP12-phagocytic component in postnatal mice development [148]. Microglia with altered phenotype respond to extracellular acidosis of the interstitium by the stimulation of P2Y1 and P2Y6 receptor which certainly influence the intracellular Ca^{2+} reservoir but not the Ca^{2+} influx channel in brain pathology [149]. Microglial activation strongly correlates with the accumulation of Tau and neurodegeneration, which is linked with reduced P2Y12 immunoreactivity in plaque or lesion areas [150, 151]. The P2Y12 and P2Y13 receptor function differentially in the converging neuro-glial signaling pathways. The P2Y12/13 activation in cortical neurons induces the ROS level through the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) during neuroinflammation [152]. But, P2Y13 KO mice showed more microglial processes and less-ramified structure, while the P2Y12 blockage leads to reduced brain surveillance with more IL1β secretion [153]. The appropriate and combinatorial selection of agonist or antagonist of purinergic receptors along with specific nucleotide function can potentially shape the microglia-mediated neuroinflammation and maintain CNS homeostasis for therapeutic benefits.

1.7 The interconnection of purinergic signaling and actin remodeling

Migratory microglia form various actin-rich structures, such as- filopodia, flat fan-shaped frontal lamellipodia which are associated with membrane ruffling and concentrated actin-ring structures called podosome for extracellular matrix degradation [154]. The activation of ERK1/2 regulates the paxillin phosphorylation and subsequent rearrangement of the localized actin network, which leads to the directional migration axis of microglia [124]. Plaque-associated microglia appeared hyper-motile, extending the process with a high speed as compared to non-plaque-associated microglia, where both were non-respondent to mechanical injury in 5xFAD mice. Whereas; the selective blockage of the adenosine A2A

receptor restored the microglial response to cell injury and hence reduced hyper-motility in the PD mice model [155]. The TGF1β induced exocytosis of ATP elevates the cellular migration and actin remodeling *via* purinergic P2X7 receptor in human lung cancer cells [156]. Extracellular ATP induces the cofilin rod along with actin remodeling *via* calcineurin-dependent cofilin phosphorylation and Ca^{2+} influx through P2X receptor for neuritic extension [157]. Long-term exposure to ATP during CNS injury (>30 min) rapidly induces microglial cytoskeleton transition and reduces phagocytic potential. While; the blockage of P2X7 resulted in reduced release of cytokines, intracellular Ca^{2+} and ATP by Aβ phagocytic microglia along with the loss of dendritic spine [158]. Therefore, of microglial functions can be intervened for the improvement of age-related neuro-synaptic cytoskeleton rearrangements, dendritic spine regeneration without altering immune activation and phagocytosis [159, 160].

Apart from inotropic purinergic receptors, metabotropic GPCR- P2Y receptors are enormously associated with the actin cytoskeleton remodeling as an immune component or migratory integral parts in a variety of cells such as- podocyte, cardiac fibroblast, neutrophil, astrocytes and microglia. The P2Y4 receptor regulates the F-actin cytoskeleton remodeling and contractile apparatus *via* the RhoA-PKA signaling pathway with increased cAMP levels in podocytes to combat oxidative stress and energy balance [161]. Similarly, microglial motility was also elevated by P2Y12 signaling, which follows through the downstream Akt phosphorylation and the IRF8 activation during ATP and C5a complement-mediated signaling induction [162]. Moreover, the P2Y12-induced microglial migration was upregulated through TLR2 pathway by the intermediate involvement of PI3K and Rac [163]. P2Y12 signaling was also found to follow through VASP phosphorylation, which regulates the formation of actin-rich focal adhesions in migratory microglia. Another report showed that the P2Y12 activation lead to the collapsing of filopodia with increased formation of bulbous actin-protrusions. On the other hand, the localized cAMP level regulates the nanoscale filopodia formation in ramified microglia relating to the homeostatic condition [164]. The previous report has emphasized that P2Y12 activation is often associated with alternatively activated microglia i.e., M2 phenotype and the $P2Y12^{+}$ microglia were located adjacent to the MS plaques area [165]. The hypertension-related chronic neuroinflammatory microglia display shorter actin-rich extensions with reduced levels of P2Y12 and CX_3CR_1 [166]. Another report has shown that P2Y12-induced microglia facilitate the formation of neuronal micro-projection and regulate the neurogenesis of pyramidal neurons in DG and CA3 regions [167, 168]. Henceforth, the appropriate agonism or antagonism of these purinergic signaling may underlie a probable therapeutic intervention in glia-associated neuroinflammation or neurodegenerative diseases (Fig. 6A).

1.8 P2Y12 signaling in neurodegenerative diseases

Initially, P2Y12 was considered as a homeostatic, non-activated phenotypic marker of microglia in CNS inflammation. Various microarray and proteomics analysis studies showed the reduced expression level of homeostatic genes, where P2Y12 is specifically associated with the homeostatic condition and modulated with neuroinflammation. But a recent study emphasized that $P2Y12^{+}$ microglia encompass a wide range of morphological phenotypes in the middle temporal gyrus of AD brain. Despite the classical markers such as- Iba1, CD68, MHCII, microglia express P2Y12 throughout their lifetime as compared to bone-derived monocytes and peri-vascular macrophages. The P2Y12/13 receptor mediates the intracellular Ca^{2+} release through IP3/ ER- Ca^{2+} pump and releases CCL_3 *via* NFAT activation [94, 169]. Moreover, the caspase1-mediated mitochondrial membrane potential was altered through P2Y12 signaling in microglia [170]. Similarly, the P2Y12 signaling in non-activated microglia also supervises the synaptic plasticity and motility of the murine visual cortex. The interconnected P2Y12 signaling with voltage-gated ion channel for outward K^{+} current and two-pore domain channel THIK-1, together mediate microglial activation, migration and prevent the IL1β release from the [171, 172]. Further, $P2Y12^{+}$

microglia were found to be present in diffused plaque area and was also evidenced to associate with activation markers such as CD68, progranulin and HLA-DR in brain [173].

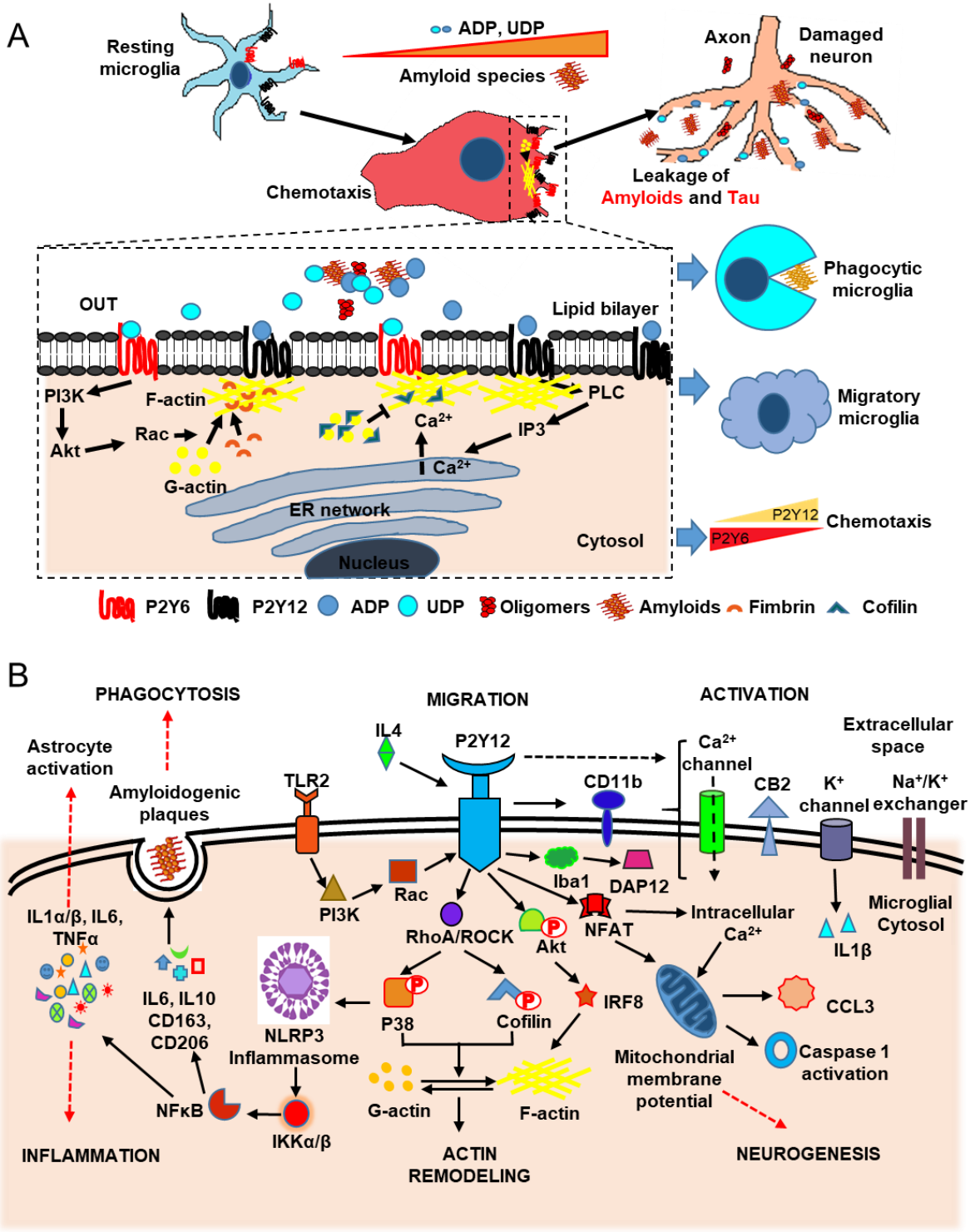

Figure 6. Purinergic P2Y-receptors mediated microglial chemotaxis, phagocytosis and inflammatory signaling in AD. A. Extracellular ADP/UDP from damaged neurons are detected by P2Y12 and P2Y6 receptors respectively, which mediate the downstream signaling *via* either PLC or PI3K cascade. **B.** P2Y12 activation resulted in the inter-connected signaling and effector function cascades in microglia, such as phagocytosis, activation, actin remodeling, inflammation, and neurogenesis along with chemotactic migration. The P2Y12 signaling leads to actin remodeling, Ca^{2+} signaling, migration, inflammation,phagocytosis and neurogenesis. *(Adopted from Das and Chinnathambi, 2020, Neuroscience)*

Purinergic P2Y12 can play a dual (beneficial and detrimental) role during the progressive stages of neurodegenerative diseases. At the initial stages of the disease, the reduced level of P2Y12 mediates a protective immunity and slows down the disease progression. However, in later stages, P2Y12 is involved in microglial activation and pro-inflammatory condition. It has been previously reported that P2Y12 regulates the microglial surveillance in CNS by inducing cellular movement, i.e., independent of cell proliferation [143]. While; the P2Y12 depletion resulted in reduced microglial surveillance, immune activation and synaptic elimination in brain [174, 175]. Moreover, microglial P2Y12 signaling performs the juxta-vascular process extension and wound closure in laser-induced BBB opening mice model [176, 177]. Similarly, the P2Y12 activation was modulated by CB2 analgesic receptor and evoked the release of inflammatory cytokines such as-IL1β, TNFα and IL6 through ROCK and NF-κB signaling cascade in cultured dorsal horn microglia during neuropathic pain [178-180]. A pharmacological inhibitor of PDE1 (calcium-dependent phosphodiesterase) blocks the P2Y12-associated migration and LPS-induced inflammation in BV2 and P2 microglia [181]. Furthermore, P2Y12 expression was upregulated in IL4-induced anti-inflammatory microglia which mediates the IL6, IL10 secretion and T_h17 response in the murine stroke model and lesion-associated MS human brain [173, 182, 183]. The activation of P2Y12/13 inhibits astrocytic activation/ inflammation by capturing excess extracellular ADP/ATP and thereby preventing the release of astrocytic cytokines- IL1α and TNFα but not IL1β. In this way, P2Y12 signaling is involved in the inflammatory state and actin-rich micro-structures formation in primary microglia [145, 169]. Therefore understanding the differential role of P2Y12 in developing stages of neurodegeneration can provide a suitable therapeutic intervention (Fig. 6B).

1.9 Endolysosomal degradation and clearance of Aβ and Tau by microglia

Microglia are the CNS-resident macrophages, which consistently perform the phagocytosis of unusual matrix deposition, improper synapses, invading pathogens and damaged neurons. The recent finding demonstrates that microglial dysfunction can be targeted as multidirectional therapy, which targets the Aβ clearance, STAT3-mediated immune modulation and the reduction of cytokine burst at the later stages of AD [174]. Moreover, it was recently shown that Tau species can be internalized by dynamin-mediated endocytosis, which is independent of actin-mediated micropinocytosis. Microglia degrade the engulfed objects, protein clumps by the lysosomal pathway and eventually lead to activation and inflammation [184, 185]. But, the prolonged activated microglia have endosomal dysfunctions and faulty lysosomal machinery, which eventually result in the release of pro-aggregant protein seeds in the interstitial milieu [186, 187]. Genome-wide association studies revealed the altered level of various endosomal trafficking and actin-interacting proteins in AD. These are involved in membrane invagination (BIN1), clathrin pit formation (SHIP1,2, PICALM), dynamin-assisted actin polymerization (SYNJ1), vesicle transportation (RIN3) and Tau toxicity (CASS4), etc [188]. Recent studies also emphasize the dysregulation of phosphatidyl-inositol signaling pathway- PI(4,5)P2 which significantly impacted Tau and Aβ pathology through the interaction with SH3 domain-proteins [109, 189, 190]. The extracellular Tau aggregates can induce Tauopathy by increasing the seeding activity through the NLRP3-ASC complex which resulted in Tau uptake, lysosomal degradation, microglial activation and neuroinflammation in the Tauopathy mice model [191]. Similarly, brain-derived Tau oligomers from AD and DLB patients were found to be internalized by the HSPG-dependent pathway while; the PSP-derived oligomeric Tau follows clathrin and caveolin-dependent endocytosis in brain cells. Moreover, the HSPG antagonism resulted in reduced Tau oligomer internalization, endolysosomal translocation, Tau hyperphosphorylation and NFTs accumulation in neurons [192]. RIPK1-mediated CST7 induction resulted in the impairment of the endolysosomal pathway and microglial degradation of Aβ by the exhibition of DAM and inflammatory phenotype in AD brain. Moreover, the late endosome-derived cathepsin-D regulates the local actin polymerization and lamellipodia extension in ADP-induced migratory microglia [193]. Activated microglia

phagocytose degenerated myelin through the CR3-mediated cofilin activation, which promotes actin polymerization and filopodia formation by down-regulating Syk [194]. Further, genetic knockdown cofilin $^{(+/-)}$ leads to reduced secretion of Aβ and plaque deposition *via* APP endocytosis in the PS1-AD mice brain. While cofilin $^{(+/-)}$ mice showed the Iba1^{+} activated microglia surrounding Aβ plaques which signifies the dual function of cofilin in AD pathology by increased neuronal production of Aβ and impaired Aβ endocytosis and clearance in microglia [195]. Upon activation, different microglial cytokines regulate the expression of matrix metalloproteases, cathepsins, heparanase for tissue hydrolysis and facilitate chemotactic migration, which can lead to phagocytic clearance of damaged cells, protein deposits and pathogens.

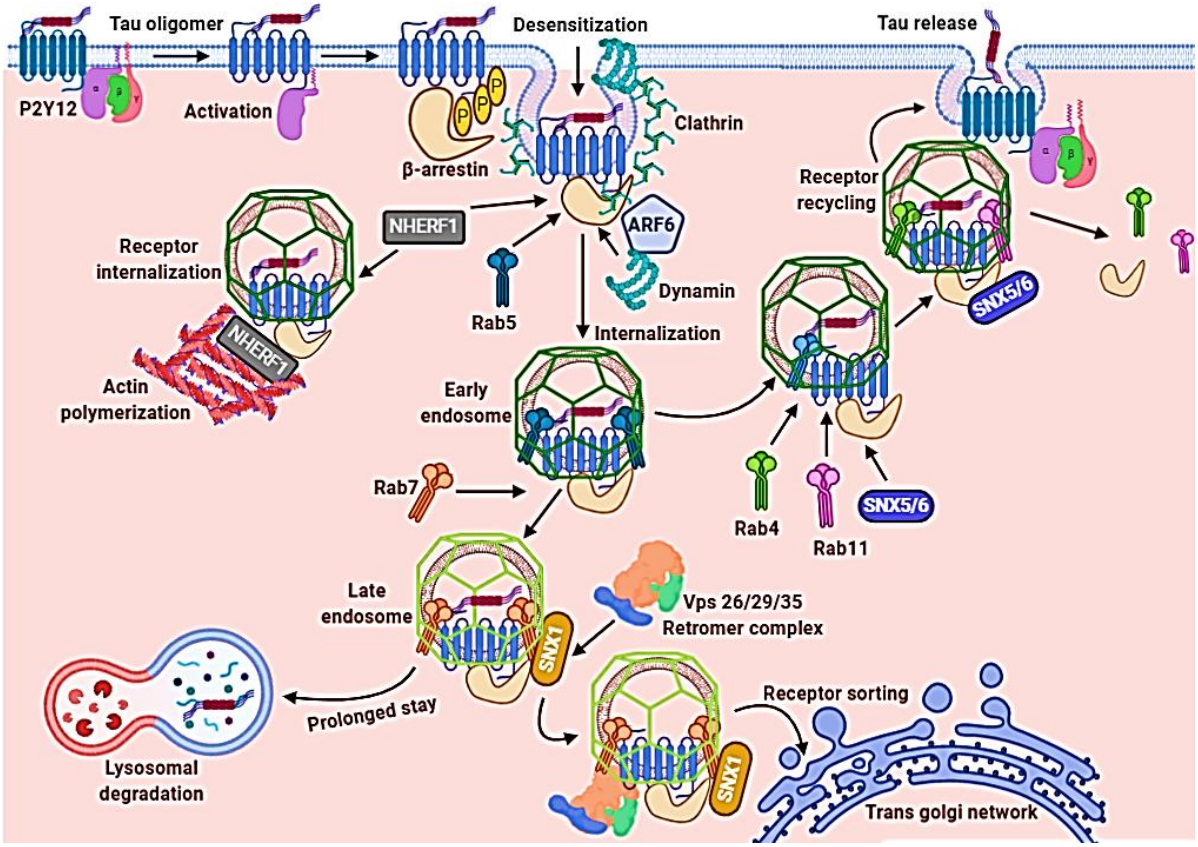

Figure 7. P2Y12-mediated Tau endocytosis in migratory microglia: A hypothesis. The probable interaction of Tau oligomers with microglial P2Y12 may result in β-arrestin1-associated receptor desensitization, clathrin-mediated endocytosis (CME) and Tau internalization. The actin-associated endosomal trafficking either may lead to lysosomal Tau clearance or release through P2Y12 recycling vesicle as seed. The recruitment of Rabs small-GTPase involves endosome maturation and fusion with the lysosome.

Extracellular endo-vesicle (EVs) contains various signaling molecules such as receptors, cytokines, integrins, lipid mediators and nucleic acids, which mediate intercellular communication, immune activation and can also spread amyloidogenic seeds contributing the progression of neurodegenerative disease [196]. Microglial P2Y6 phagocytose the Aβ-associated live neurons and cause Aβ/ Tau-induced neuronal death, synaptic loss and memory deficit in the P301S Tau mice model [197]. Similarly, Li *et al.,* showed that P2Y4 performs the fluid-phase pinocytosis of Aβ in an ATP-dependent manner in rat microglia while, P2Y6 facilitates the 'phagoptosis' of live neurons by primary microglia [147, 198]. Various reports showed that the P2Y and P2X2 activation regulate the secretion of lysosomal ATP which communicates with ER and lysosomal Ca^{2+} homeostasis in the functioning of monocyte, mouse dorsal horn spinal cord glia [199, 200]. The small GTPase Rab5 regulates the cell surface expression and dynamin-dependent endocytosis of P2X4 receptor and its downstream ATP-induced potentiation in HEK293T cells [201]. Moreover,

the hetero-oligomerization of P2Y1 and P2Y11 is required for the ligand-induced internalization of the metabotropic P2Y11 receptor and its downstream Ca^{2+} oscillation in astrocytoma and HEK293T cells [202]. Therefore, targeting the dysfunctional endolysosomal pathway and purinergic signaling activation can be a few of the most important events for the identification of plaques and the clearance of deposits by migratory microglia (Fig. 7).

1.10 Therapeutic approaches targeting neuroinflammation in AD

AD is the most prominent among all types of age-associated dementia, which contributes to about 50-70% of all neurodegenerative cases. Further, various risk factors, hereditary links, genetic variants of gene alleles, and other co-morbidities such as- diabetes, earlier brain injury, depression, hypertension, etc. can contribute to the progression of AD. In the year 2019, World Health Organization (WHO) launched a dementia plan “Global Plan of Action on the Public Health Response to Dementia 2017–2025”, which would focus on research, awareness program, diagnosis, care and treatment of dementia. Despite the advancements in research and disease treatment strategies, there is no obvious medicine or cure for the occurrence and progression of AD [203]. The current treatments include symptomatic improvement and targeting behavioral and cognitive betterment for patient management strategies. There are four medicines used for AD, which are three acetylcholine esterase inhibitors (Rivastigmine, Donepezil, Galantamine) and an NMDA receptor inhibitor (Memantine). In the last decades, various clinical trials are ongoing that target amyloid deposition, mAb against Aβ, γ-secretase and BACE-1 inhibitors and Tau phosphorylation/aggregation inhibitors or Tau-based immunotherapy (Alzforum 2019) [204]. In last decades, the intermediate oligomeric species gained attention as therapeutic targets for their high reactivity, synaptic occurrences, neurotoxicity, and immune activation. Moreover, the exponential accumulation of Aβ and Tau oligomers was evident in the progressive AD brain with a direct correlation with synaptic loss and cognitive impairment. Therefore, several drugs, mAbs and immunotherapy are in trials that target the intermediate soluble oligomers and pre-fibrillar species. The mAb targeting Aβ peptide/ oligomers/ proto-fibrils such as Crenezumab (phase III clinical trial- NCT02670083), Aducanumab (phase III clinical trial- NCT02477800 and NCT02484547), 5E3 mAb depicted clinical cognition improvements and slowing disease progression, but also associated with side effects of amyloid-related imaging abnormalities (ARIA) [205-207]. A recent article reviewed four Aβ immunotherapy clinical trials where it was evident that the mAbs targeting soluble Aβ oligomers/ proto-fibrils showed better clinical efficacy in those AD patients who carry ApoE4 risk allele and have a higher concentration of Aβ oligomers in brain. In past decade, the Aβ-targeting drugs and strategies have failed in various clinical trials while the current studies focused on Tau-induced microglial response, inflammatory outcomes and AD-associated risk factor genes [208]. Several mAbs are in clinical trials which recognize the different regions of Tau protein or PTMs-Tau or specific confirmation species. For eg., AADvac1 (repeat domain), mAb BIIB076 (pan-Tau), BIIB092 and RO 7105705 (N-terminal truncated fragment), UCB0107 (235-246) and LY3303560 (MC1: 313-322) and ACI35 (pS396) target either p-Tau or proline-rich domain or repeat domains which are involved in pathological PTMs, kinase activity sites and involved in aggregation [209-211]. An immunotherapy against Tau oligomers- TOMA mAbs did not succeeded to reduce Tau depositions, behavioural deficit in Tg4510 mice model [212]. The co-treatment of GRP78 (chaperone) and TOMA (mAbs) together can offer an intervening therapeutic strategy in Tauopathy [213]. The death-associated molecular patterns (DAMPs) which are released from dying neurons into the extracellular space such as cardiolipin, prothymosin α, HSP27, HSP20, binding immunoglobulin proteins (BiP), αβ crystallin can activate the neighboring glial cells which can be considered as glial target to treat neuroinflammation [214]. The current diagnostic approaches for AD include the detection of Aβ and pTau as biomarker in CSF or the Positron emission test (PET) and MRI scan of brain atrophy and amyloid deposits. These diagnostic techniques are either invasive or very expensive and remain limited in large-scale preclinical studies. Biomarkers can verify many minute pathological details for clinical

diagnosis, early detection, progression and experimental outcomes of drug trials. The biomarkers generally used for diagnosis of AD include- the CSF and plasma level of Aβ, pTau, their oligomeric forms and the ratio of oligomers and monomer. It has been already observed that the ELISA-based biomarker study varies enormously from lab to lab, concentration level and procedure, which sometimes remain undetectable in patient's blood samples. Similarly, the MRI study of the plaque area is unreliable because some drugs show side effects like- ARIA (Amyloid-related imaging abnormalities) and mild cognitive deficit. The detection of small Aβ oligomer (10-25 KDa) from the CSF sample is difficult by ELISA [215]. Multiple recent studies developed radioactive compounds such as 18F-PM-PBB3, PI2620, R0948, etc. for specific detections of various forms of Tau deposits in AD, FTLD, PSP, CBD patients' brain and by ocular PET imaging [216, 217]. The Aβ oligomers functionalized with silica nanoparticles can be used as a potent standard in Aβ-oligomer detection from CSF as diagnostic approaches [218]. Therefore in the current scenario, various non-invasive techniques and their cumulative efficacy can be studied such as- RGC's health in ocular neuropathy, and arterial spin labeling for better understanding and accurate detection of the biomarker in AD severity and progression [219].

Aims and objectives of the study

The main pathophysiological aspects of AD are the extracellular accumulation of Aβ plaques, intracellular and extracellular Tau NFTs and microglial activation with oxidative damage, inflammatory outburst, synaptic loss and progressive cognitive decline. Although the pathological deposition of Aβ plaques and Tau fibrils are thought to be enormously detrimental in the occurrences and progression of AD, recent understanding emphasized the age-related and early accumulation of intermediate Tau oligomers in the MCI and severe AD patients' brain. Various brain-derived or *in vitro*-prepared Tau oligomers were highly reactive and shown to mediate neurotoxicity, synaptic dysfunction, cognitive decline and microglial (astrocytes) activation/ inflammation, which proves them as a potent candidate for therapeutic targeting. Till now, various mutated or truncated Tau protein was used for the *in vitro* preparation of oligomers which showed an elevated aggregation propensity. Due to the structural fragility, the stabilization of the intermediate Tau oligomers is another difficult aspect in avoiding further aggregation or degradation during the *in vitro* experimental phase. So, here we have standardized the preparation and stabilization of full-length Tau oligomers *in vitro*. Next, we are interested to study the immune-activation potential of Tau oligomers in microglia. The DAMs were reported to function differently in AD conditions. For eg. The DAMs showed altered phenotype, tissue migration/ invasion, abrupt activation, cytokine-chemokine secretion, altered phagocytic and degradation potential. Moreover, DAMs were also depicted to spread the Tauopathy at different locations of brain by releasing seed species generated through improper proteolytic degradation. Purinergic signaling plays important roles in microglial migration at the site of injury, activation, phagocytosis and debris clearance and balanced inflammation. Therefore, we are interested to study the morphological changes, migration, actin cytoskeleton remodeling and endo-lysosomal clearance in Tau oligomers-induced migratory microglia.

The objectives of the study include-

1. The preparation and characterization of full-length Tau (hTau40wt) oligomers
2. The internalization of Tau oligomers by activated microglia
3. The remodeling of membrane-associated actin, microtubule network, migration/invasion in Tau oligomers-induced microglia.
4. The P2Y12-associated formation of actin microstructures for *in vitro* Tau deposits clearance by migratory microglia.
5. The P2Y12-associated endo-lysosomal trafficking and degradation of Tau oligomers in microglia.

Materials and Methods

1. Materials
1.1 List of chemicals

Sr. No	Chemical/Reagent/Media	Company/Suppliers
1	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT reagent)	Sigma
2	4′,6-diamidino-2-phenylindole (DAPI reagent)	Thermo Fisher Scientific
3	8-Anilinonaphthalene-1-sulfonic acid (ANS dye)	Sigma
4	Acrylamide	Invitrogen
5	ADP	Sigma
6	Alexa647-C2 maleimide	Invitrogen
7	Ammonium persulphate (APS)	MP Biomedicals
8	Ampicillin	MP Biomedicals
9	BES	Sigma
10	Bicinchoninic acid (BCA)	Sigma
11	Bis-acrylamide	Invitrogen
12	Bovine Serum Albumin (BSA std)	Sigma
13	Bradford Reagent	Bio-Rad
14	Bromophenol blue	MP Biomedicals
15	Calcium chloride dihydrate	Sigma
16	Cell culture 24-well inserts	Thermo Fisher Scientific
17	Clarity™ Western ECL Substrate	Bio-Rad
18	Clopidogrel bisulphate (IP grade)	Gift
19	Coomassie brilliant blue R-250	MP Biomedicals
20	Copper-coated carbon grids for TEM 400 mesh	Ted Pella, Inc
21	Copper sulfate (II)	Sigma
22	Dimethyl sulfoxide (DMSO)	MP biomedicals
23	Dithiothreitol	Calbiochem
24	Ethanol	MP Biomedicals
25	Ethylene glycol tetraacetate (EGTA)	MP Biomedicals
26	Fetal Bovine Serum	Thermo Fisher Scientific
27	Glacial acetic acid	MP Biomedicals
28	Glass Coverslip (12 mm diameter)	Blue star
29	Glutaraldehyde	Sigma
30	Glycerol	MP Biomedicals
31	Glycine	Invitrogen
32	Guanidinium HCl	Invitrogen
33	Heparin (MW~17500 Da)	MP Biomedicals
34	Horse serum	Invitrogen
35	IPTG	MP Biomedicals
36	Isopropanol	MP Biomedicals
37	Lactate dehydrogenase release assay kit	Thermo Scientific Scientific
38	LB Agar	Invitrogen
39	LB Broth	Invitrogen/HiMedia
40	L-glutamine	Invitrogen
41	Magnesium chloride hexahydrate	MP Biomedicals
42	MES hydrate	Sigma
43	Methanol	MP Biomedicals
44	Paraformaldehyde	Invitrogen
45	Penicillin-Streptomycin	Invitrogen
46	Phalloidin-Alexa488	Invitrogen
47	Phenylmethylsulfonylfluoride (PMSF)	MP Biomedicals

48	Phosphate buffer saline (PBS, cell biology grade)	Invitrogen
49	Pierce Co-Immunoprecipitation Kit	Thermo Fisher Scientific
50	Polysorbate 20	MP Biomedicals
51	Polyvinylidene fluoride membrane	Merck Milipore
52	Potassium chloride	MP Biomedicals
53	Potassium phosphate dibasic trihydrate	MP Biomedicals
54	Potassium phosphate monobasic anhydrous	MP Biomedicals
55	Precision Plus ProteinTM Dual Color Standards	Bio-Rad
56	Protease inhibitor Cocktail	Roche
57	RIPA buffer	Thermo Fisher Scientific
58	RPMI 1640 media	Invitrogen
59	Sodium azide	MP Biomedicals
60	Sodium chloride	MP Biomedicals
61	Sodium dodecyl sulfate	Sigma
62	Sodium hydroxide	MP Biomedicals
63	Sodium phosphate dibasic anhydrous	MP Biomedicals
64	Sodium phosphate monobasic mono hydrate	Sigma
65	TCEP (tris(2-carboxyethyl)phosphine)	Thermo Fisher Scientific
66	TEMED	Invitrogen
67	Thioflavin S	Sigma
68	Tris base	BIO-RAD
69	Tris HCl	Invitrogen
70	Triton X 100	Sigma
71	Trypan blue	Invitrogen
72	Trypsin-EDTA	Invitrogen

1.2 List of antibodies

Sr. No	Antibodies	Company/Suppliers
1	A11 Oligomer specific antibody (AHB0052)	Thermo Fisher Scientific
2	anti-guinea pig secondary antibody Alexa flour-488 (A11073)	Thermo Fisher Scientific
3	anti-mouse secondary antibody conjugated with Alexa flour-488 (A-11001)	Invitrogen/ Thermo Fisher Scientific
4	Arp2 monoclonal antibody (703394)	Thermo Fisher Scientific
5	Beta-actin loading control (BA3R) (MA515739)	Thermo Fisher Scientific
6	Extracellular P2Y12 antibody (PA5111827)	Thermo Fisher Scientific
7	Goat anti-mouse - alexa fluor 555 (A28180)	Thermo Fisher Scientific
8	Goat anti-mouse IgG HRP (32430)	Thermo Fisher Scientific
9	Goat anti-rabbit - alexa fluor 488 (A-11008)	Thermo Fisher Scientific
10	Goat anti-rabbit IgG (H+L) Cross-adsorbed secondary antibody HRP (A16110)	Thermo Fisher Scientific
11	Goat anti-rabbit IgG (H+L) Cross-adsorbed secondary antibody with Alexa Fluor 555 (A-21428)	Thermo Fisher Scientific
12	Goat anti-rabbit IgG (H+L) Cross-adsorbed secondary antibody with Alexa Fluor 555 (A-21428)	Thermo Fisher Scientific
13	Goat anti-rabbit IgG HRP (31460)	Thermo Fisher Scientific
14	Iba1 Polyclonal antibody (PA5-27436)	Thermo Fisher Scientific
15	Lamp2A antibody (51-2200)	Thermo Fisher Scientific
16	P2Y12 antibody (4H5L19) (702516)	Thermo Fisher Scientific
17	Pan Tau (K9JA) (A0024)	Dako
18	Phospho-Tau (Thr212, Ser214) (MN1060)	Thermo Fisher Scientific

19	Rab11A antibody (3H18L5)	Thermo Fisher Scientific
20	Rab5 antibody (MA5-32150)	Thermo Fisher Scientific
21	Rab7 antibody (PA5-52369)	Thermo Fisher Scientific
22	Rabbit anti-Goat IgG - Alexa Fluor 594 (A27016)	Thermo Fisher Scientific
23	Tau antibody (T46) (13-6400)	Thermo Fisher Scientific
24	TKS5 (SH3PXD2A) polyclonal antibody (PA5-58168)	Thermo Fisher Scientific
25	α-tubulin monoclonal antibody (DM1A)	Thermo Fisher Scientific
26	β-arrestin1 antibody (395000)	Thermo Fisher Scientific
27	Prolonged diamond anti-fade mounting media	Thermo Fisher Scientific

2. Equipments and softwares

2.1 Equipments

Sr. No	**Instrument/Equipment**	**Suppliers**
1	AKTA Pure FPLC system	GE Healthcare
2	Amersham Imager 600	GE Healthcare
3	Amersham Semi Dry blotting apparatus	GE Healthcare
4	Analytical weighing balances	Mettler Toledo
5	Autoclave	Spire
6	Avanti JXN26 High-speed Centrifuge	Beckman Coulter
7	BioSafety cabinet/Clean bench	Thermo Fisher Scientific
8	CO_2 incubator	Thermo Fisher Scientific
9	Dry bath	Genei
10	Far-UV CD spectrometer J-815	Jasco
11	Forma 900 series -80°C	Thermo Scientific
12	Gel rocker	Benchmark
13	Haemocytometer	Rohem India
14	Heraeus Incubator	Thermo Scientific
15	Heratherm Hot Air Oven	Thermo Scientific
16	High-Speed Centrifuge 5804R	Eppendorf
17	Homogenizer	Constant Systems Ltd.
18	Jeol JEM F200 High resolution- Transmission Electron Microscope	JEOL Ltd.
19	Laminar Air Flow	Microfilt
20	Magnetic Stirrer	Genei
21	Microcentrifuge 5418 R	Eppendorf
22	Microplate reader Infinite 200 PRO	Tecan
23	MiliQ Unit Direct 16	Millipore
24	Mini Spin Plus Table top centrifuge	Eppendorf
25	Optima XPN10 Ultracentrifuge	Beckman Coulter
26	pH meter Five Easy plus	Mettler Toledo
27	Shaker Incubator (H1010-MR)	Benchmark Scientific
28	Shaker Incubator Multitron Standard	Infors HT
29	SimpliNano (Nanodrop)	GE Healthcare
30	T20 Transmission Electron Microscope	Tecnai
31	Vacuum Pump	Millipore
32	Vortexer mixer	Genei
33	Water bath	Genei
34	Zeiss Axio observer 7 microscope with Apotome 2.0	Zeiss

2.2 Softwares

Sr. No	Software	Developer
1	BIORAD Quality one 4.6.6	BIO RAD
2	ImageJ /Fiji	NIH
3	MS office (Excel)	Microsoft
4	Sigma Plot 10.0	Systat software
5	Spectra Manager™	Jasco
6	Zeiss Image processing Software (Zen 2.3)	Zeiss
7	Zeiss Imaging Software	Zeiss
8	Inkscape	Inkscape Project
9	BioRender image illustrator	BioRender.com

3. Methods

3.1 Tau protein expression and purification

The Tau protein expression and purification were standardized previously in our lab [220]. In brief, we cultured the BL21* *E.coli* bacteria for expressing Tau protein and purified by cation-exchange chromatography which was followed by size-exclusion chromatography (Fig. 8A).

Reagents and buffers:

Cell lysis buffer (pH 6.8)	Dialysis buffer (pH 6.8)
50 mM MES	20 mM MES
1 mM EGTA	50 mM NaCl
2 mM $MgCl_2$	1 mM EGTA
5 mM DTT	1 mM $MgCl_2$
1 mM PMSF	2 mM DTT
Protease inhibitor cocktail	0.1 mM PMSF

Ion Exchange Chromatography buffers		Size-Exclusion Chromatography (SEC) buffer		
Buffer A (pH 6.8)	**Buffer B (pH 6.8)**	**10X Phosphate buffered saline (PBS) stock (1000 mL)**		
20 mM MES	20 mM MES	**Molarity**	**Quantity**	**SEC buffer**
50 mM NaCl	1 M NaCl	1.37 M NaCl	80 g	1X PBS pH 7.4
1 mM EGTA	1 mM EGTA	27 mM KCL	2 g	2 mM DTT
1 mM $MgCl_2$	1 mM $MgCl_2$	100 mM Na_2HPO_4	14.2 g	
2 mM DTT	2 mM DTT	18 mM KH_2PO_4	2 g	
0.1mM PMSF	0.1 mM PMSF			

Protocol:

1. The BL21* *E.coli* strain glycerol stock containing full-length Tau- hTau40WT expressing plasmid was taken from -80°C refrigerator and inoculated into the 100 ml LB media containing 100 µg/ml ampicillin as ‘Primary inoculum’. The primary inoculation was incubated at 37°C overnight in a shaker incubator.

2. The next day, the secondary inoculation was done for 6 liters of culture at a ratio of primary: secondary inoculum- 1:100, along with ampicillin. The secondary culture was incubated at 37°C 180 rpm in a shaker incubator till OD_{600} reached around 0.6.
3. The culture was induced with 0.5 mM IPTG and incubated for 4 hours at 37°C in a shaker incubator.
4. After the incubation, the bacterial cells were harvested by centrifuging at 4500 rpm for 10 minutes at 4°C.
5. The cell pellet was weighed, snap-freezed with liquid nitrogen and stored at -80°C refrigerator.
6. The bacterial pellet was resuspended in the lysis buffer and vortexed to obtain a homogenous suspension. Then, the cell lysis was carried out in the constant cell disruption system at 15 Kpsi pressure.
7. The cell lysate was collected, centrifuged and supplemented with 0.5 M NaCl and 5 mM DTT. The cell lysate was then heated at 90°C for 20 minutes and cooled down for precipitating out other well-folded cellular proteins. But, the natively disordered soluble Tau becomes heat-resistant and remains unaffected from precipitation.
8. The cooled lysate was centrifuged at 40,000 rpm 4°C for 45 minutes in an ultracentrifuge. The supernatant containing the Tau protein was carefully separated and the pellet was discarded.
9. The supernatant containing Tau protein was subjected to dialysis overnight by changing the buffer twice.
10. Next, the dialyzed sample was subjected to ultracentrifugation at 40,000 rpm 4°C for 45 minutes to remove the particulate or precipitated matter. The supernatant lysate was filtered and kept on ice. The lysate was loaded carefully onto the super-loop and attached with the chromatographic system AKTA.
11. For cation exchange chromatography, the Sepharose (SPFF) fast flow manually packed 24 mL column was equilibrated with buffer A.
12. After the column equilibration, the lysate was pumped into the ion-exchange column and the column was washed 4 times with buffer A to remove the non-specifically bound proteins.
13. Tau protein was eluted with a linear gradient of buffer B.
14. The fractions under the peak were collected and subjected to SDS-PAGE for accurately identifying the Tau protein-containing fractions.
15. The Tau monomer protein-containing fractions were pooled, and concentrated up to 1-2 ml for the SEC.
16. The SEC, the Hi-Load Superdex 75 pg (16/600) column was pre-equilibrated with SEC buffer and the concentrated protein was loaded in the column.
17. The Tau protein was eluted and the obtained SEC fractions were subjected to SDS-PAGE analysis.
18. The Tau monomer-containing fractions were concentrated, aliquot and stored at -80°C.
19. The protein concentration was estimated by Bicinchoninic Acid (BCA) assay.

3.2 Preparation, and stabilization of Tau oligomers and Tau aggregates

Tau oligomers' preparation and stabilization were standardized in this study, while aggregates preparation was previously standardized in our lab [220]. In case of Tau oligomers preparation, various buffers were trailed such as BES (pH 7.4), Tris-base buffer (pH 8), PBS (pH 7.4) at various time points. Finally, the globular oligomers were found to be formed in PBS buffer at 12 hours of incubation. For stabilization, 0.01% glutaraldehyde was used for protein cross-linking and then subjected to buffer dilution/ SEC.

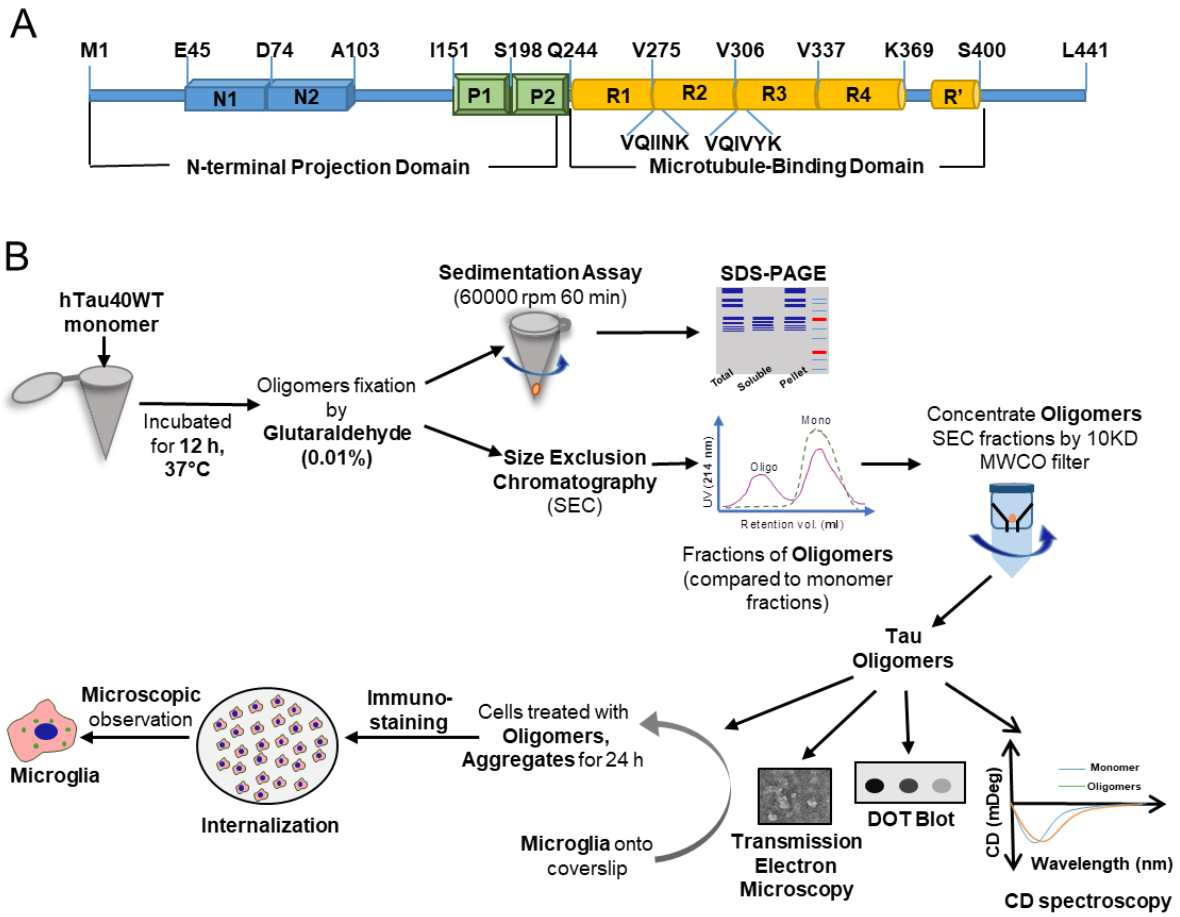

Figure 8. Preparation, characterization and internalization of Tau species. A. Full-length Tau (hTau40wt) has a flexible N-terminal domain and a C-terminal domain. The two-hexapeptide motif (VQIINK and VQIVYK) in the repeat region plays a crucial role in Tau oligomerization and further aggregation. **B.** Diagrammatic representation illustrates the workflow for oligomers preparation and characterization by various biochemical and biophysical techniques. The prepared Tau oligomers are explored in microglial internalization and activation assay by immunofluorescence microscopy.

Reagents:

Tau oligomers preparation		**Tau aggregates preparation**	
Components	**Final concentration**	**Components**	**Final concentration**
Tau Monomer	100 µM	Tau Monomer	100 µM
PBS buffer (pH 7.4)	1X	BES buffer (pH 7.4)	20 mM
NaCl	25 mM	NaCl	25 mM
Sodium azide	0.01%	Sodium azide	0.01%
DTT	2 mM	DTT	1 mM
Heparin (17.5 kDa)	25 µM	Heparin(17.5 kDa)	25 µM
Protease inhibitor cocktail	From 25X to 1X	Protease inhibitor cocktail	From 25X to 1X

Protocol:

1. The Tau protein aliquots were taken from the -80°C storage and thawed. The protein was subjected to ultracentrifugation at 60,000 rpm for 60 minutes at 4°C.
2. The supernatant was collected to ensure only monomeric soluble fractions for in vitro oligomerization.

3. The 100 µM Tau monomer was diluted in respective buffer and incubated with DTT for 10 minutes to maintain reducing/monomeric condition.
4. Then Tau monomer was induced by the polyanionic factors heparin at 25 µM concentration (Tau: heparin, 4:1), along with the other enlisted components.
5. The assembly buffer containing Tau was thoroughly mixed and incubated for 12 hours at room temperature in case of oligomers, and at 37°C for 7 days for the formation of fibrillar aggregates.
6. After the incubation, the Tau oligomers were stabilized by adding 0.01% glutaraldehyde and incubated for 10 minutes at room temperature in dark.
7. The oligomers were then subjected to PBS buffer exchange (20 times) by centrifuging at 3800 rpm for 2 hours through 5 KDa molecular cut-off filters.
8. The oligomers were then aliquoted and stored at -80°C.
9. The concentration of Tau oligomers and aggregates was measured by BCA assay.

3.3 Bicinchoninic Acid (BCA) assay for protein estimation

The concentration of Tau protein was measured by BCA assay which is standardized in our lab and appropriate for Tau protein. As Tau protein lacks the tryptophan residues, which is essential for the measurement of concentration by protein estimation methods.

Reagents:

BSA protein standard	1 mg/mL
Bicinchoninic acid	solution
$CuSO_4$	Solution
Ultrapure MilliQ water	

Protocol:

1. The BSA standards were prepared in duplicates in ultrapure MilliQ water as given below.
2. Tau monomer is diluted in ultrapure MilliQ water (1:100, 1:200 and 1:400) in duplicates.
3. The BCA: $CuSO_4$ reagent was mixed at a ratio of 50:1 and prepared freshly at a volume of 200 µL per sample.
4. 25 µL of standards and protein dilutions were thoroughly mixed with 200 µL of the BCA reagent and added to 96 well plate. The plate was incubated at 37°C for 1 hour.
5. The absorption was measured at 562 nm in a spectrophotometer and the Tau protein concentration was obtained from the BSA standard graph in MS Excel.

3.4 Biochemical and Biophysical characterization of Tau monomer, oligomers and aggregates

The stabilized Tau oligomers and fibrillar Tau aggregates in comparison with monomer was characterized by various biochemical and biophysical techniques which are described below.

3.4.1 Size exclusion chromatography

The stabilized Tau oligomers were subjected to SEC to remove the monomeric fraction from the oligomer-containing sample.

Reagents:

1X PBS (pH 7.4)
1 mM DTT

Protocol:

1. The Tau oligomers were diluted in PBS buffer and loaded onto the AKTA chromatographic system by syringe injection.
2. The Sephadex 200 Increase, 24 ml column SEC column was pre-equilibrated in SEC-PBS buffer.
3. The oligomeric fractions were found to be separated from monomeric fractions.
4. The oligomeric fractions were collected and concentrated by centrifuging at 3800 rpm through 5 KDa molecular cut-off filters.
5. The concentration of the oligomers was checked by BCA assay.

3.4.2 SDS-PAGE analysis

SDS-Poly acrylamide gel electrophoresis was to study the molecular weight of Tau monomer, oligomers and fibrillar aggregates without the influence of charge.

Reagents:

SDS Gel running buffer		**Lammelli buffer/6X loading dye**	
Component	**Molarity**	**Component**	**Molarity**
Tris base	25 mM	Tris-Cl pH 6.8	300 mM
Glycine	192 mM	SDS	12%
SDS	0.1%	Bromophenol blue	0.6%
		Glycerol	60%
		DTT	600 mM

Preparation of Resolving Gel (For 5 ml)		**Preparation of Stacking** (For 1 ml)	
Component	**Quantity (ml)**	**Component**	**Quantity(ml)**
Autoclaved MilliQ water	1.9	Autoclaved MilliQ water	0.68
40% Acrylamide solution	1.7	40% Acrylamide solution	0.17
1.5 M Tris buffer pH 8.8	1.3	1 M Tis buffer pH 6.8	0.13
10 % SDS	0.05	10 % SDS	0.01
APS 10% solution	0.05	APS10% solution	0.01
TEMED	0.002	TEMED	0.002

Staining solution (1000 ml)		**Intensive destaining solution (1000 mL)**		**Normal destaining solution (1000 mL)**	
Component	**Quantity**	**Component**	**Quantity**	**Component**	**Quantity**
CBB R-250	1 g	Methanol	500 mL	Methanol	50 mL
Methanol	450 mL	Acetic acid	100 mL	Acetic acid	75 mL
Glacial acetic acid	90 mL				

Protocol:

1. The required percentage of the resolving and stacking gels were made as per the table.
2. The BIO-RAD gel casting units along with the plates were placed properly and gel was casted.
3. The samples were diluted in 6X loading dye. The sample was then loaded onto the wells of the gel.
4. A 90 V was applied in a vertical electrophoresis unit in presence of gel running buffer.
5. The gel is run till the tracking dye reaches 90% of the gel and the gel is removed.
6. The gel was stained with a staining solution for 30 minutes on a rocker.
7. Then the gel was first washed with the intensive destaining solution for 20 minutes and then for normal destaining solution up-to the clear visibility of protein bands.
8. The gel was scanned in a white background by HP computer scanner.
9. The gels can be used for western blot before staining steps were done.

3.4.3 Western blotting analysis

Western blotting analysis helps to confirm the presence of Tau protein in the oligomers containing SEC fraction along with Tau monomer by the use of specific Tau antibody pan-Tau K9JA antibody.

Reagents:

Transfer buffer (freshly prepared)		**1X PBST (pH 7.4)**		**Stripping buffer (pH 2.8) For 1000ml**		**Ponceau S stain (200 mL)**	
Compo.	**Molarity**	**Compo.**	**Quantity**	**Compo.**	**Molarity**	**Compo.**	**Quantity**
Tris base	25 mM	PBS	1X	Glycine	15g	PonceauS	0.4 g
Glycine	192 mM	Tween 20	0.1%	SDS	1g	Glacial acetic acid	2 mL
Methanol	20%			Tween 20	10ml		

Protocol:

1. The oligomers-containing SEC fractions were run in SDS-PAGE at 90V.
2. The PVDF transfer membranes were cut in the 8 cm X 7 cm dimensions.
3. The transfer buffer was prepared and stored at 4°C.
4. The filter paper stacks were cut and soaked in the transfer buffer in a tray.
5. After the electrophoresis, the gel was soaked in a transfer buffer.
6. The PVDF membrane was activated in 100% methanol and then equilibrated in transfer buffer.
7. The 4 stacks of soaked filter papers were placed on the dry blot platform followed by the membrane.
8. The gel was placed on the membrane. The gel once placed on the membrane should not be moved.
9. Another 4 stacks of soaked filter paper was placed on the gel and the transfer buffer was added over the sandwich.
10. The air bubbles were removed by gentle rolling with glass rods.
11. The lid of the Semi-dry blot transfer (Amersham biosciences) apparatus was closed and the current was set to 200 mA for 3 hours.
12. The blot was put in a blocking buffer containing 5% skimmed milk in PBST for 1 hour at room temperature.
13. The blot was incubated in primary antibody K9JA for 1 hour.
14. The membrane was washed with PBST 3 times for 10 minutes each to remove unbound antibody.
15. The blot was incubated with respective HRP-conjugated secondary antibody.
16. The membrane was washed with PBST 3 times for 10 minutes each to remove unbound antibody.
17. The blot was developed using ECL (Enhanced chemiluminescence) reagent.

3.4.4 Fluorescence assay

The internal property of the Tau monomer, oligomers and aggregates can be estimated by various fluorescence assay. Tau monomer is an intrinsically disordered protein, in a soluble state it forms random-coil structures. But, during oligomerization/aggregation, the intermolecular interaction leads to the formation of various ordered confirmations- β-sheet structures. Here, we used 2 types of fluorescence assays- Thioflavin-S (ThS) and 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorescence which would be used for comparative analysis of various Tau species.

3.4.4.1 Thioflavin-S (ThS) assay

ThS preferentially binds to the β-sheet structures in the protein. Here, during oligomerization/ aggregation Tau acquires β-sheet structures where ThS can bind and give a fluorescence signal. The comparative ThS fluorescence of various Tau species *i.e.,* oligomers and aggregates and control monomer can be a determinant of relative β-sheet content in the protein species.

Reagents:

Component	**Concentration**
Tau species	2 µM
Thioflavin S	8 µM
Ammonium acetate pH 7.0	50 mM

Protocol:

1. For the fluorescence assay, ThS dye was diluted to 8 µM concentration from the stock solution of 200 µM in 50 mM ammonium acetate buffer.
2. The various Tau species- monomer, oligomers and aggregates were diluted in ammonium acetate buffer at a final concentration of 2 µM. Therefore, Tau: ThS is maintained as 1:4 ratio.
3. The control blank was taken where no protein samples were added.
4. The mixture was mixed thoroughly and added to the 384 black well plates in triplicates for each reaction mixture.
5. The plate was incubated at room temperature for 10 minutes and the fluorescence reading was taken in the Tecan Infinite M200 Pro plate reader.
6. The excitation/emission was adjusted to 440/521 nm at 100 gain 25 flashes.

3.4.4.2 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorescence assay

ANS fluorescence dye binds to the hydrophobic patches in the protein occurring during protein unfolding and misfolding. Here, during oligomerization/ aggregation, Tau undergoes various structural conformation changes with exposed hydrophobicity where ANS bind and give a fluorescence signal.

Reagents:

Component	**Concentration**
Tau species	2 µM
ANS	40 µM
Ammonium acetate pH 7.0	50 mM

Protocol:

1. For the ANS fluorescence assay, ANS dye was diluted to 40 µM concentration from the stock solution in ammonium acetate buffer (pH 7.0).
2. The various Tau species- monomer, oligomers and aggregates were diluted in ammonium acetate buffer at a final concentration of 2 µM. Therefore, Tau: ANS is maintained as 1:20 ratio.
3. The control blank was taken where no protein samples were added.
4. The reaction mixtures were added to the 384 black well plates in triplicates for each reaction mixture.
5. The plate was incubated at room temperature for 20 minutes and the fluorescence reading was taken in the Tecan Infinite M200 Pro plate reader.
6. The excitation/emission was adjusted to 390/475 nm at 100 gain 25 flashes.

3.4.4 Sedimentation assay and SDS-PAGE analysis

Sedimentation assay of Tau oligomers by ultracentrifugation can determine the nature of the *in vitro* prepared oligomers either as soluble or insoluble. The soluble oligomers would be present in the supernatant or insoluble oligomers would be in pellet fractions, after

ultracentrifugation. The subsequent SDS-PAGE analysis would be the determinant of the nature of the oligomers in terms of SDS detergent solubility.

Protocol:
1. In vitro prepared Tau oligomers of 10 µl were placed in a centrifuge tube.
2. The oligomers were subjected to ultracentrifugation at 60,000 rpm for 60 minutes.
3. The supernatant fraction was carefully collected and the pellet fraction was further dissolved in 10 µl PBS buffer.
4. Further, 10 µl of the oligomers sample was taken as 'total', without centrifugation.
5. Total, supernatant and pellet samples were mixed with 6X loading dye.
6. The oligomeric samples were run in SDS-PAGE for determining the soluble/insoluble nature of the oligomers and their relative molecular weight by using a protein molecular marker.

3.4.5 Dot Blot assay

Dot blot assay is a simplistic version of western blot, which is highly used to detect a specific type of protein and also determine the binding capacity of antibody to specific epitopes in molecular biology. Here, we used Dot blot study to detect the comparative binding of various Tau species- monomer, oligomers and aggregates to oligomer-specific A11 antibody in comparison with total Tau-K9JA antibody.

Reagents:

1X PBST (pH 7.4)		**Blocking buffer**
Component	**Quantity**	**Component**
PBS	1X	1X PBST (pH 7.4)
Tween 20	0.1%	Skimmed milk 10%

Protocol:
1. The Nitrocellulose membrane was cut in the required shape and activated by a wash with PBST buffer.
2. The wet nitrocellulose membrane was air-dried completely and placed onto a plastic sheet to prevent sample smudging.
3. Tau monomer, oligomers and aggregates were diluted at a final concentration of 1 mg/ml in PBS buffer.
4. The 5 µl of each samples (monomer, oligomers and aggregates) were spotted onto the dried membrane and allowed to dry completely.
5. The sample-containing membrane was blocked with a blocking buffer for 1 hour at room temperature in gel rocker.
6. The A11 and K9JA antibody dilutions were prepared in blocking buffer.
7. The blots were incubated with oligomer-specific A11 antibody (1:1000 dilution) for overnight binding at 4°C and for K9JA at room temperature for 1 hour.
8. The blot was washed with PBST for 3 times, 10 minutes each in gel rocker.
9. The blots were incubated with HRP-conjugated secondary antibody for 1 hour.
10. The blots were further washed with PBST for 3 times, 10 minutes each in gel rocker.
11. The blots were developed by using ECL solutions in Amersham blot imager.

3.4.6 CD spectroscopy

CD Spectroscopy is used widely to detect the secondary structure of proteins. As Tau is a natively unfolded protein, it acquires random-coil structures. But, during the process of aggregation/oligomerization, Tau undergoes a conformational transition with localized β-sheet structures in the repeat region. The CD spectroscopic analysis was carried out for monomer, oligomers and aggregated Tau to identify the comparative accumulation of β-sheet structure in various protein species.

Reagents:

Components
50 mM Phosphate buffer (pH 6.8)
Ultrapure MilliQ water
6M guanidium hydrochloride solution
100% methanol
Nitrogen gas

Protocol:

1. The Jasco J-815 CD spectrometer was attached with a nitrogen gas cylinder and purged with nitrogen gas to create an inert environment.
2. The spectra for phosphate buffer (pH 6.8) was acquired and set as a baseline for the following Tau sample spectra.
3. The Tau monomer, oligomers and aggregates were diluted in the phosphate buffer at a final concentration of 3 µM (0.15 mg/ml).
4. A quartz cuvette with 1 mm path length was properly cleaned with guanidium hydrochloride solution, 100% methanol and Milli-Q water to remove the left-over samples during the acquiring.
5. The spectra were recorded in a cuvette at 1 mm path length, 1 nm bandwidth and 100 nm/min scan speed at 25°C.
6. The spectra were recorded in the range of 190-250 nm with an average of 5 acquisitions.
7. The CD spectra were plotted in Sigma plot 10.0 with data-smoothening if needed.

3.4.7 Transmission electron microscopy (TEM) and High resolution-TEM

Transmission electron microscope is a very powerful tool to identify the structure of proteins in a very high resolution by the use of a high-energy electron beam. The shorter wavelength electron beam passes through the sample and the transmitted electrons are used to obtain the image. Here, we used the TEM and HR-TEM techniques for the accurate identification of the size and shape of the Tau oligomers along with fibrillar aggregates by employing negative staining.

Reagents:

Component
2% Uranyl acetate
Ultrapure MilliQ water
4% glutaraldehyde solution

Protocol:

1. The Tau oligomers and aggregates were diluted with Milli-Q water at a final concentration of 5 µM and 2 µM, respectively.
2. The 10 µl of the Tau samples were placed in a drop onto a paraffin sheet. Similarly, 10 µl of glutaraldehyde solution, Milli-Q water and uranyl acetate stain were placed onto the paraffin in a series.
3. The 400 mesh carbon-coated copper grids were placed onto the Tau sample by facing the dark side of the grid towards the protein sample.
4. The grids were allowed to soak with Tau samples for 5 minutes. After incubation, the excess protein solution was gently removed with a piece of filter paper.
5. The grids were placed next onto the glutaraldehyde solution for 30 seconds, to fix the sample to achieve better contrast during staining.
6. The grids were given MilliQ water wash for 30 seconds.
7. The grids were then stained in 2% uranyl acetate solution for 5 minutes and dried completely before scanning.
8. The air-dried grids were scanned on TECNAI T20 electron microscope at 200 KV and JEOL JEM-F200 Electron Microscope at 120 KV for the HR-TEM study (Fig. 8B).

3.5 Preparation of Alexa647-C2-labelled Tau monomer and oligomers

For the time-dependent phagocytosis assay, the Tau monomer and oligomers were labeled with Alexa647 fluorophore which will specifically indicate the internalization and relative endosomal localization of extracellular Tau species in microglia. Alexa is a synthetic pH-resistant fluorescence dye that gives an accurate signal from inside the cell compartments. The Alexa^{647} dye was attached to a C2-maleimide label, which specifically interacts with the cysteine residue of the protein in reducing condition. As Tau monomer contains 2 cysteine residues which can be eventually tagged by Alexa^{647}-C2-maleimide.

Reagents:

Components	**Molarity**
Tau monomer and oligomers	100 µM
PBS (pH 7.4)	1X
TCEP solution (Stock 1M)	1mM
Alexa647-C2-maleimide (Stock 10 mM)	200 µM

Protocol:

1. Tau monomer and oligomers were diluted in PBS buffer at a final concentration of 100 µM.
2. Tau monomer was mixed with 10 molar excess concentration of TCEP (tris(2-carboxyethyl)phosphine) for 10 minutes at room temperature.
3. Both the Tau monomer and oligomers were mixed with Alexa647-C2-maleimide solution drop-by-drop and by gentle tapping.
4. The solutions were then kept at 4°C overnight in a shaker-mixer of 600 rpm.
5. The next day, the Alexa-tagged Tau protein was subjected to buffer exchange with 10 times excess PBS for 2 cycles, for the removal of excess unbound Alexa dye.
6. The buffer exchange was done by centrifugation with 3 KDa molecular cut-off filter at 3800 rpm for 1 hour at 4°C.
7. The Alexa-tagged Tau monomer and oligomers were collected, aliquoted and stored at -80°C fridge.
8. The concentration of the monomer and oligomers were measured by BCA assay, as described previously.

3.6 Mammalian cell culture and maintenance

For the cell biology-based studies, N9 microglial cell line: CVCL_0452 was used. N9 is a retrovirus-transformed mouse glial cell line that is widely used for neurobiology and neuroimmunology studies.

Reagents:

Components	**concentration**
Cell culture media-	
RPMI 1640 media	
Fetal bovine serum (FBS)	10%
Pen-strep antibiotic cocktail (Stock 100X)	1X
Anti-anti cocktail (Stock 100X)	1X
For cell passaging-	
PBS solution (Cell biology grade)	1X
Trypsin-EDTA (0.25%) 10X	1X

Protocol:

Cell growth:

1. The N9 microglial cells were maintained in RPMI 1640 media supplemented with 10% FBS, 1X pen-strep and an anti-anti cocktail mixture.
2. The cells were incubated at 37°C in a CO_2 incubator with 95% humidity.
3. The cells were passaged after reaching 80-90% confluency in the culture dish.

Cell passage:
1. After reaching the desired confluency, cells were passaged by removing the growth media and washing with 1X PBS.
2. The trypsin EDTA solution was added to the culture dish and incubated at 37°C for 5 minutes which allows the cells to detach from the culture dish.
3. The reaction of trypsin was stopped by adding the growth media containing 10% FBS.
4. The cell suspension was mixed homogeneously and collected in 15 ml centrifuge tube and centrifuged at 800 rpm for 5 minutes to pellet down the cells.
5. The supernatant was discarded and the cell pellet was resuspended in fresh growth media.
6. The cells were split to a fresh culture dish at a ratio of 1:3.
7. The culture dishes were incubated at 37°C in 5% CO_2 incubator with 95% humidity.

3.7 Cell viability assay

Cell viability assay is an important tool to study the biocompatibility of a single mixture of compounds in a dose-dependent manner on a particular/ variety of cells for therapeutic intention. There are various classes of cell viability assays like dye exclusion (trypan blue), colorimetric, Flow cytometry assay. Here, we used the MTT and LDH assay to study the toxicity of various Tau species- monomer, oligomers and aggregates in N9 cells. The MTT and LDH assay would provide a determining specific concentration at which we can further perform various assays without impairing the cell viability.

3.7.1 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

MTT assay is a widely used colorimetric assay for determining dose-dependent cellular toxicity by measuring the mitochondrial function of cells. MTT is a tetrazolium salt, which can easily permeabilize into the cell, but is converted into formazan crystals by mitochondrial NADP(H) oxidoreductase enzyme. These crystals are solubilized by DMSO and the intensity of color developed is measured at 500-600 nm. The more metabolically active cells mean more amount of MTT formazan color development with lesser cytotoxicity.

Reagents:

Growth RPMI media (10% FBS)
Basal RPMI media for treatment (0.5% FBS)
1X PBS
Trypan blue solution (0.4%)
MTT solution
100% DMSO

Protocol:
1. Confluent N9 cells were trypsinized and suspended in fresh RPMI media (10% FBS).
2. The cells were diluted with trypan blue dye solution at 1:10 ratio and counted the cell number by hemocytometer.
3. The N9 viable cell number was calculated.
4. Cells were mixed with an adequate amount of fresh RPMI media (10%) and seeded at a density of 10,000 cells/ well in a 96-well plate. The cells were incubated at 37°C 5% CO_2 overnight.
5. The next day, these cells were treated with varying concentrations of Tau species (monomer, oligomers and aggregates) in Basal RPMI media (0.5% FBS) from 0.125 µM

to 10 µM, along with ATP as a negative control in triplicates. The treated cells were incubated for 24 hours at 37°C 5% CO_2.

6. Post-treatment, the cells were washed with PBS and treated with 0.5 mg/mL of MTT solution in RPMI basal media. The MTT-treated cells were incubated for 3 h in a CO_2 incubator.
7. The formazan crystals were dissolved in 100 µl of DMSO/ well. The absorbance of the purple-coloured solution was measured at 590 nm in TECAN Infinite Series Pro M200 spectrophotometer.

3.7.2 Lactate dehydrogenase (LDH) leakage assay

Lactate dehydrogenase (LDH) enzyme is a housekeeping gene, physiologically present in the cell cytoplasm. The membrane leakage caused by the activity of various toxic molecules leads to the extracellular release of this enzyme. LDH leakage assay follows two steps coupled enzymatic reaction for measurement. In the first step, LDH catalyzes the conversion of lactate to pyruvate and thus NAD to NADH. In a second step, diaphorase uses the NADH to reduce tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to red formazan. We utilized LDH assay to determine the effect of Tau treatment on N9 cells to determine cytotoxicity via membrane leakage.

Protocol:

1. N9 Cells were seeded at the density of 10,000 cells/well in RPMI media (10% FBS) and incubated overnight at 37°C 5% CO_2.
2. Tau monomer, oligomers and aggregates were used to treat N9 cells at various concentrations (0.5, 1, 5µM) for 24 hours in basal RPMI media.
3. LDH release assay was carried out according to the manufacturer's protocol (Pierce, Thermo Scientific).
4. The %cytotoxicity was quantified by using a standard graph and maximum LDH release control, as mentioned in the manufacturer's protocol.

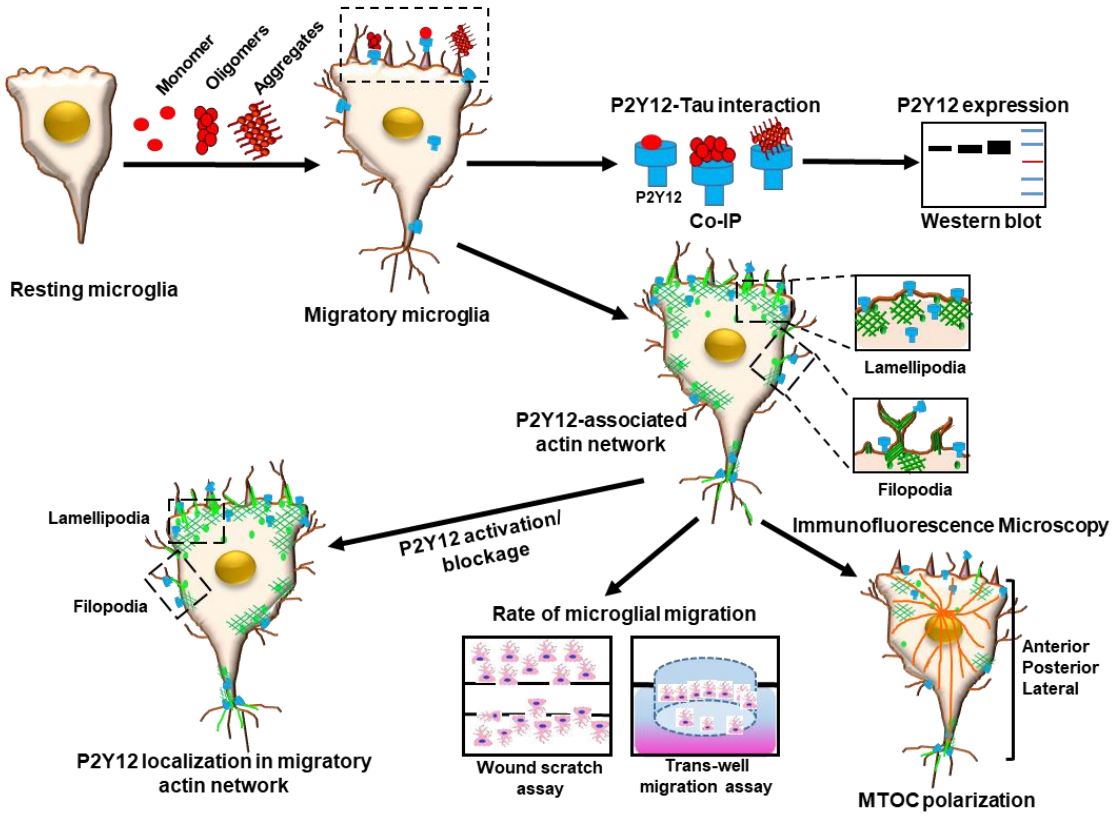

Figure 9. Tau-induced microglial migration and formation of actin microstructures through P2Y12 signaling. The diagrammatic representation depicts the workflow of Tau-induced microglial

migration, actin remodeling and also during P2Y12 signaling activation. The N9 microglia were exposed to various forms of Tau species- monomer, oligomers and aggregates to study P2Y12-Tau physical interaction by Co-IP and receptor expression by WB. The migratory actin structures, tubulin-MTOC polarization, wound-scratch and trans-well migration were studied in Tau-induced microglia by immunofluorescence and phase-contrast microscopy. Moreover, the activation and inhibition of P2Y12 signaling were explored to study actin remodeling as lamellipodia and filopodia formation in migratory Tau-induced microglia.

3.8 Western blot assay for protein expression

Western blot is widely used to study the relative expression level changes in various treatment groups from cells and whole tissue lysate. Here, we want to study the expression level of microglial proteins such as Iba1 (Activation marker), P2Y12 (Purinergic receptor), Actin-binding proteins- Arp2 and TKS5, along with house-keeping controls- Actin or tubulin in microglia.

Protocol:

1. The N9 cells were passaged and seeded at a density of 3,00,000 cells/well in 6-well plates and incubated overnight at 37°C 5% CO_2.
2. The cells were treated with 1 µM concentration of Tau monomer, oligomers and aggregates along with ATP/ADP (50 µM) in basal RPMI media for 24 hours at 37°C 5% CO_2.
3. After the treatment, the media was removed and the cells were washed with PBS.
4. The cells from various treatment groups were trypsinized and pelleted by centrifuging at 800 rpm for 10 minutes.
5. The supernatant was discarded and the cell pellets were kept on ice.
6. The cell pellets were dissolved in 100 µl of RIPA buffer per sample and vortexed in every 10 minutes for complete cell lysis. The cell lysis was done for 30 minutes on ice.
7. The cell lysates were centrifuged at 12,500 rpm for 20 minutes at 4°C, for the removal of insoluble cell debris and nucleic acids.
8. The supernatants were carefully collected without hampering the pellet.
9. The concentrations of each cell lysate were measured by Bradford assay for equal loading (concentration) onto the SDS-PAGE and western blotting.
10. The SDS-PAGE and western blot were carried out as described previously.
11. The following dilutions of the primary antibodies were used- Iba1 (1:500), P2Y12 (1:1000), Arp2 (1:1000), TKS5 (1:1000), Tubulin (1:2000), Actin (1:2000).
12. The blots were incubated overnight with primary antibody and for 1 hour for the secondary antibody.
13. The blots were thoroughly washed with 1X PBST and developed by ECL solution.
14. For the house-keeping control blot, the target protein blot was stripped twice with stripping buffer (pH 2.5) for 15 minutes each in gel rocker.
15. Then the blots were neutralized with PBST and further blocked with blocking buffer.
16. The blots were further stained with Actin/ tubulin antibody ad developed.
17. The band intensity of the target protein and their relative housekeeping control protein were measured by BIO-RAD quality one software. Then the relative expression level changes in various Tau-treated groups were quantified and represented in comparison with cell control.

3.8.1 Bradford assay for protein estimation

Bradford's assay uses the binding principle of coomassie brilliant blue G-250 dye to the carboxyl groups of protein molecules which leads to the formation of the blue color.

Reagents:

Components	Concentration
BSA protein standard	1 mg/mL
Bradford's reagent	

Ultrapure MilliQ water	

Protocol:
1. The BSA standards were prepared in duplicates in ultrapure Milli-Q water.
2. The cell lysates were also diluted at 1:10 ratio in ultrapure Milli-Q water in duplicates.
3. The Bradford reagent was diluted in water at 1:4 ratio.
4. The 10 µL of the standards and lysate dilutions were mixed with 200 µL of the Bradford reagent and added to the 96 well plates.
5. The plate was taken for reading at 595 nm in a spectrophotometer plate reader.
6. The concentration was determined from the standard BSA graph in MS-Excel spreadsheet.

3.9 Co-immunoprecipitation (Co-IP) assay

Co-IP is an important tool to specifically detect the protein-protein interaction in the cell/biological system. If two proteins particularly interact with each other, that complex can be easily precipitated by using a specific antibody to that interacting partner(s). By targeting a known protein by precipitating antibodies, one can pull out other interacting protein partners which are not previously known. Therefore, it is of immense importance to identify new protein-protein interactions or unknown members of any protein complex by this molecular biology tool.

Reagents:
Pierce Co-immunoprecipitation kit, Thermo.

Protocol:
1. N9 cells were seeded onto 100 mm dishes at a density of 10^7 cells and incubated for 24 hours.
2. The cells were harvested and subjected to immunoprecipitation according to the manufacturer's protocol.
3. In brief, The N9 cells were lysed with the co-IP lysis buffer for 30 minutes and the cell lysate concentration was measured by Bradford assay, as described previously.
4. The 10 mg concentration of cell lysate was mixed with 1 µM concentration of Tau monomer, oligomers and aggregates separately in a rotor at room temperature for 3 hours.
5. The amino-linked resins were coupled with pan-Tau K9JA antibody at a concentration of 10 mg/ml, by reductive amination method. Various controls were taken to signify the non-specific binding, such as- without antibody bead control and isotype IgG antibody control.
6. The Tau-containing cell lysates were then mixed with antibody-coupled resin columns and incubated for overnight binding at 4°C in the rotor.
7. The precipitated Tau along with various protein partners were eluted by using 50 µl of a non-reducing elution buffer containing 0.1M glycine (pH 2.5), as per the protocol. The elution buffer did not interfere with the antibody fragments which were covalently linked with the resin.
8. The eluted fractions were subjected to protein estimation by nano-drop instruments and followed by western blot analysis, as described previously.
9. The primary antibody dilutions were used for blotting- P2Y12 (1:1000) and then the blot was stripped and further confirmed by K9JA antibody (1:8000).

3.10 Immunofluorescence assay and Epifluorescence microscopy

Immunofluorescence assay is an important tool for visualizing the intracellular localization of molecules, their subcellular translocation and colocalization. It employs the labeling of specific proteins with primary antibodies and visualization by fluorescently tagged secondary antibodies. Here, we employed immunofluorescence assay and Epifluorescence microscopy to study the levels, localization, internalization and colocalization of various intracellular proteins in Tau-treated microglia (Fig. 9).

Reagents:

Component	Concentration
Fixative- Paraformaldehyde solution in PBS	4%
Permeabilizing buffer- PBST	1X PBS (pH 7.4)
	Triron X100 (0.2%)
Blocking buffer- Horse serum in PBS	2% Horse serum
Antibody solution	Antibody+ Blocking buffer
Washing solution-1X PBST	1X PBS (pH 7.4)
	Triton X100 (0.2%)
DAPI solution	300 nM
Mounting media	

Protocol:

1. N9 cells were maintained in RPMI (10% FBS) growth media.
2. Then, N9 cells were passaged, trypsinized and counted in trypan blue by hemocytometer.
3. The sterilized 12 and 18 mm glass coverslips were placed in the 24-well plates and exposed to UV light for surface sterilization.
4. For immunofluorescence studies, 20,000 cells were seeded per coverslip in a 24-well culture plate overnight at 37°C 5% CO_2.
5. The cells were then treated with Tau monomer, oligomers and aggregates at 1 µM concentration and ATP/ADP 50 µM concentration in basal RPMI media (0.5% FBS) for different hours for different experiments (mentioned below).
6. The treatment time was varying for different experiments which would be mentioned in later subdivisions.
7. After the incubation, the culture media was removed and the cells were fixed with ice-chilled 4% paraformaldehyde solution for 20 minutes in dark.
8. Then, the cells were permeabilized with permeabilizing buffer for 10 minutes.
9. The cells were then washed with 1X PBS for complete removal of the paraformaldehyde solution.
10. The cells onto the coverslips were blocked with blocking buffer for 1 hour.
11. Cells were incubated with primary antibodies with different dilutions (mentioned-below) at 4°C overnight.
12. Further, cells on coverslips were washed with 1X PBST three times, each for 10 minutes.
13. The coverslips were incubated with respective secondary antibodies tagged with Alexa fluorophore for 1 hour.
14. The coverslips were further washed with 1X PBST three times, each for 10 minutes.
15. The cells were counterstained with nuclear stain DAPI for 10 minutes.
16. The coverslips were mounted in anti-fade mounting media, then properly labeled and kept for complete drying overnight.
17. The coverslips were observed under a 63X oil immersion objective lens in Zeiss Axio Observer 7.0 Apotome 2.0 microscope using ZEN pro software.
18. The images were analyzed by Zeiss 2.3 microscopic image analysis software and ImageJ/Fiji software (mentioned-below).

3.10.1 Internalization of Tau oligomers and aggregates by actin remodeling in activated microglia

The experiments performed by using immunofluorescence assay include- Internalization of Tau oligomers and aggregates, membrane-associated actin remodeling and Iba1 localization in the actin network etc. The experiments were performed using the protocol mentioned above. For these experiments, below steps were modified-

1. The Tau treatment (oligomers and aggregates) was given for 24 hours.
2. The primary antibody dilutions were as follows- A11 (1:100), T46 (1:250), β-actin (1:250), Iba1(1:100).
3. The percentage of phagocytic cells was quantified by $A11^+$ cells out of total cells/ field from multiple fields (n=10), in oligomers and aggregate-treated groups.
4. The % of cells showing lamellipodia and filopodia were quantified by lamellipodia$^+$ or filopodia$^+$ cells out of total cells/ field from multiple fields (n=10), in oligomers and aggregate-treated groups.
5. The absolute intensity of A11, actin and Iba1 and the corresponding microglial area (n=30) were quantified by Zen 2.3 software.

3.10.2 P2Y12-actin localization, MTOC polarization in Tau-induced microglia

These experiments were performed by using the immunofluorescence assay protocol with some modifications which include-

1. The 1 µM of Tau treatments- monomer, oligomers and aggregates were given for 24 hours.
2. The primary antibody dilutions used were as follows P2Y12 (1:250), Phalloidin-Alexa488 (1:40), β-actin (1:250), α-tubulin (1:500).
3. The % of lamellipodia and filopodia-bearing cells was quantified by lamellipodia+ or filopodia+ cells out of total cells/ field from multiple fields (n=14), in oligomers and aggregate-treated groups.
4. Pearson's coefficient analysis of colocalization between P2Y12 and actin was quantified from multiple fields (n=20) in various treatment groups.
5. In the clopidogrel-mediated actin remodeling experiment, multiple fields (n=14) were quantified for the absolute intensity measurement of P2Y12 and Phalloidin by Zen 2.3 software. The filopodia and P2Y12-Factin colocalization were calculated from multiple cells (n=70) in different treatment groups by Zen 2.3 and ImageJ software.
6. For, the MTOC polarization assay, multiple cells (n=65) from multiple fields were counted in various Tau treatment groups.

3.10.3 Tau-induced formation of actin microstructures

These experiments were performed by using immunofluorescence assay protocol with some modifications and the quantification methods include-

1. For the Arp2, TKS5 and P2Y12-associated actin remodeling experiment, Tau monomer, oligomers and aggregates of 1 µM concentration were treated for 24 hours along with 50 µM of ADP.
2. Fore, time-dependent podosome cluster formation experiment, N9 cells were treated with oligomers at various time points: 1, 2, 6 and 12 hours. After the incubation, cells were immediately fixed with paraformaldehyde.
3. The primary antibody dilutions used in this experiment were as follows- P2Y12 (1:100), Phalloidin-Alexa488 (1:40), Arp2 (1:100), and TKS5 (1:100).
4. For the quantification for mean fluorescence intensity of Arp2, TKS5 and P2Y12 in actin microstructures were measured in multiple cells in various fields (n=50) by using ZEN 2.3 software. The area of cells containing podosome was quantified in multiple podosome-bearing cells (n=40) among various Tau and ADP-treated groups.
5. The numbers of podosome+ cells (n=13 fields), filopodia+ cells (n=22 fields), % of cells with different podosome rearrangements (n=28) and time kinetics of podosome clustering in oligomers exposure (n=10 fields) in various Tau treated groups were counted in and plotted (Fig. 9).

3.10.4 Tau deposits degradation assay

To study the in-vitro deposits/plaque degradation by migratory microglia, we mimicked the situation of plaque deposition in the brain by coating the glass coverslips with pathological Tau species- monomer and oligomers. Then, the microglia were allowed to degrade the Tau

deposits at various times and the degradation spots were observed in colocalization with P2Y12-associated actin remodeling (Fig. 10).

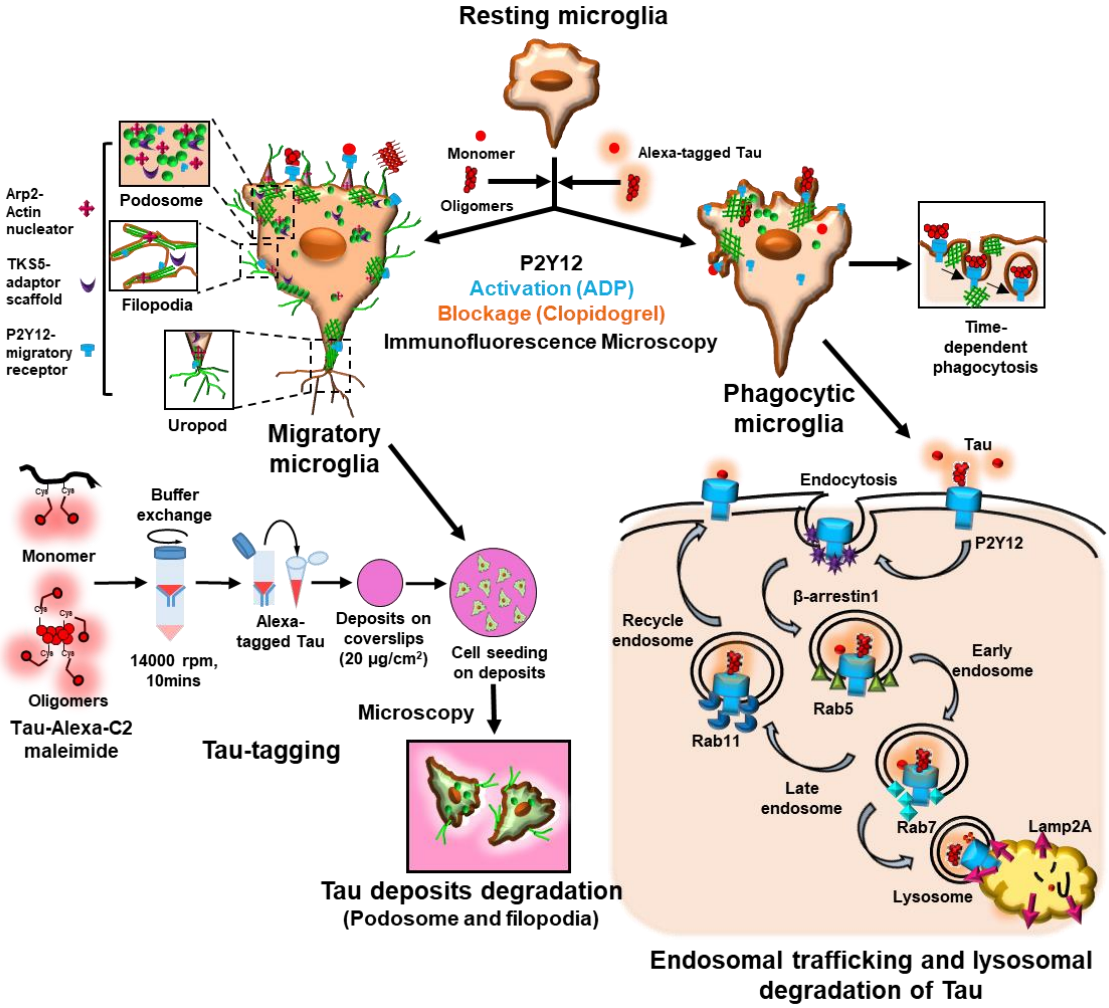

Figure 10. P2Y12-mediated Tau deposits degradation and receptor-mediated endocytosis of Tau in migratory microglia. The diagram demonstrates the workflow of P2Y12-mediated Tau deposit degradation and Tau endocytosis in microglia. The hTau40wt Tau monomer and oligomers were tagged with Alexa647-C2 maleimide and coated onto the glass coverslips as Tau depositions. Next, the time-dependent phagocytosis of Tau and its endosomal trafficking followed by lysosomal degradation were studied in P2Y12 signaling-induced/ inhibited microglia.

Protocol:

1. The Alexa^{647} tagged-Tau monomer and oligomers were diluted in PBS.
2. The 18 mm coverslips were coated with Alexa^{647}-Tau at a final concentration of 10 µg/cm^2 and incubated overnight at 4°C.
3. After incubation, the excess solution was aspirated by vacuum and the coverslips were air-dried at 37°C.
4. The coated coverslips were surface-sterilized by UV exposure for 30 minutes in a laminar hood.
5. The Tau-Alexa^{647} coated coverslips were washed with PBS and neutralized with RPMI media containing 10% FBS for 15 minutes.
6. The N9 cells were seeded at a density of 50,000 cells/ well for Tau deposit degradation.
7. The cells were incubated for 8 hours and 24 hours for both Tau monomer and oligomers-deposits degradation.
8. After incubation, the cells were fixed with 4% paraformaldehyde and followed for immunofluorescence staining protocol as described previously.

9. The primary antibody dilution used for staining were phalloidin-alexa488 (1:40), TKS5 (1:100), Arp2 (1:100), and P2Y12 (1:100)
10. The microscopic images were taken in Zeiss Axio observer with Apotome2 fluorescence microscope at 63X oil immersion objective.
11. The quantification of microscopic images for mean fluorescence intensity of Arp2, TKS5 and P2Y12 was done from various cells (n=100 cells) for different test groups by using ZEN 2.3 software and plotted.
12. From various microscopic fields, the quantification was performed for- % cells with deposit degradation (n=40 fields), relative area of Tau degradation/total cell area (n=45 fields) and P2Y12 activation/blockage-related Tau degradation (n=30 fields).
13. The colocalization analysis of Arp2, TKS5 and P2Y12 with Phalloidin by Pearson's coefficient in podosome and filopodia-associated Tau degradation area (n= 25 cells) was quantified in ADP and clopidogrel treated groups.

3.10.5 Time-dependent and P2Y12-dependent Tau endocytosis assay

The P2Y12-mediated endocytosis via F-actin polymerization and endo-lysosomal localization of Tau was studied by immunofluorescence study.

Protocol:

1. The N9 cells were seeded on 12 mm glass coverslips at a cell density of 10000 cells/ well in 24 well plates and incubated overnight at 37°C 5% CO_2.
2. For time-dependent phagocytosis assay, the cells were treated with 1 µM of Tau monomer and oligomers for 10, 30, 60 and 120 minutes along with untreated cell control.
3. For P2Y12 activation/blockage-associated Tau endocytosis assay, N9 cells were treated with Tau monomer and oligomers for 2 hours in presence of either ADP (50 µM) or clopidogrel (2 µM).
4. To study the consecutive event of Tau endosomal localization and lysosomal degradation, microglia were treated with Tau for 5, 10 and 30 minutes.
5. After incubation, the cells were immediately washed with PBS and fixed with 4% paraformaldehyde. The subsequent steps of immunofluorescence were then followed.
6. The primary antibody dilutions used in this study- Extracellular-P2Y12 (1:250), Phalloidin-alexa^{488} (1:40), β-arrestin-1 (1:200), Rab5 (1:100), Rab7 (1:200), Rab11 (1:100) and Lamp2A (1:100) antibody.
6. In the time-dependent phagocytosis, the percentage of Tau positive phagocytic cells was quantified from multiple fields (n=20), in Tau monomer and oligomers-treated groups at various time points. The mean Tau fluorescence intensity from monomer positive and oligomers positive cells was measured from various fields at various time points using ZEN 2.3 software (n=90 cells).
7. For P2Y12 signaling-dependent phagocytosis assay, % phagocytic cells for both monomer and oligomers were quantified from multiple fields (n=32 fields). The Tau fluorescence intensity from monomer positive and oligomers positive phagocytic microglia were measured from various cells (n=101 cells).
8. The P2Y12 and β-arrestin fluorescence signals from membrane vs internal were quantified from n=50 positions, by drawing a square onto the membrane and placing the same box at the cell interior position using Zen 2.3 software. Pearson's co-efficient between Tau-P2Y12 was quantified from n=15 fields by ImageJ software.
9. Similarly, Rab5-Tau colocalization was analyzed from n=134 locations, Rab7-Tau colocalization from n= 100 cells, Lamp2A-Tau colocalization from n=143 cells and Rab11-Tau colocalization were carried out from n=115 areas of the microglia by Pearson's coefficient analysis by using ImageJ software (Fig. 10).

3.11 Migration assay

Cell migration assay is an important determinant of the rate of migration and execution of migration to a selective microenvironment by a particular type of cell to function accurately. Cell migration plays an important role in various cellular processes- embryonic development,

neuronal cell migration and nervous system development, tissue homeostasis and immune cell migration and tissue invasion; also in the pathological state- such as cancer cell metastasis, Wiskott-Aldrich syndrome and osteoporosis, etc. Microglia as prime-most CNS immune cells surveil the tissue environment and also migrate at the site of injury by detecting chemical gradients. Here, we were interested to study the in-vitro microglial migration and invasion by detecting the extracellular Tau as a chemoattractant by intervening in the P2Y12 signaling.

3.11.1 Wound-scratch assay (2D)

Wound scratch assay is a widely used simple technique to determine the speed and polarity of migration in a time-dependent manner by detecting the relative amount of wound closure in a cell monolayer. In our study, we used the wound-scratch assay to quantify the rate of microglial migration in various Tau- monomer, oligomers and aggregates and ATP treatment. Similarly, the rate of migration was further measured for Tau-induced microglia in P2Y12 activation and blockage condition by ADP (50 µM) and Clopidogrel (2 µM).

Protocol:

1. The N9 cells were seeded at a density of 5,00,000 cells/well in a 6-well plate and incubated overnight.
2. The microglial monolayer was scratched in three lines/ well by 200 µl pipette tips in various treatment groups.
3. The cells were treated with 1 µM concentration of Tau monomer, oligomers, aggregates and ATP (50 µM). Similarly, in P2Y12 signaling-dependent migration assay, the microglial monolayer was treated with various combinations such as cell control, monomer, oligomers, ADP, Clopidogrel, ADP+ monomer, ADP+ oligomers, Clopidogrel+ monomer, Clopidogrel+ oligomers.
4. The treated microglial monolayer was incubated for 24 hours.
5. The phase-contrast microscopic images were taken at various time intervals from 0 to 24 hours in ZEN Axio observer 7 microscope at 20X magnification.
6. The wound lengths were analyzed and measured from the images in different positions at different time intervals.
7. The % of wound closure was calculated in comparison with cell control by using the following formula-

   [The mean wound closure in a particular treatment (at t=0-24 hours)/ the mean wound closure of cell control (at t=same hours)]*100

   Where, the mean wound closure= [(the wound length at t=0 hour) - (the wound length at t=specific time 2, 4, 6…24 hours)]

8. The experiment was performed thrice, and multiple values were taken from single treatment groups for wound closure (n=12).

3.11.2 Trans-well migration assay (3D)

Tran-well migration assay is also called the Boyden's chamber assay, which is a widely used selective migration assay technique that allows the invasion of migratory cells through the porous membrane by following the chemical gradient. In this assay, the migratory cells were placed in an upper chamber containing a porous membrane (5 µm pore size) where the chemoattractant was placed in the lower chamber. The main advantage of this test is selectivity while the disadvantage of this study was prolonged exposure of chemoattractant can equilibrate in both the chamber. Here, we used the trans-well migration or 3D migration assay to study the Tau-induced microglial invasion and also in terms of P2Y12 signaling activation and blockage condition.

Reagents:

Components
Cell culture inserts (5 µm pore size)- 24 well format
4% paraformaldehyde solution
1X PBS (cell biology grade)
Autoclaved milliQ water
0.2% crystal violet stain

Protocol:

1. N9 microglia were seeded at a density of 50,000 cells/inserts, on the upper well of the chamber of 24-well plate format.
2. The lower chambers were loaded with 1 µM of Tau monomer, oligomers, aggregates and 50 µM of ATP. The chamber was incubated for 24 hours.
3. Similarly, in the P2Y12 signaling-induced/ inhibited microglial trans-migration, the ADP (50 µM) and Clopidogrel (2 µM) were mixed with the microglial cell suspension and added to the top chamber. But, the Tau species were added similarly at the bottom chamber. The assembly was incubated for 24 hours
4. After the incubation, the cell inserts were removed, carefully washed with PBS and placed in the well containing 4% paraformaldehyde solution. The lower surface of the inserts would contain migrated microglia.
5. The fixed migrated cells were stained with 0.2% crystal violet solution and washed with Milli-Q water to remove excess stain.
6. The upper surface of the inserts containing non-migrated cells was removed by using a cotton swab.
7. The lower surfaces of the inserts were imaged by ZEN Axio observer 7 microscope at 20X magnification under a bright-field microscope.
8. The numbers of migrated cells per field were counted (n=12 fields) in different treatment groups.

3.12 Statistical analysis

All experiments were performed in three biological replicates and each measurement for every experiment was taken in triplicate. Statistical analyses were performed for biochemical/ biophysical assays, western blots and microscopic quantification by using one-way ANOVA. All the data points were represented in the graph. The statistical significance of the datasets was compared among various treatment groups by multiple-correlation correction and analyzed by Tukey-Kramer's criteria at a 5% level of significance. For microscopic analysis, several data points from multiple fields were analyzed and plotted. The data point numbers for each experiment were mentioned in the respective experimental protocol. The test groups such as monomer/ oligomers/ aggregates were compared with untreated cell control and the p-values were mentioned within the figures. In ADP or Clopidogrel-mediated actin remodeling, Tau deposit degradation and the phagocytosis assay, the ADP/Clopidogrel+ Tau species-treated groups were compared to only ADP/Clopidogrel-treated group as well as only monomer/oligomers groups. For eg. ADP+ Tau monomer vs. ADP and only monomer, Clopidogrel+ oligomers vs. Clopidogrel and only oligomers, etc. Hence the p-values were quantified and depicted within the figures. The results were considered significant if the mean difference between groups is greater than calculated Tukey's criterion (X-X`>T).

Chapter 2

Preparation and characterization of Tau- monomer, oligomers and aggregates and internalization by activated microglia

2.1 Background

The microtubule-associated protein Tau, here the full-length Tau contains two domains- the N-terminal projection domain to maintain the soluble form of Tau and the C-terminal repeat domain, which interacts with microtubules for tread-milling and axonal transport. In AD, various PTMs of Tau resulted in increased intermolecular interaction which leads to the formation of oligomers, higher-order aggregates and further the NFTs [221-223]. Oligomers are secreted from neurons *via* various mechanisms such as passive transport, exocytosis, cell-to-cell connection formation and along with neurotransmitters [224, 225]. Although the oligomers are the unstable intermediate species, it acts as a seed component for further aggregation and mediates neurotoxicity and inflammatory activity [51, 226, 227]. Multiple studies showed the elevated level of granular Tau oligomers (5-50 nm) by almost four times in the AD patient's brain as compared to the age-matched control group [49, 228]. Mirbaha *et al.,* showed that the trimeric Tau oligomers were the minimum units for cellular engulfment and propagation for high seeding potential. The high molecular weight (HMW >670 kDa) globular Tau oligomers, as observed by size-exclusion chromatography, were endocytosed most effectively and were immunoreactive to oligomer-specific antibody [229]. Furthermore, the extracellular oligomers exposure can induce the aggregation of intracellular Tau by seeding in the HEK293T-Tau cell model [230]. Microglia as the prime immune cells in the brain maintain the ramified structure with a small cell body and long extensions for surveilling the microenvironment. But upon tissue damage or plaque deposition, microglia transform into the ameboid structure by retracting its cellular processes and form flat protrusion for migration and accumulation at the site of injury [231, 232]. Another study showed that the rat primary microglia and BV2 microglia phagocytosed the mutant Tau oligomers upon LPS stimulation [233]. Similarly, microglia were shown to internalize the hyperphosphorylated Tau oligomers by antibody-mediated and complement-mediated opsonization for degradation [234, 235]. Funk et al., also evidenced that the BV2 microglia, but not the neurons internalized HMW Tau oligomers (>20 mers) by antibody-mediated opsonization which can be considered important immunotherapy in Tauopathies [236]. The activated microglia migrate by the cytoskeletal network remodeling such as actin, tubulin and intermediate filaments, with the accumulation of calcium-binding adaptor protein- Iba1, which can mediates actin cross-linking, phagocytosis of protein debris and regulates inflammation [237, 238]. A previous but related study showed the preparation of low n-oligomers of mutant truncated Tau which showed an importance in the field of AD for the generation of Tau-specific immunotherapy [239]. The main challenges for studying the full-length Tau oligomers are their instability and lack of homogeneity during the experiments. Therefore, the *in vitro* preparation and stabilization of hTau40wt oligomers was the prime most important in the current field. Here, we have standardized the preparation and stabilization of hTau40wt oligomers and characterized by various biochemical and biophysical techniques. Then, the immunoreactive nature of these Tau oligomers was studied in N9 microglial cells by phagocytosis, membrane-associated actin remodeling and Iba1-related microglial activation.

2.2 Preparation and stabilization of HMW full-length Tau oligomers

Oligomers are very unstable, short-lived intermediate species which appeared during Tau aggregation. But at the same time, Tau oligomers were found to be accumulated in the AD patient's brain in an age-dependent manner, relating further NFTs formation and cognitive decline [51, 240]. The isolated Tau oligomers from AD brain showed cytotoxicity at a lower concentration in neuroblastoma cell line, which was decreased by oligomer-specific Tau monoclonal (TOMA) antibody [241]. Henceforth, the selective trapping and degradation of these neurotoxic Tau oligomers by microglia would be an important strategy to combat AD pathology. To prepare and stabilize in vitro Tau oligomers, full-length hTau40WT monomers were induced for oligomerization with polyanionic co-factor heparin in PBS (pH 7.4) for 12 hours at room temperature. The Tau aggregates were prepared by inducing heparin in BES buffer (pH 7.4) at 37°C for 3 days, as described previously by our group [242].

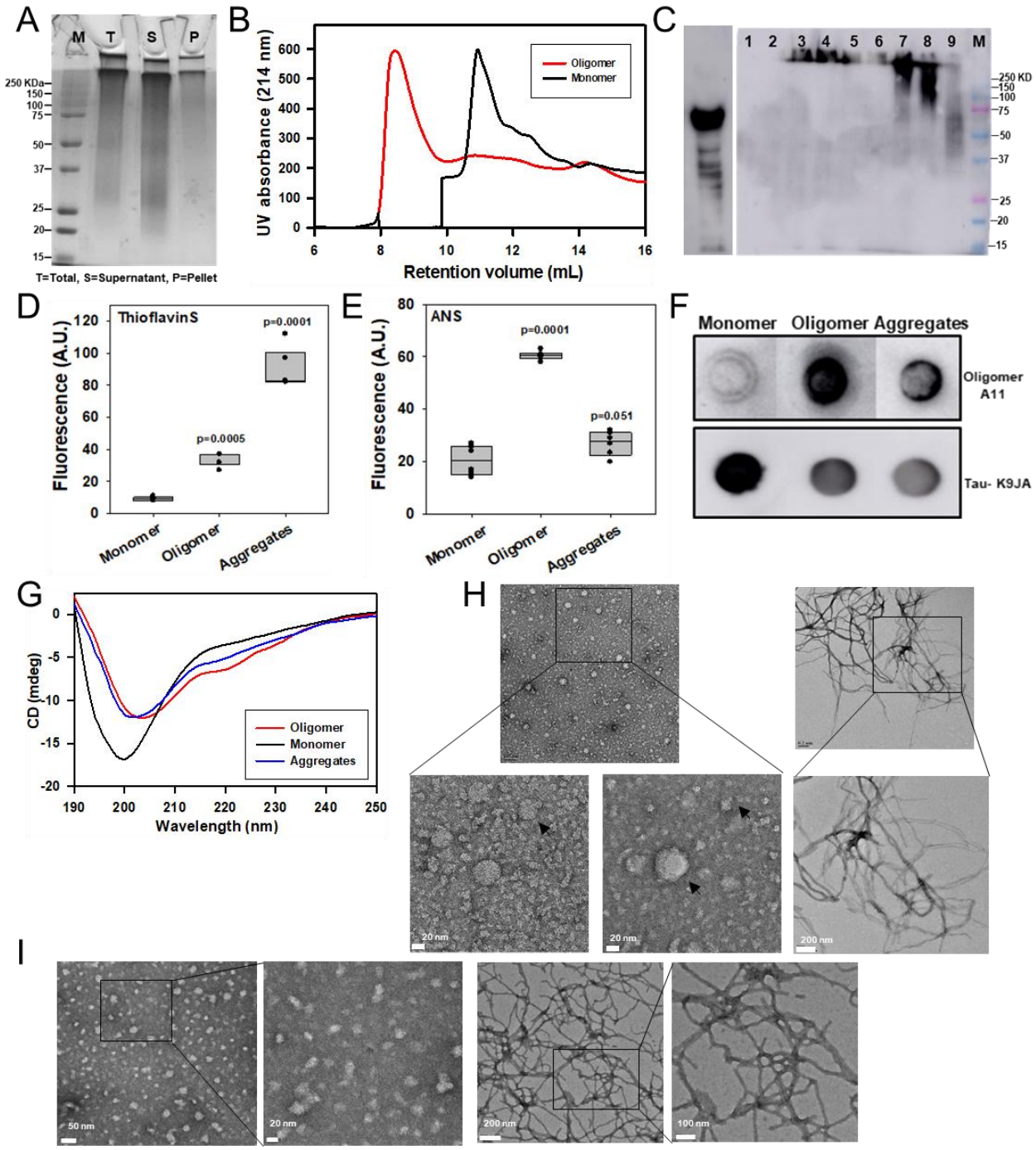

Figure 11. Biochemical and biophysical characterization of Tau species- monomer, oligomers and aggregates. A. The full-length Tau (hTau40wt) oligomers were subjected to sedimentation assay, which showed the presence of soluble HMW oligomers by SDS-PAGE. **B.** The stabilized Tau oligomers were separated by SEC. **C.** SEC fractions containing oligomers were confirmed by K9JA WB. **D.** The prepared Tau oligomers contained less cross-β structure than aggregates by ThS assay. **E.** Oligomers showed increased exposed hydrophobicity by ANS assay. **F.** Tau monomer, oligomers and aggregates were checked by dot blot staining where oligomers showed maximum A11 reactivity. **G.** CD spectroscopic studies revealed a spectral shift which denotes the transition from random coil to the β-sheets structure during oligomerization. **H, I.** Tau oligomers formed globular structures and aggregates showed fibrillar structure by TEM and HR-TEM study.

The hTau40wt oligomers (from here, Tau oligomers) were then subjected to sedimentation assay, followed by SDS-PAGE which depicted the maximum presence of soluble HMW Tau oligomers in the solution (Fig. 11A). After that, Tau oligomers were stabilized by 0.01%

glutaraldehyde for 10 minutes for cross-linking. When the oligomers-containing solution was allowed to pass through SEC, it was eluted out at a lower retention volume of 8.2 ml as compared to monomer at 12.1 ml retention volume which signified the formation of HMW Tau oligomers (Fig. 11B). Then, the oligomers-containing fraction was collected and concentrated by 5 KDa molecular cut-off filters at 3800 rpm for 3 hours. In an alternative method, the cross-linked Tau oligomers were directly subjected to buffer exchange by 20 times excess PBS for 3 hours by centrifuging at 3800 rpm. Finally, the concentration of the oligomers was checked by BCA assay and stored at -80°C.

2.3 Characterization of Tau oligomers by biochemical and biophysical techniques

The prepared Tau oligomers were characterized by various biochemical methods such as western blot, dot blot analysis and multiple fluorescence assays, and biophysical techniques such as Transmission electron microscopy (TEM), High resolution-TEM and CD spectroscopy. The oligomers-containing SEC fractions were subjected to WB with a total pan-Tau K9JA antibody which showed the formation of HMW oligomers (>250 KDa) (Fig. 11C). Then in the fluorometric assay, Tau oligomers were characterized by Thioflavin-S (ThS) and ANS fluorescence assay which determines the formation of cross-β structure and surface exposed hydrophobic patches on the protein species during Tau aggregation, respectively. As the oligomers are the intermediate species in the aggregation process, the ThS fluorescence of oligomers was significantly lower than the pre-formed Tau aggregates. Similarly, the natively unfolded monomer showed a very less ThS fluorescence because of the lack of secondary structures (Fig. 11D). Next, the Tau oligomers showed an increased ANS fluorescence by two-fold as compared to monomer and even Tau aggregates, which signified the exposure of hydrophobic patches on the surface of the oligomers but that buried inside the fibrillar-aggregates. Collectively, the fluorescence assay depicted the formation of hydrophobic Tau oligomers with a reduced content of inside β-sheet structures. But the mature Tau fibrils have a higher extent of internal β-sheet structures and less surface hydrophobic residues (Fig. 11E). Furthermore, the oligomers along with monomers and aggregates were characterized by oligomer-specific A11 antibody and total-Tau K9JA antibody in dot blot analysis. The Tau oligomers were immune-stained with A11 antibody more than the monomer and performed aggregates of 1 mg/ml concentration. Similarly, all the Tau species (Monomer, oligomers and aggregates) were equally stained with the total-Tau K9JA control antibody staining (Fig. 11F). CD spectroscopy helps to understand the global secondary conformations of proteins. Tau monomer showed a minimum ellipticity at 200 nm, which is an indication of random coil structures. But Tau oligomers and aggregates showed the minimum ellipticity at 204 nm and a growing ‘shoulder’ at 220 nm, which indicates the formation of partial β-sheet structures (Fig. 11G). Previously, the truncated and repeat domain Tau oligomers were shown to form globular structures, therefore we were interested to study the morphology of our Tau oligomers [233, 239]. The TEM and HR-TEM analysis revealed that the full-length Tau oligomers formed heterogeneous globular structures, sizes ranging from 5-50 nm. The Tau aggregates formed elongated fibrillar structures and soluble Tau monomer showed no specified structure by TEM study (Fig. 11H, I) [243].

2.4 Internalization of Tau oligomers and aggregates by N9-Microglia *via* membrane-associated actin remodeling

The released soluble Tau oligomers from neurons can be considered an early event of Tauopathy, which can act as an activation signal for microglia [243]. Previous studies showed that extracellular Tau can be internalized by 6-O-heparan sulfate proteoglycan and by the involvement of lipid microdomain [244]. To study the internalization of Tau oligomers by N9-microglia, the cells were exposed to oligomers and aggregates for 24 hours and the internalized Tau species were studied by A11 and K9JA antibody-associated immunofluorescence staining. The fluorescence microscopic study revealed the presence of intracellular and surface-adhered $A11^{+}$ oligomers in microglia as compared to the untreated

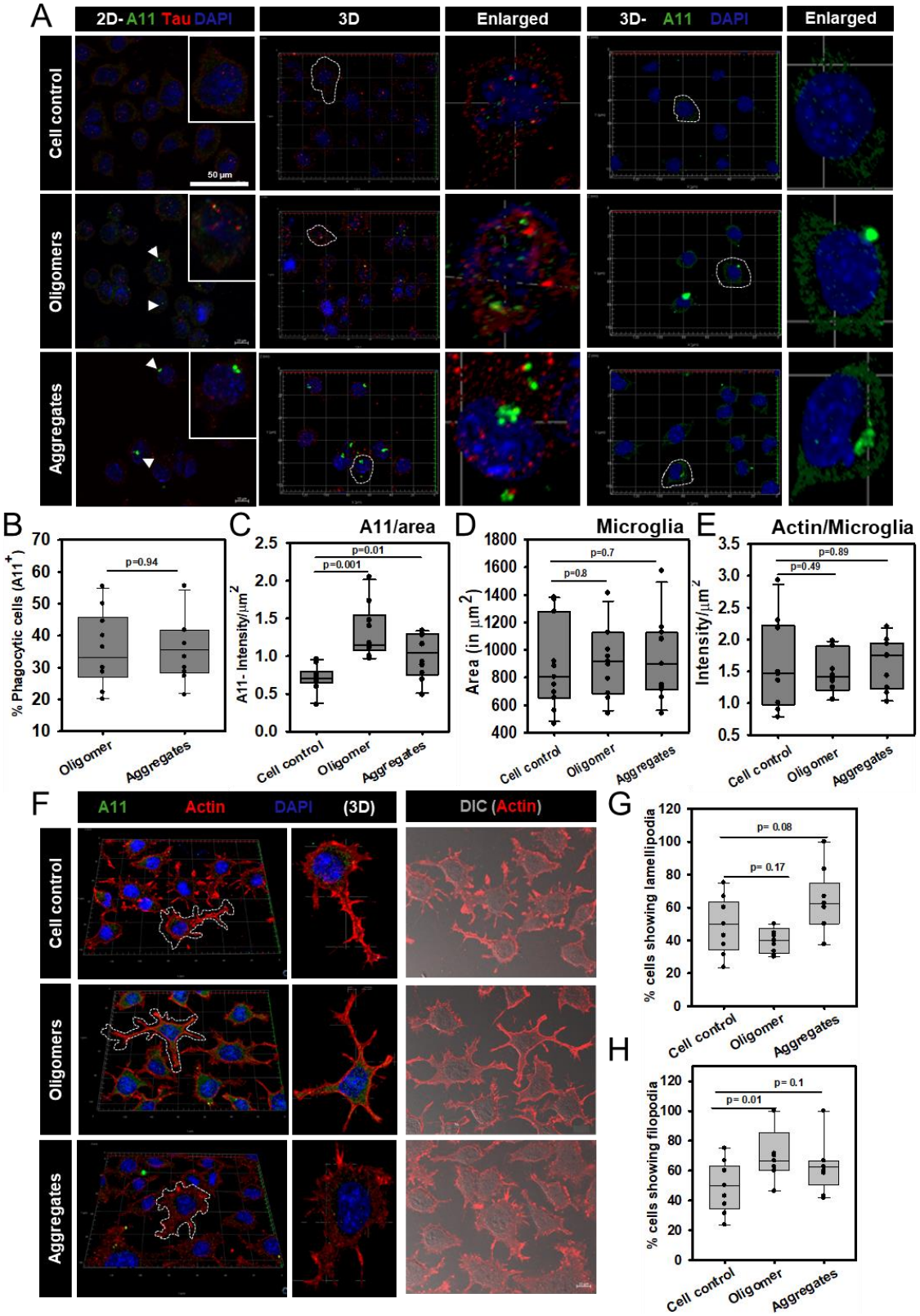

Figure 12. Phagocytosis of Tau oligomers by membrane-associated actin remodeling. A. N9 microglia internalized extracellular Tau oligomers and aggregates at 24 hours **B.** The percentage of $A11^{+}$ phagocytic microglia was equal in Tau oligomers and aggregates-exposed groups. **C.** The A11-intensity revealed the increased phagocytosis of oligomers than aggregates by microglia. **D, E.** Microscopic quantification depicted the unaltered actin intensity and the area of microglia in Tau-exposed microglia. **F.** Extracellular Tau oligomers and aggregates exposure induced the formation of membrane-associated actin network. **G, H.** Tau aggregates-exposed microglia formed increased fan-

shaped lamellipodia structure while the oligomers-treated group has increased actin micro-spikes called filopodia.

group. Microglia were found to internalize both the Tau species- oligomers and aggregates equally at 24 hours as observed by $A11^{+}$ microglial population by 3D image depiction (Fig. 12A). As the pre-formed aggregates contain heterogeneous species, *i.e.* both oligomers and mature fibrils, we observed the presence of $A11^{+}$ microglia in the aggregates-treated group (Fig. 12B). The A11 intensity from the phagocytic microglial cells was increased upon oligomers and aggregates exposure than the cell group (Fig. 12C). But, there were no significant changes in the microglial area upon Tau internalization (Fig. 12D).

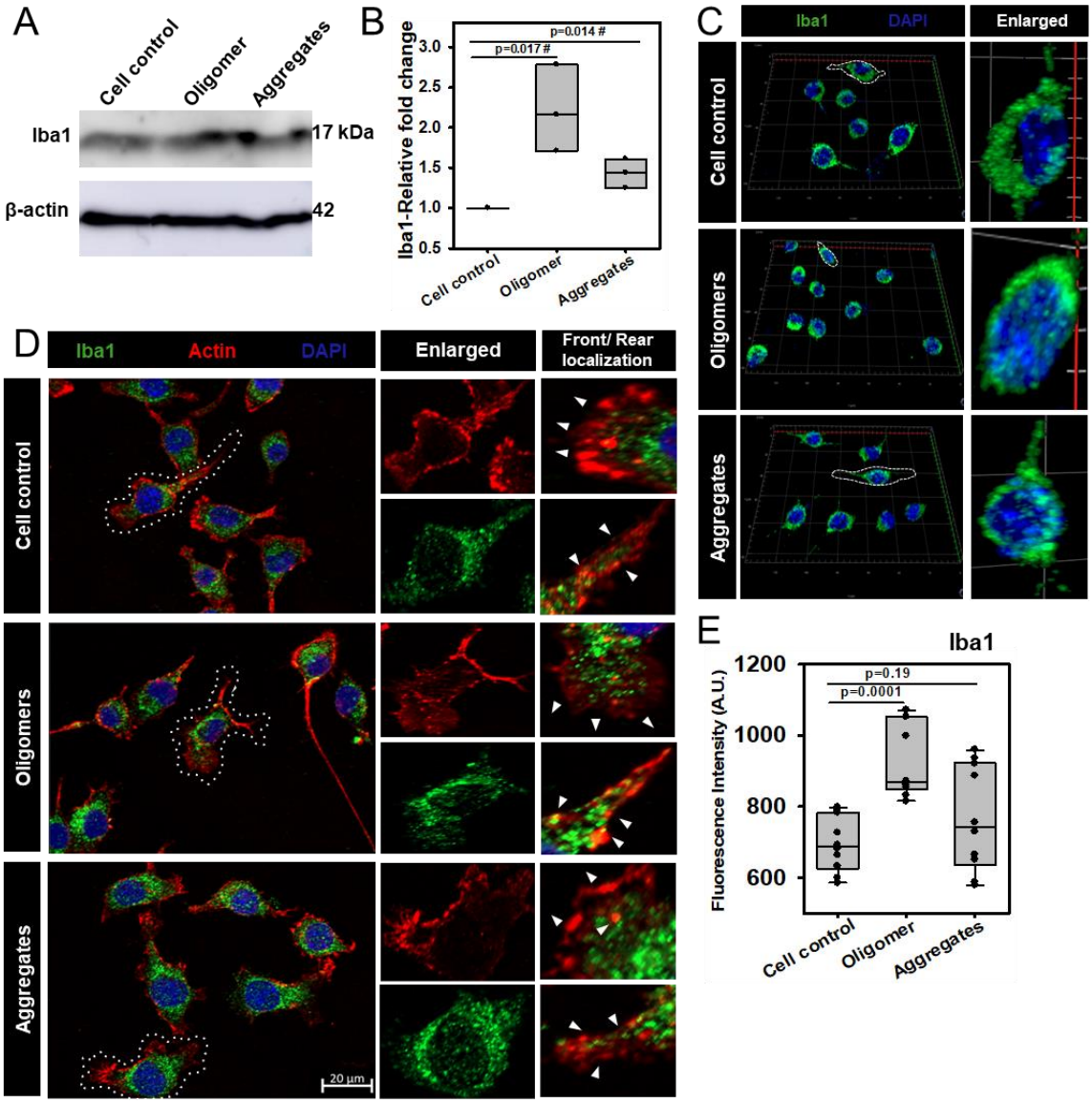

Figure 13. Tau-induced microglial activation by Iba1 expression and colocalization in actin network. A. Tau oligomers and aggregates-exposed microglia elevated the Iba1 protein level more than cell control, by WB. **B.** The quantification of Iba1 WB, followed by post-hoc Tukey's test (#: ns) signified the unaltered level of Iba1 in various Tau-treated groups than cell control. **C.** Tau oligomers-exposed microglia induced the cytoplasmic relocation of Iba1 protein. While; aggregates treatment localized Iba1 in the peri-membrane localization **D.** The immunofluorescence microscopic study revealed the accumulation of Iba1 with actin network in Tau-induced microglia. The 'arrow heads' indicated the colocalization points of actin and Iba1. **E.** An increased fluorescence level of Iba1 was quantified in oligomers-exposed microglia than aggregates treatment.

Actin is one of the basic cytoskeletal networks involved in various cellular processes such as cell structure maintenance, migration, matrix remodeling and phagocytic clearance [99]. To check the mechanism of Tau internalization by microglia, the actin network was stained with β-actin antibody for Tau oligomers and aggregates treated groups. The microscopic images and related fluorescence quantification revealed that the actin levels normalized to the surface area remained unchanged, signifying the rearrangement of the actin network for phagocytosis of extracellular Tau species and probably for migration (Fig. 12E, F). Further, we observed that Tau aggregates exposure resulted in the formation of actin-rich flat membrane extension *i.e.,* lamellipodia-like structure (Fig. 12G). While, oligomers treated microglial population was shown to increase actin micro-spikes, can be termed as filopodia-like structure, which signified the concomitant microglial migration and phagocytic uptake of extracellular Tau species (Fig. 12H). These results suggest that microglia could exhibit the phagocytosis of extracellular Tau oligomers for clearance and mediate migration by membrane-associated actin remodeling.

2.5 Iba1 upregulation and colocalization with actin network in Tau-induced microglia

Tau oligomers can act as an activation signal for microglia by various receptors and induce the secretion of cytokines and chemokines [245]. A calcium-binding protein, Iba1 was found to be unregulated by hTau40 overexpression and involved in actin remodeling in microglia [246]. In the western blot study and quantification of relative fold change, we found that the Iba1 level was unregulated in the Tau oligomers treated group than aggregates exposure for 24 hours. But, the statistical significance analysis revealed that the change in Iba1 level was insignificant among the Tau-treated groups (Fig. 13A, B). Interestingly, IF study depicted that Tau oligomers exposure induced the cytoplasmic localization of Iba1 in microglia than cell control. While the aggregates exposure showed the spread localization of Iba1 across the cell and in extensions (Fig. 13C). Furthermore, the colocalization of Iba1 in the membrane-associated actin network was studied to understand the signaling overlapping of microglial activation and migratory actin remodeling. In our study, we found that the actin-crosslinking and Ca^{2+}-associated protein Iba1 was found to colocalize more with actin at the rear end of Tau-oligomers exposed microglia than the aggregates exposure (Fig. 13D). The frontal actin-rich lamellipodia were observed to have less Iba1 and actin colocalization in all Tau-treatment groups (Fig. 13E). These observations suggested that extracellular Tau oligomers can drive the Iba1 signaling-mediated microglial activation and associated actin remodeling for phagocytosis in the early-stages of AD.

2.6 Summary

In this study, we have prepared and stabilized full-length Tau oligomers which are globular in structures and immune-reactive to oligomer-specific A11 antibody. These Tau oligomers were effectively phagocytosed by microglia which leads to the microglial activation by Iba1 and membrane-associated actin remodeling specifically filopodia-like microstructures. Hence, the microglia-associated actin cytoskeleton remodeling for phagocytosis and rapid clearance of extracellular Tau oligomers can be intervened as a therapeutic strategy in Alzheimer's disease.

Chapter 3

P2Y12-driven chemotaxis and actin network remodeling in Tau-induced microglia

3.1 Background

Oligomers are the most reactive species, which can interact with various cellular processes for eg. microtubule treadmilling, membrane leakage, impaired neurotransmitter release, cargo trafficking, amyloidogenesis and even synaptic loss [247-249]. Resting microglia maintain the ramified state for continuously sampling the microenvironment and performing synaptic surveillance [231]. But, the extracellular protein deposits, here Tau can be sensed by microglia, which in turn, can be transformed into amoeboid state [93]. The interaction of amyloids and microglial receptors leads to receptor-mediated endocytosis and clearance of extracellular protein aggregates [148, 151, 186, 234]. Depending on the activation state, either 'pro' or 'resolving', microglia modulate the cytoskeletal network- actin and tubulin for migration, phagocytosis and immune effector functions [237, 245]. Microglia can sense and migrate along with the released chemical gradients from affected neurons such as- excess ATP, glutamate, protein aggregates and chemokine-fractalkine, etc [49, 93]. P2Y receptors play an essential role in platelet aggregation and targeted therapeutically in hematological and cardiovascular diseases. But in recent times, P2Y and P2X GPCRs have gained a lot of interest in neurodegeneration for their inter-connected signaling outcome in various pathological and physiological processes [250-252]. A recent study showed that microglia formed the P2Y12-dependent somatic junction for the maintenance of neuronal health *i.e.,* regulated by Ca^{2+} signaling [253]. On contrary, Clopidogrel-mediated inhibition of P2Y12, exhibits the effective clearance of Aβ-associated dead neurons and performs behavioral improvement in the neurodegeneration-induced Zebrafish larvae model [254]. A recent report showed that an amyloidogenic protein α-synuclein can act as a chemoattractant and mediates the microglial migration *via* interaction with β1 integrin [255]. Another report emphasized the interaction of extracellular Tau and fractalkine receptor- CX3CR1 leads to Tau internalization in migratory microglia [256]. In this scenario, we will explore the chemotactic potential of various Tau species- monomer, oligomers and aggregates in microglial migration, microtubule-organizing centre (MTOC) polarization and the formation of P2Y12-associated actin structures. Then, the role of P2Y12 signaling (activation/ blockage) would be elucidated for the actin network remodeling in Tau-induced migratory microglia.

3.2 Cytotoxic Tau oligomers interact with microglial P2Y12 and localize in membrane-associated actin network

To study the chemoattracting property of Tau, N9 microglia were exposed to various Tau species for 24 hours and the expression and interaction were studied by western blot and Co-IP. Here, we found that the microglial P2Y12 expression level was elevated by 12 folds by monomer exposure, while the oligomers and aggregates induced the P2Y12 expression about 8 folds as compared to cell control (Fig. 14A, B). In the Co-IP study between Tau species and microglial cell lysate (containing P2Y12), P2Y12 was found to interact with various Tau species and become precipitated with Tau-specific K9JA antibody. The P2Y12 receptor interacted more with Tau oligomers as compared to aggregates and monomer, which may indicate the intense binding capacity of oligomers to this purinoceptor (Fig. 14C, D). It was found for the first time that extracellular Tau can directly interact with microglial P2Y12 and induced its cellular expression. These findings can in turn inter-relate a dual potential of this purinergic receptor P2Y12 in microglial migration and Tau phagocytosis.

Further we studied the localization and function of the P2Y12 receptor in membrane-associated actin structure as a component of active microglial migration for Tau

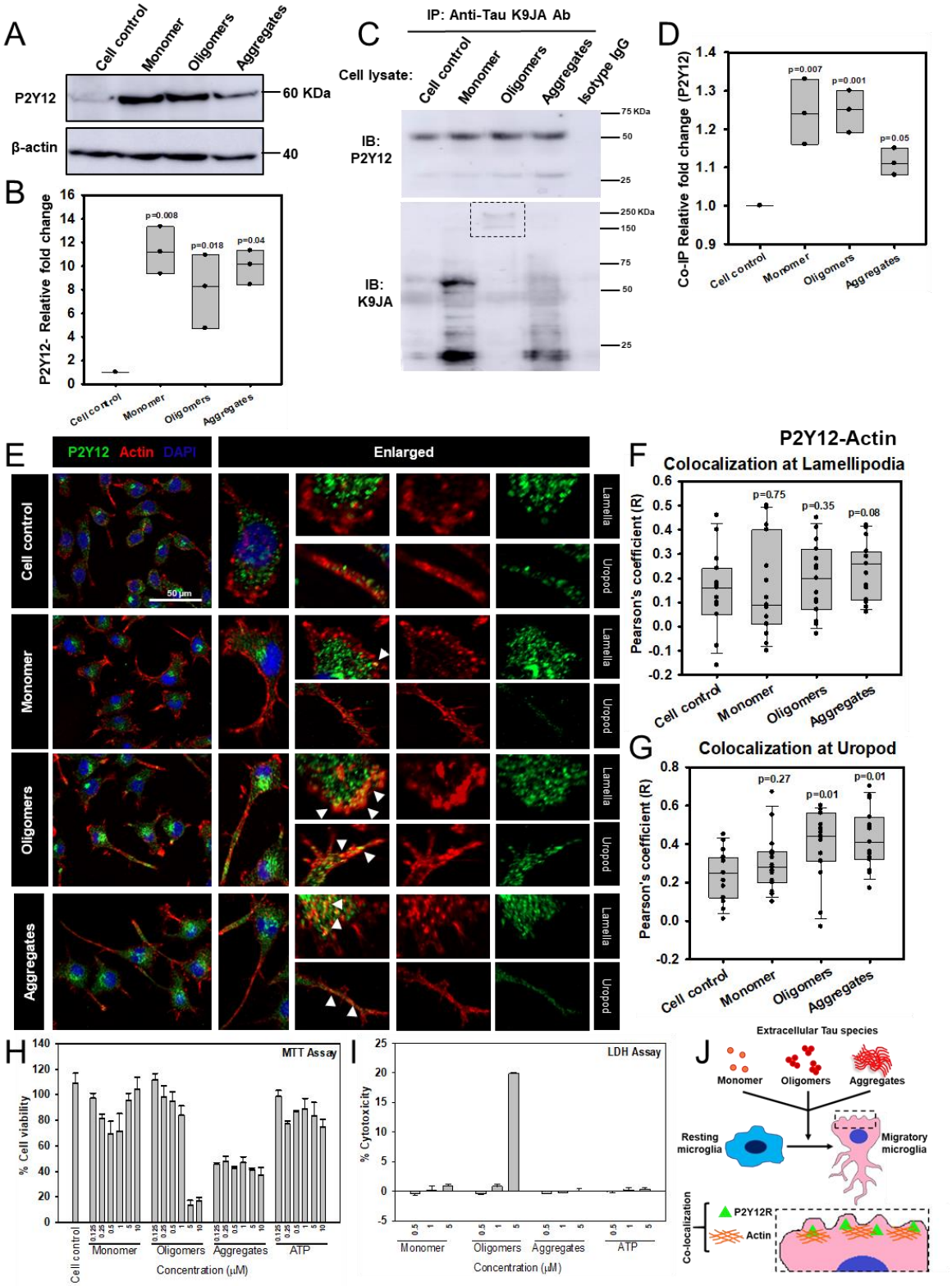

Figure 14. Extracellular Tau interacts with P2Y12 and induces its expression and localization in actin network. A. Extracellular Tau monomers, oligomers and aggregates induced the P2Y12 receptor expression in N9 microglia by WB study. **B.** Densitometry analysis estimated the increased level of P2Y12 by Tau treatment than cell control. **C.** Various Tau species can interact with P2Y12, as observed by the co-precipitation **D.** Tau monomer and oligomers precipitated the P2Y12 receptor more than aggregates. **E.** Tau oligomers induced the colocalization of P2Y12-actin more than aggregates and monomer exposure. The arrow heads indicated the colocalization points. Scale bar 50 µm. **F.** P2Y12 and actin colocalization in lamellipodia was unaltered in Tau-exposed microglia by

Pearson's coefficient (R). **G.** Oligomers and aggregates exposure increased the P2Y12- actin colocalization (R) in uropod than monomer. **H.** Oligomer induced the cytotoxicity in microglia by MTT assay. **I.** Oligomers can induce membrane leakage by LDH assay from microglia. **J.** Hence, the extracellular toxic Tau species induced the elevated expression and colocalization of P2Y12 with actin-rich lamellipodia and uropod.

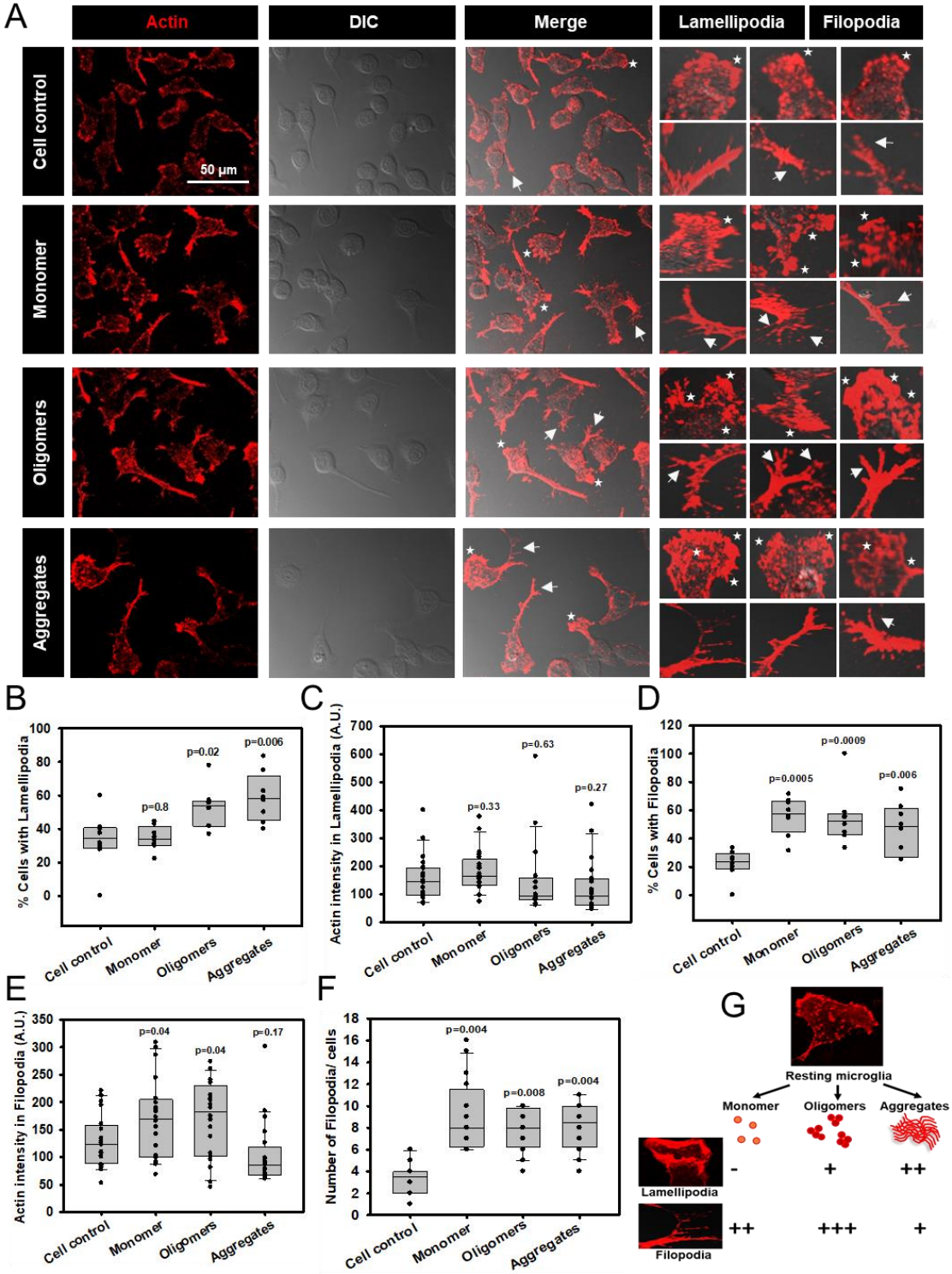

Figure 15. Tau oligomers induce filopodia formation, but aggregates increase lamellipodia in migratory microglia. A. Tau oligomers and aggregates-exposed microglia increased the formation of lamellipodia and filopodia than monomer. The 'star' sign indicated the lamellipodia and the 'arrow' indicated the filopodia. Scale bar 50 µm. **B.** Tau oligomers and aggregates induced the percentage of cells-bearing lamellipodia more than monomer. **C.** The actin intensity in lamellipodia remained

unaltered among all Tau-exposed groups. **D.** Extracellular Tau exposure accounted for more filopodia-bearing cells than cell control. **E.** Actin intensity in filopodia was increased in Tau-exposed microglia. **F.** Tau exposure induced microglial filopodia extensions from microglia. **G.** Lamellipodia bearing microglia was more in aggregates exposure while oligomers treatment induced filopodia formation.

phagocytosis. Here, the P2Y12 was observed to be localized with membrane-associated actin structures more in Tau oligomers-exposed microglia than in the aggregates and monomer-treated groups (Fig. 14E). The co-localization of P2Y12 and actin was more in the microglial rear end called 'Uropod' than lamellipodia in both Tau oligomers and aggregates treated groups (Fig. 14F, G). The cytotoxicity of Tau oligomers and aggregates was studied in N9 cells by MTT and LDH leakage assay. Extracellular Tau oligomers were found to be toxic at 5 and 10 µM concentrations, while aggregates were toxic to microglial cells even at lower concentrations, as observed by MTT assay (Fig. 14H). Similarly, Lactate dehydrogenase (LDH) leakage assay indicates the consequence of membrane leakage by the presence of toxic amyloids. Tau oligomers showed membrane leakage even at 5 µM concentration, while other Tau species monomer and aggregates were non-toxic to microglia in terms of LDH release (Fig. 14I). Together, these results suggested that the cytotoxic Tau oligomers can intervene in the purinergic P2Y12 signaling for membrane-associated actin remodeling and microglial migration (Fig. 14J).

3.3 Tau oligomers induce filopodia formation while aggregates influence lamellipodia

The morphological changes of microglia were studied in terms of actin remodeling upon Tau induction. Here, we found that extracellular Tau oligomers and aggregates induced the accumulation of localized actin networks in microglial lamellipodia and the formation of branched filopodia, compared to monomer exposure and cell control (Fig. 15A). By quantifying the microscopic images, we found that lamellipodia-bearing microglia were increased by 20% in oligomers and aggregates exposure than monomer-treated population (Fig. 15B). But, the total fluorescence intensity of the actin in microglial lamellipodia remained unchanged in various Tau-treated groups than in cell control (Fig. 15C). Next, the filopodia-bearing microglial populations were also increased by 40% upon Tau exposure, which was the maximum in monomer exposure than the HMW oligomers and aggregates (Fig. 15D). Similarly, the actin intensity in filopodia was increased in monomer and oligomers exposed to microglia than in aggregates treatment (Fig. 15E). Moreover; the number of protruding filopodia from microglia was also increased in all Tau-exposed groups as compared to cell control (Fig. 15F). These results indicated that extracellular Tau monomer facilitated only filopodia formation. However, the HMW oligomers and aggregates exhibited microglial actin remodeling through the formation of lamellipodia and filopodia growth(Fig. 15G).

3.4 ATP induces filopodia formation and localizes P2Y12 in lamellipodia in Tau-exposed microglia

Here, we were interested to understand the synergistic function of ATP and extracellular Tau in actin remodeling by P2Y12 signaling. It was observed that only ATP induced the formation of microglial lamellipodia and filopodia and also elevated the P2Y12 localization in remodeled actin network (Fig. 16A). When microglia were treated with Tau monomer and oligomers along with ATP, it was observed that the actin intensity was increased in microglial lamellipodia and uropod as compared to only Tau-treated groups. While the synergistic aggregates and ATP treatment did not exhibit actin remodeling in migratory microglia (Fig. 16B, C). Similarly, various Tau species, such as monomer, oligomers and aggregates influenced the P2Y12 localization at the microglial uropod. But, the co-treatment of Tau oligomers and ATP induced the P2Y12 intensity in uropod while, monomer and aggregates reduced the P2Y12 level in microglia (Fig. 16D, E). In terms of filopodia extension, Monomer and aggregates along with ATP were found to induce the percentage of filopodia-bearing microglia, while oligomers+ ATP was associated with a reduced number of filopodia^{+} cells.

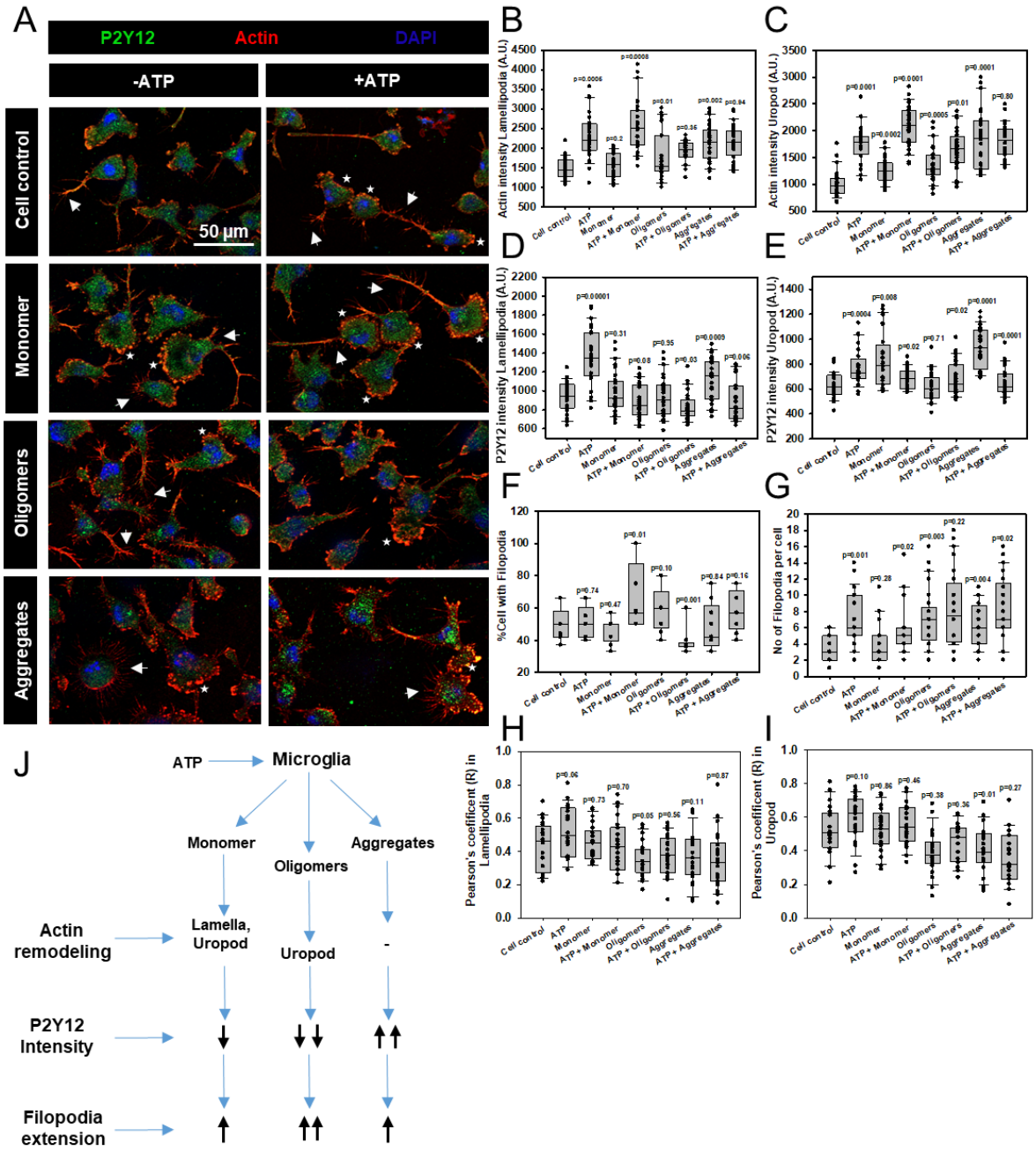

Figure 16. Tau facilitates filopodia formation and localized actin polymerization in ATP-induced migratory microglia. A. The synergistic treatment of Tau and ATP induced P2Y12-localized actin remodeling in migratory microglia. Scale bar 50 µm. **B, C.** ATP exposure induced the actin polymerization (intensity) more in microglial lamellipodia and uropod along with the monomer and oligomers than aggregates. **D, E.** P2Y12 was accumulated more in lamellipodia and uropod upon ATP exposure. But in Tau oligomers+ ATP co-treatment, P2Y12 intensity was increased in only microglial uropod. **F, G.** Tau monomer and aggregates along with ATP induced the filopodia-bearing microglia and the number of filopodia per cell. **H, I.** The P2Y12-actin colocalization (R) depicted no significant changes in synergistic Tau+ ATP-treated microglia. **J.** extracellular Tau oligomers and monomer increased the ATP-induced actin remodeling and filopodia extensions with reduced P2Y12 levels in migratory actin structures of microglia.

Also, we observed that the number of filopodia per cell was increased in ATP and Tau oligomers and aggregates treated groups. But, when Tau species were exposed to ATP-induced microglia, only monomer and aggregates induced the numbers of filopodia further as compared to the oligomers-treated group (Fig. 16F, G). To determine the colocalization of

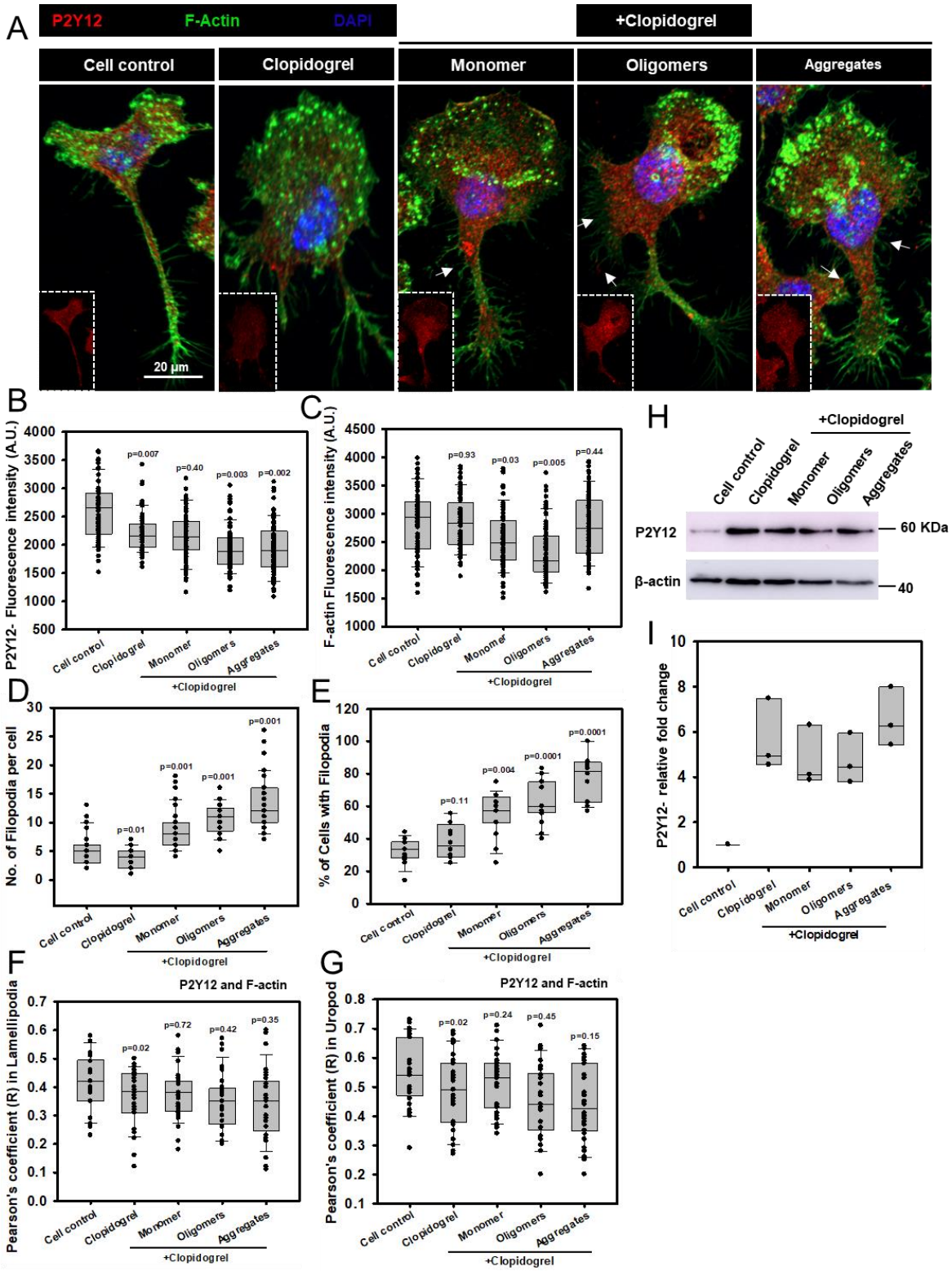

Figure 17. Tau induces microglial actin-rich filopodia formation, redundant of P2Y12 signaling. **A.** Clopidogrel reduced the P2Y12 level and actin remodeling in migratory microglia. But, the extracellular Tau restored the filopodia extensions, even upon P2Y12 blockage. The arrow indicates the filopodia extensions and P2Y12 (red) was shown in the inset. Scale bar 20 µm. **B, C.** Clopidogrel treatment has significantly reduced the P2Y12 fluorescence level in microglia with the unaltered level of F-actin intensity. **D, E.** Clopidogrel significantly reduced the formation of filopodia per cell; while the filopodia number and filopodia^{+} microglia were increased by the co-treatment with extracellular Tau species. **F, G.** Clopidogrel exposure reduced the colocalization of P2Y12-F-actin in lamellipodia and uropod which remained unaltered by Tau exposure by Pearson's coefficient (R). **H, I.** The WB study

and densitometry showed the increased expression level of P2Y12 upon Clopidogrel and Clopidogrel+ Tau co-treatment conditions.

P2Y12 in microglial lamellipodia and uropod, we performed Pearson's coefficient colocalization test. Here, we observed that ATP induced the P2Y12-actin colocalization in uropod, but the synergistic Tau species+ ATP exposure did not induce the P2Y12 and actin colocalization in lamellipodia and filopodia. Moreover, P2Y12-actin was always colocalized positively at lamellipodia and uropod in migratory microglia (Fig. 16H, I). Hence, Tau oligomers modulated the filopodia and uropod-associated actin remodeling in ATP-induced microglia, while aggregates preferred the P2Y12-localized lamella formation during migration (Fig. 16J).

3.5 Tau species modulate the filopodia-mediated actin remodeling, redundant of P2Y12 signaling

To study the role of P2Y12 signaling in microglial actin remodeling, Clopidogrel was used to inhibit the P2Y12 signaling for 24 hours. Here, we observed that Clopidogrel exposure reduced the number of filopodia extensions but not the lamellipodia arrangement in migratory microglia as compared to cell control. However, when the microglia were treated with extracellular Tau species along with Clopidogrel, the filopodia extensions were restored in microglia (Fig. 17A). But, the P2Y12 fluorescence level remained consistently low in Clopidogrel and Tau-exposed microglia while, the F-actin intensity remained unchanged in all treatment groups (Fig. 17B, C). Further, the quantification of microscopic images revealed that extracellular Tau species- monomer, oligomers and aggregates significantly induced the percentage of filopodia^{+} cells as well as the number of filopodia per cell, even upon blockage of P2Y12 signaling (Fig. 17D, E). Moreover, the colocalization of P2Y12 and actin in lamellipodia and uropod by Pearson's coefficient test revealed the insignificant alteration of colocalization in Clopidogrel-treated microglia. Also, the exposure of extracellular Tau species did not replenish the P2Y12-actin colocalization in migratory microglia (Fig. 17F, G). Furthermore, the western blot study showed that Clopidogrel exposure induced the expression level of P2Y12 receptor as compared to untreated control, while the P2Y12 level was upregulated even in Tau+ Clopidogrel exposed condition (Fig. 17H, I). Together, these results signify that the blockage of P2Y12 signaling resulted in more P2Y12 receptor expression which may contribute to the replenishment of Tai-induced actin remodeling in microglia. Hence, the extracellular Tau oligomers can influence the P2Y12-mediated and filopodia-associated actin remodeling but seem redundant to only P2Y12 signaling pathway.

3.6 Tau oligomers and aggregates enhance microglial chemotaxis, but monomer reduces transmigration

Tau species and ATP exposure were found to be involved in actin-mediated migratory structures remodeling in microglia, therefore the wound scratch assay can determine the rate of microglial migration *via* P2Y12 signaling. Microglia were exposed to extracellular Tau species, ATP and the wound closure was measured in a time-dependent manner up to 24 hours by phase contrast microscopic imaging. ATP acts as a positive regulator of chemotaxis. Here, we observed that Tau aggregates exposure has induced a rapid wound closure at 6 and 12 hours in migratory microglia, as compared to cell control. Monomer exposure showed an initial delayed migration at 6 hours, which was then increased from 12 to 24 hours and reached to equal wound closure at the final timepoint similar to aggregates. Tau oligomers-treated microglia have shown a constant elevated rate of migration in a time-dependent fashion, which resulted in the maximum wound closure at 24 hours (Fig. 18A). Altogether, it was evidenced that Tau oligomers and aggregates act as chemoattractants in microglial migration compared to aggregates (Fig. 18B). To understand the microglial invasion in response to extracellular Tau, the trans-well migration assay was performed along with ATP as a positive regulator. Here, we found that Tau oligomers-induced the microglial trans-well migration quite similar to ATP. Although, Tau aggregates induced the microglial 2D migration by wound-scratch assay, but the microglial invasion through

membrane-pore was not significant compared to cell control (Fig. 18C). Moreover, Tau monomer was found to reduce the number of invaded cells through the porous membrane during microglial chemotaxis. As a positive regulator of microglial migration, ATP has induced the trans-well as well as wound closure migration in microglia (Fig. 18D). Together, these results emphasized that among all the Tau species, oligomers can act as a better chemoattractant for the induction of microglial migration and tissue invasion (Fig. 18E).

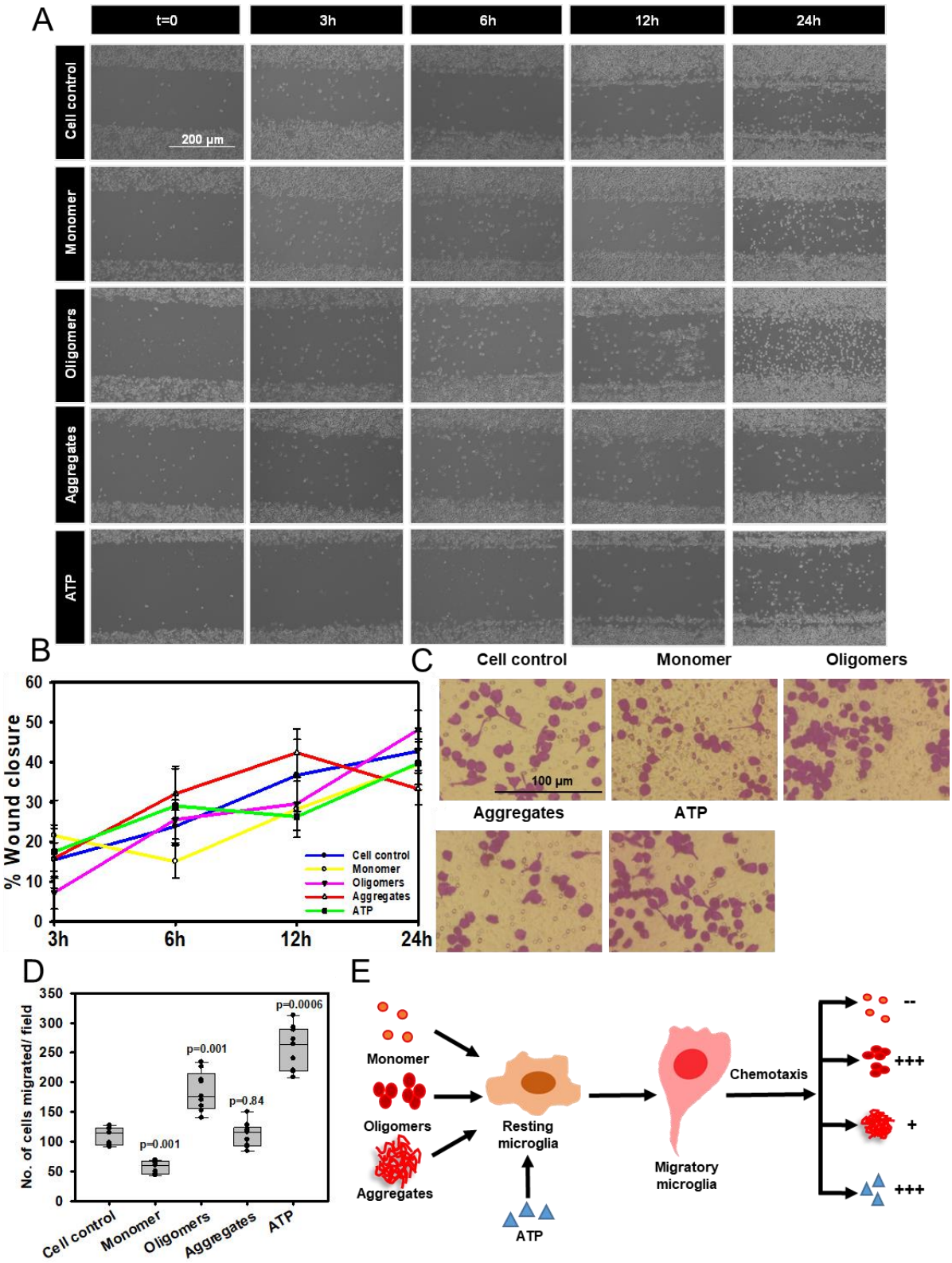

Figure 18. Tau oligomers elevate microglial migration and invasion, compared to aggregates

and monomer. A. Microglial monolayers were scratched and treated with various Tau species and ATP for time-dependent migration. Scale bar 200 µm. **B.** Tau aggregates exposure showed a prompt wound closure at 6 and 12 hours, oligomers showed a constant increased rate of migration with maximum wound closure at 24 hours. **C.** Microglia migrated maximally through the trans-well plate in response to oligomers similar to ATP. But, Tau monomer reduced the number of trans-migrated microglia. Scale bar 100 µm. **D.** Tau oligomers and ATP induced the maximum microglial invasion, while monomer reduced the invasion, as compared to cell control. **E.** Soluble oligomers have the highest capacity to induce microglial wound closure and invasion among all Tau species.

3.7 Tau exposure influences MTOC polarization in migratory microglia

Along with actin-associated migratory structures, the tubulin network and the position of the microtubule-organizing centre (MTOC) determine the migration axis and direction of the motile immune cells (Fig. 19A). To study the structure of microtubule network in microglial migration, the Tau-exposed N9 cells were stained with α-tubulin antibody and observed under fluorescence microscope.

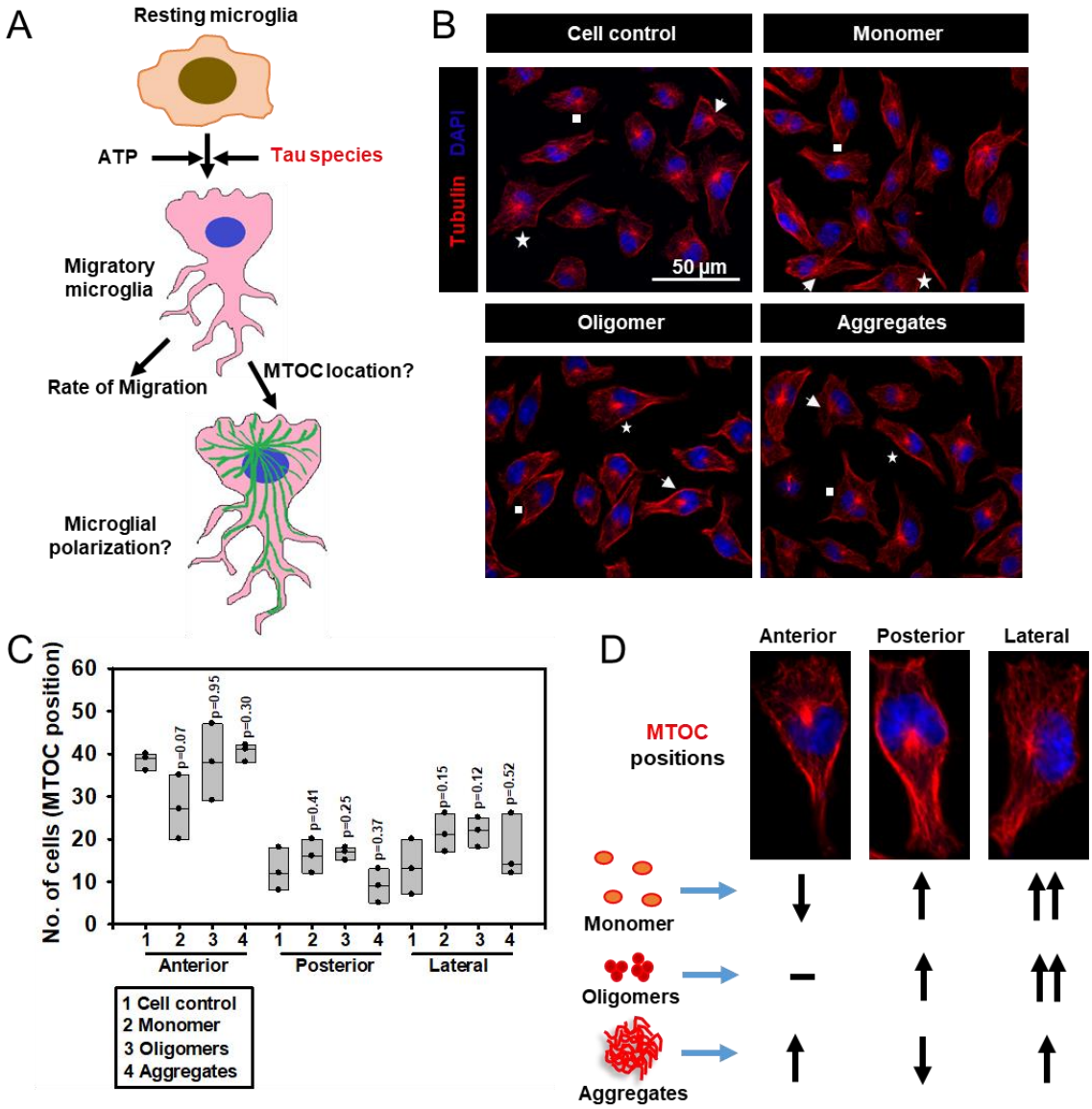

Figure 19. Tau oligomers and aggregates influence MTOC polarization in migratory microglia. A. Here, we hypothesized the MTOC polarization can relate to the Tau-induced migration. **B.** MTOC was visualized by α-tubulin staining in microglia and scale bar 50 µm. The star sign indicates the anterior MTOC, the arrow indicates the posterior MTOC and the box indicates the lateral MTOC position. **C, D.** Tau monomer exposure has induced more lateral and reduced anterior MTOC

position, while oligomers preferred all MTOC positions during microglial migration. But, the Tau aggregates led to a more lateral MTOC position and reduced posterior MTOC position for migration.

In our study, we found that Tau oligomers and aggregates exposure is majorly associated with the anterior position of MTOC in polarized microglia, which may relate to the rapid forward movement for the phagocytosis of Tau (Fig. 19B). The monomer exposure led to almost equal amount of anterior, posterior and lateral MTOC positions, which may signify a delayed migration and phagocytosis associated with microtubule network. But, the Tau aggregates exposure has resulted in more anterior but less posterior and few lateral MTOC positions which may relate to the migratory, phagocytic and inflammatory phenotype of microglia [257] (Fig. 19C). Hence, it is evident that extracellular Tau oligomers-induced the microglial migration through the modulation of various membrane-associated actin structures and tubulin networks by interacting with purinergic P2Y12 receptor-signaling (Fig. 19D).

3.8 Summary

During the study of Tau-induced microglial migration, we found for the first time that extracellular Tau can directly interact with purinergic P2Y12 receptor and mediates the formation of $P2Y12^{+}$ remodeled actin structures, especially filopodia and polarize MTOC localization in migratory microglia. ATP, as an activator of P2Y12 signaling, enhances the lamellipodia-filopodia formation and P2Y12 localization as an active component of migration in Tau-induced microglia. Therefore, the direct interaction of Tau and P2Y12 receptor might facilitate various significant inter-connected cellular functions in terms of P2Y12-mediated signal transduction, directional chemotaxis and receptor-mediated phagocytosis of extracellular Tau species for effective deposits clearance in the scenario of Tauopathy.

Chapter 4

Microglial degradation of Tau deposits by P2Y12-mediated podosome and filopodia formation

4.1 Background

Microglia rearrange the membrane-associated actin network for directional migration, tissue invasion, phagocytosis, vesicular trafficking and immune effector function mediation [97, 258, 259]. Previously, the microglia were reported to migrate toward the area of plaque accumulation, neuronal damage and inflammation in various neurodegenerative diseases [260]. Various actin microstructures were formed in migratory immune cells which include-lamellipodia, filopodia, podosome, invadopodia, focal adhesion-stress fibers and cortical actin sheet, etc [84, 261]. Lamellipodia is a fan-shaped frontal edge of the cell, containing cross-linked and branched actin organization which is firmly attached to the cortical substratum layer to generate tensile force for forwarding movement. The filopodia are finger-like projections with parallel-bundled actin networks which are involved in adherence, object trapping, and mechano-transduction [262]. Podosome are the protrusive ventral actin structure with a cross-linked actin core, surrounded by vinculin ring and adhesion receptors such as integrins [86]. Podosome are dynamically formed at the cell periphery in contact with focal adhesion and eventually relocate to the lamellipodia for matrix adhesion, degradation/remodeling for migration, and tissue invasion [263-265]. Various actin structures including podosome are formed by the induction of extracellular growth stimuli, immune activation, tissue invasion or protein deposit degradation by immune cells [266]. The Src kinase and TKS5 scaffold protein interconnect the extracellular signaling to the intracellular effector functions by regulating actin flux, nucleation and podosome positioning during migration or tissue evasion [260]. P2Y12 pathway is also associated with the Ca^{2+} signaling and the Ca^{2+} ion channels, Iba1 and calmodulin proteins as the integral component of podosome to mediate directional migration in microglia [267, 268]. The previous report showed that the impaired P2Y12 signaling and actin-associated microglial migration become hampered due to aging [150]. Therefore, the structural organization of podosome and other actin structures in association with Arp2 nucleator and TKS5 adaptor colocalization would be studied in Tau-induced microglial migration. Moreover, the involvement of the P2Y12 receptor and its signaling activation or blockage would be emphasized in relation to migratory actin structures, and actin-associated Tau deposits degradation by microglia.

4.2 Tau oligomers induce the formation of Arp2-localized F-actin-rich actin structures

Here, we studied the structural formation of individual actin microstructures such as podosome, filopodia and uropod in association with actin nucleator protein Arp2. We observed that Tau exposed microglia induced the formation of podosome structures colocalized with Arp2 in frontal lamellipodia. The podosome accumulated more upon Tau oligomers exposure than monomer and ADP as a P2Y12 signaling activation control. The 3D microscopic images revealed the accumulation of $Arp2^{+}$ podosome at more amount in Tau oligomers-treated microglia than monomer (Fig. 20A). The quantification of microscopic images depicted that Tau oligomers have induced the number of podosome-bearing microglia by 20% more than the monomer exposure and untreated cell control (Fig. 20B). Furthermore, the podosome-associated area was also increased by both the Tau species-monomer and oligomers, in migratory microglia as similar to ADP (Fig. 20C). The western blot study showed no significant changes in the expression of Arp2 protein in Tau-induced or ADP-induced microglia (Fig. 20D, E). Next, we found that extracellular Tau and ADP induced the Arp2-decorated filopodia and branched uropod formation in microglia, emphasizing more cortical adhesions. The microscopic image quantification showed that the number **of** filopodia from individual cells were increased by two times in Tau monomer exposure and three times in oligomers exposure than in cell control (Fig. 20F, G) Similarly, the Tau oligomers reduced the level of Arp2 fluorescence in the uropod, which may signify the rapid actin turnover and nucleation at the lamella from the uropod required for induced migration (Fig. 20H). Therefore, the extracellular Tau oligomers may facilitate the Arp2-associated actin rearrangements in podosome and filopodia by the altered actin turnover towards lamella from uropod for migration and active phagocytosis (Fig. 20I).

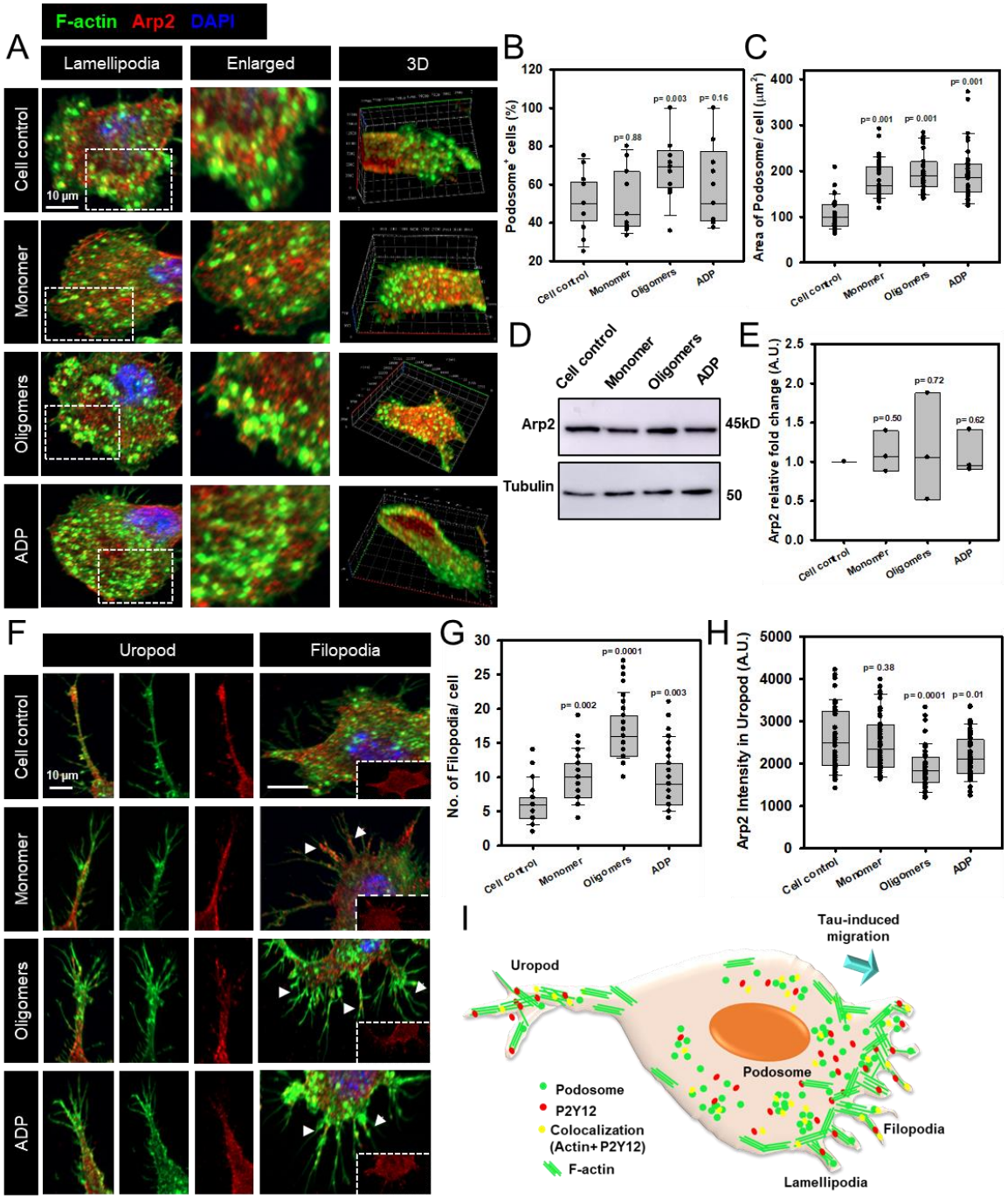

Figure 20. Tau oligomers induce the formation of podosome, filopodia and uropod, colocalized with actin nucleator protein Arp2. A. Extracellular Tau oligomers induced more podosome accumulation in lamellipodia than monomers in association with Arp2. Scales bar 10 µm. **B.** Tau oligomers have induced the podosome$^+$ microglia than monomer. **C.** The podosome-associated area has increased twice in Tau-exposed microglia than cell control. **D, E.** The Arp2 expression level remained unaltered in Tau and ADP exposure by WB and densitometry analysis. **F.** Tau oligomers facilitated the Arp2$^+$ filopodia and branched uropod structures in migratory microglia. Scale bar 10 µm. **G.** Tau monomer, oligomers and ADP increased the filopodia number in microglia. **H.** The Arp2 level was reduced from uropod upon Tau oligomers exposure **I.** Tau oligomers induced the accumulation of podosome and filopodia by localizing Arp2 at lamellipodia in migratory microglia

4.3 Tau oligomers induce podosome clusters in lamellipodia

The western blot study showed that the TKS5 protein level was unchanged in various Tau treatments and ADP-induced migratory microglia, although the podosome area has

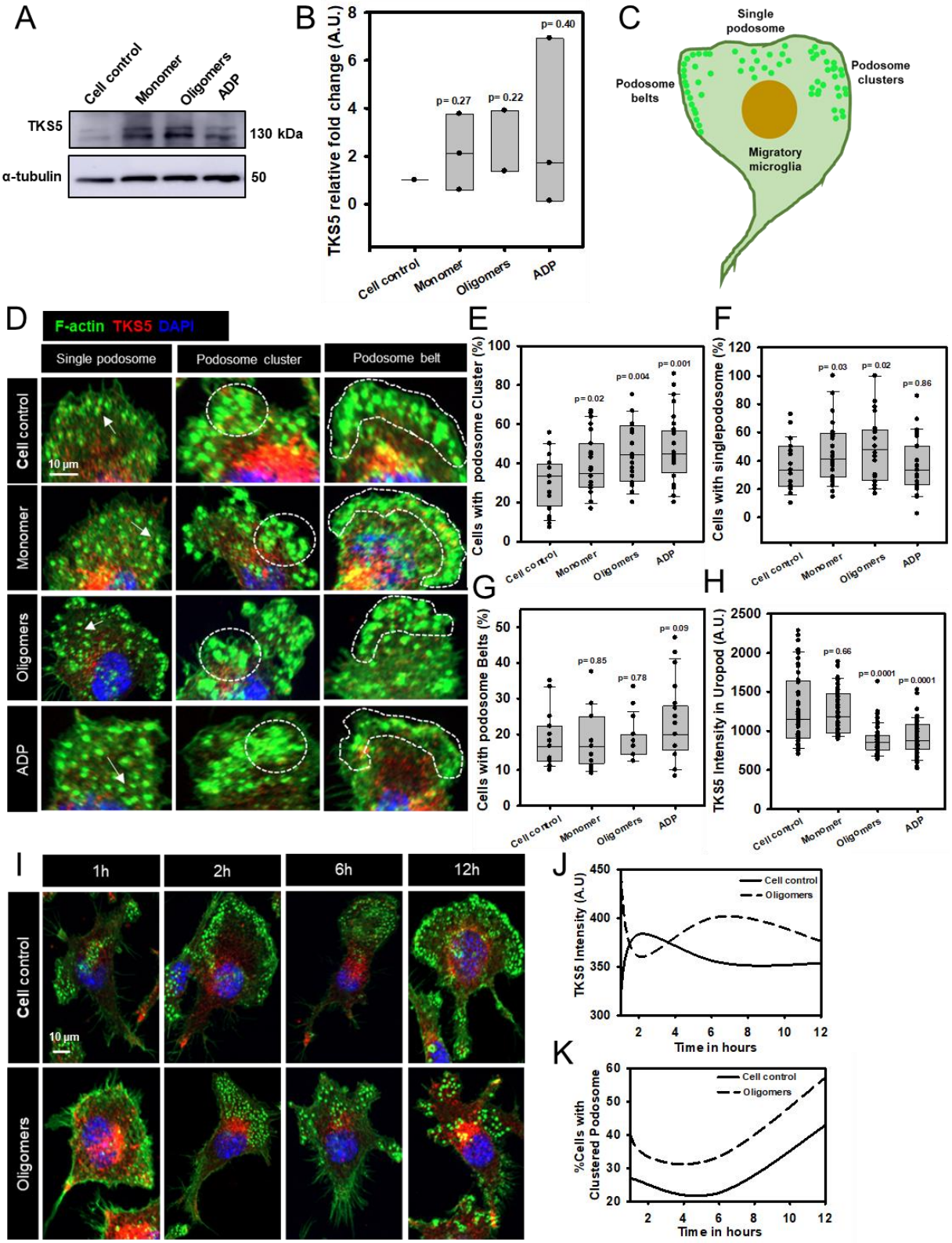

Figure 21. Extracellular Tau induces the accumulation of podosome clusters in microglial lamellipodia. A, B. The western blot analysis showed insignificant changes of TKS5 expression upon Tau and ADP treatment. **C.** Microglia displayed various rearrangements of podosome. **D.** The podosome clusters were more evident in oligomer-treated microglia than monomer exposure. Scale bar 10 µm. **E.** The clustered podosome accumulated more in lamellipodia upon Tau Tau exposure than cell control. **F, G.** The single podosome arrangement and podosome belts remained unaltered in Tau-treated groups. **H.** TKS5 intensity in uropod was decreased in Tau-induced microglia. **I.** Extracellular Tau oligomers induced the accumulation of clustered podosome in microglial

lamellipodia in a time-dependent manner. Scale bar 10 µm **J, K.** TKS5 intensity and the clustered podosome were increased from 4 hours of oligomers exposure as compared to cell control.

increased significantly in Tau/ADP-induced conditions (Fig. 21A, B). In migratory and immune cells, various podosome rearrangements can be witnessed, such as podosome clusters, belts, rosettes, and single podosome. However, the exact functions and occurrences of these individual podosome organizations were not yet known [260] (Fig. 21C). In our study, we observed that microglia formed various podosome structures- clusters, belts, and single podosome upon extracellular Tau oligomers and ADP stimuli (Fig. 21D). From the microscopic images, we quantified that podosome clusters-containing microglial cell have significantly increased by 20% upon Tau oligomers and ADP treatment as compared to monomer and cell control (Fig. 21E). While the other podosome arrangements- single podosome and belts, became elusive in Tau/ADP-induced microglia (Fig. 21F, G). Like Arp2 intensity, the TKS5 fluorescence was also reduced in the uropod of Tau/ADP-treated microglia (Fig. 21H). These results might emphasize the skewed turnover of actin-associated proteins- Arp2 and TKS5 from uropod to lamellipodia for podosome and filopodia formation during Tau-induced microglial migration and Tau degradation. Furthermore, we have studied the time-dependent podosome cluster formation, colocalized with TKS5 in Tau-oligomers-induced microglia (Fig. 21I). Here, we found that the podosome clusters^{+} microglia started accumulation from the 6th hour of Tau oligomers exposure till 12 hours, compared to the untreated control. Similarly, the TKS5 fluorescence intensity was found to be elevated time-dependently *i.e.,* from 4 to 8 hours of oligomers exposure in migratory microglia (Fig. 21J, K). Hence, it is evident that microglia preferentially organize the podosome cluster formation in lamellipodia and elevate the TKS5 level at early hours of Tau oligomers-induced migration.

4.4 Tau oligomers influence the P2Y12^{+} podosome and filopodia formation in microglia

In our study on Tau oligomers-induced microglial migration, P2Y12 was found to be colocalized with the F-actin-rich podosome structures at lamellipodia. The 3D microscopic images of microglial lamellipodia depicted that Tau oligomers have induced the accumulation of P2Y12^{+} clustered podosome at the front edge while monomer exposure resulted in single podosome formation (Fig. 22A). Therefore, the spline-fluorescence intensity quantification at the podosome clusters showed the maximum colocalization points of F-actin-P2Y12 at 14 µm distance, in Tau oligomers-exposed microglia (than monomer) (Fig. 22B). This result suggests that this purinoceptor P2Y12, which dictates the microglial directionality during chemotactic movements, can be an integral component of podosome-mediated adhesion and matrix remodeling. Next, we studied the localization of P2Y12 receptor in filopodia and rear uropod in Tau-induced migratory microglial. Here, we further observed that extracellular Tau oligomers and ADP induced the P2Y12-associated filopodia formation and the P2Y12 localization in branched uropod structures of migratory microglia (Fig. 22C). Microscopic quantification showed that extracellular Tau oligomers increased the percentage of microglia bearing P2Y12^{+} filopodia by almost 20% as compared to monomer-treated and cell control. Moreover, the localization of P2Y12 was increased in microglial rear ends-uropod upon oligomers exposure, alike Arp2 and TKS5 (Fig. 22D, E). Therefore, these observations might suggest that the P2Y12 localizes in various actin structures for cell adhesion in Tau oligomers-induced microglial migration.

4.5 Tau aggregates induces podosome cluster formation, but displaces TKS5 and P2Y12 from podosome

In order to identify the extracellular Tau aggregates-induced actin remodeling, microglia were treated with Tau fibrils and monomers as a control for 24 hours. Tau monomer and aggregates did not alter the expression level of Arp2 and TKS5 scaffold protein in microglia as observed by WB study (Fig 23A-D). But, the preformed Tau fibril-induced the localization of Arp2 in F-actin-rich podosome and filopodia structures of microglial lamellipodia,

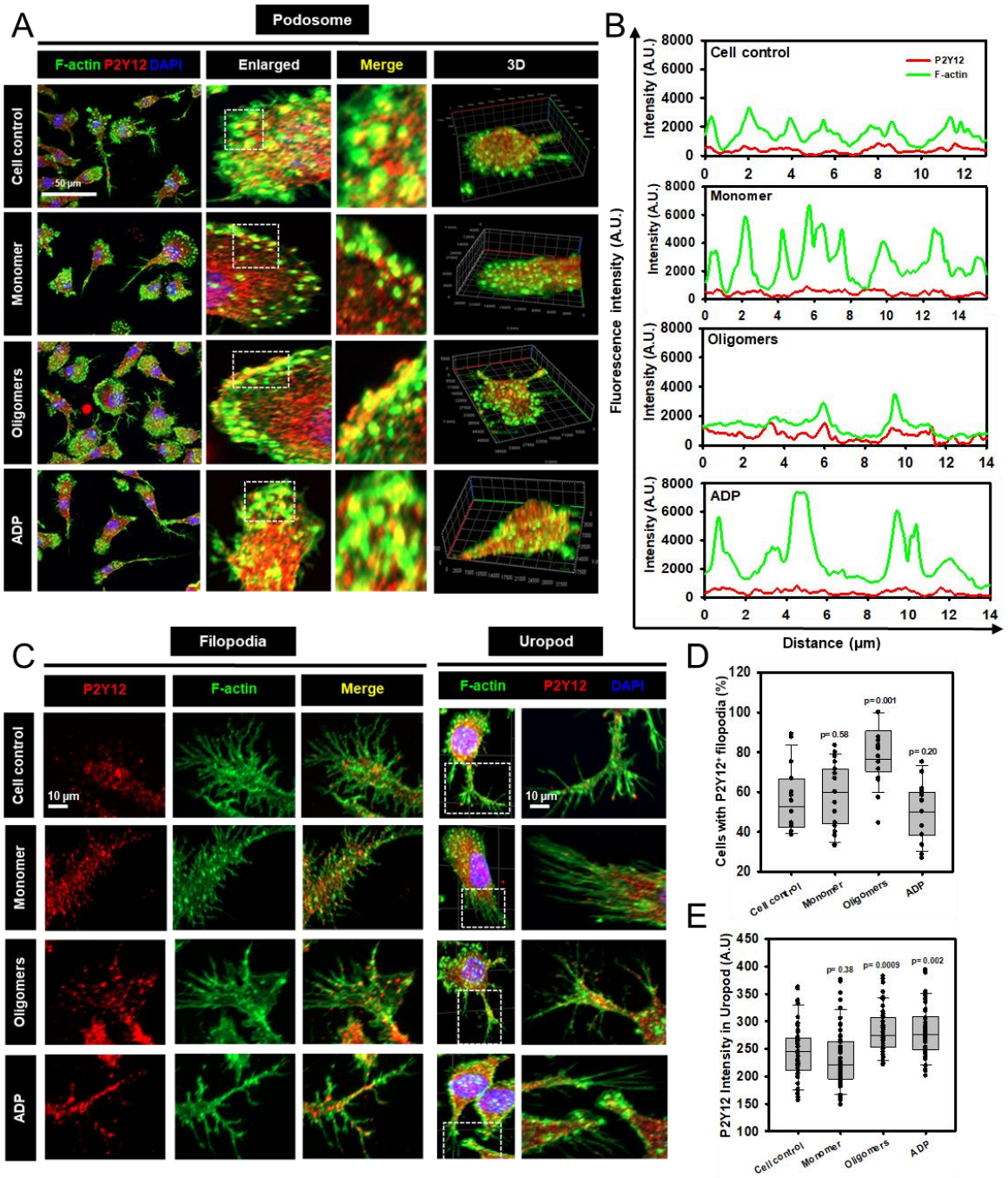

Figure 22. P2Y12 localizes in actin structures in Tau-exposed migratory microglia. A. P2Y12 receptor was colocalized with F-actin-rich podosome in lamellipodia during Tau-induced microglia migration. Scale bar 10 µm. **B.** Spline intensity of podosome-rich lamella (box marked area) was quantified in Tau-treated groups for the colocalization of P2Y12 and F-actin. **C.** P2Y12 was colocalized with F-actin in filopodia and branched uropod of microglia upon Tau oligomers exposure. Scale bar 10 µm. **D.** $P2Y12^{+}$ $filopodia^{+}$ microglia population has significantly increased upon oligomers exposure than monomer. **E.** P2Y12 intensity has increased in uropod by Tau oligomers and ADP exposure in migratory microglia.

compared to monomer treatment (Fig. 23E). During the process of mechanosensing, individual podosome appeared at protruding lamella. Then, multiple podosome got merged to form podosome clusters and belts to induce the ECM remodeling. Rosettes are circular podosome arrangement that involves in matrix remodeling and migration in smooth muscle cells and endothelial cell [262]. Here, microglia formed various podosome arrangements such as- podosome rosettes, belt, clusters and single podosome upon extracellular Tau

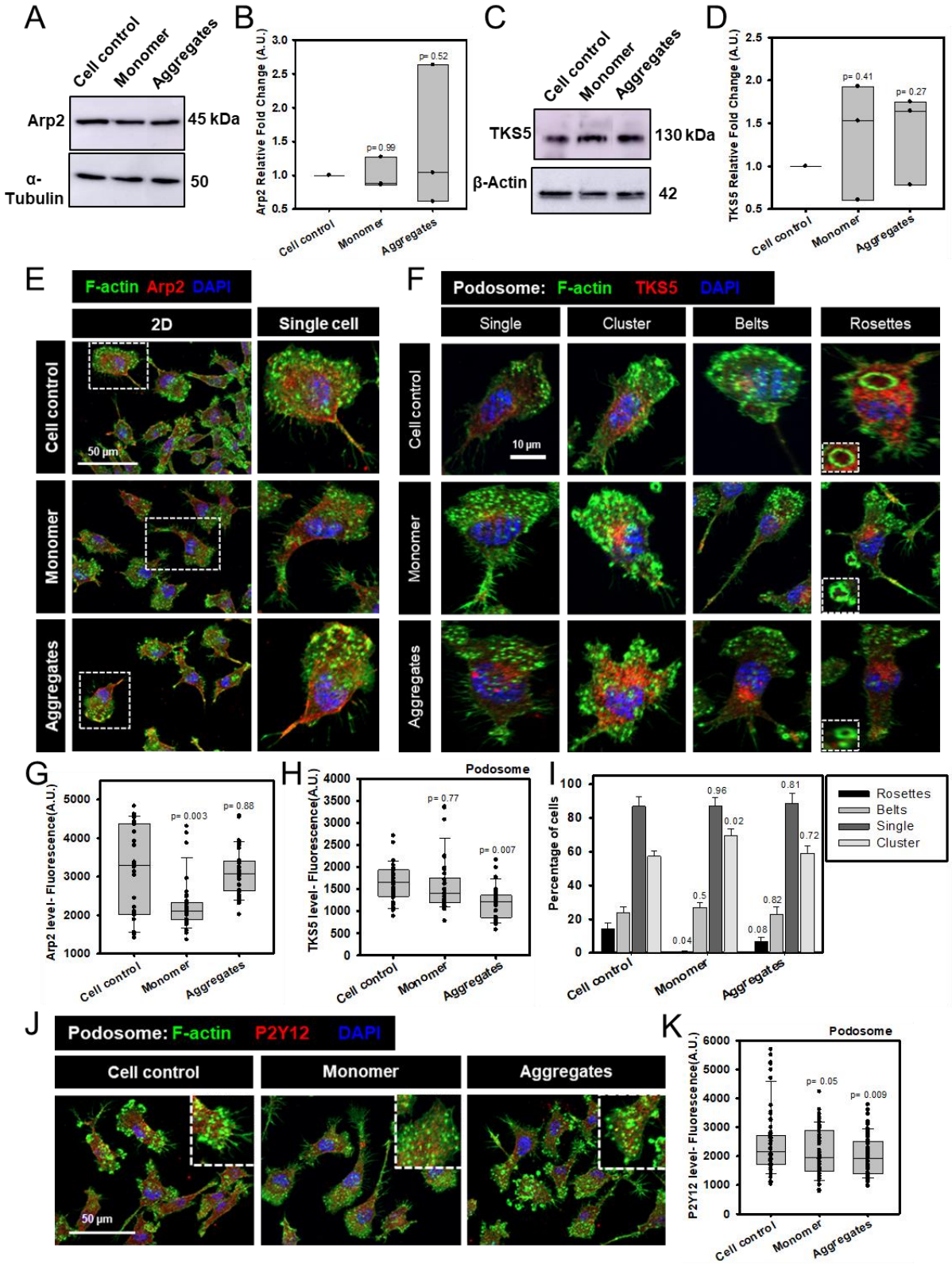

Figure 23. Actin remodeling in Tau aggregates-induced microglia. A-D. Tau aggregates did not alter the expression of the Arp2 and TKS5 proteins by WB in N9 microglia. **E.** Tau aggregates induced the formation of Arp2-localized podosome in microglia. Scale bar 50 µm. **F.** Tau monomer and aggregates displayed various podosome rearrangements in lamella. Scale bar 10 µm. **G.** The microscopic quantification showed a reduced level of Arp2 in monomer-exposed microglia than aggregates. **H.** TKS5 fluorescence level was reduced in Tau aggregates-exposed microglia than monomer. **I.** Tau monomer induced podosome clustering and reduced the rosettes bearing microglia. **J.** The formation of $P2Y12^{+}$ podosome was evidenced in Tau-induced microglia. Scale bar 50 µm. **K.**

P2Y12 intensity was reduced from podosome-containing areas upon Tau exposure in migratory microglia.

treatment (Fig. 23F). The quantification of microscopic images revealed that the Arp2 fluorescence level from individual microglia became decreased in monomer treatment but remain constant upon Tau aggregates exposure (Fig. 23G). Similarly, the aggregates exposure has reduced the level of TKS5 fluorescence from podosome structures in migratory microglia than the monomer-treated groups (Fig. 23H). In our study, we quantified that extracellular Tau monomer has induced the podosome clusters formation and reduced the rosettes structures in migratory microglia. But, Tau aggregates did not alter the occurrences of podosome cluster- or rosette-bearing microglia (Fig. 23I). Moreover, we found that Tau monomer and aggregates exposure resulted in P2Y12-localized podosome and filopodia formation in microglia by immunofluorescence microscopy. But, quantitatively the P2Y12 fluorescence level was reduced from the podosome-associated area in Tau monomer and aggregates-exposed microglia (Fig. 23J, K). Therefore, it is evident that Tau aggregates alike oligomers can induce podosome accumulation during microglial migration, but displaced the actin-binding proteins- Arp2 nucleator, TKS5 scaffold and also purinergic P2Y12 receptor from podosome structure in microglia. Hence, the pathway of actin remodeling and P2Y12-mediated chemotaxis might be divergent in Tau aggregates-induced microglial migration.

4.6 The inhibition of P2Y12 signaling reduces the Tau-induced microglia migration and invasion

Previously we showed that extracellular Tau oligomers act as a better chemoattractant in the induced microglial wound closure and trans-migration. To identify the involvement of P2Y12 signaling in Tau-induced microglial migration, P2Y12 signaling was activated and blocked by ADP and Clopidogrel, respectively, then the migration was measured by % wound closure and trans-migrated cell/ field. Here, we observed that both monomer and oligomers have induced the wound closure *i.e.,* microglial migration by almost two times (40%), alone and together with ADP as compared to cell groups (20%). While the blockage of P2Y12 signaling by Clopidogrel significantly reduced the % wound closure by 25% as compared to the ADP-treated groups (40%) (Fig. 24A). However, the Clopidogrel exposure has reduced the microglial migration when treated along with both the Tau species- monomer at 25% and oligomers at 20%, as compared to only Tau and ADP+Tau treatment groups (Fig. 24B). Next, we studied the trans-well migration of microglia in response to Tau along with ADP and Clopidogrel exposure. Interestingly, we found that the Tau oligomers exposure increased the microglial trans-migration by two times as similar to only ADP and ADP+ oligomers-group than cell control. But, the Tau monomer itself has reduced the microglial invasion to half than cell control, which became significantly increased by three times in the ADP+ monomer treatment condition (Fig. 24C). The P2Y12-blockage led to a reduced level of microglial trans-migration than ADP induction. But, the Tau oligomers could reverse the level of trans-migration (around 1.5 times) even upon P2Y12-blocked conditions (Fig. 24D). Hence, these results may signify that P2Y12 signaling positively influences microglial migration and invasion, but only the Tau oligomers might have the potential to intervene in P2Y12-mediated chemotaxis during disease conditions.

4.7 Microglia degrade Tau monomer more than oligomers *via* $P2Y12^{+}$ podosome and filopodia formation

The role of P2Y12 receptor in migratory actin structures- podosome and filopodia and in protein degradation are a matter of investigation. To mimic the scenario of plaque degradation, the fluorescence-tagged Tau monomer and oligomers were coated onto the coverslips and then microglia were allowed to degrade the protein deposits. The hollow zone of degradation in colocalization with actin microstructures can be considered and quantified to understand the microglia-mediated Tau clearance. In our study, we observed that microglia degraded the Tau monomer and oligomers deposits through the $P2Y12^{+}$ F-actin-

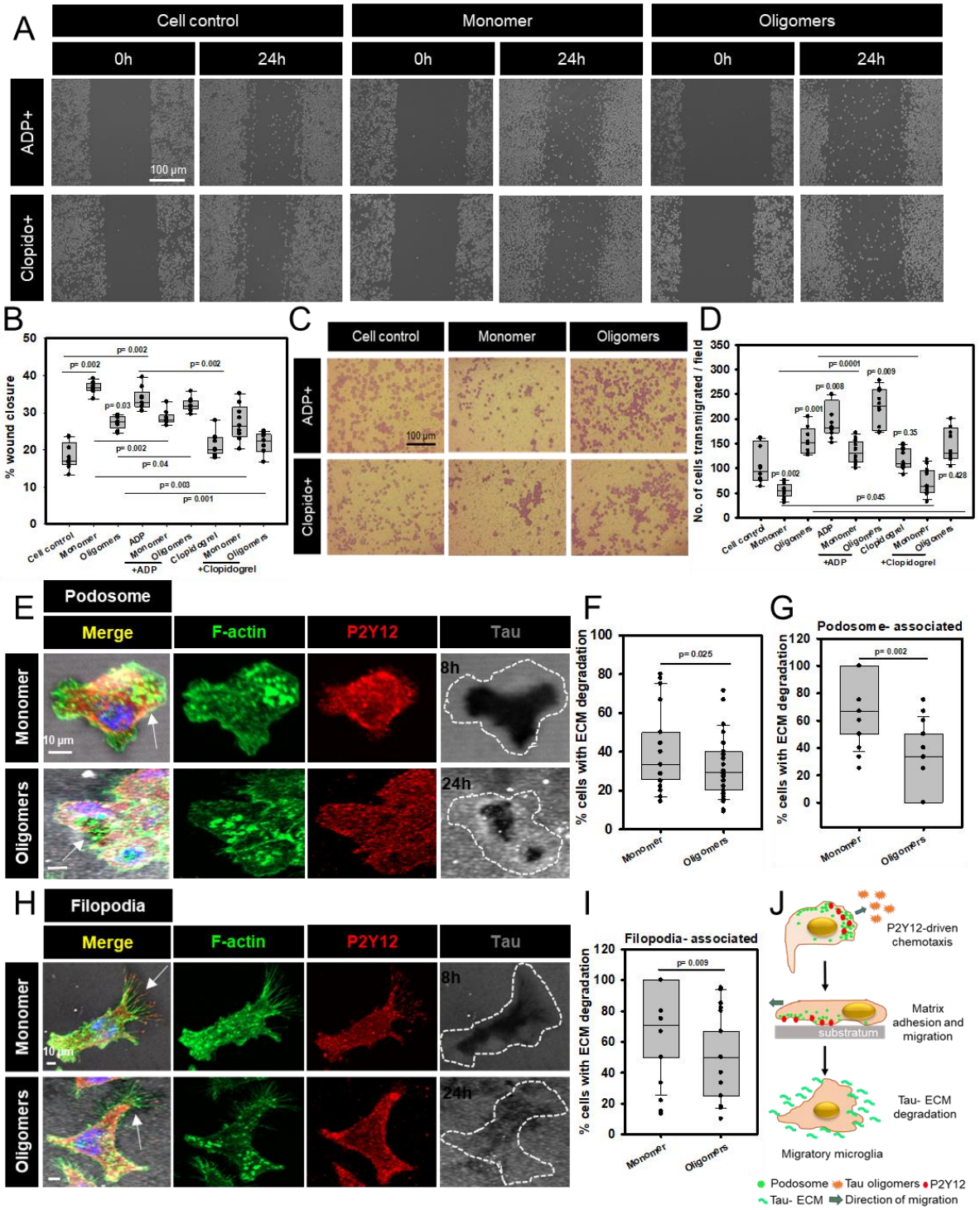

Figure 24. P2Y12 signaling influences microglial migration and Tau-deposits degradation. A, B. Tau and ADP, together and separately induced the microglial migration as quantified by %wound closure at 24 hours. Clopidogrel reduced microglial migration but, Tau+ Clopidogrel co-treatment replenished the microglial migration. Scale bar 100 µm. **C, D.** Clopidogrel exposure has significantly reduced the microglial invasion, which was eventually restored by Tau oligomers exposure. Tau monomer induced the microglial trans-migration only in ADP induction. Scale bar 100 µm. **E.** Microglia degraded Tau monomer deposits by 8 hours and Tau oligomers deposits by 24 hours through the formation of the F-actin-rich $P2Y12^{+}$ podosome. Scale bar 10 µm. **F, G.** Microglia preferentially degraded Tau monomer deposits more than oligomers by $P2Y12^{+}$ podosome. **H, I.** Microglia degraded Tau monomer more than oligomers deposits by $P2Y12^{+}$ filopodia formation. Scale bar 10 µm. **J.** Microglial P2Y12 signaling mediated various functions which include- Tau-induced chemotaxis, matrix adhesion, migration and Tau deposits degradation via actin microstructures- podosome and filopodia formation.

rich podosome formation, which were colocalized with a hollow degradation area. Interestingly, we found that monomer deposits were degraded after 8 hours of cell seeding while; the degradation of oligomers deposits were not started by microglia at 8 hours. Furthermore, the microglia started the degradation of oligomers deposits after 24 hours of cell seeding (Fig. 24E). But, at that incubation time, the monomer deposits were completely degraded. Quantitatively, microglia preferred to degrade Tau monomers more than Tau oligomers, as 41% and 33% of the microglial populations were positive for monomer and oligomers deposit degradation, respectively (Fig. 24F). Among the degradation^{+} population, 72% of the microglia were associated with monomer-deposit degradation but only 26% of the microglia could degrade Tau oligomers through the formation of podosome (Fig. 24G).

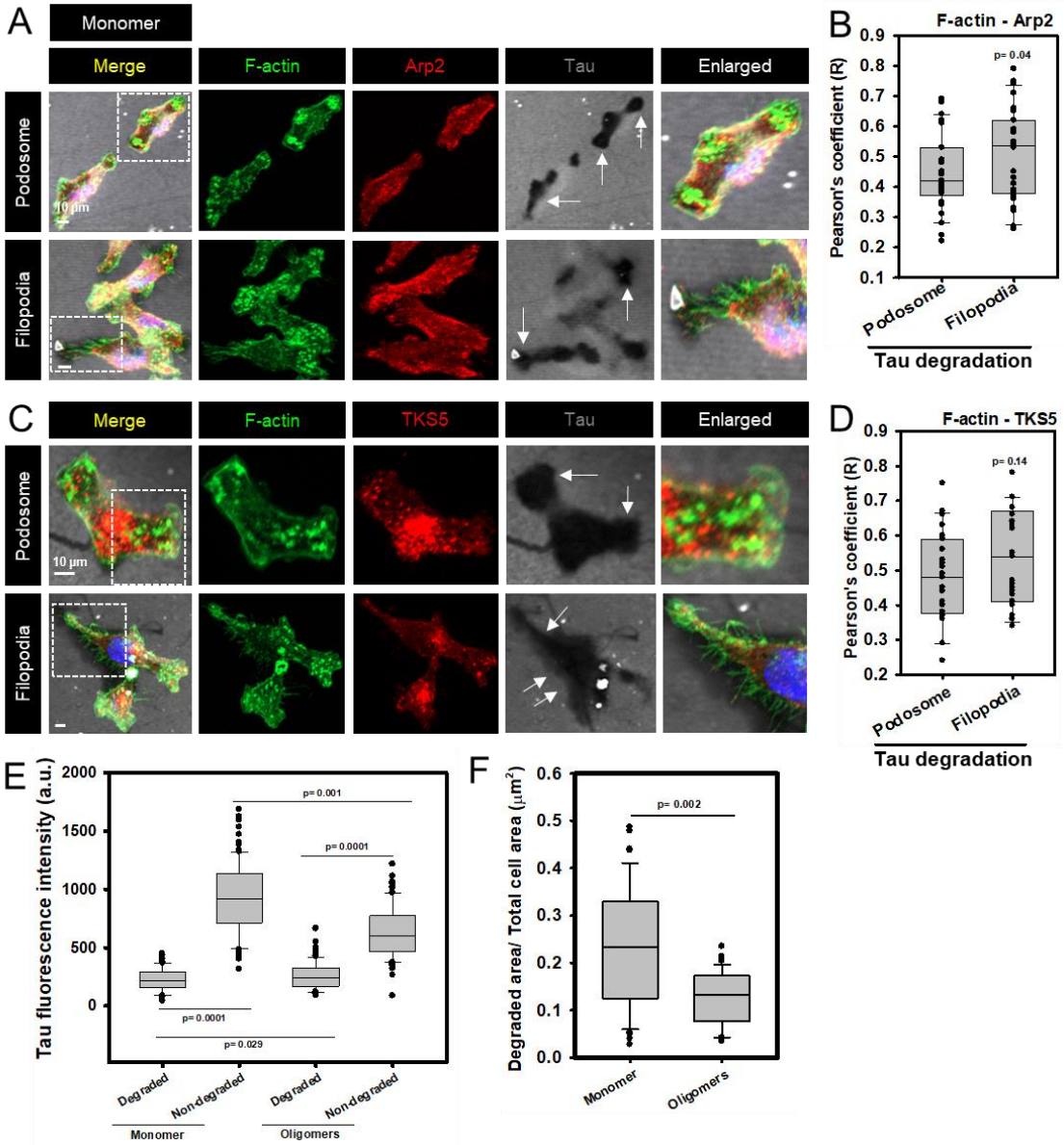

Figure 25. Microglia preferentially degrade Tau monomer deposits *via* Arp2 and TKS5-localized podosome and filopodia formation. A. Microglia degraded Tau monomer deposits through the Arp2^{+} podosome and filopodia formation and scale bar 10 µm. **B.** Arp2 was found to be colocalized more with filopodia than podosome at the Tau degradation area. **C, D.** Similarly, TKS5 adaptor protein became colocalized with podosome and filopodia at the site of Tau deposits degradation where the colocalization of F-actin and TKS5 did not alter. scale bar 10 µm. **E.** Particularly, Tau monomer was significantly degraded more than oligomers in microglia-mediated deposit degradation. **F.** The quantification of the relative degraded area/ total cell area revealed that monomer-deposits were degraded more than oligomers deposits.

Similarly, we investigated the occurrences of filopodia in the Tau deposit degradation. So, we found for the first time that microglial filopodia colocalized with P2Y12, performed the degradation of Tau deposit (both monomer and oligomers). Quantitatively, 70% of microglia degraded the Tau monomer deposit while; 50% of the cells were positive for oligomers deposit degradation through the formation of filopodia (Fig. 24H, I). Cumulatively, these results signify that P2Y12 signaling can also participate in the podosome and filopodia-associated substrate adhesion as well as microglial migration to mediate plaques degradation, here Tau deposits (Fig. 24J).

4.8 Microglia degrade Tau deposit by Arp2-localized podosome and filopodia formation

Microglial podosome and filopodia, which were associated with Arp2, were evidenced to be localized in the Tau-deposit degradation area (Fig. 25A). The Pearson's coefficient analysis showed that the filopodia contained more colocalization of Arp2 and F-actin than podosome during microglia-mediated Tau deposit degradation (Fig. 25B). Similarly, the TKS5-associated podosome and filopodia degraded the Tau deposit. But, the TKS5 and F-actin colocalization in the degradation-associated podosome and filopodia did not alter in microglia (Fig. 25C, D). Thus, it is evident that filopodia-mediated Tau degradation might require faster actin nucleation by Arp2 than podosome-mediated degradation. For better understanding, we further quantified the Tau fluorescence intensity from degraded vs. non-degraded spots within the microglia. Then, we found that the Tau monomer and oligomers intensity was significantly decreased in the Tau-degraded area as compared to non-degraded spots. The monomer fluorescence was comparatively reduced more than the oligomers intensity in the deposits degradation area (Fig. 25E). Further, the quantification of the relative Tau-degraded area (degradation area/Total cell area) depicted that the monomer deposits were degraded significantly more than the oligomers-deposits (Fig. 25F). Hence, these results signify that microglia preferentially degrade Tau monomer deposits than the bulky toxic oligomers which can relate to the complicated elimination and subsequent accumulation of oligomers in AD conditions.

4.9 The P2Y12 signaling blockage reduces Tau-deposit degradation

Microglial P2Y12 dictates the actin-mediated directional migration, filopodia formation and secretes various proteases for matrix remodeling to reach the damage site [164, 257, 269]. Here, we were further interested to study the function of P2Y12 signaling in Tau deposits degradation. We observed that the ADP-induced microglia showed increased filopodia formation during Tau deposits degradation, but the blockage of P2Y12 signaling by Clopidogrel did not alter the level of remodeled actin structures (Fig. 26A). Microscopic quantification revealed that the P2Y12 signaling inhibition resulted in a reduced level of Tau-deposits degradation while; ADP induction did not alter extracellular Tau degradation (Fig. 26B). Similarly, the podosome and filopodia were colocalized with Arp2 and TKS5 in the Tau deposits degradation area, which correlated actin nucleation and podosome formation in microglia (Fig. 26C, D). The colocalization analysis depicted that Clopidogrel induction lead to more accumulation of Arp2 with F-actin. At the same time, the ADP-mediated activation resulted in less TKS5 localization in podosome and filopodia in Tau-degrading microglia (Fig. 26E, F). Therefore, the innovative functions of this P2Y12 receptor in chemotaxis, Tau interaction, integration in actin structure and deposits degradation can be intervened therapeutically in the microglia-mediated Tau clearance in AD.

4.10 Summary

This study aimed to understand the various functions of P2Y12 receptor in Tau oligomers-induced microglial migration and actin remodeling. We found that extracellular Tau oligomers facilitate the Arp2 and TKS5-localized podosome clusters and filopodia formation through

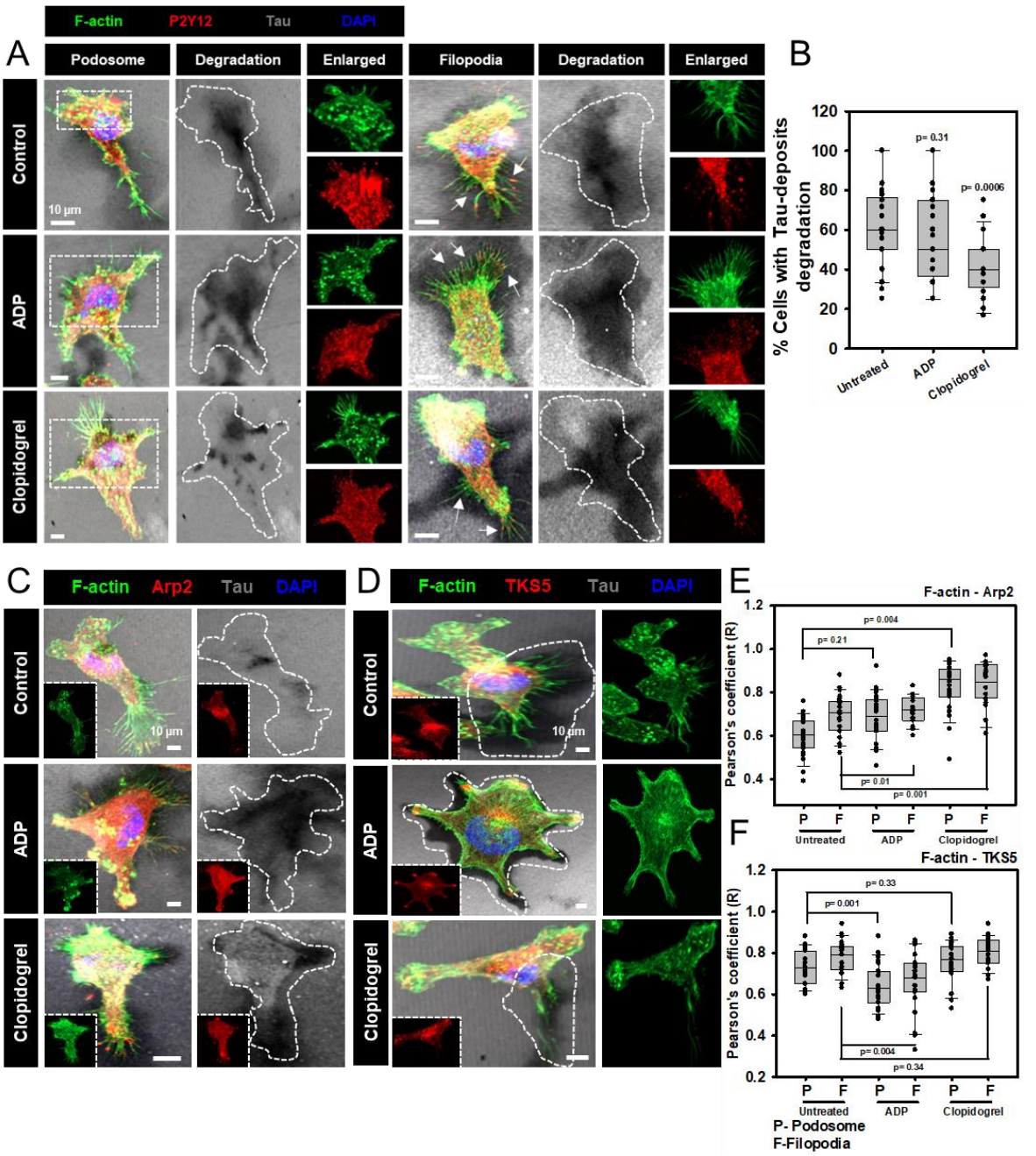

Figure 26. Blockage of P2Y12 signaling reduces Tau deposit degradation. A, B. P2Y12 activation by ADP has induced more filopodia formation for Tau deposit degradation. Microglia degraded Tau deposits as small scattered spots upon Clopidogrel treatment. Scale bar 10 µm. **C, D.** P2Y12 activation resulted in more Arp2-localized filopodia formation and reduced TKS5 localization in podosome and filopodia during Tau deposit degradation. Scale bar 10 µm. **E, F.** Pearson's coefficient analysis showed that P2Y12 inhibition led to increased Arp2-F-actin colocalization but, P2Y12 activation resulted in reduced TKS5-F-actin colocalization in podosome and filopodia formation for Tau degradation.

the involvement of P2Y12 signaling. Moreover, the P2Y12 signaling not only influences the microglial migration and invasion but also regulates the Tau-deposits degradation through the formation of F-actin-rich podosome and filopodia. Hence, the P2Y12 signaling was demonstrated to mediate various functions in Tauopathy-associated microglia, those are-migration, actin remodeling, phagocytosis and the clearance of Tau deposits in AD condition.

Chapter 5

P2Y12-mediated endocytosis, vesicle trafficking and lysosomal degradation of Tau in migratory microglia

5.1 Background

The intermediate Tau oligomers are short-lived but highly reactive species during aggregation, which causes neuronal toxicity, synaptic loss, cognitive decline and neuroinflammation [270]. In age-related neurodegeneration, microglia migrate towards the damaged neurons by transforming from ramified to amoeboid phenotype to exhibit directional migration, inflammation and phagocytic clearance [103]. Purinergic signaling plays multi-functional roles important in CNS homeostasis, microglial directional migration, synaptic surveillance and phagocytosis [259]. Microglial purinergic receptor P2Y12 forms cellular junction and bulbous actin-tip extension to maintain neuronal health through cAMP, regulates Ca^{2+} signaling-related migration and diminished immune activation [165, 253, 271]. Similarly, it was reported that the DAMs were associated with impaired P2Y12 signaling and actin-associated migration due to aging [272]. Other purinoceptors- P2Y6 and P2Y4 were reported to perform phagocytosis and liquid-phase macropinocytosis of Aβ for the clearance of plaque deposits in brain [198, 273]. Extracellular Tau can traverse through the neuroanatomical network by prion-like propagation and spread Tauopathy in a seed-dependent manner [274]. Various studies showed that the abnormalities of endolysosomal pathways and impaired processing of Aβ and Tau can directly contribute to the development of AD and other neurodegenerative diseases [275, 276]. On the other hand, the activation of endosomal pathways would be beneficial for trapping and clearance of extracellular amyloids to prevent disease progression. Previously, it was shown that the GPCR-associated desensitization protein β-arrestin1 was upregulated in AD brain and induced Aβ pathology and behavioral deficit through γ-secretase complex assembly in the transgenic mice model [277]. Similarly, β-arrestin1 along with β2-adrenergic receptor and metabotropic glutamate receptor2 exhibited the pathogenic Tau accumulation, microtubule network destabilization and autophagic flux impairment in FTLD patient's brain and AD mice model [278]. Moreover, various endocytosis-regulating proteins- BIN1, PICALM and SYNJ1 were found to directly interact with Tau which can emphasize the coupled endocytosis or altered subcellular localization of pathological Tau, leading to the clearance [279, 280]. Previously, we showed that microglia phagocytose extracellular Tau oligomers by membrane-associated actin remodeling [281]. In this scenario, we would study the role of P2Y12 receptor in Tau endocytosis in association with localized F-actin network and by the β-arrestin-1-mediated desensitization process. Further, the endocytic localization with early (Rab5), late (Rab7) and recycle vesicle (Rab11) and lysosomal (Lamp2A) degradation of monomer and oligomers were studied by fluorescence microscopy. Therefore, this study would emphasize the potential of microglia in receptor-mediated Tau endocytosis and cellular trafficking which is important to emphasize on the pathoprotein clearance in Tauopathy condition.

5.2 Microglia phagocytose Tau monomer faster than oligomers by P2Y12 and localized F-actin network

Previously, we showed that microglia phagocytose extracellular Tau oligomers and aggregates by actin remodeling and induce migration/invasion and MTOC polarization [282]. Here, we further studied the time-dependent P2Y12-mediated endocytosis of Tau oligomers in association with localized F-actin networks in migratory microglia. With increasing time, Tau was found to be phagocytosed via P2Y12-associated actin polymerization by microglia. Tau monomer was phagocytosed maximally at 10 and 30 minutes of exposure, while, the Tau oligomers were internalized maximally at 30 and 60 minutes (Fig. 27A). The orthogonal projection of microscopic images revealed that Tau monomer and oligomers were colocalizing with P2Y12 and F-actin in XZ and YZ planes during internalization (Fig. 27B). The microscopic quantification of % of Tau^{+} microglia revealed that 90% of the microglial population was phagocytic for both Tau monomer and oligomers' at 10 and 30 minutes of

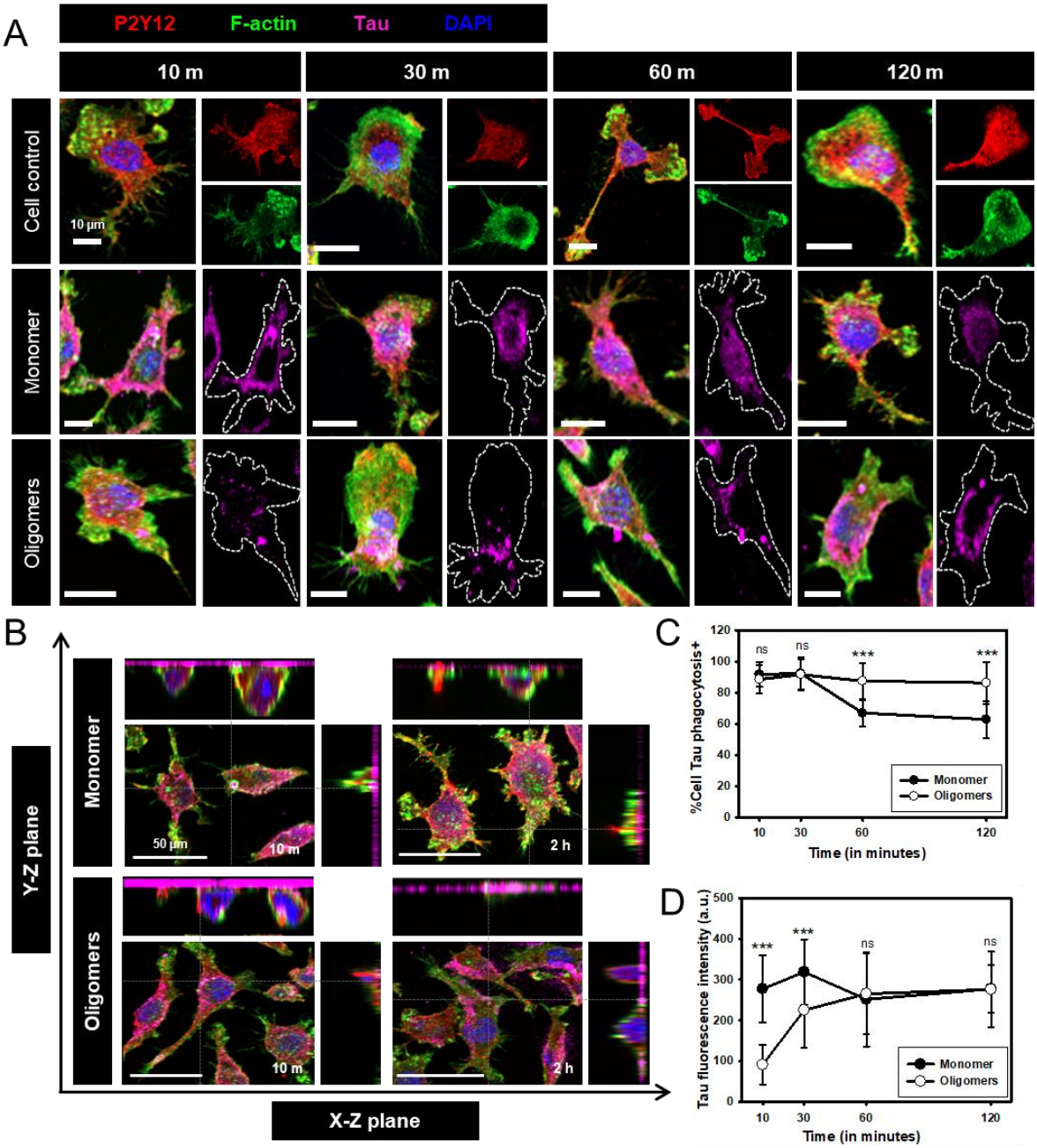

Figure 27. P2Y12-associated endocytosis of Tau monomer and oligomers, via actin polymerization. A. Microglia phagocytosed the extracellular Tau monomer and oligomers in a time-dependent manner. Scale bar 10 µm. **B.** The orthogonal projection of microscopic images revealed the colocalization of internalized Tau along with P2Y12 and F-actin, near the cell membrane and inside the cytoplasm in XZ and YZ planes. Scale bar 50 µm. **C.** At the initial time, 90% of the microglia internalized both monomer and oligomers. But with time, Tau monomer$^+$ cells were markedly reduced, while oligomers$^+$ cells remained constant. **D.** Monomer fluorescence intensity was more than oligomers at initial time, but monomer intensity was decreased and oligomers intensity was increased time-dependently.

exposure. But at later time points, only 60% of the microglia was Tau monomer$^+$ while; oligomers$^+$ microglial population remained consistent at 90% till 60 and 120 minutes (Fig. 27C). Moreover, the time-dependent fluorescence intensity of Tau from individual microglial cells showed that the Tau signal was increased by three times in monomer-exposed groups than oligomers' after 10 and 30 minutes of exposure. But, the fluorescence signal of monomer was dropped from 60 minutes while; oligomer's signal was continuously increasing till 60 minutes and become stagnant at 120 minutes (Fig. 27D). These results suggest that

the Tau monomer undergoes a faster and elevated level of phagocytosis as compared to oligomers by P2Y12-associated F-actin localization in migratory microglia.

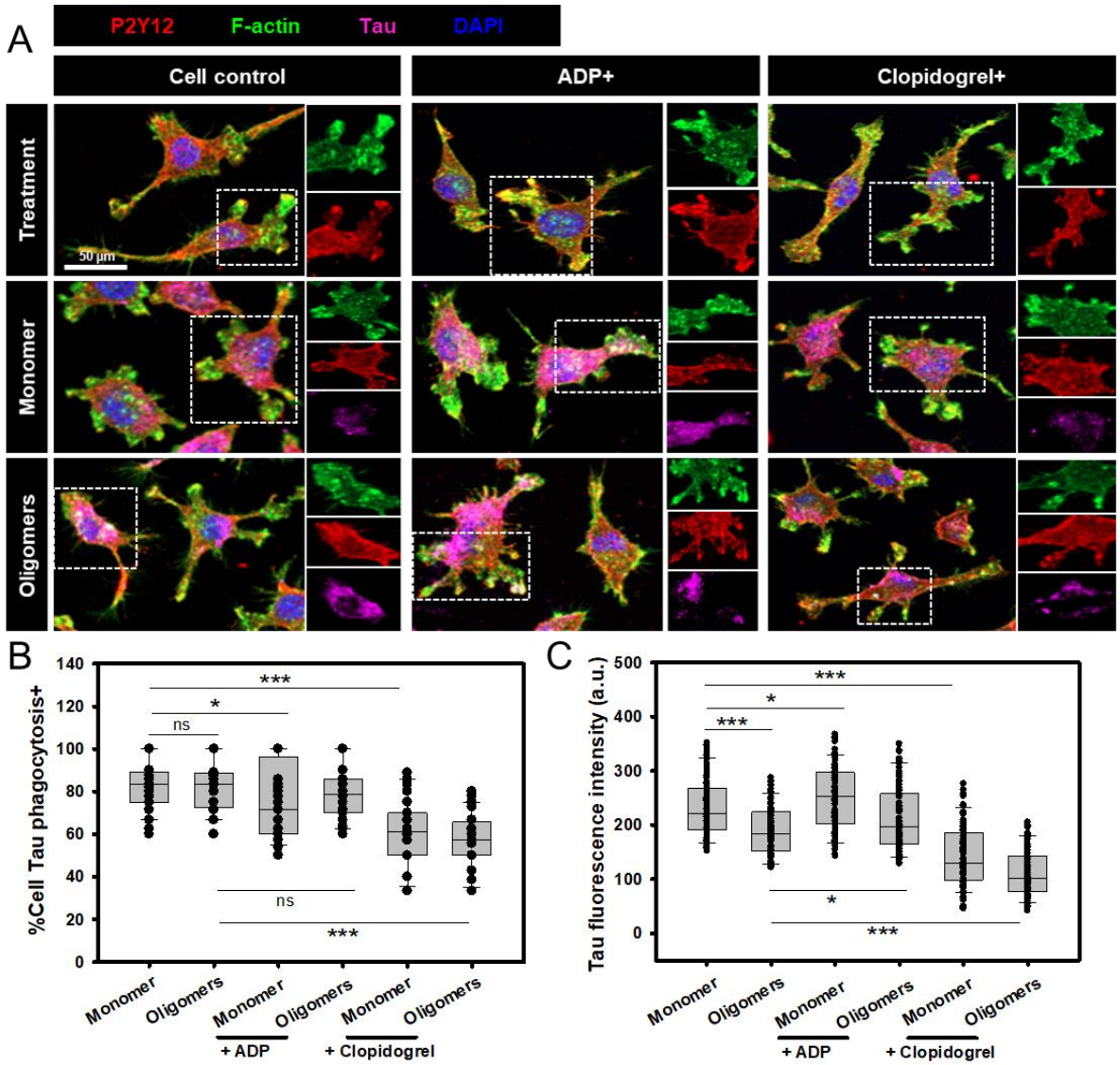

Figure 28. Signaling blockage of P2Y12 reduced the level of Tau endocytosis in microglia. A. P2Y12 activation influenced the Tau phagocytosis, while Clopidogrel significantly reduced the Tau internalization. Scale bar 50 µm. **B.** Clopidogrel exposure has reduced the phagocytic microglial population by 20% for both monomer and oligomers internalization. **C.** ADP induction resulted in increased Tau intensity in monomer and oligomer-exposed microglia. Clopidogrel reduced the Tau intensity by 50% from phagocytic microglia.

5.3 The inhibition of P2Y12 signaling reduces the Tau phagocytosis

Previously, we found that P2Y12 interacts with extracellular Tau, while the inhibition of P2Y12 signaling affects the Tau deposit degradation without affecting actin microstructures [283]. Here, we performed the Tau phagocytosis assay for 2 hours by activating or blocking the P2Y12 signaling with ADP and Clopidogrel, respectively. Interestingly, we observed that the induction of P2Y12 signaling has increased the amount of Tau phagocytosis at 2 hours. Similarly, the blockage of P2Y12 signaling by Clopidogrel has significantly reduced the level of Tau endocytosis, for both monomer and oligomers (Fig. 28A). The quantification of % Tau^{+} microglia from microscopic images revealed that Clopidogrel has reduced the population of Tau+ phagocytic cells i.e., around 60% as compared to only Tau and ADP-induced phagocytic cells >80% (Fig. 28B). Similarly, Tau fluorescence intensity from microglial cells depicted that ADP induction has increased the fluorescence signal of

monomer and oligomers upon P2Y12-mediated endocytosis. But, Clopidogrel treatment has diminished the fluorescence signal of monomer and oligomers by half in Tau^{+} microglia (Fig. 28C). These results signify that P2Y12 signaling can regulate the event of Tau phagocytosis in association with actin remodeling in migratory microglia.

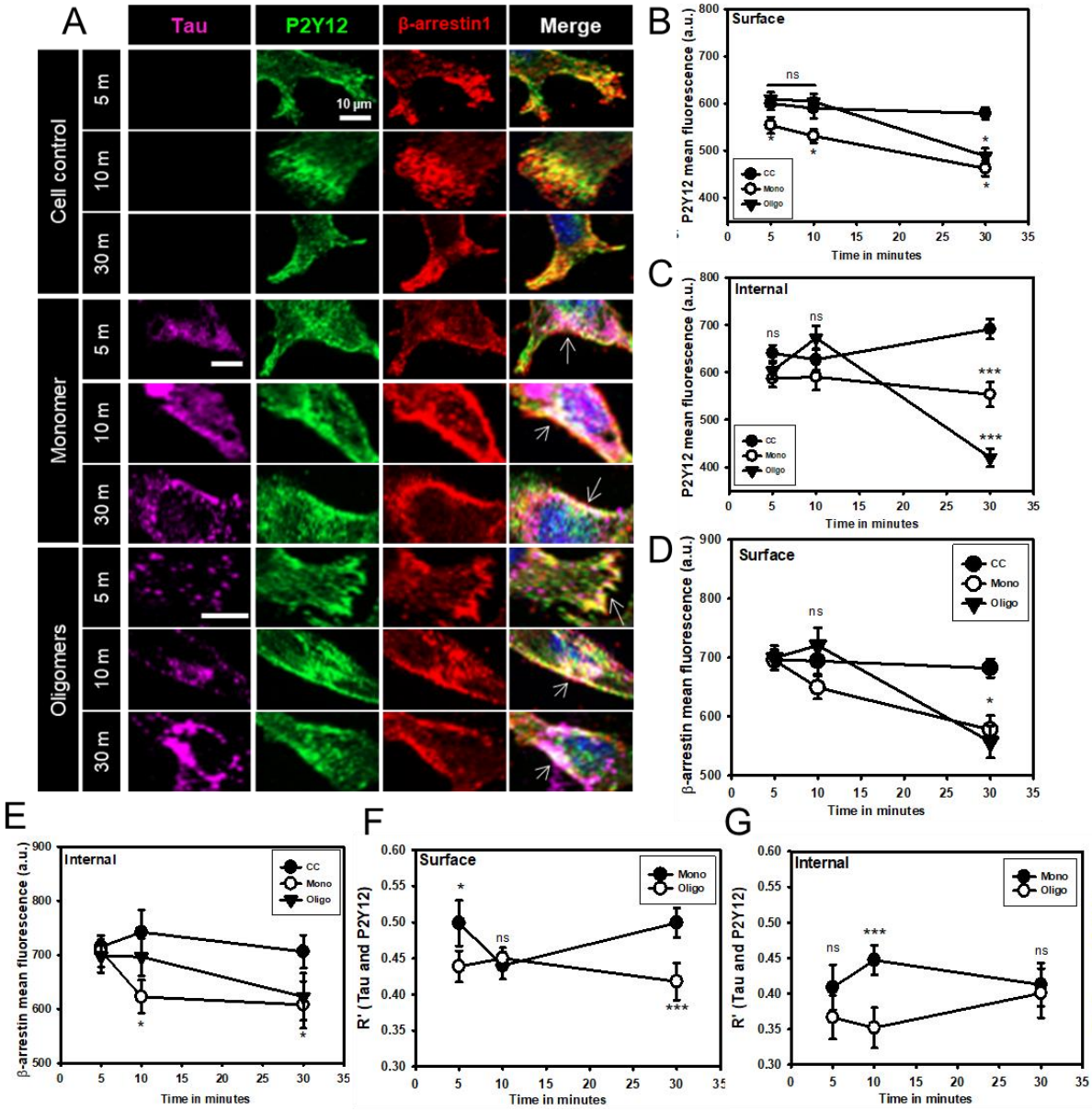

Figure 29. Microglia internalize Tau by P2Y12-mediated β- arrestin1-associated receptor desensitization. A. Tau monomer and oligomers were endocytosed with P2Y12 in a time-dependent manner, which was colocalized with β-arrestin1. The arrowhead indicates the colocalization and scale bar: 10 µm. **B, C.** P2Y12 fluorescence signal from microglial cell surface vs cytoplasmic were evaluated for both Tau monomer and oligomers-exposure in a time-dependent manner **D, E.** Similar to P2Y12, β-arrestin-1 surface signal and cytoplasmic signals were quantified in monomer and oligomers exposure time-dependently **F, G.** Pearson's coefficient analysis showed that colocalization between Tau with P2Y12 on the cell surface vs cytoplasm at different time points where monomer showed better colocalization with P2Y12.

5.4 Microglia endocytose Tau by P2Y12- and β-arrestin1-associated desensitization

As microglia perform P2Y12-GPCR mediated endocytosis of Tau, we hypothesized that Tau internalization might follow through the β-arrestin1-associated receptor desensitization process. To study the early events of P2Y12-mediated endocytosis, microglia were exposed

to Tau monomer and oligomers for 5, 10 and 30 minutes and the cellular location of Tau was studied in association with P2Y12 and β-arrestin-1. Both the Tau monomer and oligomers were found to be endocytosed with P2Y12, in association with β-arrestin-1. Tau monomer was found to be endocytosed maximally at 10 minutes while oligomers were found to be colocalized with P2Y12 and β-arrestin-1 maximum at 30 minutes of exposure. The three-point colocalization of Tau, P2Y12 and β-arrestin-1 was shown in the figure by pointed arrowheads (Fig. 29A). At 5 minutes, Tau and P2Y12 were found localize near to the cell membrane, and then migrate towards the cell body at later exposure time by cellular trafficking. The quantification of microscopic images showed the depletion of P2Y12 signal from the cell surface at 10 minutes and increase at 30 minutes in monomer and oligomers' endocytosis. Tau monomer exposure resulted in more P2Y12 signal loss from the cell surface than oligomers, starting from 5 minutes (Fig. 29B). The internal cytosolic signal of P2Y12 remained stable by oligomers endocytosis, but for monomer, the P2Y12 signal was slightly increased at 10 minutes but decreased at 30 minutes (Fig. 29C). Similar to P2Y12, the β-arrestin1 surface signal was decreased at 30 minutes upon both the Tau- monomer and oligomers endocytosis. But unlike P2Y12, the β-arrestin1 cytosolic signal started reducing from 10 minutes of Tau exposure (Fig. 29D, E). The Pearson's coefficient analysis between Tau and P2Y12 depicted that monomer was colocalized more with P2Y12 than oligomers at the cell surface at 5 and 30 minutes. But at cytosol, P2Y12 was more colocalized with monomer at 10 minutes and with oligomers at 30 minutes (Fig. 29F, G). These results may emphasize that the Tau monomer follows faster and periodic uptake, while; the bulky oligomers were associated with delayed internalization by P2Y12 and β-arrestin-1-mediated endocytosis in microglia.

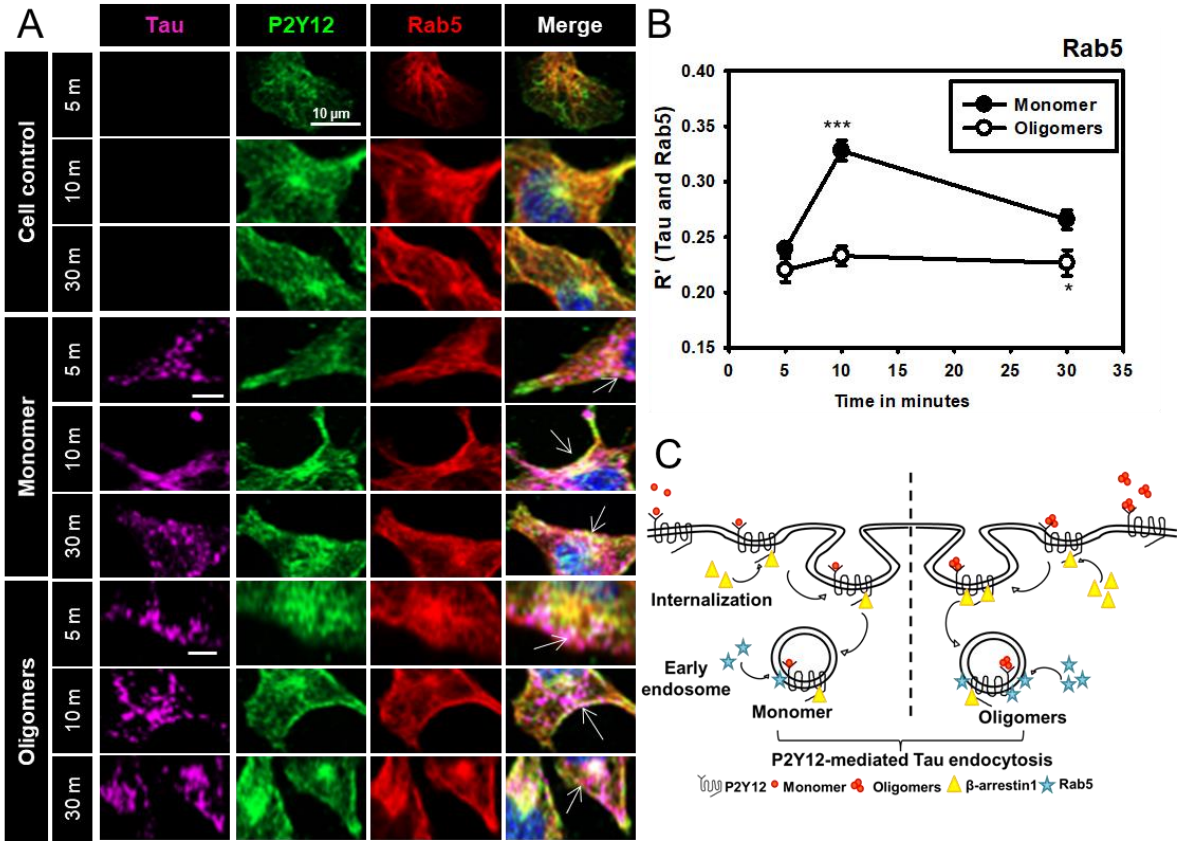

Figure 30. Microglia targets Tau monomer to Rab5+ vesicle faster than oligomers upon P2Y12-mediated internalization. A. Tau monomer and oligomers were found to be colocalized with Rab5^{+} early endosomes during the P2Y12-associated internalization. Scale bar: 10 µm. **B.** Pearson's coefficient depicted that monomer was colocalized more with Rab5 than oligomers. **C.** Extracellular Tau monomer and oligomers were endocytosed with P2Y12 receptor through the involvement of β-arrestin1 and followed by Rab5^{+} early endosomal vesicles.

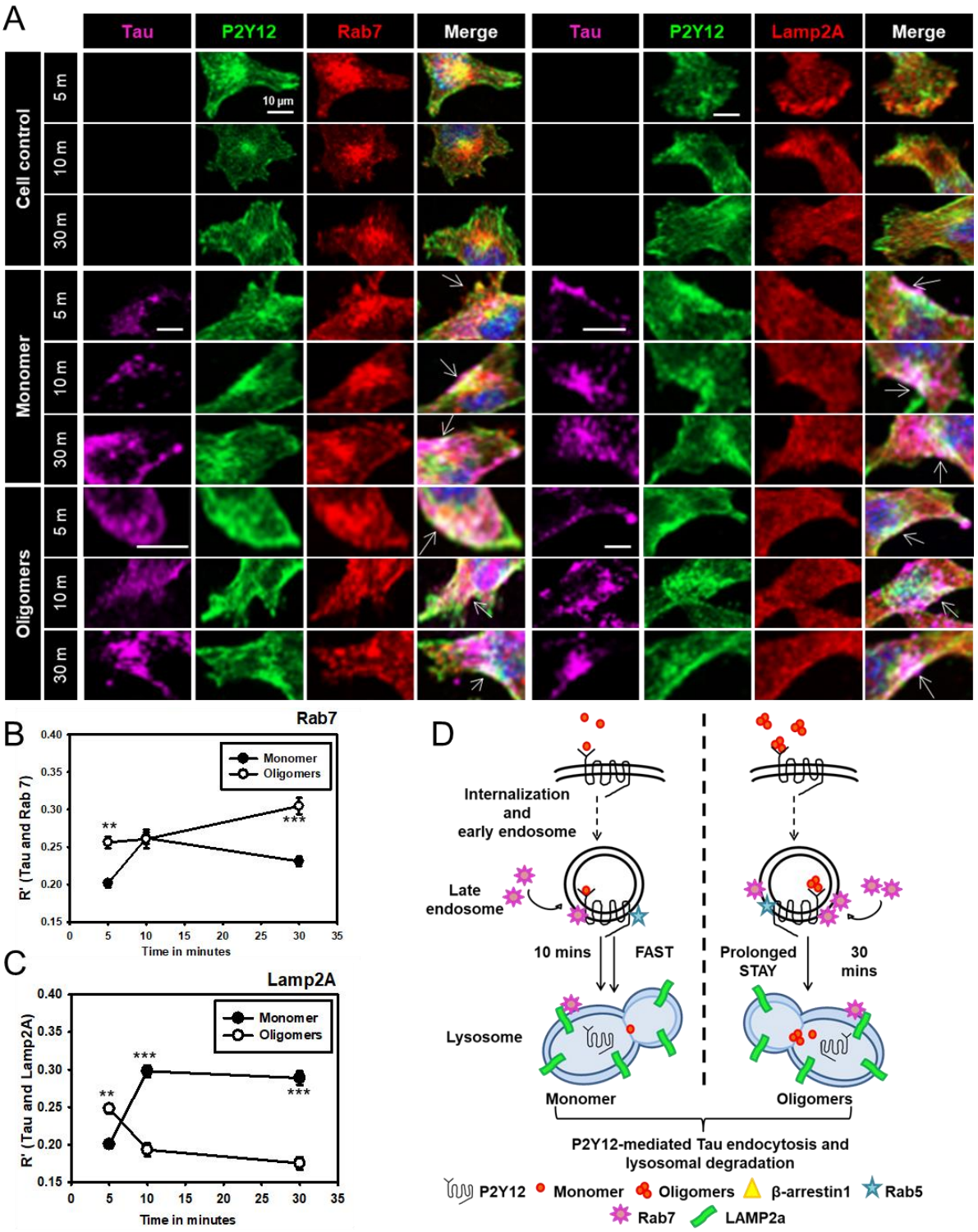

Figure 31. Tau oligomers accumulate in Rab7+ late endosomal vesicles, but monomer follow lysosomal degradation. A. Tau monomer and oligomers were trafficked through Rab7^{+} late endosomes and following the lysosomal degradation as observed by the colocalization with Tau and Lamp2A. Scale bar: 10 µm. **B.** Tau monomer was colocalized with Rab7 maximally at 10 minutes. But, Tau oligomers and Rab7 colocalization were consistently increasing till 30 minutes. **C.** Monomer was following the Lamp2A colocalization consistently, mediating steady-state lysosomal degradation. Tau oligomers were overcoming the lysosomal degradation by continuously decreasing colocalization with Lamp2A. **D.** Tau monomer was following faster late endosomal trafficking (Rab7) and undergoing steady-state lysosomal degradation (Lamp2A). But, oligomers stayed with Rab7^{+} late endosome for a prolonged period (>30 minutes), also bypassing the lysosomal degradation.

5.5 Tau localizes with P2Y12 at Rab5$^+$ early endosomes

To study the endocytic trafficking of Tau with P2Y12 receptor, microglia were exposed to Tau monomer and oligomers in a time-dependent manner. Both the Tau species- monomer and oligomers were found to be localized at Rab5$^+$ early endosomal vesicles along with P2Y12. Tau monomer was observed to colocalize with Rab5 and P2Y12 more at 10 minutes, while oligomers were localized maximally at 30 minutes (Fig. 30A). The Pearson's coefficient analysis between Tau and Rab5 depicted that monomer was localized more within early endosomal vesicle at 10 minutes but started depleting at 30 minutes of exposure. On the other hand, oligomers were localized similarly with Rab5 at 10 and 30 minutes which may signify the slower cellular trafficking of Tau oligomers (Fig. 30B). Therefore, it is evident that microglia phagocytose and process the Tau monomer$^+$ endosomes faster than oligomers in association with Rab5$^+$ vesicles (Fig. 30C).

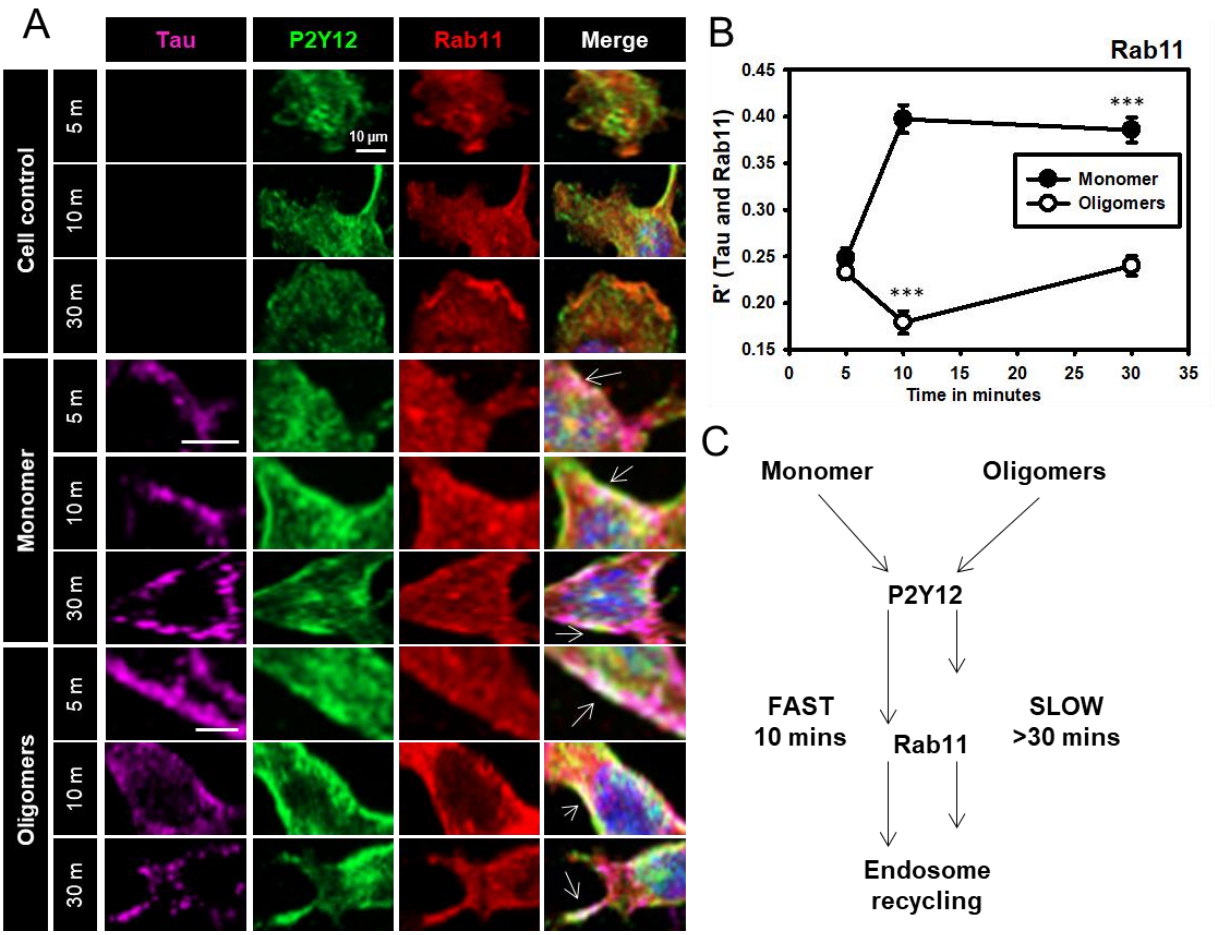

Figure 32. Monomer containing endosomal Rab11+ vesicles recycle more than oligomer+ vesicles. A. Tau monomer and oligomers were colocalized with Rab11-recycling endosomes near to the cell periphery. Scale bar 10 µm. **B.** Tau monomer was colocalized more with Rab11 endosomes at 10 and 30 minutes as compared to oligomers. **C.** Both Tau monomer and oligomers were recycled through Rab11 containing endosomes in association with P2Y12. Monomer recycled faster (10 minutes) than oligomers (>30 minutes).

5.6 Tau oligomers accumulate in Rab7$^+$ endosomes but monomer degrades in lysosomes

Next, we studied the late endosomal and lysosomal localization of Tau along with P2Y12 by late endosomal marker- Rab7 and lysosome marker- lysosome-associated membrane protein 2A (Lamp2A). To follow downstream endosomal trafficking, Tau monomer and oligomers were also found to be localized with Rab7 in a time-dependent manner. Tau

monomer was localized with Rab7$^+$ vesicles along P2Y12 at 10 minutes near the membrane that migrated to the cell interior at 30 minutes. Tau oligomers were evidenced to localize with Rab7 comparatively more than monomer at different time points 5, 10, and 30 minutes (indicated by arrowheads) (Fig. 31A). After the late endosomal location, Tau monomer and oligomers were both observed to localize with the Lamp2A. Monomer showed more colocalization with Lam2A-lysosomes than oligomers along with P2Y12 at various time intervals (Fig. 31A). Pearson's coefficient analysis revealed that Tau oligomers were more colocalized with Rab7 than monomer at various time points. Tau monomer and Rab7 colocalization was maximum at 10 minutes which was further reduced at 30 minutes. But, the oligomers-Rab7 colocalization was continuously increased from 5 up to 30 minutes of exposure (Fig. 31B). Further, Lamp2A and Tau colocalization showed an opposite pattern as compared to Rab7-Tau groups. Tau monomer was colocalized with Lamp2A, maximally at 10 minutes and remained constant till 30 minutes. But, the oligomers showed a continuously decreasing colocalization pattern with Lamp2A starting from 10 to 30 minutes (Fig. 31C). These interesting observations suggest that Tau monomer follows the P2Y12-associated endocytic trafficking, starting from Rab5, then Rab7 vesicles and lastly to the lysosomal degradation. On contrary, Tau oligomers exhibit a slower internalization and endocytosis processing which is followed by a prolonged stay at Rab7$^+$ late endosomes and are less likely to be targeted for lysosomal degradation (Fig .31D).

5.7 Tau monomer colocalize with Rab11$^+$ recycling endosome more than oligomers

To study the Tau release from the microglial endosomal pathway, we studied the colocalization of Tau with Rab11$^+$ recycling endosomal vesicles in a time-dependent manner. Both Tau monomer and oligomers were found to be colocalized with Rab11 near to the cell membrane at various time points (Fig. 32A). But comparatively, Tau monomer was more associated with Rab11 at 10 and 30 minutes of exposure than oligomers. On the other hand, Tau oligomers showed a reduced level of colocalization with Rab11 at 10 minutes which then become increased slightly at 30 minutes (Fig. 32B). This observation may signify that a relatively more P2Y12-endosomal recycling of Tau monomer-containing vesicles, while the complete endosomal processing of Tau oligomers was delayed including Rab11$^+$ P2Y12 receptor recycling (Fig. 32C).

5.8 Summary

Here we defined the interaction and internalization of extracellular Tau by microglia to mediate rapid migration, activation and degradation for the pathoprotein clearance. Microglia phagocytose Tau monomer and oligomers by P2Y12-mediated and β-arrestin-1-associated endocytosis along with localized F-actin network. Tau monomer with P2Y12 followed faster endocytic trafficking and was ultimately destined for lysosomal degradation. But, Tau oligomers become accumulated at late endosomal vesicles in microglial cytosol and became resistant to lysosomal degradation. Therefore, the Tau oligomers may probably be degraded by alternative pathways such as autophagic induction. Since the capturing and degradation of pathological Tau species by microglia are of immense importance in pathoprotein clearance, the P2Y12-mediated endolysosomal pathway, migration and actin remodeling, that can be intervened as a therapeutic strategy in AD.

Chapter 6
Discussion

6.1 Phagocytosis of full-length globular Tau oligomers by N9 microglia

Tau is a microtubule-associated protein that mainly functions in microtubule network stabilization, treadmilling and axonal transport in the neuron. But, in pathological conditions, Tau undergoes various PTMs, truncation, proteolytic cleavage and many genetic variants contribute to the aggregation, accumulation as NFTs and prion-like propagation [284]. The reactive pathological Tau species are secreted, aggregated in synaptic puncta and travel through the neuro-anatomically connected neurons where it acts as a conformational seed template for endogenous healthy Tau aggregation [44, 285]. Moreover, the extracellular presence of Tau aggregates triggers the glial activation and deleterious neuroinflammatory signaling cascade in CNS. In the process of Tau aggregation, the first oligomerization is the rate-limiting step. But once achieved, it exponentially increases the aggregation steps by primary and secondary nucleation. It has been recently emphasized that the soluble oligomeric/ proto-fibrillar species are much more reactive, toxic and neuroinflammatory in neurodegenerative diseases. These reactive oligomeric species are associated with various pathological events in CNS such as interaction with endogenous proteins and their aggregation, blockage of signaling cascade/ neurotransmitter, inhibition of vesicular/ endosomal trafficking, and lastly activation of the glia [51]. Hence, the trapping and clearance of the early intermediate oligomers would be the most important way to prevent their propagation into healthy neuronal circuits [286]. Tau is a highly soluble, intrinsically unfolded protein, which lacks a particular ordered structure. The N-terminal projection domain helps in solubilization while; the C-terminal repeat domain is involved in microtubule interaction [287]. Moreover, the intermediate proline-rich domain is involved in various protein interactions and also regulates the repeat domain-mediated Tau oligomerization. In the diseased condition, various PTMs, truncation of Tau and the prevalence of many poly-anionic factors initiate the Tau disassembly from microtubule and self-oligomerization. Recent reports showed an exponential increase of granular Tau oligomers during AD progression, which can aggregate the normal Tau in healthy mice brain [5, 35]. Various reports emphasized the *in vitro* preparation of truncated or mutant Tau oligomers which showed an elevated aggregation propensity. For eg., truncated Tau (151-391) forms globular oligomers *in vitro*, which were phagocytosed by LPS-activated microglia [83]. Similarly, RDΔK280 Tau oligomers were found to attain distinct granular structures lacking β-sheet confirmation, which leads to ROS production and synapto-toxicity in hippocampal neurons [239]. Although the full-length Tau is maximally expressed in adult CNS, it is tedious for in vitro oligomerization of its structural fragility. The isolated oligomers are transient and are associated with difficulties during the performance of in vitro experiments [288]. Therefore, the *in vitro* preparation and stabilization of Tau oligomers are of great concern to study their biochemical and biophysical properties and further *in vitro* cell biology-based experiments. Here, we prepared the full-length Tau oligomers by inducing them with poly-anionic factor heparin for 12 hours and then stabilized with 0.01% glutaraldehyde. The stabilized formed oligomers were purified by SEC and confirmed by western blot of SEC fractions. The ThS and ANS fluorescence studies revealed that the oligomers contain less β-sheet structure with increased exposed surface hydrophobicity. Moreover, the high-resolution electron microscopic study showed the presence of heterogeneous globular Tau oligomers, sizes ranging from 5-50 nm which are of a similar kind as reported previously [228]. The assembly of relative β-sheets structures during oligomerization was confirmed by CD spectroscopy [289]. Therefore, we hypothesized that the extracellular presence of these oligomers can induce microglial phagocytosis and related activation pathway, which mimics the early pathological situation of AD condition.

Microglia are the prime immune cells in the CNS that play a crucial role in pre/postnatal neurodevelopment, circuitry management in the adult brain, neuronal repair and tissue homeostasis [5]. Microglia maintain synaptic health, neurotransmitter recycling and metabolic regulation through the long cellular process and small soma. Upon sensing extracellular protein oligomers/aggregates, microglia become activated, retracts their

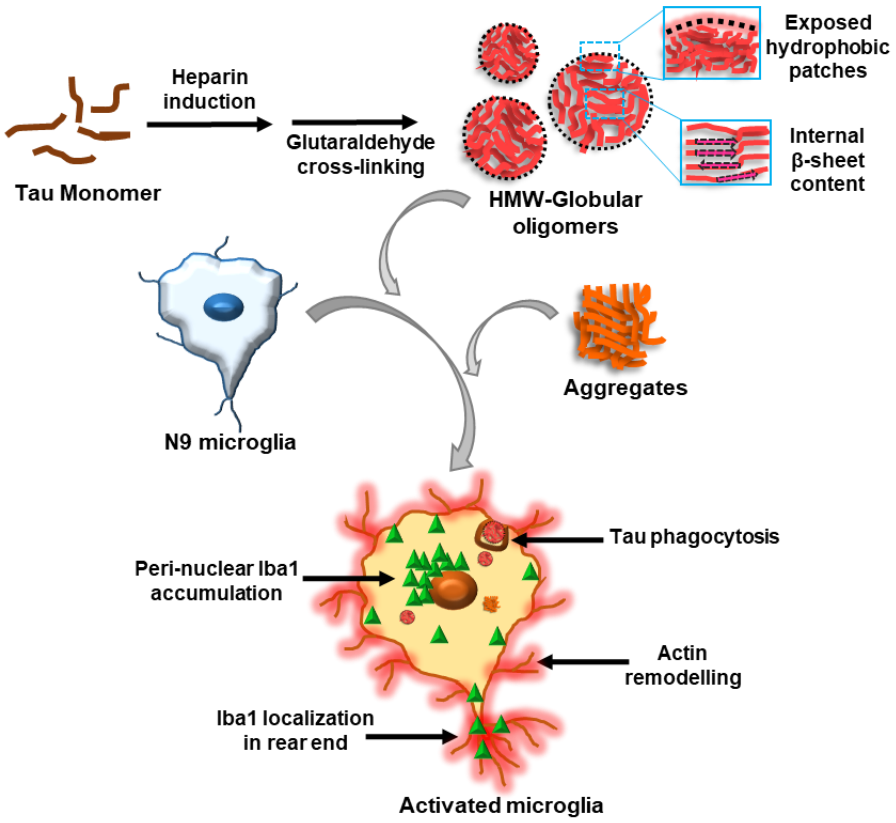

Figure 33. Phagocytosis of Tau oligomers *via* Iba1⁺ membrane-associated actin network by activated microglia. Tau oligomers were prepared *in vitro* and stabilized, which showed the globular structure with increased surface hydrophobicity and less β-sheet content. Tau oligomers and pre-formed aggregates were phagocytosed by microglia by actin remodeling. Moreover, the extracellular Tau oligomers and aggregates have induced the microglial activation by unregulated Iba1 expression and colocalization with remodeled actin cytoskeleton network.

processes and secrete various cytokines and chemokines. Various membrane receptors and complement factors are involved in the recognition of DAMPs to initiate phagocytosis and clearance of debris [235, 290]. Activated microglia engulf the amyloid-containing neurons flagging phosphatidylserine signals [291, 292]. But, the excessive synaptic engulfment resulted in gradual synaptic loss and progressive cognitive impairment. Moreover, the improper proteolysis of phagocytosed materials resulted in the excretion of seed entities for further aggregation which can lead to the microglia-mediated transmission of Tauopathy [292]. In our study, we have shown that the extracellular Tau oligomers and aggregates were phagocytosed by N9 microglia for clearance. Actin is a dynamic cytoskeletal network, which is involved in cellular migration, phagocytosis, exo/endocytosis and immune effector functions. In the migratory cells, the G-actin rapidly converted into F-actin at the growth end, which makes a tensile strength and pushes the membrane for forward movement [293]. While, the concomitant depolymerization from F-actin to G-actin increases the membrane fluidity for rapid phagocytosis of extracellular particles [257, 294]. Hence, membrane-associated actin remodeling regulates various microglial functions like- migration, matrix adhesion and phagocytosis by the involvement of many receptor and actin-binding proteins- CD36, ROCK, cofilin, vinculin, etc [258, 294, 295]. In our study, we observed the formation of filopodia-like actin structures in oligomers-exposed microglia. While the aggregates-exposed microglia bear more flat actin-rich protrusions, which is called lamellipodia, without altering the actin expression and the cellular area. These observations may emphasize the membrane-associated actin remodeling in phagocytic microglia for Tau clearance. Iba1 is a calcium-binding adaptor protein, which functions not only as a microglial marker in CNS, but also performs the actin cross-linking in migratory immune cells [249, 296]. Previous reports

showed that the Iba1 level becomes upregulated during neuronal injury which correlates with the accumulation of activated microglia at the site of injury [246, 297]. Similarly, Iba1 was found to remodel actin bundling by interacting with L-fimbrin and inducing phagocytic cup formation [93, 298]. In our study, we evidenced the increased level and localization of Iba1 with the actin-rich membrane projection in Tau oligomers-exposed microglia. Similarly, the 3D microscopic images revealed the cytosolic relocation of Iba1 upon Tau oligomers exposure which may signify the Ca^{2+}-related migration and rapid Tau clearance by phagocytic microglia. The elevated microglial migration and Iba1-associated actin rearrangements can sight the interplay of various signaling cascades in phagocytic and proteostasis amyloid clearance and further mediate the innate-adaptive immune response. The molecular mechanisms of Tau phagocytosis and improvement of directional mobility in microglia will also help to identify the probable immune targets in the treatment of Tauopathy (Fig. 33).

6.2 P2Y12-driven actin remodeling, MTOC polarization and migration by Tau oligomers-induced microglia

Microglia orchestrate various receptors for sensing and engulfing the extracellular protein deposits and for directional chemotaxis [299]. It has been previously reported that microglia express the P2Y12 receptors throughout their lifetime which are involved in cellular directionality, migration and process extension in non-activated conditions [150, 182]. Single-cell RNA sequence analysis showed that the DAMs have a reduced expression of various homeostatic genes (Olfml3, Tmem119, GPR34, P2Y12 and CX3CR1) and induced expression of inflammatory molecules (IL1β, TNFα, NO, ApoE, TREM2 and ROS) in AD mice model [174, 300]. The DAMs also play a deleterious role in the spreading of Tau seed in different brain locations by live neurons phagocytosis, migration and exhibiting neuroinflammation [151, 301]. Here, the prepared Tau oligomers possess heterogeneous globular structures which were toxic and then phagocytosed by $Iba1^{+}$ microglia *via* membrane-associated actin remodeling. The extracellular release of Tau oligomers can function as a chemoattractant for microglial clearance of protein deposits [292, 302]. Here, we hypothesized that extracellular Tau monomer and oligomers can act as soluble chemoattractants while the HMW aggregates mimic the Tau deposition in vitro microglial culture. In our study, extracellular Tau species- monomer, oligomers and aggregates induced the microglial P2Y12 expression. In order to identify the signaling cross-talk of microglial Tau internalization, actin-remodeling and increased P2Y12 receptor expression, we performed Co-IP study between Tau-P2Y12 and also immunofluorescence study for cellular colocalization. The microglial P2Y12 was found to be precipitated with Tau species which may suggest the function of extracellular Tau in P2Y12-mediated chemotaxis. In particular, P2Y12 relatively interacted more with Tau oligomers and monomer as compared to aggregates, which depicted Tau oligomers as more potent chemoattractant than HMW aggregates. In IF study, P2Y12 was observed to colocalize with migratory actin structures, specifically with filopodia upon Tau oligomers exposure. This may signify that extracellular Tau can intervene the P2Y12 signaling and induce microglial migration through the formation of membrane-associated actin microstructures. Remodelled actin structures include- frontal lamellipodia which exhibits tensile forces for forward movement, filopodia is involved in matrix adhesion, while the rear end uropod helps in cellular retraction from the matrix surface [93]. In an interesting report, α-syn protein was shown to induce microglial migration by interacting with CD11b and remodelled the cortactin-regulated actin scaffold into lamellipodia [255]. Moreover, the P2Y12 redistribution on the membrane link to the filopodia formation towards the site of injury, while the P2Y12 mutation results in reduced number of cellular processes and non-specified synaptic engulfment of in seizure-induced epileptic brain [167, 182]. Another report contradicted that the P2Y12 activation collapses the filopodia but increases the bulbous actin structures in immune-surveilling microglia [164]. In our study, we found that extracellular Tau oligomers and aggregates induced the lamellipodia and filopodia-bearing microgliaand the filopodia numbers/ cell, which may

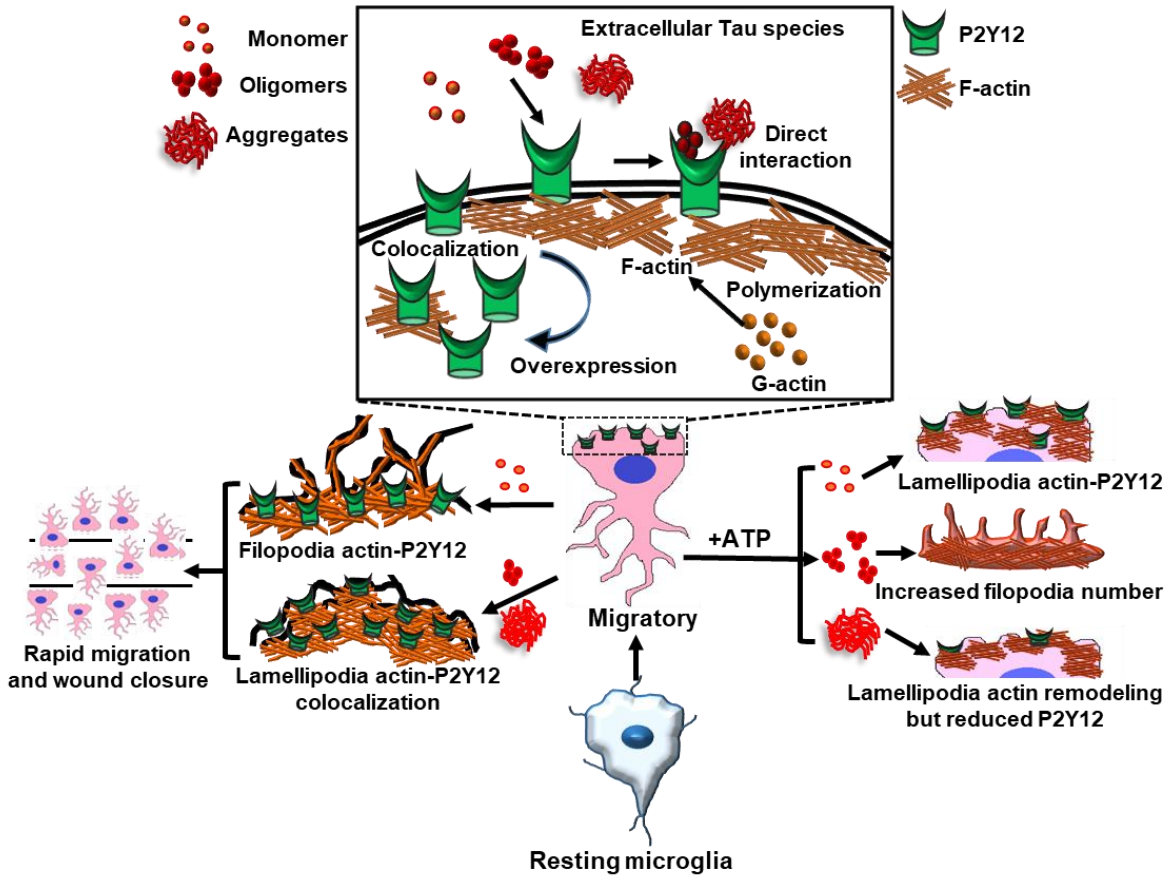

Figure 34. Tau-induced microglial chemotaxis and actin remodeling by interacting with P2Y12 signaling. Extracellular Tau species- monomer, oligomers and aggregates can directly interact with microglial P2Y12 and induce its cellular expression which leads to the membrane-associated actin remodeling for P2Y12-associated microglial migration. Tau oligomers and aggregates showed differential microglial actin remodeling upon co-administration with/ without ATP. Among all extracellular Tau species, oligomers demonstrated a better potential as a chemoattractant in wound closure and Trans-invasion in P2Y12-dependent migration during Tauopathy.

correlate the accumulation of actin microstructures for migration and internalization of Tau. It has been well-proven that the extracellular gradient of ATP/ADP acts as a signal for microglial chemotaxis toward the injured neurons [121]. Upon binding of ATP in the ligand-binding pocket of P2Y12, the signal passes through the intracellular release of Ca^{2+} from ER reservoirs, which can act as the signal for migration [94, 182]. The previous report emphasized that the $P2Y12^{+}$ tufted microglia were present at the boundary of the plaque deposited area, but upon reaching, microglia transformed into $HLA\text{-}DR^{+}$ inflammatory phenotype [173]. Moreover, P2Y12/13 was reported to induce the cellular processes through the RhoA/ROCK-mediated P38-MAPK signaling cascade in rat spinal microglia. Therefore, the reorganization of actin and tubulin cytoskeletal networks may contribute to the differential cellular functions in microglia [180]. In our study, the synergistic exposure of ATP with oligomers resulted in an increased level of P2Y12 and actin remodeling in the uropod. Further, the co-treatment of ATP and monomer resulted in increased actin remodeling in lamellipodia and uropod with an increased amount of filopodia extension. While the aggregates with ATP mediate an increased level of filopodia formation with a decreased level of P2Y12 in microglial actin structures. These may signify that Tau oligomers interact with microglial P2Y12 signaling and induced actin remodeling in ATP-induced migratory microglia. But Tau aggregates induced the microglial actin remodeling as filopodia formation by ignoring the P2Y12 interaction for Tau phagocytosis. To emphasize further the P2Y12-mediated actin remodeling in Tau-induced migratory microglia, P2Y12 signaling was selectively blocked by the potent antagonist Clopidogrel. The blocking of P2Y12 signaling reduced the P2Y12 level and actin remodeling, particularly the filopodia extensions in

microglia. However, the number of extending filopodia got restored by concomitant Tau exposure in microglia. Therefore, these results can signify that extracellular Tau regulates the actin/ filopodia remodeling in migratory microglia, which is redundant to P2Y12 signaling.

During synaptic health maintenance as well as immune surveillance in CNS, microglia display a dynamic migration and tissue invasion in the neuronal network [91, 303]. Microglia can traverse through small tissue crevices by secreting various actin-associated proteases to reach at site of neuronal injury [257, 304]. The chemical gradient can act as a 'search me' signal for microglia for the identification of the plaque region [101]. Upon reaching the protein deposits, DAMs transformed into a phagocytic and inflammatory state [300]. Therefore, the rate of microglial migration and invasion can be a determining factor for altered activation in neurodegenerative conditions [64, 176]. In our study, we found that similar to ATP, the soluble Tau oligomers as well as HMW aggregates act as better chemoattractants than monomer as observed by the elevated rate of wound closure in microglial monolayer. Moreover, the extracellular Tau species can form the chemotaxis gradient in brain tissue organization for attracting microglia. Hence, the trans-well migration assay would identify the potential of Tau oligomers and other species as a chemoattractant in the microglial invasion. Here, we observed that Tau oligomers potentially induced microglial invasion through membrane pores similar to ATP, while; monomer reduced the trans-migration. Thus, the gradient of ATP along with Tau oligomers can dictate the microglial migration and invasion to the protein deposit area by involving P2Y12 signaling and actin remodeling [121, 122]. The microtubule network and MTOC position also regulate and shape the migration of immune cells. The previous report showed that IL-4-induced microglia orients the MTOC in both anterior and posterior positions, relating to directional migration for tissue repair. But the LPS stimulation disorganizes the microtubule network with no preferential MTOC position [257]. It has been also reported that the neutrophil prefers the anterior MTOC position for rapid movement and phagocytosis of the pathogen [305]. Here, we connect the time-dependent P2Y12-mediated microglial chemotaxis with microtubule network reorganization, in response to various Tau exposures. Aggregates exposure induces the rapid migration for wound closure and favors the anterior MTOC position, which may indicate the phagocytic phenotype of microglia. But oligomers mediate the increased migration at a constant rate with all preferable MTOC positions, which may signify the phagocytic/ inflammatory phenotype of microglia in response to soluble toxic oligomers. Therefore, the direct interaction of Tau and P2Y12 receptor might facilitate the ATP-mediated actin remodeling, microglial migration and chemotactic invasion for P2Y12-mediated Tau phagocytosis and clearance in the scenario of Tauopathy (Fig. 34).

6.3 Microglial chemotaxis and Tau deposits degradation by P2Y12-associated filopodia and podosome formation

Cell migration and invasion play a pivotal role in various physiological functions such as embryonic development, combating infection, and repairing injury. But the elevated cell migration can result in carcinogenesis, immune disease, genetic disorders (Wiskott Aldrich Syndrome, Frank-Ter Harr Syndrome) and neurodegenerative diseases like- AD [85, 306]. Microglia were observed to clear the cofilin rods near the Aβ plaques and neuropils, which is independent of p-Tau accumulation [307]. As mentioned earlier, microglia remodel the membrane-associated actin network through the formation of various microstructures such as lamellipodia, filopodia, podosome, cortex layer, focal adhesions, etc. The podosome, lamellipodia, and filopodia in immune cells function in the surveillance, tissue remodeling, ECM degradation, cytokine release and even antigen recognition [308-310]. Here, we emphasized that microglia induced the formation of various migratory actin structures such as podosome, filopodia and uropod by incorporating Arp2 for rapid nucleation during Tau oligomers-induced migration. Moreover, the actin turnover was skewed towards the frontal lamellipodia with podosome and filopodia, as seen by a reduced level of Arp2 and TKS5 from uropod in Tau-induced migratory microglia. Migratory cells can form various podosome rearrangements in a physiological state, e.g., osteoclasts formed a single podosome-rich

area, which further matured into podosome belts [311]. The clustered podosome are continuously formed by periodic fusion and fission for mechano-transduction in macrophages [312]. Similarly, podosome rosettes formation was initiated by integrin signaling, which is regulated through PIP2 and N-WASP cascade[98, 265]. In our study, extracellular Tau exposure was associated with various podosome structures in microglia such as- podosome belts, clusters, rosettes and single podosome. Among all, the TKS5-localized clustered podosome was induced in Tau oligomers-exposed microglia in a time-dependent manner.

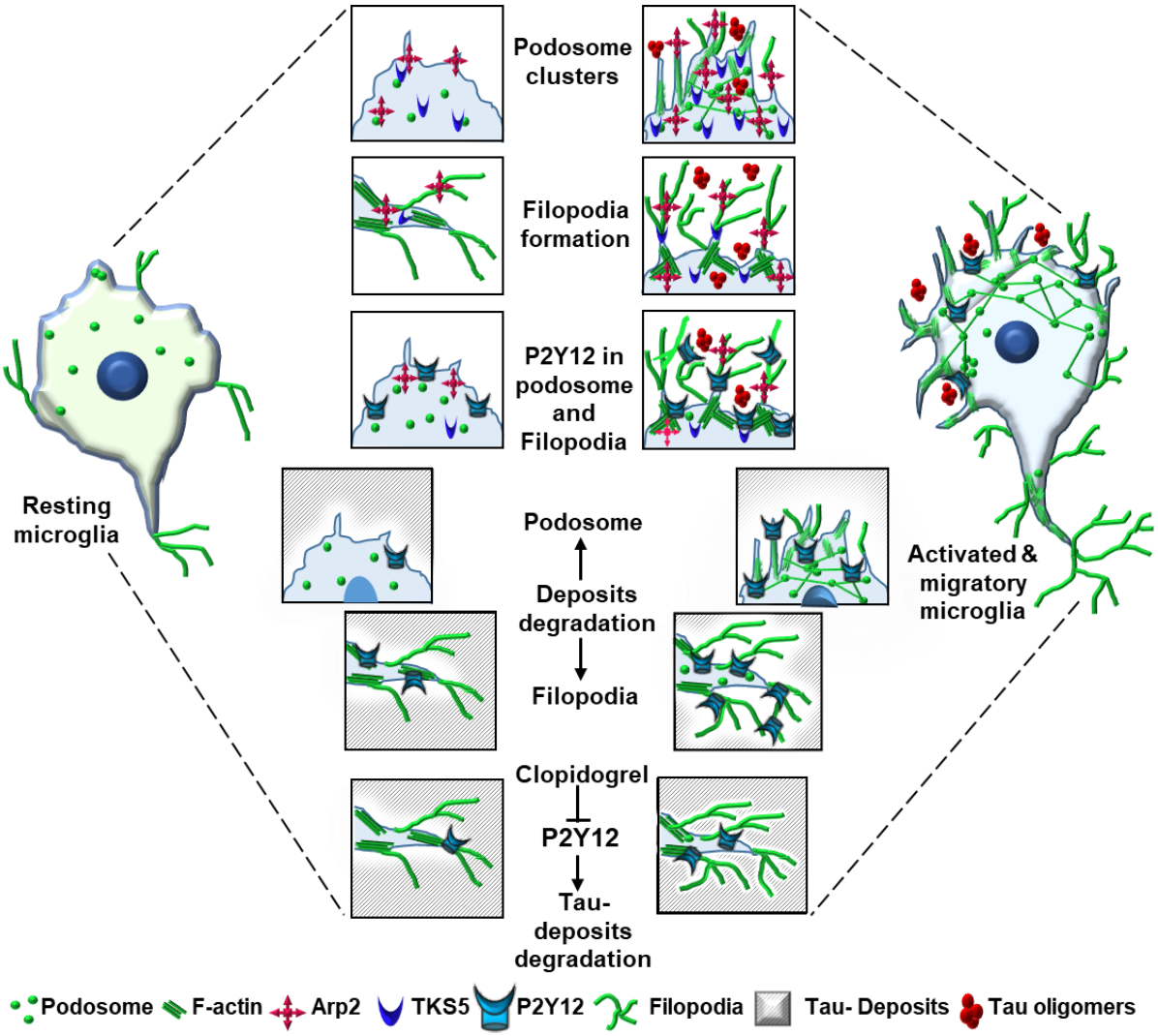

Figure 35. Microglia degrade Tau monomer and oligomers deposits by the formation of P2Y12-mediated podosome and filopodia. Extracellular soluble Tau oligomers have induced the Arp2, TKS5 and P2Y12-localized podosome clusters and filopodia formation in migratory microglia with increased migration and invasion. Tau monomer and oligomers deposits can be degraded by microglial P2Y12^{+} filopodia and podosome formation, depending on P2Y12 signaling activation/ blockage. Hence, the differential functioning of P2Y12 signals in Tau-induced microglial chemotaxis and deposits clearance *via* podosome and filopodia-associated actin remodeling can be intervened therapeutically.

The P2Y12 signaling is thought to be associated with homeostatic microglia, which function in chemotaxis, process extensions and neuronal health surveillance. As also, the DAMs showed a reduced expression of various homeostasis genes, like- P2Y12 and CX_3CR_1 in AD and Tauopathy mice model [306]. Moreover, the P2Y12 level was diminished near the p-Tau/ aggregated plaque area with an increased number of Iba1^{+} microglia in AD brain and mice model [313]. On the other hand, many reports showed that the P2Y12^{+} microglia were surrounding the Aβ and Tau plaques in Tauopathy mice brain [313]. But, the direct function

and occurrences of P2Y12 in the migratory actin network in terms of deposit clearance have not been explored yet [95, 267]. In our study, we observed that the extracellular Tau oligomers have induced the localization of P2Y12 receptor in F-actin-rich podosome, branched uropod and elevated filopodia number in migratory microglia. Therefore, the co-occurrence of P2Y12 in actin network may depict the signaling coupling of microglial chemotaxis and podosome formation to eliminate extracellular Tau oligomers from the microenvironment. Similar to metastatic cells, the smooth muscle cells, endothelial cells, and immune cells show podosome-mediated ECM degradation and cell migration [314-316]. Moreover, the actin ring containing podosome interacting with vinculin and integrins, termed as podonuts, are involved in ECM degradation and migration for invasion [317]. Previous reports showed that P2Y12 can interact with β1-integrin to mediate microglial chemotaxis and tissue invasion. While; the blockage of P2Y12 resulted in reduced cytokine production and neuroprotection in ischemic stroke [318, 319]. In our study, it has been evidenced that both the Tau monomers and oligomers have induced microglial migration (wound closure), which was influenced by the activation and blockage of P2Y12 signaling. Similarly, Tau oligomers have induced the microglial invasion which was significantly reduced by the Clopidogrel exposure. Hence, these studies signify that extracellular Tau oligomers can directly influence microglial chemotaxis and invasion by interfering with P2Y12 signaling and chemotaxis in Tauopathy.

Previously, it was reported that phagocytic microglia mediate oxidative injury, antigen presentation and T-cell activation in the active lesion of multiple sclerosis. But in resolving stages, microglia transformed back into $P2Y12^{+}$ $TMEM119^{+}$ homeostatic phenotype near the inactive plaque regions [320]. In order to understand the podosome/ filopodia-associated Tau deposits degradation and corresponding P2Y12 signaling association, we mimicked the plaques deposition scenario by immobilizing Tau monomer/ oligomers onto the coverslip and then allowing the microglia for time-dependent degradation. In our study, we depicted that microglia can degrade the immobilized Tau deposits by Arp2 and TKS5-associated podosome and filopodia formation in association with P2Y12 receptors. Interestingly, microglia showed a preferential degradation of Tau monomer's deposits (8 hours) than oligomers' (24 hours), as observed by the dilatory effect of oligomers degradation with the lesser area and fluorescence intensity of degraded Tau. Moreover, the Clopidogrel-induced P2Y12 signaling blockage led to the reduction of Tau deposits degradation. Therefore, the P2Y12 signaling can influence Tau-induced microglial chemotaxis and Tau deposits clearance via podosome and filopodia-associated actin remodeling (Fig. 35).

6.4 P2Y12-mediated endocytic trafficking, cytosolic accumulation and lysosomal degradation of Tau oligomers in migratory microglia

Extracellular Tau follows the transcellular spreading in CNS *via* various pathways such as macropinocytosis, clathrin-dependent endocytosis, nanotube, lipid raft formation and HSPGs-dependent phagocytosis, which are depending on the various Tau species and recipient cell type [280, 321]. A previous report emphasized that monomeric Tau entered the neuron *via* HSPG-dependent actin-associated macropinocytosis, while aggregated Tau was internalized by dynamin-dependent endocytosis [184]. Moreover, it was reported that reactive astroglia phagocytoses Aβ oligomers but not hyperphosphorylated Tau via clathrin-mediated endocytosis (CME) and by F-actin polymerization through the phagocytic receptors in hippocampal dystrophic synapses [322]. Other groups also showed Aβ phagocytosis by astroglial CD36 and CD47 receptors *via* actin modulation [323]. In our study, we observed that extracellular Tau monomer and oligomers were internalized by P2Y12 receptor-mediated endocytosis in a time-dependent manner. Whereas, monomer was found to be endocytosed faster and more consistently than oligomers in migratory microglia. Previously, it was shown that the upregulation of Aβ endocytosis and subsequent accumulation recapitulate the age-associated synaptic loss in neuron. Similarly, the CME of Aβ monomer and oligomers occurred by dynamin2 in neuro2a and endothelial cells. While, the blockage of CME and actin polymerization directly reduce the Aβ endocytosis by decreasing the

association of clathrin and PICALM complex [187, 324]. Moreover, the haploinsufficiency of PICALM leads to the accumulation of pathogenic p-Tau and NFTs with elevated autophagic markers in the FTLD tau mice brain [325]. As our study showed the direct interaction of Tau oligomers and P2Y12 and thereby emphasized the microglia-mediated phagocytosis and deposits clearance *via* actin remodeling; we further wanted to study the P2Y12 receptor-mediated Tau endocytosis and its subcellular localization. We found that the ADP-mediated P2Y12 activation has induced Tau phagocytosis, while Clopidogrel exposure significantly reduced the P2Y12-associated Tau endocytosis in microglia. These signify that P2Y12 signaling is directly involved in Tau-induced chemotaxis as well as receptor-mediated Tau endocytosis in microglia.

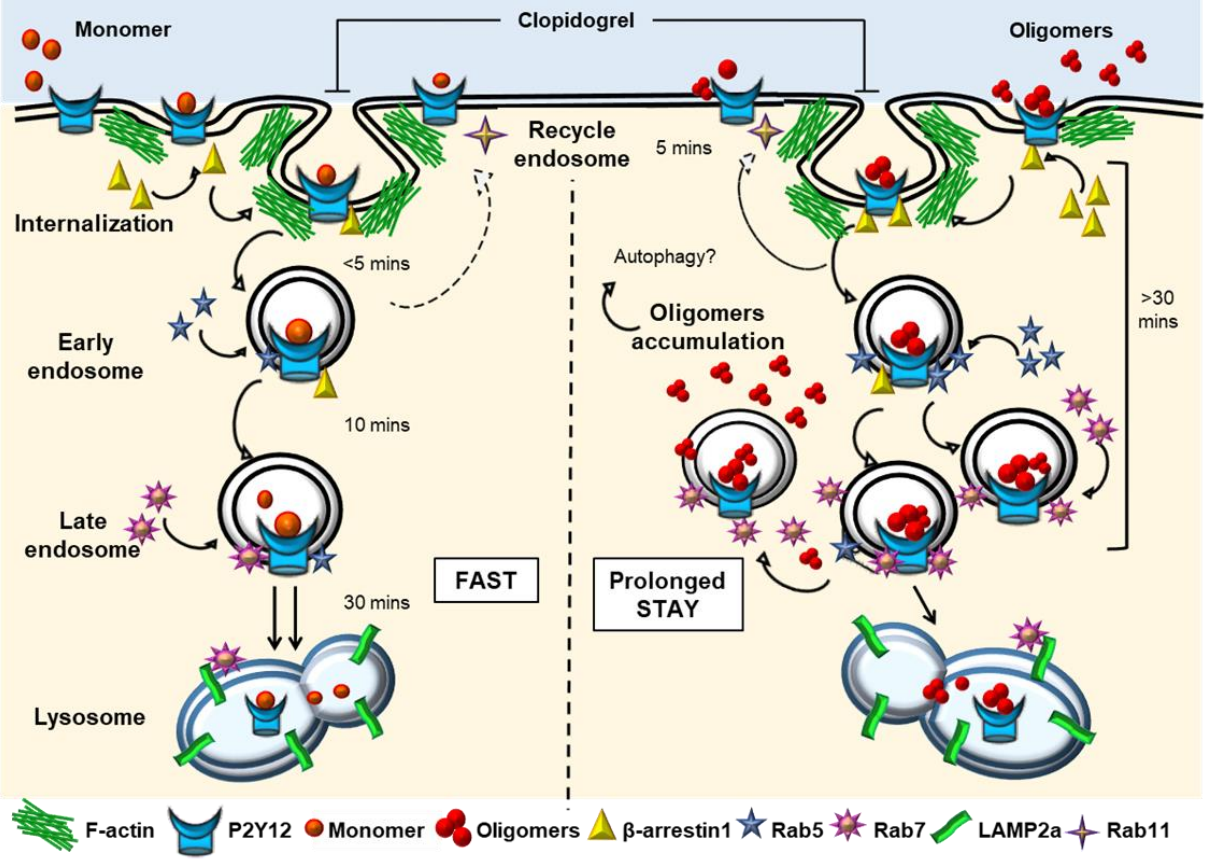

Figure 36. Microglia internalizes Tau by P2Y12 receptor-mediated endocytosis, following vesicle trafficking and lysosomal degradation. Extracellular Tau monomer and oligomers were internalized by P2Y12 receptor-mediated endocytosis colocalized with F-actin network, through the β-arrestin1-associated desensitization pathway. Clopidogrel significantly reduces Tau phagocytosis which signifies the bi-functional nature of P2Y12 in migration and Tau endocytosis. Tau monomer was internalized and followed the endosomal trafficking and degradation faster than oligomers. Internalized oligomers exhibited a prolonged stay in Rab7-late endosomes, become resistant to lysosomal degradation and accumulated in the microglial cytoplasm.

Endosomal dysfunction is one of the most prominent pathophysiological scenarios, where the Aβ and Tau-induced Rab5 activation, endosomal mistrafficking and vesicle enlargement were evident in the progressive stages of AD. The impaired Rab5 signaling can induce disputed synaptic plasticity, neurotransmitter endocytosis, Tau hyperphosphorylation and Aβ accumulation in AD [326]. Furthermore, microglia were shown to internalize Aβ by LC3-associated endocytosis (LANDO) by forming $Rab5^{+}$ $clathrin^{+}$ vesicles which subsequently lead to aggregates clearance [327]. In our study, we found that Tau monomer and oligomers were internalized by P2Y12-dependent endocytosis through β-arrestin1-localized receptor desensitization. Similarly, Tau monomer was observed to follow internalization and early Rab5-endosomal trafficking faster than bulky Tau oligomers, which signifies the size-

dependent differential endosomal trafficking of various Tau species in migratory microglia. A report found that the Aβ internalization was resulted in the enlargement and accumulation of endosomal vesicle which subsequently lead to the lysosomal dysfunction and synaptic loss in aged neurons [190]. Moreover, Burrinha *et al.,* reported that the dynamin-dependent endocytosis of Tau aggregates damaged the endosomal vesicle membrane that eventually leaked the vesicular content into the cytosol, relating the seed propagation [328]. Interestingly, we also found that endocytosed Tau oligomers were accumulated in Rab7-containing late endosomal vesicles for prolonged periods (>30 minutes) than the monomeric species (10 minutes maximally). These may signify that the monomer follows smooth endosomal trafficking, but the reactive oligomers may mediate vesicular leakage or cytoplasmic release for spreading Tauopathy. The previous report stated that BIN1 and clusterin chaperon were shown to interact with Tau aggregates which subsequently followed endocytosis and cytoplasmic leakage, leading to the spreading of Tauopathy [329, 330]. In our study, Tau monomer followed timely endosomal trafficking and was destined for lysosomal degradation. But, Tau oligomers were found to be delayed for lysosomal localization and resistant to degradation. Therefore, the dilatory endosomal trafficking and cytoplasmic accumulation of Tau oligomers can probably trigger either autophagy pathway as alternative machinery for degradation in microglia. Otherwise, the accumulated Tau oligomers in the cytoplasm and reduced amount of oligomers deposits degradation by migratory microglia may contribute to the spreading of Tauopathy. Henceforth, the trapping and clearance of the toxic intermediate chemotactic Tau oligomers by P2Y12-mediated endolysosomal pathway in migratory microglia can be targeted by immunotherapeutic intervention in AD (Fig. 36).

Conclusion and Future direction

Conclusion

Alzheimer's disease is associated with the gradual accumulation of Aβ plaques and Tau NFTs in brain parenchyma, synaptic loss, cognitive decline and glia-mediated neuroinflammation, leading to neurodegeneration, dementia and other clinical symptoms. In AD, the microtubule-associated protein loses its affinity from tubulin upon various PTMs and mutation, leading to oligomerization, aggregation and neuronal accumulation as NFTs. The accumulated Tau-NFTs exhibit impaired axonal transport, neurotransmitter releases, membrane leakage, mitochondrial potential loss and neuronal apoptosis. The released Tau species act as a potent DAMP signal by surveilling microglia which initiates phagocytic clearance and results in the phagocytosis of live NFTs-bearing neurons and excessive inflammation. During Tau aggregation, various intermediate species are formed in the early stages of disease for *eg.* soluble oligomers which are short-lived but highly reactive, neurotoxic and activators of microglial pro-inflammation. Isolated brain-derived Tau oligomers are unstable species which is challenging for *in vitro* experiments and hence, the preparation of stable full-length Tau oligomers was a major concern. Microglia as surveilling immune cells in the brain can recognize the presence of p-Tau or oligomers in the extracellular milieu. Various microglial or astrocytic surface receptors were identified for the internalization and clearance of pathological Aβ/ Tau species such as scavenging receptors, RAGEs, phagocytic receptors- CD206, CD163, complement receptors, Fcγ receptors, purinergic GPCRs, *etc.* Extracellular pathogenic Tau can also be sensed as a danger signal by microglia to mediate acute migration, activation and inflammation for the pathoprotein clearance. Recently, the purinergic P2Y12 receptor gained an interest in neurodegeneration for neuro-glial communication, actin-associated process extension, microglial chemotaxis towards plaque deposition and balanced inflammation. Here, we have addressed the versatile functions of P2Y12 receptor signaling in Tauopathy-associated microglia. Based on our findings, it can be concluded that-

- The in vitro-prepared stabilized globular Tau oligomers have an immune-reactive potential. Extracellular Tau oligomers and aggregates can induce microglial activation (Iba1) and membrane-associated actin remodeling upon internalization. These oligomers may have some potential to be utilized as a diagnostic biomarker and can be translated for the production of immunotherapeutic mAb.
- The extracellular Tau oligomers were cytotoxic to microglia and induce membrane leakage at high concentrations. Extracellular Tau can directly interact with microglial P2Y12 receptor, induced its expression and elevate the actin-associated $P2Y12^{+}$ lamellipodia and filopodia extensions in migratory microglia. Further, the rate of wound closure, tissue invasion and MTOC polarization were increased in Tau oligomers-induced chemotactic microglia.
- Furthermore, Tau oligomers induced the Arp2 and TKS5-associated podosome and filopodia formation and also localized P2Y12 receptors in migratory actin structures. The P2Y12 signaling activation/blockage directly influences Tau-induced microglial migration, invasion and deposit degradation *via* actin structure formation. These beneficial roles of P2Y12 in microglial chemotaxis, actin network remodeling and Tau deposits clearance can be investigated further in AD mice models or AD brain samples.
- Tau monomer and oligomers were phagocytosed by P2Y12 signaling-mediated and β-arrestin1-associated internalization process *via* a localized F-actin network in microglia. Tau monomer followed faster endocytic trafficking and lead to lysosomal degradation. But, Tau oligomers accumulated with late endosomal vesicles and became resistant to degradation. Therefore, the P2Y12-mediated chemotaxis, endolysosomal targeting and Tau degradation can be intervened as a microglia-based therapeutic strategy in AD (Fig. 37).

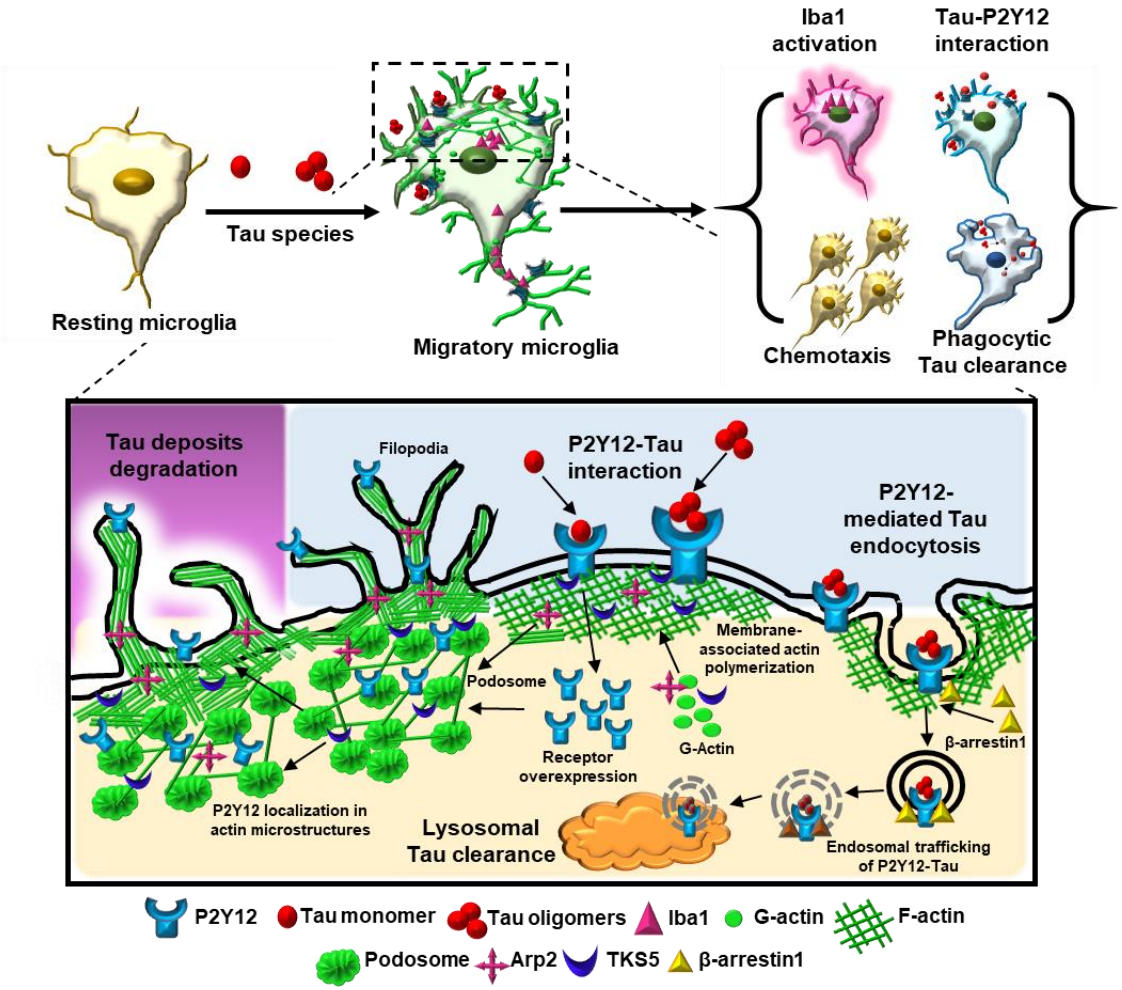

Figure 37. P2Y12 mediated migration, internalization, endosomal trafficking and differential degradation of pathological Tau through the formation of migratory actin microstructures. Extracellular Tau species induced the migratory state of microglia with the stimulation of various functions such as Iba1-associated activation, membrane-associated actin remodeling via podosome, filopodia formation, and chemotaxis. Tau-P2Y12 physical interaction leads to phagocytic and deposits clearance of Tau in activated microglia via actin microstructures. Therefore, the differential functionality of P2Y12 signaling pathway in microglial activation, chemotaxis, Tau lysosomal and deposit clearance *via* actin remodeling can be considered as therapeutically intervened in the field of Tauopathy.

Future direction

The clearance of early pathological protein species/deposits by the microglial involvement is of immense importance in the scenario of AD-like neurodegenerative diseases. Various microglial receptors and risk factors are involved in the pathoprotein accumulation, reception and clearance. Here, we investigated the purinergic GPCR- P2Y12 in terms of microglial chemotaxis, actin remodeling, oligomeric pathoprotein clearance *in vitro* cell culture model system. Hence, the role of this purinoceptor and its genetic knockout/mutation can be studied in the AD mice model or human brain for the identification of microglial beneficial function. Similarly, the *in vitro* prepared Tau oligomers can be studied for induction of Tauopathy by seeding effect in normal mice models. Further, the immunoreactive potential

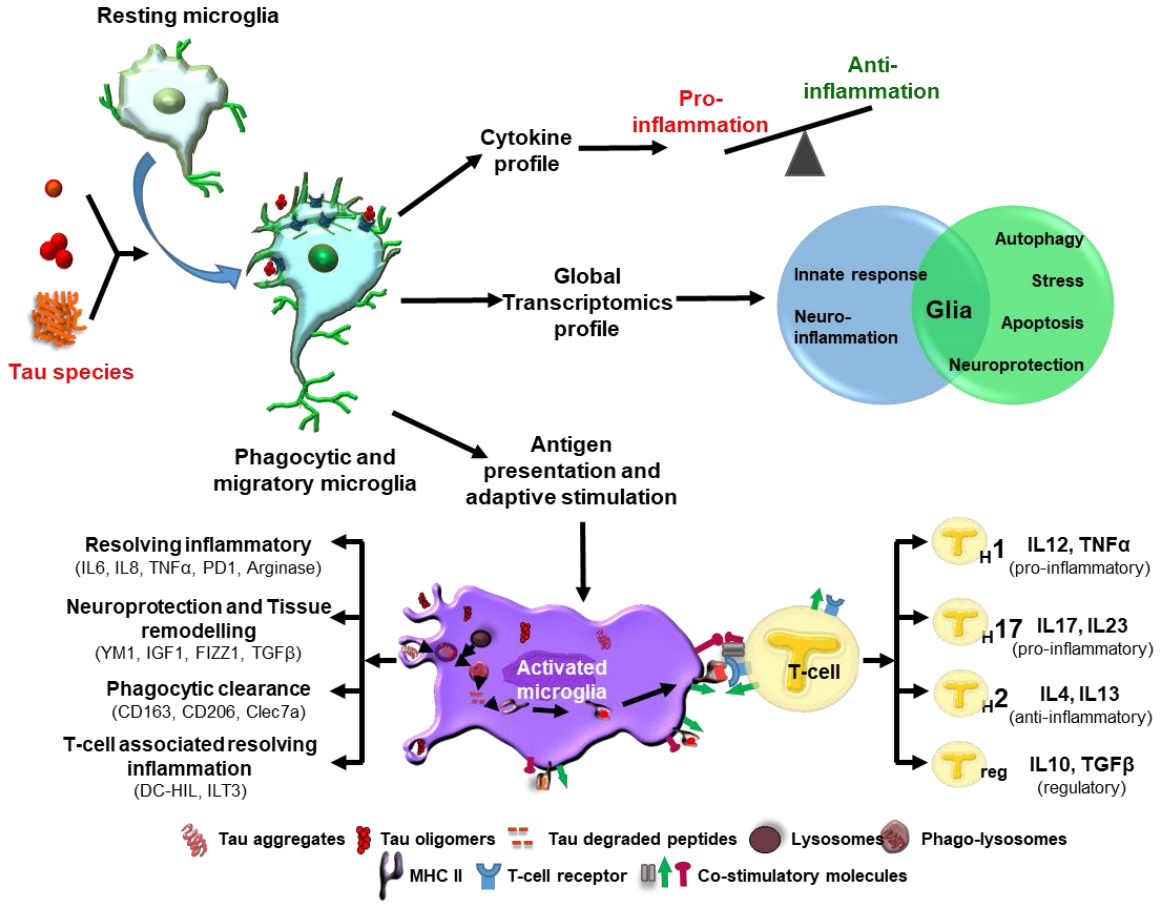

Figure 38. Cytokine profile, immune activation and conversation between microglia and T-cell during antigen presentation in AD. Apart from the microglial Tau phagocytosis, clearance and P2Y12-mediated migration via actin remodeling, the cytokine profile of the Tau-induced microglia can be studied to understand the differential pattern of pro/ anti-inflammatory phenotype in Tauopathy. Moreover, the global transcriptomics profile or sc-transcriptome can be studied to reveal the interconnected signaling pathway of immune activation vs. autophagy vs. neuroprotection. On the other hand, microglia-mediated processing of engulfed Tau (monomer vs. oligomers vs. aggregates) and further generation of pathological epitopes can be studied to identify MHC II loaded peptides in normal vs. AD diseased conditions. Similarly, the approach can be performed in vivo and would be useful to identify the T-cell-mediated immune response and microglia-mediated neuroprotection in the consequence of antigen presentation in CNS.

of these oligomers can be explored in terms of cytokine profiling in microglia or astrocytes. The transcriptomics profiling of *ex-vivo* or *in vivo* microglial fractions can provide insight into the neuroinflammation and metabolic functioning of DAMs. Lastly, the microglia-mediated antigen presentation can be studied in vitro and *ex-vivo* for pathological *vs.* physiological Tau peptide epitope identification and T-cell-associated adaptive stimulation. Moreover, the bipartite conversation between Tau antigen expressing microglia/ infiltrated macrophages and T-cell can identify a beneficial/ detrimental scenario of CNS in AD brain. The prevention or cure of AD is a multi-targeted approach that may involve the intervention of various pathways. Therefore, targeting the oligomeric species by CNS-resident microglia with balanced phagocytic and inflammatory phenotypes may help to prevent synaptic loss, and neurodegeneration with improved cognition in AD (Fig. 38).

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ABSTRACT

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Title of the thesis: Purinergic receptor P2Y12 involves in full-length Tau oligomers-induced microglial chemotaxis, phagocytosis and endocytic trafficking via filopodia-associated actin remodeling.

Alzheimer`s disease (AD) is an age-associated neurodegenerative disease, which is associated with progressive synaptic loss, cognitive impairments, chronic neuroinflammation and behavioral problems. The pathophysiological scenario of AD includes the misfolding and mis-sorting of two proteins: Amyloid-β as extracellular plaques and microtubule-associated protein Tau as neurofibrillary tangles (NFTs). During the process of aggregation, various intermediate species are generated which are released from affected neurons and propagated for further aggregation of endogenous normal protein as seed component. The protein oligomers are short-lived but, highly reactive species that mediate neurotoxicity, inflammation and cognitive decline. The preparation, isolation and detection of oligomers are of immense importance in the current field of designing therapeutics and diagnostics. In this study, we have prepared stable full-length Tau (hTau40wt) oligomers and characterized by various biochemical and biophysical techniques. The in vitro prepared Tau oligomers showed exposed surface hydrophobicity, less β-sheet content and heterogeneous globular structure (5-50 nm). Then, we explored the immune-reactive potential of oligomers on N9 microglia. The disease-associated microglia (DAMs) can mediate improper phagocytosis, oxidative damage, pro-inflammation and impaired actin-associated migration. Here, we found that extracellular Tau oligomers directly interacted with purinergic receptor P2Y12 leading to elevated microglial migration, Iba1-associated activation and phagocytosis of Tau *via* remodeling membrane-associated actin structures such as filopodia, lamellipodia and podosome. Moreover, P2Y12 signaling has impacted Tau deposit degradation by the formation of Arp2 and TKS5-associated actin microstructures. Lastly, we evidenced P2Y12-mediated endocytosis of Tau monomer and oligomers and subsequent endosomal trafficking and lysosomal degradation. Tau monomer was found to be endocytosed with P2Y12 faster than oligomers, then follow endosomal processing and consistent lysosomal degradation. But, oligomers accumulated in the $Rab7^{+}$ late endosomal vesicle and became resistant to lysosomal localization. These functions of P2Y12 in microglial chemotaxis, actin remodeling and Tau clearance can be intervened as therapeutic strategies in AD.

1) **List of publication(s) in SCI Journal(s) (published & accepted) emanating from the thesis work, with complete bibliographic details.**

1. **Das, R.** and Chinnathambi, S.*, 2021. Microglial remodeling of actin network by Tau oligomers, via G protein-coupled purinergic receptor, P2Y12R-driven chemotaxis. **Traffic**, 22(5), pp.153-170.
2. Chidambaram, H., **Das, R**. and Chinnathambi, S.*, 2022. G-Protein coupled Purinergic P2Y12 receptor interacts and internalizes TauRD-mediated by membrane-associated actin cytoskeleton remodeling in microglia. **European Journal of Cell Biology**, p.151201.
3. **Das, R.** and Chinnathambi, S.*, 2022. Microglia degrade Tau oligomers deposit via Purinergic P2Y12-associated podosome and filopodia formation and induce chemotaxis. Available at SSRN: https://ssrn.com/abstract=3919950 .

2) List of Papers with abstract presented (oral/poster) at national/international conferences/seminars with complete details.

1. Poster presentation: **JCB-JEM 2021 Symposium on Neurodegeneration**, online (26-27th April, 2021). **Das, R.** and Chinnathambi, S.*, 2021. Tau oligomers induce P2Y12R-driven microglial chemotaxis and phagocytosis via membrane-associated actin remodeling.
   Abstract: Alzheimer's disease (AD) is age-related neurodegenerative disease which is associated with misfolded protein aggregation, synaptic deformation and chronic inflammation by microglia in brain. Tau oligomers are released from damaged neurons via exosomes, neurotransmitters and membrane leakage etc. Extracellular oligomers and aggregates initiate microglial activation and convert surveilling state into inflammatory state. Microglia sense extracellular Tau through various receptors for chemotaxis and phagocytosis. In our study, we prepared a stable heterogenous globular Tau oligomers range from 5-50 nm, which are phagocytosed by activated A11+ Iba1high microglia. The purinergic receptor P2Y12R involves in neuro-glial communication and microglial chemotaxis towards plaque deposition. Interestingly, we found that Tau directly interacted with microglial P2Y12R and induced its expression. Then, the effect of extracellular Tau oligomers were studied in P2Y12R-mediated actin remodeling and rate of migration in ATP-induced migratory microglia. Also, the reorientation of microtubule network was studied in association with active chemotaxis. Microglial P2Y12R found to colocalize with membrane-associated actin network as a component of migration in response to Tau oligomers. As an inducer of P2Y12R, ATP facilitated the P2Y12R localization more in lamellipodia and filopodia during accelerated microglial migration. While, the inhibition of P2Y12R signaling by potent antagonist, clopidogrel impeded the filopodia-associated actin remodeling, but become restored upon Tau-induced microglial migration. The direct interaction of Tau with P2Y12R would facilitate the signal transduction in both way i.e., directional chemotaxis and receptor-mediated phagocytosis. These unprecedented findings emphasize that microglia incorporate P2Y12R in membrane-associated actin structure to perceive rate of chemotaxis and Tau phagocytosis in Tauopathy.
2. **National Science Day, CSIR-National Chemical Laboratory 2019. Das, R.** and Chinnathambi, S.*, 2019. Internalization of Full-length Tau Oligomers by Activated Microglia.
   Abstract: Alzheimer's Disease (AD) is associated with accumulation of intracellular Tau tangles within the neurons and extracellular Amyloid-β plaques deposition in brain

parenchyma which altogether resulted in synaptic loss and neurodegeneration. Extracellular concentration of oligomeric and aggregated proteins initiate the microglial activation and converts synaptic surveillance into destructive inflammatory state. Although Tau oligomers are unstable species, it was shown to mediate neurotoxicity and glial pro-inflammation. Due to the fragility of oligomers, in vitro experiments become challenging and hence the stability of the full-length Tau (HTau40WT) oligomers is major concern. In this study, we have prepared and stabilized HTa40WT oligomers in PBS (pH 7.4) which is purified by size exclusion chromatography. These oligomers have formed heterogeneous globular structures (5-50nm) as seen by transmission electron microscopy which was further characterized by oligomer-specific A11 antibody. Immunocytochemistry studies for oligomers treatment has evidenced with A11+ Iba1high activated microglia, suggesting the principle of phagocytosis. Also, the microglia were observed with remodeled filopodia-like actin structures upon the exposure of oligomers and aggregated Tau, relating the microglial migration for engulfment.

3. **National Science Day, CSIR-National Chemical Laboratory 2021. Das, R.** and Chinnathambi, S*., 2021. Tau oligomers remodel microglial actin network for chemotaxis via P2Y12R purinergic receptor.
   Abstract: Alzheimer's disease (AD) is an age-related neurodegenerative disease with misfolded protein aggregation, synaptic deformation and chronic inflammation by microglia in brain. Tau oligomers can be released from damaged neurons via exosomes, neurotransmitter and membrane leakage etc. Microglia can sense the extracellular Tau through various receptors to mediate chemotaxis and phagocytosis. The purinergic receptor P2Y12R is involved in neuro-glial communication and microglial chemotaxis towards the plaque deposition. The effect of extracellular Tau oligomers were studied in P2Y12R-mediated actin remodeling, in the rate of migration and reorientation of microtubule network in ATP-induced microglial cells. The extracellular Tau directly interacted with P2Y12R and also induced its expression in microglia. Microglial P2Y12R colocalized with remodeled membrane-associated actin network as a component of migration in response to Tau oligomers. As an inducer of P2Y12R, ATP facilitated the localization of P2Y12R in lamellipodia and filopodia during accelerated microglial migration. The direct interaction of Tau oligomers with microglial P2Y12R would facilitate the signal transduction in both ways i.e., directional chemotaxis and receptor-mediated phagocytosis. These unprecedented findings emphasize that microglia can incorporate P2Y12R in modulated membrane-associated actin structure and perceive the rate of chemotaxis in Tauopathy.

3) A copy of all SCI publication(s), emanating from the thesis.

Received: 21 August 2020 | Revised: 5 January 2021 | Accepted: 25 January 2021 | Published on: 18 February 2021
DOI: 10.1111/tra.12784

ORIGINAL ARTICLE

Traffic WILEY

Microglial remodeling of actin network by Tau oligomers, via G protein-coupled purinergic receptor, P2Y12R-driven chemotaxis

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Funding information
Council of Scientific and Industrial Research, India, Grant/Award Number: MLP101726; CSIR-National Chemical Laboratory, Grant/Award Number: MLP029526

Abstract

Alzheimer's disease (AD) is associated with age-related neurodegeneration, synaptic deformation and chronic inflammation mediated by microglia and infiltrated macrophages in the brain. Tau oligomers can be released from damaged neurons via various mechanisms such as exosomes, neurotransmitter, membrane leakage etc. Microglia sense the extracellular Tau through several cell-surface receptors and mediate chemotaxis and phagocytosis. The purinergic receptor P2Y12R recently gained interest in neurodegeneration for neuro-glial communication and microglial chemotaxis towards the site of plaque deposition. To understand the effect of extracellular Tau oligomers in microglial migration, the P2Y12R-mediated actin remodeling, reorientation of tubulin network and rate of migration were studied in the presence of ATP. The extracellular Tau species directly interacted with P2Y12R and also induced this purinoceptor expression in microglia. Microglial P2Y12R colocalized with remodeled membrane-associated actin network as a component of migration in response to Tau oligomers. As an inducer of P2Y12R, ATP facilitated the localization of P2Y12R in lamellipodia and filopodia during accelerated microglial migration. The direct interaction of extracellular Tau oligomers with microglial P2Y12R would facilitate the signal transduction in both way, directional chemotaxis and receptor-mediated phagocytosis. These unprecedented findings emphasize that microglia can modulate the membrane-associated actin structure and incorporate P2Y12R to perceive the axis and rate of chemotaxis in Tauopathy.

KEYWORDS
actin remodeling, Alzheimer's disease, chemotaxis, microglia, migration, P2Y12R, Tau oligomers

1 | INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease, which is associated with the accumulation of extracellular amyloid-β plaques and Tau protein as neurofibrillary tangles (NFTs) in the brain.[1] The astrocytes and microglia-mediated neuroinflammation in the central nervous system (CNS) exacerbate the massive oxidative damage, synaptic loss and propagation of seed species to healthy cells.[2,3] Tau is a microtubule-associated protein that is mainly involved in axonal microtubule stabilization and synapto-trafficking in neurons.[4] In AD, the accumulation of Tau majorly affects neurons,[5] while in glial tauopathies, Tau aggregates were accumulated in the perinuclear region of microglia, oligodendrocytes and astrocytes.[6-8] In disease condition, Tau has been post-translationally modified by phosphorylation, glycation, acetylation, methylation and glycosylation, and so forth, which contribute to NFTs formation and subsequent escape

from neurons.[4,9] During aggregation, oligomerization is the rate-limiting step but once initiated; it accelerates the formation of Tau fibrils.[10] Oligomers are the most reactive species, which can intervene in various cellular processes such as microtubule treadmilling, membrane leakage, neurotransmitter release, faulty synapses, cargo trafficking and amyloidogenesis.[11-15] Hence, targeting and sequestering of toxic Tau oligomers and fibrils by microglia is the prime most concern in neurodegeneration.

Microglia are the brain-resident macrophages which generally maintains the ramified structures with long processes, continuously sampling the tissue microenvironment.[16] But, the extracellular presence of Tau can be diagnosed by microglia, which in turn, retracts its surveilling extensions into amoeboid structures.[17] Microglia sense the released chemical gradients from affected neurons and phagocytose the proteinaceous plaques and damaged axons.[17,18] By encountering the patho-proteins, microglia become activated[19] and express various cytokines, chemokines and complement factors. The interaction of amyloids and immune receptors leads to the receptor-dependent endocytosis and clearance of extracellular protein depositions.[20-23] Upon differential activation pattern, either "classical" or "resolving," microglia modulates its cytoskeletal network of actin and tubulin for active migration and phagocytosis.[19,24] The engulfed plaques can be degraded by activated microglia; therefore, the peptides can be presented with MHC-II to the infiltrated T-cells, leads to the adaptive immune response in CNS.[1] Damaged neurons are the source of extracellular protein aggregates as well as energy nucleotides (ATP, ADP, UDP, etc.).[25] Microglia mediates chemotaxis by various surface receptors, among which purinergic G-protein coupled receptors (GPCRs) are the most abundant in brain parenchyma.[26,27] Purinergic receptors are broadly classified into two groups, such as P2X ion receptors and metabotropic P2Y GPCRs.[28,29] The P2X receptors are two-transmembrane ligand-gated ion channels, which are mainly involved in ion homeostasis. But, P2Y receptors are seven-transmembrane GPCRs. P2Y receptors play an essential role in platelet aggregation, while this group of GPCRs has gained recent interest in neurodegeneration because of their occurrences only in myeloid progenitor cells and in chemotaxis.[30-32] Hence, chemical-driven migration is the most important step in the initiation of misfolded protein clearance and protective immunity by pathology-associated microglia.[33,34]

A recent report suggested that α-synuclein aggregates can act as a chemoattractant and mediates the microglial migration via β1 integrin-dependent manner.[35] Extracellular ADP induces the microglial chemotaxis and membrane ruffling through the activation of Extracellular signal-regulated kinase 1/2 (ERK1/2) and β-arrestin signaling cascade, which leads to the formation of paxillin-containing lamellipodia structures.[36] Upon binding of ATP on the ligand-binding pocket of P2Y12R, the signal passes through the intracellular release of Ca^{2+} from endoplasmic reticulum reservoirs, which can act as the signal for migration.[37,38] Therefore, the disorganization of actin and tubulin cytoskeletal networks may contribute to the hampered cellular function in microglia.[39] The P2Y12/13R activation in cortical neurons has been shown to induce the ROS level through the activation of Nuclear factor erythroid 2-related factor 2 (Nrf2) during neuroinflammation.[40] Microglia mediates the neuroprotection through the formation of P2Y12R dependent somatic junction, which is regulated by Ca^{2+} signaling in neurons.[41] Similarly, Li et al., showed that P2Y4R regulates the uptake of fluid-phase amyloid-β by pinocytosis in ATP-dependent manner in rat microglia.[42] In contrast, the P2Y6R is shown to facilitate the "phagoptosis" of live neurons by primary microglia.[43] Clopidogrel, an inhibitor of P2Y12R, plays a significant role in the clearance of amyloid-β-induced dead neurons and behavioral improvement in neurodegeneration induced *Zebrafish larvae* model.[44]

In this scenario, we are interested to elucidate the role of Tau oligomers and fibrils as chemoattractant in microglial migration. The formation of P2Y12R-containing remodeled actin cytoskeletal network, migration rate and correlation of microtubule-organizing center (MTOC) with the microglial polarization would emphasize on Tau exposed microglia. Then, either activating or blocking of P2Y12R signaling would explore the further remodeling of microglial actin network upon extracellular Tau species-mediated chemotaxis.

2 | RESULTS

2.1 | Tau forms stable globular oligomers, cytotoxic to N9 microglia

Intracellular accumulation of Tau as NFTs and its subsequent escape from neurons are the prominent scenario of Tauopathy, which converts the patrolling microglia into activated phagocytic phenotypes.[45] Tau is a microtubule-associated protein, which contains two domain N-terminal projection domain and C-terminal microtubule-binding domain.[46] The repeat region contains two hexapeptides which are responsible for Tau oligomerization (Figure 1A).[47] Tau monomer was found to be oligomerized at 12 hours of incubation with heparin, while the formation of Tau fibril was evidenced after 4 days of incubation, by SDS-PAGE (Figure 1B). Upon oligomerization, Tau protein exposes the surface hydrophobicity and accumulates core β-sheet structure, as evidenced by increased 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence and Thioflavin S (ThS) fluorescence, respectively.[48,49] Tau aggregates were found to be distinct as compared to oligomers with increased ThS fluorescence but less ANS fluorescence, which determines the abundance of core β-sheets and buried hydrophobicity, respectively (Figure 1C). The immunoreactivity of Tau oligomers was more with the A11-oligomer specific antibody as compared to monomeric and aggregated form (Figure 1D). Moreover, the Transmission electron microscopy (TEM) study revealed the globular Tau oligomers have size ranging from 5 to 50 nm and Tau aggregates as long fibril (Figure 1E). In order to understand the cytotoxic potential of stable Tau oligomers and aggregates, N9 cells were exposed to extracellular Tau species for 24 hours. Extracellular Tau oligomers were found to be more toxic than the aggregates species to N9 microglial cells. The Thiazolyl blue tetrazolium bromide (MTT) assay showed that the Tau oligomers at 5 μM concentration had shown less than 20% cell viability, while the aggregated Tau showed >40% cell viability

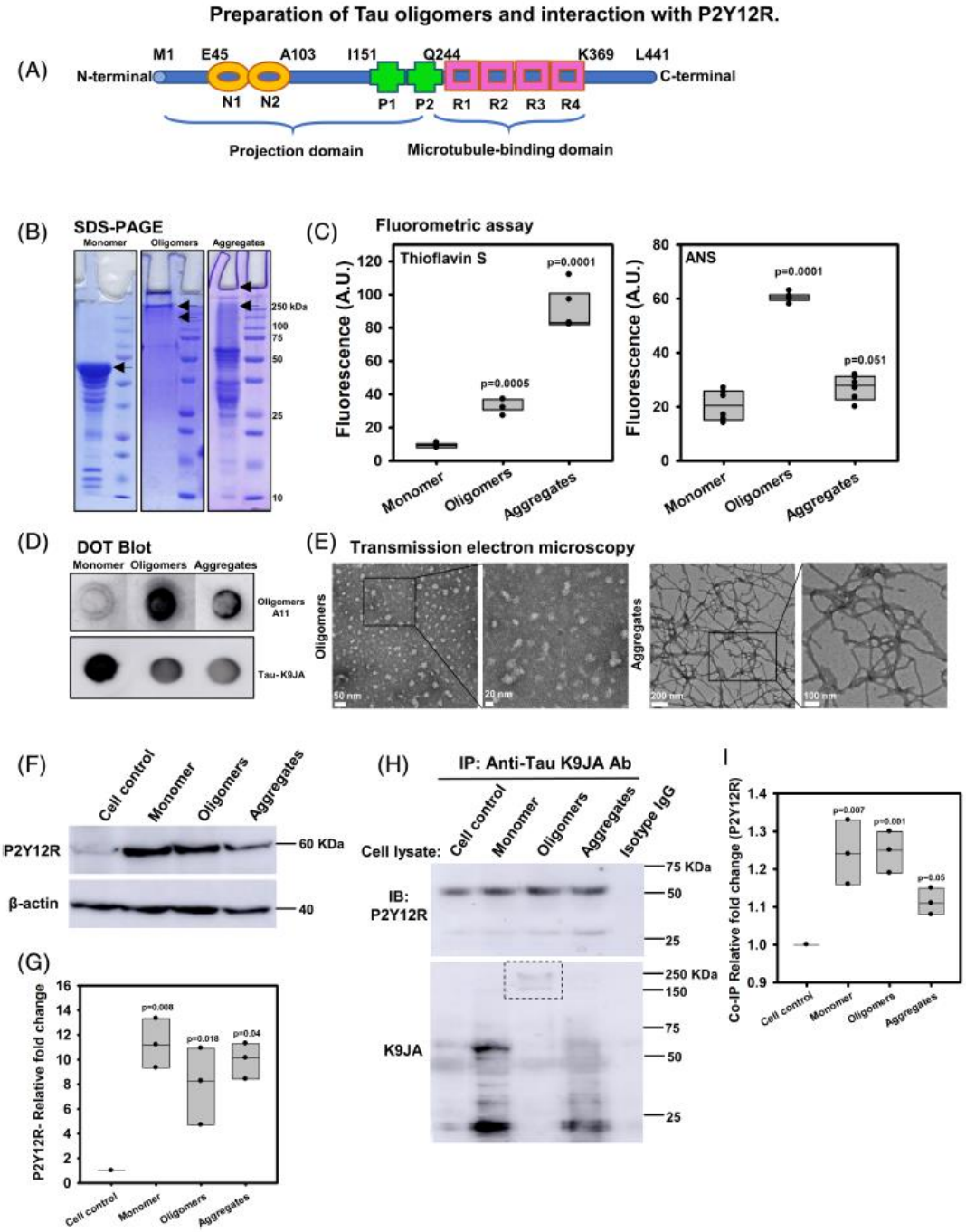

FIGURE 1 Preparation of Tau oligomers and interaction with P2Y12R. A, Microtubule-associated protein Tau comprises two domains, N-terminal projection domain and C-terminal repeat domain. The projection domain contains two inserts, while the repeat domain contains four repeat regions. B, Recombinant human Tau40wt (hTau40) was oligomerized by polyanionic heparin, followed by glutaraldehyde fixation. SDS-PAGE analysis showed the formation of oligomers at 150 to 250 kDa MW. At the same time, the aggregates formed a smear throughout the well. C, Fluorometric analysis showed the formation of oligomers with less β-structure and high hydrophobic patches as compared to aggregates and monomer. D, Dot Blot analysis confirmed the presence of A11-reactive oligomers in comparison with total Tau K9JA staining. E, TEM analysis showed the formation of heterogeneous globular oligomers. In contrast, the aggregates formed tangles in nature. F, Extracellular Tau monomers and oligomers have induced the protein expression of P2Y12R more in N9 microglia as compared to aggregates exposure. G, Densitometric analysis has estimated around 12 folds increase in P2Y12R protein expression upon monomer exposure while the oligomers and aggregates induced the receptor expression around 8 folds and 10 folds, respectively. H, Co-immunoprecipitation of microglial P2Y12R with external recombinant Tau species showed the maximum interaction of P2Y12R-Tau in oligomers exposure, which is followed by monomer and aggregates. I, Densitometric analysis showed about 1.3 fold more precipitation of P2Y12R from Tau monomer and oligomers-exposed microglia while, the aggregates treatment resulted in 1.1 fold changes in P2Y12R precipitation

at the same concentration on N9 cells (Figure S1A). Similarly, Lactate dehydrogenase (LDH) leakage assay is a determinant of membrane leakage because of the abundance of toxic amyloids. *Tau* oligomers showed membrane leakage even at 5 μM concentration, while the same concentration of Tau aggregates was non-toxic to N9 cells in terms of LDH release (Figure S1B). Together, these results suggested that extracellular globular Tau oligomers could induce cytotoxicity and membrane leakage more than fibrillar aggregates on N9 cells.

2.2 | Extracellular Tau interacts with P2Y12R and induces its expression

Upon encountering extracellular amyloidogenic protein, activated microglia altered the expression of membrane receptors to migrate at the location and subsequent phagocytic clearance.[1] When extracellular Tau are released from damaged or NFTs bearing neurons, microglia can sense Tau species as chemoattractant.[50] Here, the microglial P2Y12R expression level was increased by 12 folds by monomer exposure while the oligomers and aggregates induced the P2Y12R expression about eight folds as compared to cell control (Figure 1F, G). In order to understand the interaction of extracellular Tau with P2Y12R signaling, the co-immunoprecipittation (co-IP) was performed between these protein partners. Interestingly, P2Y12R was found to interact with toxic Tau species as precipitated by pan Tau-K9JA antibody. The microglial P2Y12R interacted more with extracellular Tau oligomers as compared to aggregates, which may signify the binding reactivity of oligomers to P2Y12R (Figure 1H, I). In this scenario, it has been observed for the first time that extracellular Tau can directly interact with microglial P2Y12R and might intervene P2Y12R-mediated chemotaxis. The direct interaction between Tau oligomers and P2Y12R may signify the dual role of this particular purinergic receptor, which might involve in microglial migration and phagocytosis.

2.3 | P2Y12R Interacts with remodeled actin network for migration

Previous reports have shown that the P2Y12R is involved in microglial morphological process extension, filopodia formation, neuro-glial somatic junction formation and excessive phagocytosis of affected neurons.[41,51] Similarly, P2Y12R was shown to induce the bulbous actin cone formation in the lamellipodia of immune-surveilling microglia.[52] In this regard, P2Y12R was observed to be localized more with a membrane-associated actin network in Tau oligomers-treated microglia as compared to the aggregates-treated group (Figure 2A). The colocalization of P2Y12R and actin was more in microglial lamellipodia than in filopodia in both Tau oligomers and aggregates treated groups (Figure 2B, C). This result suggests that P2Y12R is driving the microglial migration by remodeling the lamellipodial actin network in response to Tau overload (Figure 2D). In order to quantify the membrane-associated actin structures in migratory microglia, we observed that lamellipodia-bearing microglia has increased around 20% in oligomers and aggregates exposure as compared to monomer treatment (Figure 3A-C). While the filopodia-bearing microglia has increased about 40%, which was maximum in monomer exposure than higher molecular weight (HMW) Tau species (Figure 3D-F). Moreover, the filopodia number per microglia has been increased extensively in monomer exposure which is followed by aggregates and oligomers treatment (Figure S2A). These results indicate that extracellular Tau monomer facilitates filopodia formation. However, the HMW oligomers and aggregates mediate the active microglial migration via directed lamellipodial growth (Figure 3G).

2.4 | ATP colocalizes P2Y12R with actin in lamellipodia upon Tau aggregates exposure

ATP is a high-energy phosphate molecule regulating various cellular processes, which includes energy metabolism, mitochondrial function, cargo movements and cellular migration, and so forth.[53] Furthermore, ATP acts as a ligand for the P2Y12R-mediated signaling pathway and mediates the microglial chemotaxis.[50] Here, we aim to mimic the situation of neuronal damage during Tauopathy by treating the microglial cell with extracellular Tau species. Also, we interested to understand the synergistic function of ATP and Tau in actin remodeling via P2Y12R signaling. It was observed that ATP-induced the actin remodeling in microglial lamellipodia and uropod, determining the microglial polarization for directional movement (Figure 4A). When microglia were treated with monomer and oligomers along with ATP, it was observed that P2Y12R was colocalized more in migratory structures (lamellipodia and uropod) as compared to only Tau-treated microglia groups (Figure S3). But the aggregates and ATP exposure facilitated the actin remodeling more at microglial uropod, while lamellipodial actin rearrangement remained unaltered (Figure 4B). Together ATP and aggregates exposure accumulated the P2Y12R at frontal lamellipodia. While, the monomer and oligomers treatment along with ATP reduces the P2Y12R localization from microglial lamellipodia (Figure 4C). Various Tau species, such as monomer, oligomers and aggregates allow P2Y12R localization at microglial uropod. But together with ATP, monomer and aggregates reduce the P2Y12R uropod localization as compared to respective control. Unlike monomer and aggregates, oligomers and ATP co-treatment induce the P2Y12R intensity synergistically in microglial locomotory actin structures (Figure S4A).

2.5 | ATP induces filopodia extension in Tau-treated microglia

In order to understand the mechanism of microglial migration in terms of filopodia extension, microglial cells were treated with Tau species together with ATP in various treatment groups. Monomer and aggregates with ATP were found to induce the number of filopodia bearing microglial cells, while oligomers with ATP was associated with

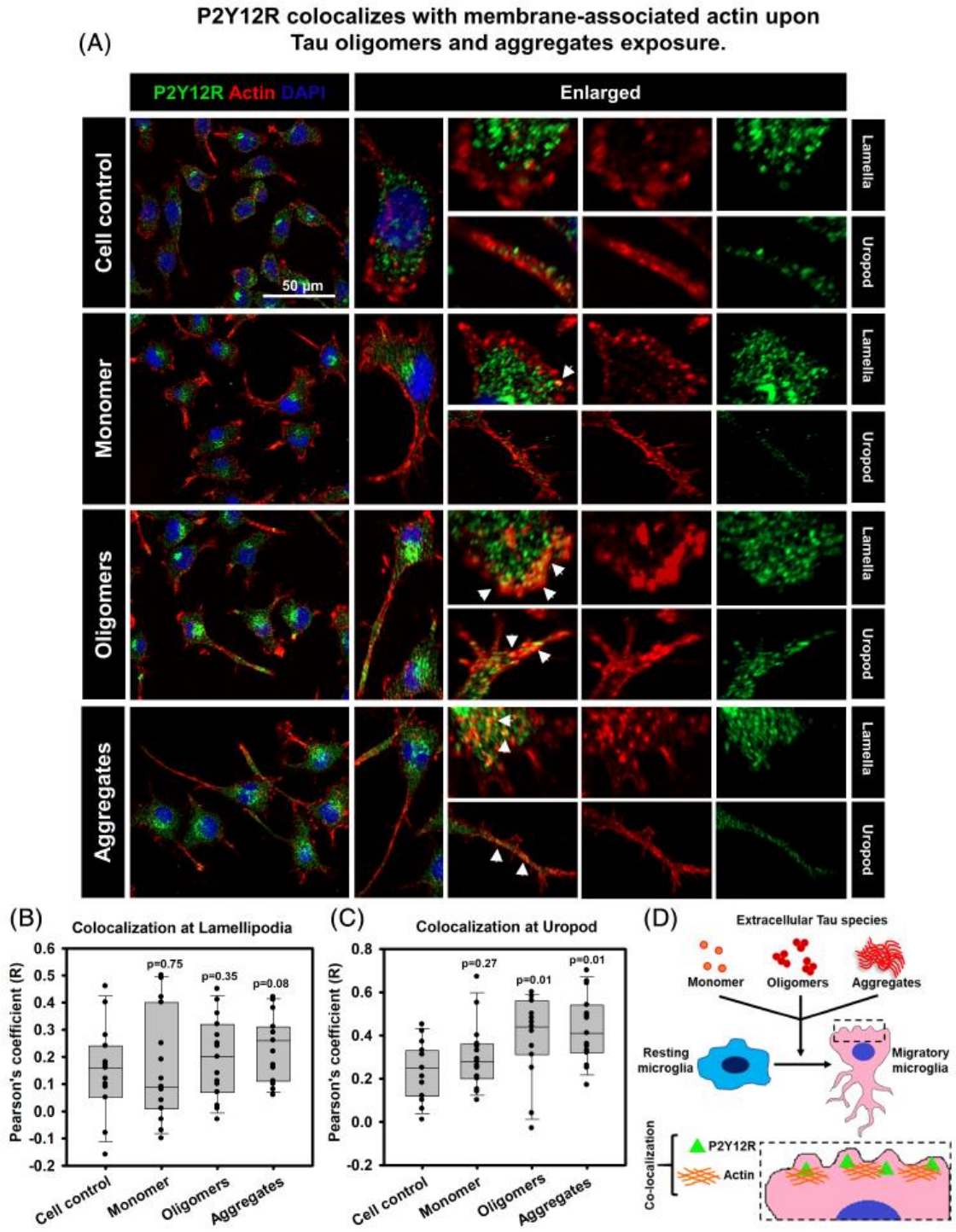

FIGURE 2 P2Y12R colocalizes with membrane-associated actin upon Tau oligomer and aggregates exposure. A, Extracellular ATP/ADP acts as a ligand for cell surface P2Y12R, which mediates the signals for rapid microglial migration. Extracellular Tau oligomers induce the maximum colocalization of P2Y12R with membrane-associated actin in lamellipodia and uropod in comparison with aggregates and monomer exposure. The arrow indicates the colocalization of P2Y12R and actin. The images were taken in 63X oil objective and scale bar 50 µm. B, P2Y12R and actin were found to be positively colocalized in lamellipodia of actively migrated microglia as observed by Pearson's coefficient (R) by ImageJ software (n = 20). C, Oligomers and aggregates exposure have increased the colocalization (R) of P2Y12R-actin in uropod whose retraction facilitates the forward movement in microglia in comparison with Tau monomer treatment. D, In summary, extracellular exposure of toxic Tau species allowed the resting microglia to transform into the migratory state, which led to the accumulation and colocalization of actin-P2Y12R into lamellipodia and uropod for forward movement

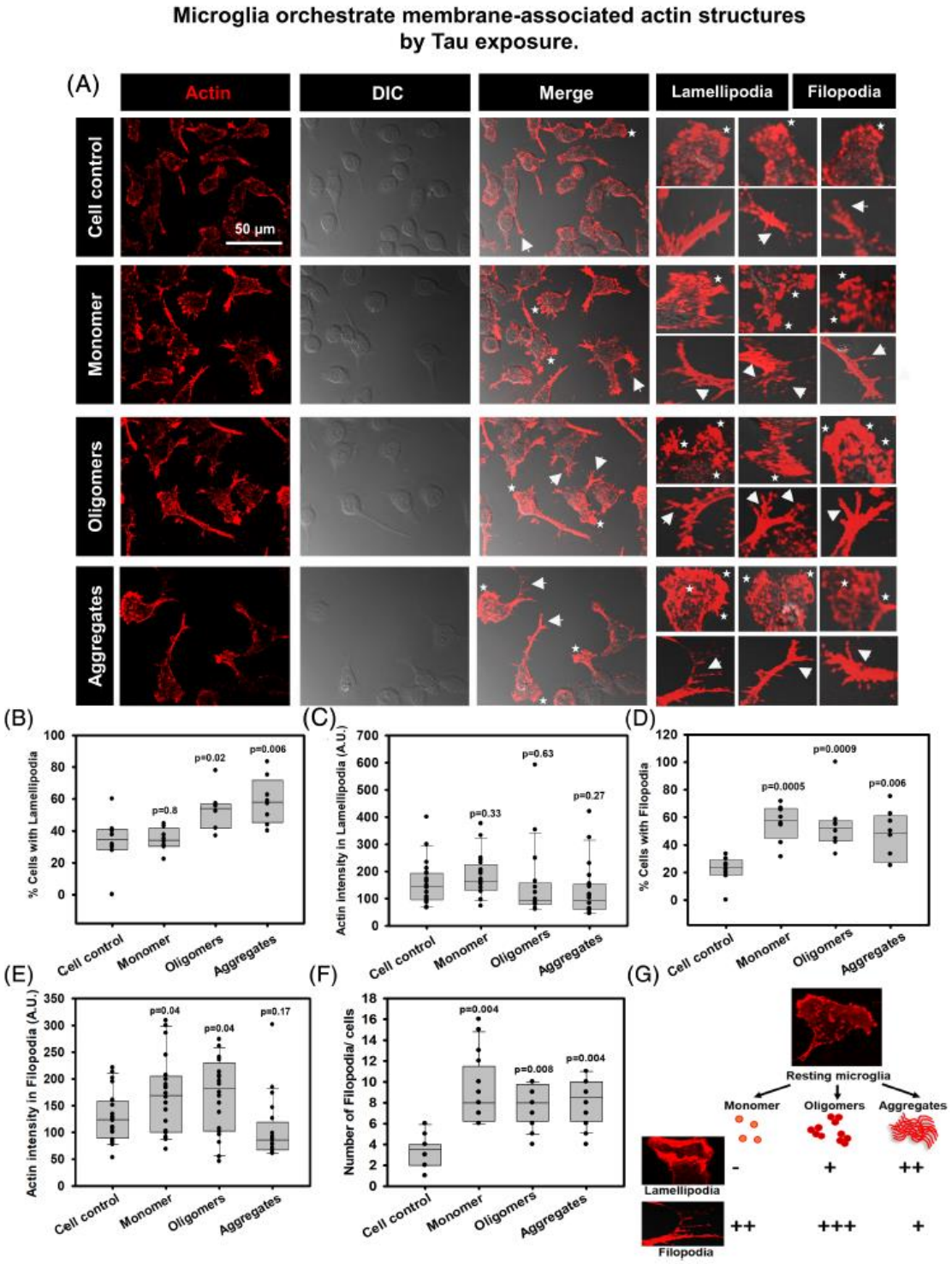

FIGURE 3 Microglia orchestrate membrane-associated actin structures by Tau exposure. A, N9 microglia increased the formation of lamellipodia with concentrated actin podosomes and more filopodial extensions upon Tau oligomers and aggregates exposure in comparison with monomer. These are the structures involved in matrix adhesion and degradation as well as for rapid migration and polarization. The star sign indicates the actin-rich lamellipodia organization and the arrow indicates the filopodia extension from migratory microglia. B, *Tau* oligomers and aggregates induced the % cells bearing lamellipodia as compared to monomer for directional migration, while, C, the actin intensity in lamellipodia remain unaltered among the various Tau-exposed groups. D, Similarly, monomer exposure accounted for more filopodia bearing cells than oligomers and aggregates, which may signify different possible directions for migration. E, Actin intensity remained unaltered among various Tau treatment groups, F, While all the Tau-exposed microglial groups showed an equal increase of filopodial extensions from migratory cells (n = 20). The images were taken in ×63 oil objective and scale bar 50 µm. The Zen 2.3 software is used for quantification. G, In summary, Lamellipodia bearing microglia was more in aggregates, followed by oligomers and monomer, but when considering filopodia-bearing cells, the maximum occurrences were with oligomers, and then followed by monomer and aggregates

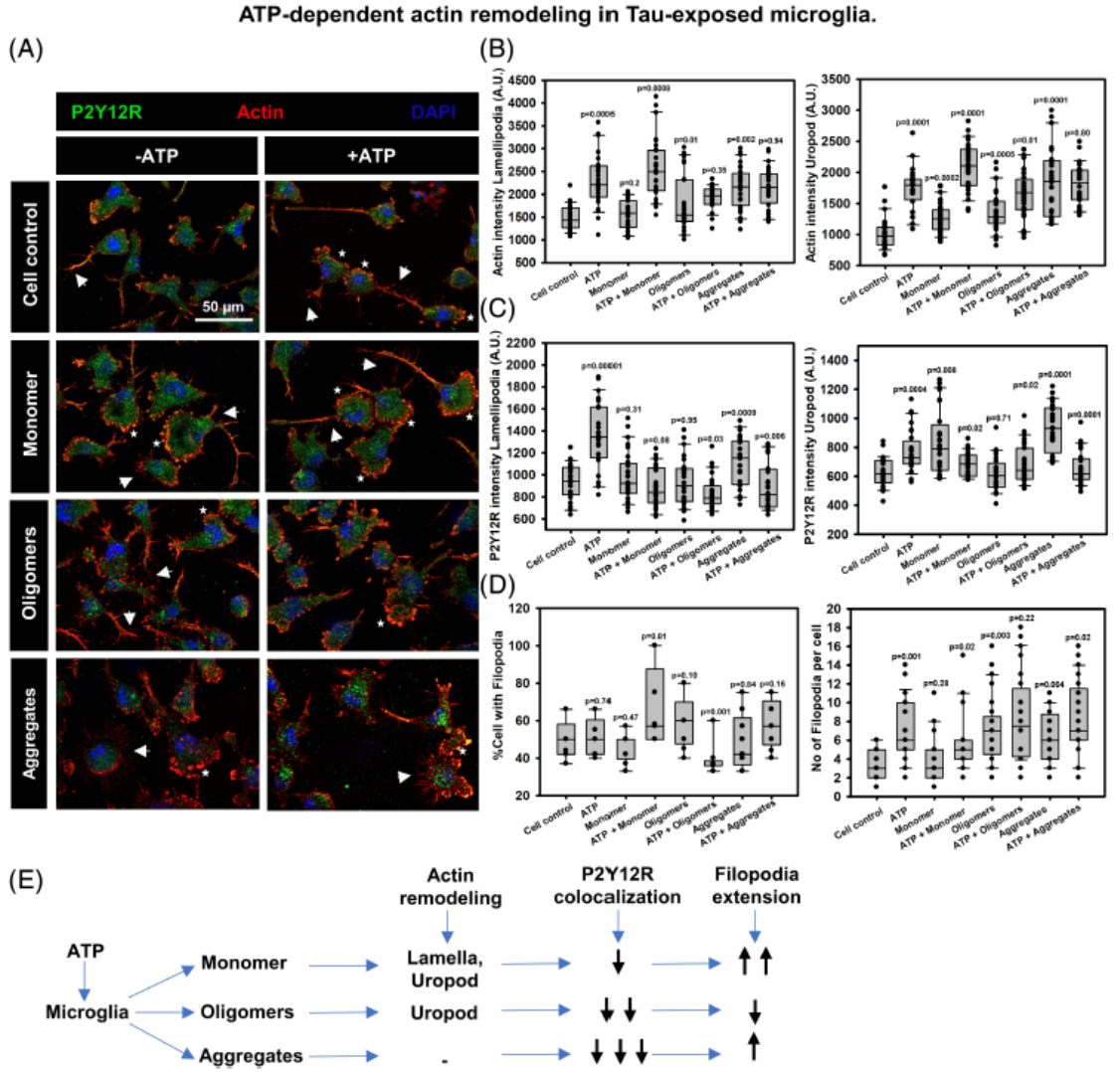

FIGURE 4 ATP-dependent actin remodeling in Tau-exposed microglia. A, Extracellular ATP acting as a chemoattractant for surveilling microglia through the activation of P2Y12R signaling led to the directional migration at the site of injury. The star sign indicates the actin-rich lamellipodia organization and the arrow indicates the filopodia extension from migratory microglia. B, ATP-induced the actin polymerization in migratory microglia along with monomer and oligomers in both lamellipodia and uropod. Similarly, P2Y12R was also found to be accumulated more in lamellipodia and uropod in synergy with monomer and oligomers while aggregates together with ATP, delocalized P2Y12R from membrane-associated actin structure. (n = 28). The images were taken in 63X oil objective and scale bar 50 µm. Zen 2.3 software is used for quantification. C, *Tau* monomer and aggregates in the presence of ATP induced the filopodia-bearing microglial cells as well as the number of filopodia from each cell while oligomers has reduced both filopodia number and associated migratory cells. D, ATP has synergistically mediated the actin remodeling in lamellipodia and uropod with extracellular Tau species. In contrast, P2Y12R colocalization with membrane-associated actin has reduced in synergistic treatment with Tau oligomers and aggregates. While, monomers and oligomers along with ATP have induced the filopodia extensions for rapid migration. E, In summary, extracellular Tau oligomers and monomer increased the ATP-induced actin remodeling and filopodia extensions with reduced membrane-associated localization of P2Y12R during microglial migration

reduced filopodia bearing cells. Similarly, the number of filopodia from microglia can relate the migration and polarization during extracellular Tau exposure. Here, we observed that the number of filopodia per cells were varying insignificantly among Tau-exposed treatment groups, along with or without ATP. ATP-induced the filopodia number more as compared to untreated microglia while all the Tau species induced filopodia formation separately, which correlates the initiation of migration (Figure 4D). When Tau species were exposed along with ATP, only monomer and aggregate-induced the filopodia numbers further in comparison with the oligomers-treated group (Figure 4E). In determining the abundance of P2Y12R with membrane-associated actin in cellular cortices, we observed that P2Y12R and actin were always positively colocalized at filopodia and lamellipodia upon Tau and ATP exposure (Figure S4B,C).

2.6 | Tau oligomers modulates actin network, redundant of P2Y12R activation

To assess the role of P2Y12R in microglial actin remodeling, clopidogrel was used to inhibit the P2Y12R signaling. Clopidogrel is a known antagonist of P2Y12R, which binds to this purinoceptor and thereby blocks the signal transduction irreversibly.[54] Here, we observed that clopidogrel exposure reduced the number of filopodia extensions from migratory microglia as compared to cell control (Figure 5A). However, when the microglia were treated with extracellular Tau species along with clopidogrel, the filopodia extensions were restored but the P2Y12R level remained consistently low (Figure 5B, C). Extracellular Tau oligomers and aggregates significantly induced the number of filopodia bearing cells as well as the number of filopodia per cells, even upon blockage of P2Y12R signaling (Figure 5D, E). Moreover, the colocalization of P2Y12R and membrane-associated actin structures in lamellipodia and uropod, decreased in clopidogrel-treated microglia (Figure S5). Also, the exposure of extracellular Tau species did not replenish their colocalization in migratory microglia (Figure 5F, G). These results signifies that the formation of migratory actin structures in response to extracellular Tau oligomers, is independent of P2Y12R pathway. Furthermore, the western blot study revealed that clopidogrel exposure induced the cellular level of P2Y12R as compared to untreated control, while the P2Y12R level became upregulated even in Tau exposed condition (Figure 5 H, I). Together, this results clearly suggested that the blockage of P2Y12R signaling resulted in the upregulation of this purinoceptor expression which may contribute to the replenishment of actin remodeling in microglia. Hence, the P2Y12R signaling is required for actin remodeling but seems redundant for microglial filopodia formation.

2.7 | Tau oligomers enhance microglial chemotaxis, but monomer reduces migration

In order to understand the microglial chemotaxis in response to extracellular Tau oligomers and aggregates, the trans-well migration assay was performed along with ATP as a positive regulator. Here, it was observed that Tau oligomers induced the trans-well migration as compared to cell control while monomer has reduced the number of migratory cells through the porous membrane during chemotaxis (Figure 6A). Although, Tau aggregates induced microglial migration by wound-scratch assay, but upon chemotactic response N9 cells migrated non-significantly by extracellular Tau aggregates (Figure 6B). As a positive regulator of microglial migration and chemotaxis, ATP has induced the trans-well microglial migration maximally as compared to untreated control. Together, it has been observed that extracellular Tau oligomers can act as a chemoattractant for induced microglial migration (Figure 6C).

2.8 | Tau oligomers and aggregates increase the rate of microglial migration

Tau species and ATP exposure are found to be involved in actin-mediated microglial migration, while wound scratch assay would give an estimation of the rate of effective migration via P2Y12R signaling (Figure 7A). The wound closure percentage can estimate microglial migration after the wound scratching on the cell monolayer. ATP acts as a positive regulator in chemotaxis, which induced the wound closure by increased time interval. Here, we observed that Tau aggregates induced rapid wound closure even from 3 hours, which continues up to 6 and 12 hours. Monomer exposure showed the initial delayed migration of microglia at 3 hours, which tend to increase from 12 to 24 hours post-scratching on the monolayer. Tau oligomers-treated microglia have shown a constant increase in migration from the initial hour, which resulted in the wound closure most effectively after 24 hours' time (Figure 7B, C). Altogether, it was evidenced that oligomers exposure helps the microglial migration maximally but slowly while aggregates possess immediate response as chemoattractant during migration (Figure S6).

2.9 | Tau exposure reorients MTOC for the polarization of microglia

The MTOC position often determines the migration axis of motile immune cells. In our study, we observed that Tau oligomers and aggregates exposure majorly accounted for the anterior position of MTOC in polarized microglia, which may relate the forward movement and active phagocytosis of toxic Tau species (Figure 8A). The oligomers exposure led to almost equal amount of posterior and lateral MTOC positions during microglial migration, which may signify the steady state migration and inflammatory response in microglia (Figure 8B). But, the Tau aggregates exposure has resulted in less posterior MTOC position and few lateral position for rapid migration. While, Tau monomer exposure has evidenced with less anterior MTOC position and more occurrences of lateral MTOC localization (Figure 8C).

3 | DISCUSSION

Tau is a soluble, natively unfolded protein, which lacks a particular three-dimensional structure. Tau has an N-terminal projection domain, which helps in solubilization and spatial arrangements while the C-terminal repeat domain is involved in interaction with microtubules.[46] Recent reports showed that a steady increase of granular toxic Tau oligomers in AD brain, which can readily aggregate the physiological Tau as plaques in healthy mice brain.[55,56] The initial release of Tau oligomers can function as chemoattractant for microglial clearance of pathoproteins.[57,58] We prepared heterogeneous globular Tau oligomers stabilized by 0.01% glutaraldehyde, containing more exposed hydrophobic patches and less β-sheet content. These Tau oligomers induced cellular toxicity and LDH leakage from N9 microglial cells. Therefore, the chasing of this Tau oligomers by microglial migration can be a crucial event to combat neurodegeneration and inflammation in CNS.[59]

Microglia orchestrate various cell surface receptors for sensing extracellular protein deposits and also chemotaxis receptors for

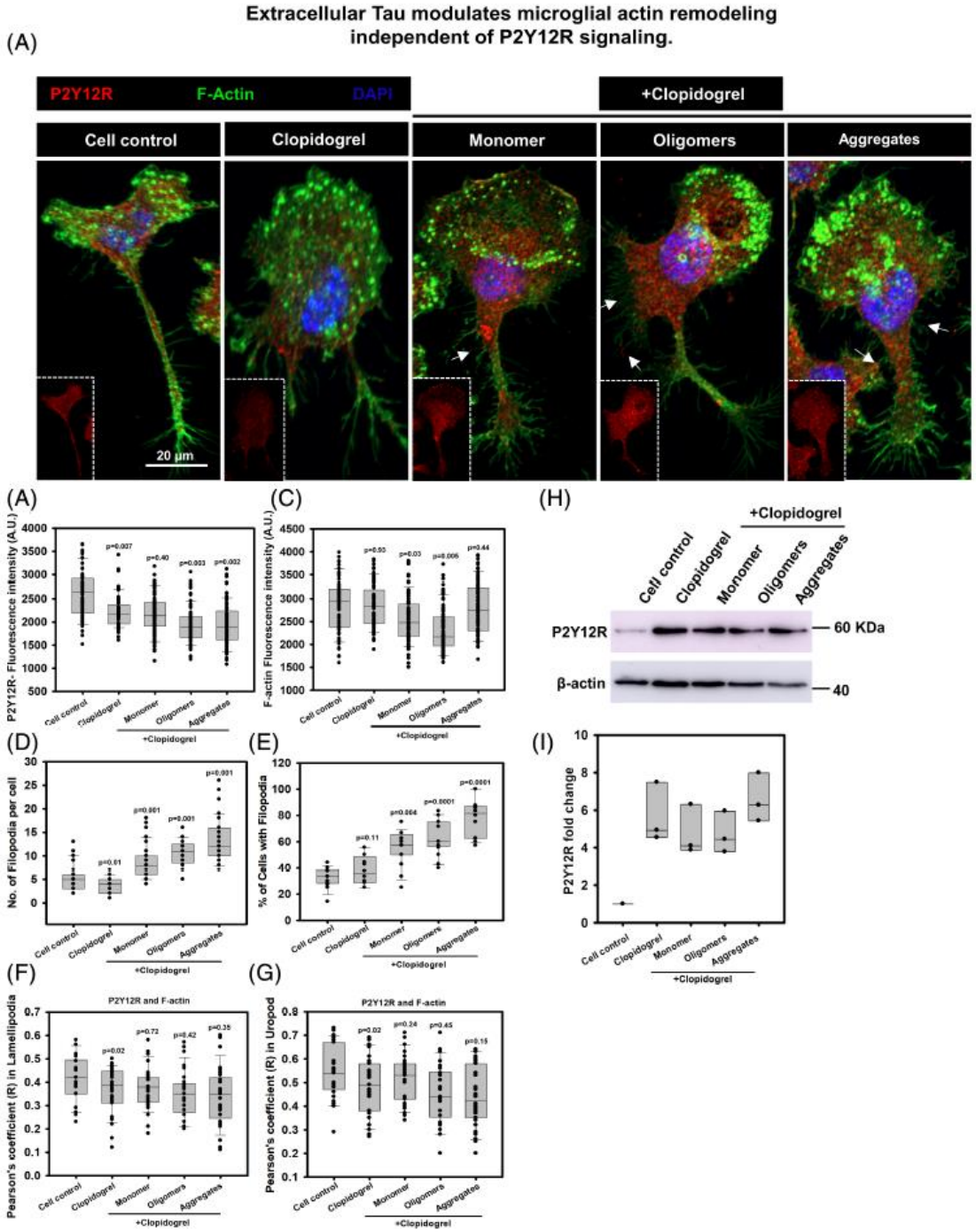

FIGURE 5 Extracellular Tau modulates microglial actin remodeling, independent of P2Y12R signaling. A, Clopidogrel as a potent antagonist of P2Y12R signaling, downregulated the level of this purinoceptor and also reduced the formation of filopodia in migratory microglia. But, the exposure of extracellular Tau oligomers and aggregates have restored the filopodia extensions, upon P2Y12R blockage. The arrow indicates the abundance of filopodia protruding out from individual microglia. Only the P2Y12R fluorescence (red) for various treatment groups were shown in inset. The images were taken in ×63 oil objective and scale bar 20 µm by the Zen 2.3 software. B, Clopidogrel treatment has significantly reduced the level of P2Y12R in microglial cells, which remained consistently low even after Tau exposure. The absolute intensity were quantified from multiple cells (n = 70) by ZEN 2.3 software and plotted. C,Clopidogrel and Tau exposure did not alter the F-actin level in microglia (n = 70). D, The blocking of P2Y12R signaling significantly reduced the formation of filopodia per cells which was replenished by co-treatment with extracellular Tau species. E, similarly, the percentage of cells bearing filopodia remained unaltered upon P2Y12R antagonism, but increased significantly by Tau oligomers and aggregates exposure (n = 70). F, Clopidogrel exposure reduced the level of colocalization of P2Y12R with F-actin in lamellipodia which remained constant even after Tau exposure. G. The Pearson's coefficient (R) for colocalization of P2Y12R and F-actin remained unaltered in microglial uropod in various treatment groups (n = 30)

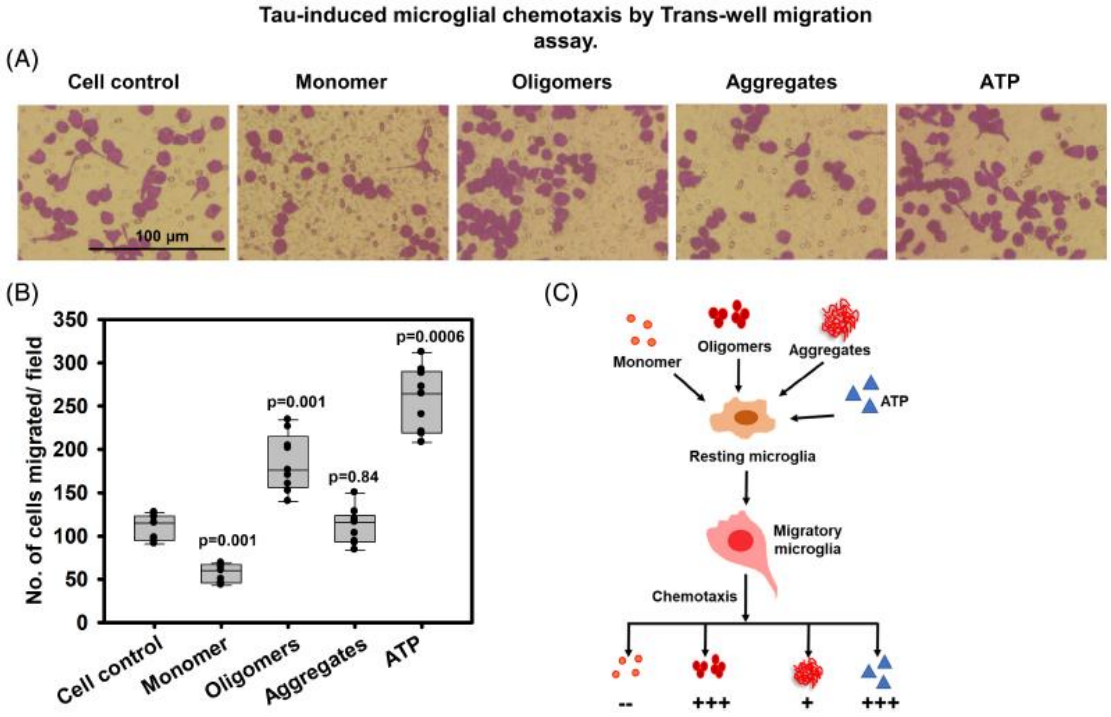

FIGURE 6 Tau-induced microglial chemotaxis by Trans-well migration assay. A, Having inherent property of migration, microglia were found to be migrated through the pore of trans-well plate. But, the addition of Tau species in lower chamber of trans-well plate, resulted in differential rate of microglial migration. Tau monomer reduced the number of microglia migrated through tans-well plate, while the oligomers have induced the maximum microglial migration as chemotaxis. B, The migration associated with aggregates exposure remained insignificant, while ATP as a positive regulator of migration, showed maximum number of migrated cells as compared to cell control. C, In summary, extracellular Tau species can act as chemoattractant where the soluble oligomers has the highest capacity to induce microglial migration among all Tau species

directional migration.[60] Among all purinergic receptors, P2Y12R gained recent attention in neurodegeneration for the expression on myeloid-originated microglial cell.[31] It has been reported that microglia highly express P2Y12R during rapid migration and non-activated condition.[61] Another report emphasized that the presence of P2Y12R^{+} tufted microglia at the boundary of plaque deposited zone, but upon reaching, microglia transformed into HLA-DR^{+} pro-inflammatory phenotype.[62] Extracellular Tau monomer and oligomers can acts as soluble chemoattractant while the HMW aggregates mimic the situation of plaque deposition in an in vitro microglial culture. Here, Tau monomer and oligomers induced the P2Y12R expression, emphasizing on P2Y12R-mediated migration. P2Y12R was also found to be colocalized more with migratory actin structures upon Tau oligomers exposure as compared to aggregates and monomer. This may signify that microglia may have switched its expression profile from non-activated to a pro-inflammatory phenotype in response to larger aggregates. The co-immunoprecipitation for Tau species and microglial P2Y12R revealed their direct interaction and role of Tau species in P2Y12R-mediated chemotaxis. In particular, P2Y12R interacts more with Tau oligomers and monomer as compared to aggregates, which signifies the role of LMW Tau species as more potent chemoattractant via P2Y12R-driven signaling cascade.

Actin is a cytoskeletal microfilament that mainly functions in cellular structural integrity, migration, invasion and ECM adhesion.[24] The polymerization of actin in lamellipodia produces tensile forces for forward movement, while depolymerization at uropod helps retraction of cell from the surface matrix.[17] Previously, we reported that the phagocytosis of globular Tau oligomers by remodeling the actin network in activated N9 microglia.[45] In another report, Parkinson's related α-synuclein protein induces microglial migration by binding with CD11b and remodeled the actin scaffold into lamellipodia by interacting with cortactin.[35] Here, the extracellular Tau oligomers and aggregates induce the lamellipodia formation, which may signify the accumulation of actin scaffold for migration and degradation of extracellular Tau species.. But, monomers were associated with filopodia extension, which determines the chemotropic sensation and matrix adhesion during microglial migration.

It has been well-proven that the extracellular gradient of ATP/ADP acts as a signal for microglial chemotaxis in response to CNS tissue injury.[25] The redistribution of P2Y12R on microglial membrane leads to the filopodia formation towards the site of injury while the P2Y12R mutation results in less number of processes and non-specified engulfment of synapses in seizure-induced epileptic brain.[37,63] In contrary, the activation of P2Y12R collapses the

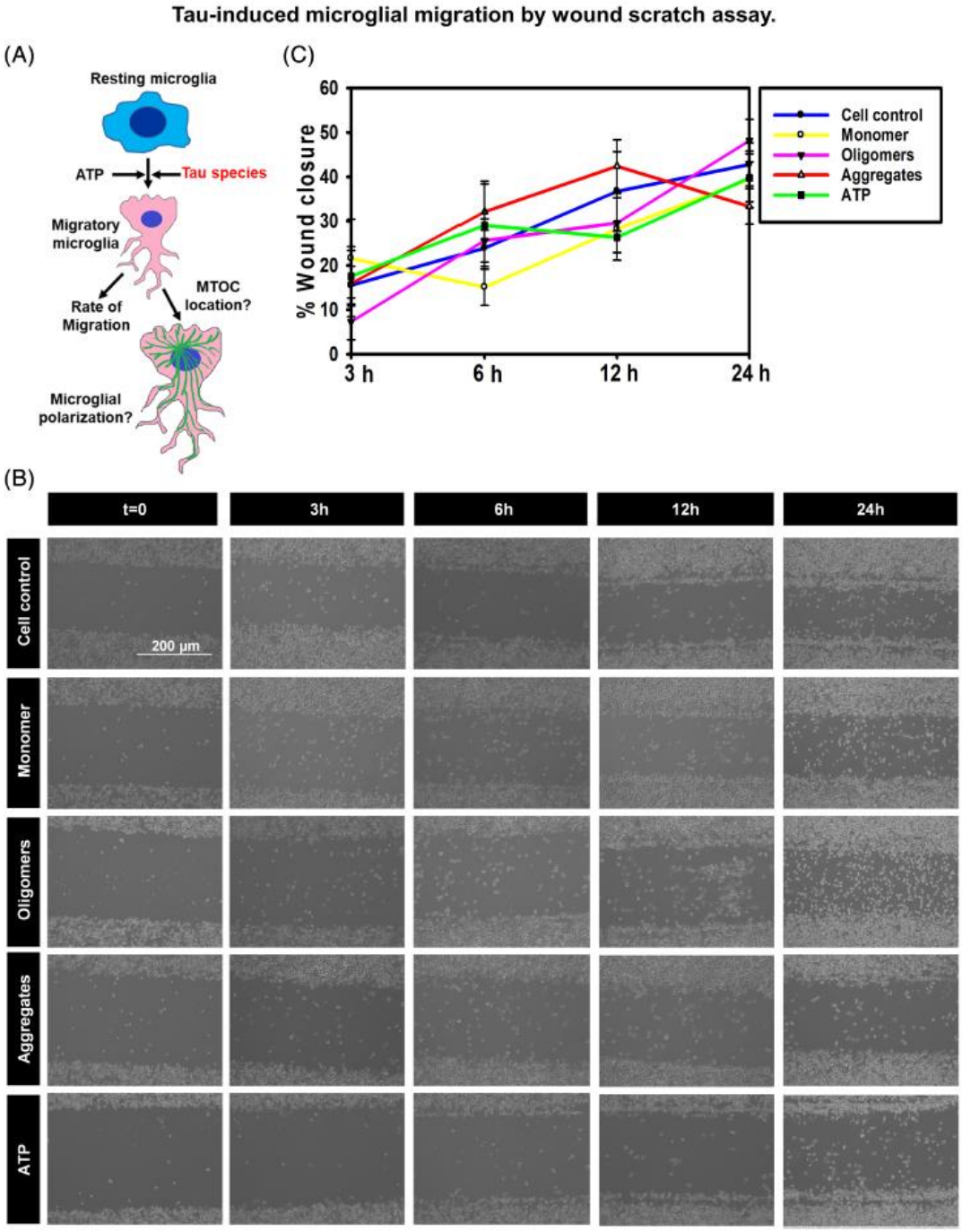

FIGURE 7 Tau-induced microglial migration by wound scratch assay. A, Upon extracellular exposure of ATP and Tau species, microglia enhanced the rapid migration via actin remodeling and chemotaxis. The rate of migration was estimated by time-dependent wound scratch assay. B, N9 microglial cell monolayers were scratched with micro tips and various Tau species were added at 0 hour time with ATP as a positive regulator of microglial migration. Tau aggregates exposure showed a prompt response in wound closure at an early time point (3 hours), while Tau oligomers showed a constant increase in the rate of migration, which reached the maximum wound closure at 24 hours' time-interval. Monomer showed an initial decrease in wound closure but slowly increased in due time. C, The phase-contrast images were taken at various time intervals at four different positions. The gap of the wounds was measured and converted into % wound closure. Oligomers closed the wound maximally but at a slow increase while Tau aggregates showed prompt wound closure at early time points. Scale bar 200 µm

filopodia and increases the bulbous actin structures in immune-surveilling microglia.[52] Moreover, P2Y12/13R induces the retraction of cellular processes through the activation of RhoA/ROCK-mediated P38-MAPK signaling cascade in rat spinal microglia.[64] The released ATP can modulate the microglial migration via either binding to P2Y12R ligand-binding pocket or by rearrangement of membrane-associated

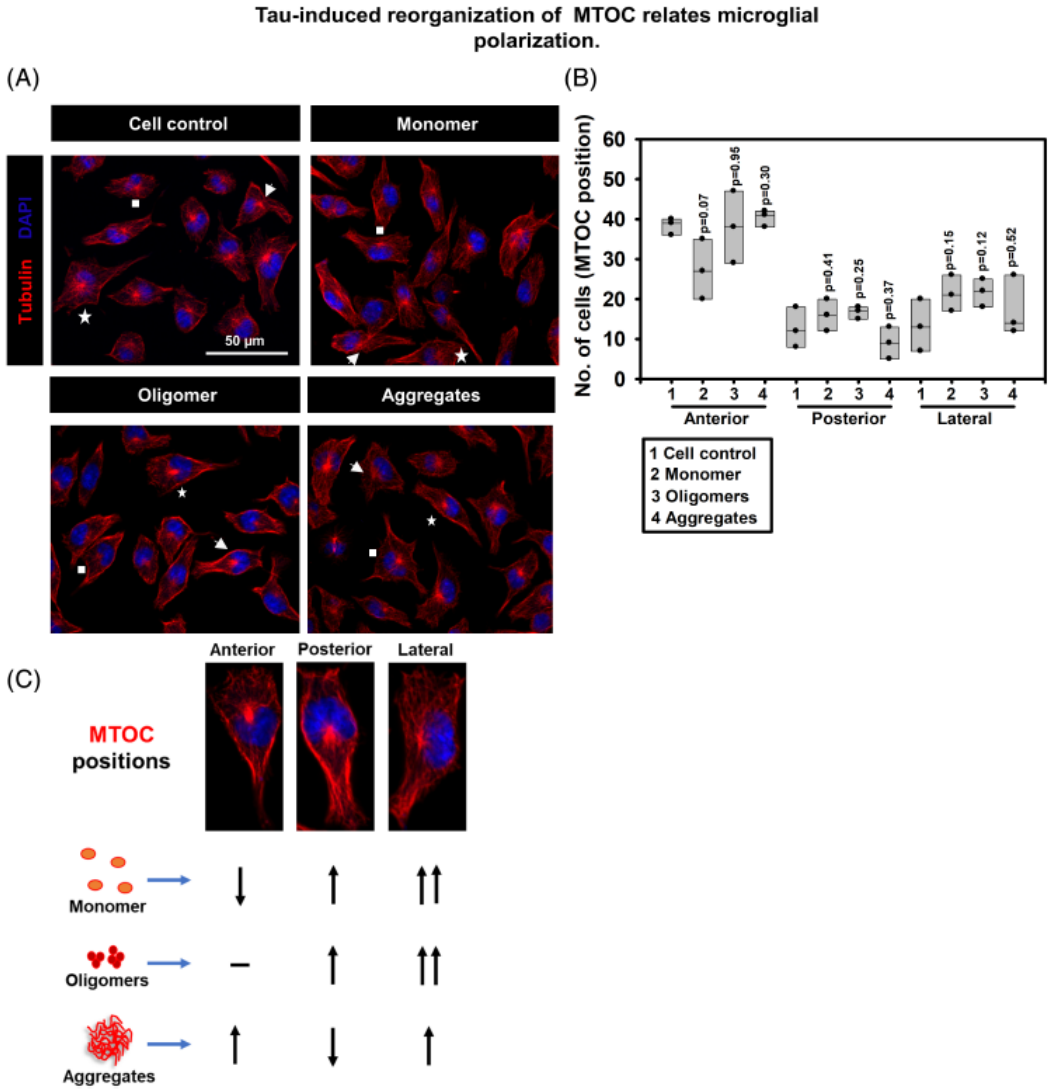

FIGURE 8 Tau-induced reorganization of MTOC relates microglial polarization. A, The reorientation of the MTOC position determined the effective way of microglial polarization. The MTOC was visualized by α-tubulin fluorescence staining in eight microscopic fields (n = 65). The images were taken in ×63 oil objective and scale bar 50 μm. The star sign indicates the anterior MTOC, the arrow indicates the posterior MTOC and the box indicates the lateral MTOC migratory microglia. Microglia were observed with prominent MTOC staining in oligomers and aggregates treatment. B, Aggregates exposure favored anterior MTOC position for migration and phagocytosis. Oligomers favored all possible MTOC positions relating to microglial inflammation. C, In summary, Tau monomer exposure has induced more posterior MTOC position, while oligomers exposure led to almost equal amount of posterior and lateral MTOC positions during microglial migration. But, the Tau aggregates led to more anterior MTOC position and reduced posterior MTOC position for migration

actin structures into lamellipodia-uropod formation.[65,66] In our study, the synergistic exposure of ATP with oligomers resulted in increased level of P2Y12R in lamellipodia and uropod. This indicates the extracellular Tau oligomers could connect the purinergic chemotaxis with orchestrated filopodial extension. The co-treatment of ATP and monomer mediates the lamellipodia actin remodeling, independent of P2Y12R while, the aggregates with ATP lacks a definite actin remodeling which may signify the transformation of migratory microglia into the phagocytic phenotype. Hence, the soluble Tau oligomers influence the P2Y12R-mediated microglial chemotaxis via actin remodeling. But HMW Tau aggregates bypass the ATP-driven P2Y12R interaction for actin remodeling in migration. In order to emphasis further on P2Y12R function in membrane-associated actin remodeling, this purinoceptor signaling was inhibited by a potent antagonist clopidogrel. Here, the blocking of P2Y12R signaling reduced the actin remodeling significantly, in particular the filopodia extensions from microglia. However, the number of filopodia got restored by subsequent Tau exposure, but not the colocalization of P2Y12R and actin in

P2Y12R inhibited microglia. Together, these signify that the inhibition of P2Y12R signaling can impede the actin remodeling partially, in microglia, upon Tau-induced migration.

As a primary immune cells in CNS, microglia display a dynamic migration and constant surveillance of the neuronal network in brain microenvironment.[21,67-70] Therefore, the rate of microglial migration and invasion can be a determinant of altered function in CNS injury and inflammation.[71,72] The soluble but reactive Tau oligomers can readily diffuse through brain parenchyma and attracts microglial chemotaxis. But, Tau aggregates can easily be deposited at particular brain region. Thereby, the identification of the plaque region can act as "search me" signal to microglia to follow the chemical gradient.[73] Microglia can even traverse through tissue crevices by secreting various proteases to reach at the damaged site.[74,75] Upon reaching to the protein deposits, microglia further transformed into phagocytic and inflammatory state.[76] Extracellular Tau species can form the chemotaxis gradient in tissue organization for attracting microglia, hence, the trans-well migration assay would identify the potency of Tau oligomers as chemoattractant. Thus, the gradient of ATP along with amyloidogenic proteins Tau dictate the microglia to reach at the protein deposited site by involving P2Y12R and actin remodeling.[25,77] The MTOC position determines the migratory state of microglia where anti-inflammatory IL-4 treatment helps the microglia to orient the MTOC in both anterior and posterior position, relating directional migration for tissue repair. But the pro-inflammatory LPS stimulation disorganizes the microtubule distribution completely with no preferential MTOC position.[74] It has been previously reported that migratory neutrophil reorients its MTOC position at anterior site for rapid movement and phagocytosis of pathogen.[78] To connect the P2Y12R-mediated chemotaxis with microtubule network reorganization, we have estimated the time-dependent microglial migration and corresponding MTOC location in response to Tau exposure. Aggregates exposure induces the rapid migration for wound closure and favors anterior MTOC position, which indicates the activation of phagocytic microglia. But oligomers mediate the slow but steady migration at a constant increased rate with all preferable MTOC position, which may signify the microglial response to globular oligomers with pro-inflammatory amoeboid phenotype.

In conclusion, we can state that extracellular Tau oligomers and other toxic species can interact with microglial P2Y12R and mediates the rearrangement of membrane-associated actin network for rapid migration. The increased lamellipodia and filopodial extension lead to the feed-forward accumulation and colocalization with P2Y12R for induced migration, invasion, MTOC repolarization and subsequent internalization in the scenario of Tauopathy (Figure 9).

4 | MATERIALS AND METHODS

4.1 | Chemicals and cell culture reagents

For the preparation of Tau protein and its oligomerization, Luria-Bertani broth (Himedia); Ampicillin, NaCl, $MgCl_2$, Phenylmethylsulfonylfluoride (PMSF), Ammonium persulfate (APS), Heparin (17 500 Da), DMSO and Methanol were purchased from MP Biomedicals; IPTG and DTT from Calbiochem; MES, BES, glutaraldehyde and SDS from Sigma; EGTA, Protease inhibitor cocktail, Tris, 40% Acrylamide, TEMED from Invitrogen. For biochemical studies, Thioflavin-S, ANS, TritonX-100 were purchased from Sigma. For cell culture studies, RPMI 1640 media, FBS, Horse serum, PBS (cell biology grade), trypsin-EDTA, Penicillin-streptomycin were purchased from Invitrogen. The coverslip of 0.17 mm for immunofluorescence study and 400 mesh copper coated carbon grids for TEM study were purchased from Blue star and Ted Pella, Inc., respectively. In immunofluorescence and western blot studies, we used the following antibodies: total pan-Tau antibody K9JA (Dako, cat no A0024), A11 Oligomers specific antibody (Thermo, cat no AHB0052), β-Actin loading control monoclonal antibody (BA3R) (Thermo, cat no MA5-15739), P2Y12R antibody (4H5L19) (Thermo 702 516), Plalloidin-Alexa 488 for F-actin (Thermo, cat no. A12379), α-tubulin monoclonal antibody (Thermo, cat no. DM1A), Goat anti-rabbit IgG (H + L) Cross-adsorbed secondary antibody HRP (Invitrogen, cat no. A16110), anti-mouse secondary antibody conjugated with Alexa flour-488 (Invitrogen, cat no A-11001), Goat anti-rabbit IgG (H + L) Cross-adsorbed secondary antibody with Alexa Fluor 555 (Invitrogen, cat no. A-21428), DAPI (Invitrogen, cat no. D1306), cell culture 24-well inserts (Thermo 140 629), Pierce Co-Immunoprecipitation Kit (Thermo 23 600) and ECL reagent (Bio-Rad, cat no. 1705060). Clopidogrel bisulfate (IP grade) is a generous gift from Mr. Prabhanjan S Giram.[79] The N9 microglial cell line no. is CVCL_0452.

4.2 | Preparation of Tau oligomers and aggregates

Tau protein was expressed in *Escherichia coli* BL21* with ampicillin antibiotic selection, while the protein expression was induced by 0.5 mM IPTG, as described earlier.[80] In brief, the bacterial cells were lysed at 15 Kpsi in the Constant cell disruption system (Constant Systems Ltd.). The lysate was heated at 90°C and dialyzed overnight at 4°C while the purification of Tau protein was done by cation-exchange chromatography, followed by size-exclusion chromatography. Tau oligomers were prepared by inducing with heparin (17.5 kDa) in PBS (pH 7.4), as described earlier.[45] After the incubation, the Tau oligomers were stabilized using 0.01% glutaraldehyde for 10 minutes. The oligomers were checked by 10% SDS-PAGE. The purified Tau oligomers were stored at −80°C. The Tau aggregates were prepared similarly as oligomerization but in BES buffer (pH-7.4) by inducing with heparin for 4 days at 37°C and similarly analyzed by SDS-PAGE.

4.3 | Biochemical and biophysical characterization of Tau species

Tau oligomers were concentrated by 10 kDa MWCO centrifugal filter by centrifuging at 3200 rpm for 2 hours. The compact cross-β structures of Tau species were characterized by Thioflavin-S at Ex/Em: 440/521 nm, while the exposure of hydrophobic patches on the surface of Tau species was quantified by ANS fluorescence at Ex/Em: 375/490 nm using a

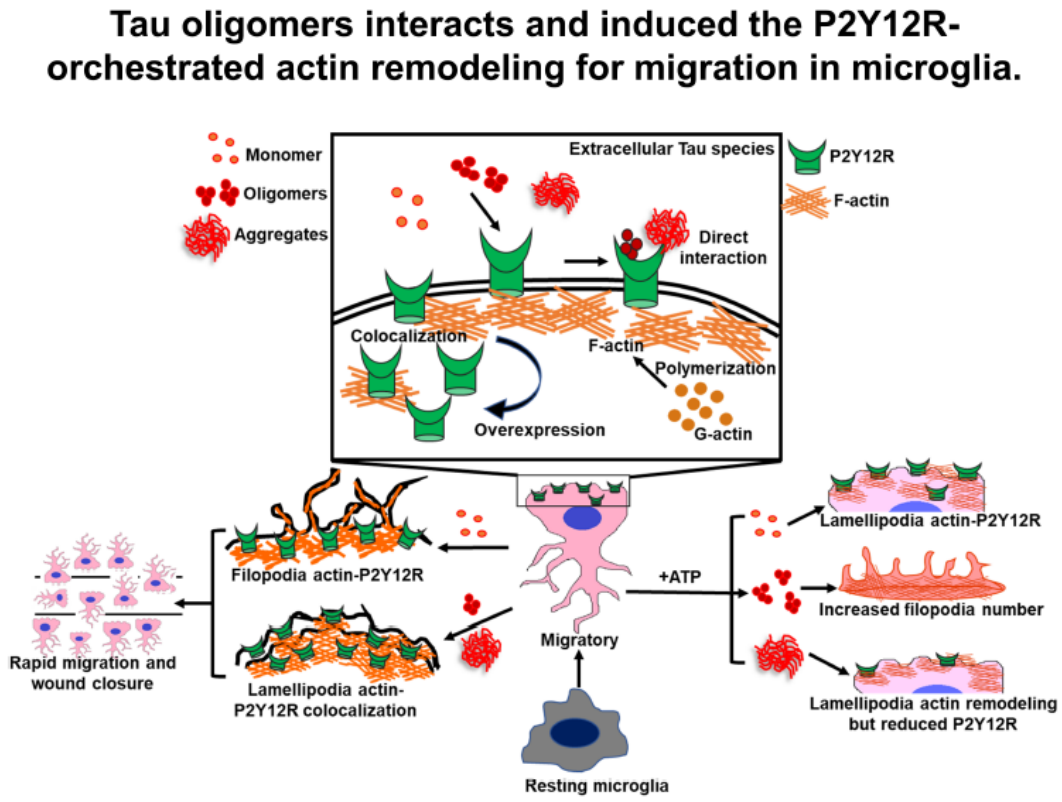

FIGURE 9 Tau oligomers interacts and induced the P2Y12R-orchestrated actin remodeling for migration in microglia. Extracellular Tau species can directly interact with microglial P2Y12R and hence induces its cellular expression. Subsequently, the membrane-associated actin fibrillation and the incorporation of P2Y12R help the microglia for directional migration. Tau oligomers and aggregates showed a different microglial phenotype and actin remodeling upon administration in the presence and absence of ATP. Monomer induced the formation of filopodia extension, while oligomers and aggregates exhibited the orchestration of P2Y12R-incorporated lamellipodia for rapid migration. ATP induced the formation of more lamellipodia in monomer and aggregates exposed microglia. In contrast, oligomeric exposure led the microglia with more number of filopodia, which demonstrated the initiation of P2Y12R-driven migration during Tauopathy

fluorescence spectrophotometer (Infinite 200 M PRO, Tecan).[81] The structure of oligomers and aggregates were observed by TEM by spotting it onto 400 mesh carbon-coated copper grids and stained with 2% uranyl acetate. The grids were dried and analyzed by Tecnai T20 at 120 kV for TEM study.[82] Tau oligomers and aggregates were spotted onto a nitrocellulose membrane at a final concentration of 2.5 mg/mL and allowed for complete drying. The blot was blocked with 5% skimmed milk buffer for an hour and probed with A11 (1:1000 dilution) and K9JA (1:8000 dilution) antibody for 1 hour. The blots were further probed with anti-rabbit secondary antibody, developed using ECL reagent and chemiluminescence was recorded by Amersham Imager 600.[45]

4.4 | Cytotoxicity assay of Tau species on microglia

N9 cell line was cultured in RPMI with 10% FBS and 100 µg/mL of penicillin-streptomycin. Ten thousand cells/well were seeded in 96-well plates. *Tau* monomer, oligomers, aggregates and ATP were added at different concentrations (from nM to µM) at a final volume of 100 µL and incubated for 24 hours at 37°C.The MTT reagent was added at a final concentration of 0.5 mg/mL in each well and incubated for 3 hours at 37°C. The MTT formazan end product was dissolved by DMSO and the absorption was measured at 570 nm in a spectrophotometer (Infinite 200 M PRO, Tecan). N9 cells were treated with different concentrations of oligomerized and aggregated Tau and ATP for 24 hours to ensure the amount of membrane leakage by Lactate dehydrogenase (LDH) release assay. The cell-free media were collected from each well and LDH leakage was measured by the formation of formazan compound, which is directly proportional to the cytotoxicity as per manufacturer's protocol (Pierce LDH Cytotoxicity Assay Kit).

4.5 | Western blot

To study the expression level of the P2Y12R receptor on Tau exposure, we treated the N9 cells ($3X10^6$ cells per group) with Tau monomer, oligomers and aggregates (45 µg/mL) separately for 24 hours.

The cells were washed with PBS and lysed with RIPA buffer and cell lysate was subjected to Bradford assay. The 75 μg of an equal amount of cell lysate were proceeding for western blot with anti P2Y12R antibody (1:1000 dilution) with β-actin loading control (1:5000 dilution) as an internal control. Then, the band intensity was quantified by using the BIORAD Quality one 4.6.6 software. The band-density of the treated group were compared with cell control group and normalized with housekeeping gene control (β-actin) (n = 3). Then, the relative fold changes were plotted for target proteins.

4.6 | Co-immunoprecipitation

N9 cells (10^7) were seeded onto 100 mm dishes and incubated for 24 hours. The cells were harvested and subjected to immunoprecipitation according to the manufacturer's protocol with some modification. In brief, The N9 cells were lysed with the co-IP lysis buffer for 30 minutes. The cell lysate protein concentration was quantified by Bradford assay. The 10 mg concentration of cell lysate was mixed with 45 μg/mL concentration of Tau monomer, oligomers and aggregates separately in a rotor at room temperature for 3 hours. The amino-linked resins were previously coupled with pan-Tau K9JA primary antibody by reductive amination reaction, along with/without antibody bead control for non-specific binding and isotype IgG control. The Tau-incorporated cell lysates were then mixed with antibody-coupled resin columns and kept at 4°C for overnight binding in the rotor. The immunoprecipitated Tau along with partner proteins were eluted by using 50 μL of a non-reducing elution buffer containing 0.1 M glycine (pH 2.5). The elution buffer did not interfere with the antibody fragments which was covalently linked with resin. The eluted fractions were subjected to western blot by P2Y12R (1:1000 dilution) and K9JA antibody (1:8000 dilution).

4.7 | Immunofluorescence microscopy

The level of P2Y12R and actin remodeling on microglia upon of Tau exposure (45 μg/mL) were checked by Immunofluorescence study along with the accumulation of actin by β-actin and P2Y12R colocalization. N9 cells (25 000 cells) were treated with Tau monomer, oligomer and aggregates separately for 24 hours. For the P2Y12R blockage experiment, N9 microglia were treated with clopidogrel at 2 μM concentration separately and together with Tau species. Then, the cells were washed with PBS thrice and fixed with chilled absolute methanol for 15 minutes and permeabilized with 0.2% TritonX-100. The cells were stained with P2Y12R (1:250), β-actin (1:500), phalloidin-alexa488 (1:40), and α-tubulin (1:500) antibody for overnight at 4°C. Then, Alexa flour-secondary antibodies were allowed to bind P2Y12R, β-actin and α-tubulin for 1 hour along with nuclear stain-DAPI (300 nM). The microscopic images were taken in Zeiss Axio observer with Apotome2 fluorescence microscope at 63X oil immersion. The quantification was done using ZEN 2.3 software and the mean fluorescence intensity was plotted for different test groups. The numbers of lamellipodia and filopodia positive N9 cells were counted in multiple fields (n = 14) and hence % cells showing remodeled actin were represented for Tau oligomers and aggregates treated groups. The absolute intensity of P2Y12R and actin of membrane-associated areas of microglia were quantified by ZEN 2.3 software and the colocalization analysis by Pearson's coefficient by ImageJ software in Tau monomer, oligomers and aggregates treated groups in multiple fields (n = 20). In the clopidogrel treatment groups, multiple fields (n = 14) were imaged for P2Y12R and phalloidin intensity by ZEN 2.3 software and the filopodia density and colocalization were calculated from various cells (n = 70) in different treatment groups.

4.8 | Microglial polarization by MTOC localization

To understand the relation of microglial motility and MTOC position, N9 cells (25 000 cells) have been stained with α-tubulin antibody (thermo) overnight and imaged in Zeiss Axio observer with Apotome2 fluorescence microscope at ×63 oil immersion. The MTOC which were positioned ahead of nucleus, were categorized as "anterior," behind the nucleus as "posterior" and beside/onto the nucleus as "lateral" in migratory microglia.[74] The experiment was performed thrice and the MTOC positions were counted and plotted for various Tau treated groups along with cell control (n = 65).

4.9 | Wound scratch assay

In order to determine the rate of migration upon Tau exposure, the N9 cells (10^6 cells) were treated with 45 μg/mL of Tau monomer, oligomers and aggregates and ATP as a positive control. Upon addition of Tau species, three scratches were made with 200 μL tips and at different time intervals (0-24 hours) the phase-contrast images were taken in Axio observer seven microscope at ×20 magnification. The wound lengths were measured in four different positions at different time interval and the % of wound closure was calculated in comparison with cell control up to 24 hours.

4.10 | Trans-well migration assay

N9 microglia were subjected to trans-well migration assay in response to extracellular Tau species as a determinant of chemotaxis. N9 cells (50 000 cells/inserts) were seeded in 24-well plate format and treated with 45 μg/mL of Tau monomer, oligomers and aggregates and ATP as a positive control in lower chamber. Upon incubation for 24 hours, the lower surface containing the migrated cells were fixed with paraformaldehyde and stained with 0.2% crystal violet solution. The upper surface containing the non-migrated cells were removed by using cotton swab and the lower surfaces were imaged at ×20 objective under bright-field microscope. The experiment was performed twice and number of migrated cells per field were counted (n = 8) in different treatment groups.

4.11 | Statistical analysis

All experiments were done in two or three biological replicates and each measurement for every experiment was taken in triplicate. Statistical analyses were performed for Fluorometric assay and microscopic quantification by using one-way ANOVA. The statistical significance of the multiple groups has been calculated by Tukey-Kramer's post-hoc analysis for multiple comparisons at 5% level of significance. In immunofluorescence microscopic analysis, several data points from 8 or 14 fields were plotted. The test groups were compared with untreated control and the P-values were mentioned within the figures. In ATP-mediated actin remodeling experiment, the ATP + Tau species treated groups were compared to only Tau treated group (For, eg, ATP+ Tau monomer vs Tau monomer, ATP + Oligomers vs Tau Oligomers, etc). The results are considered significant if the mean difference between groups is greater than calculated Tukey's criterion (X-X' > T).

ACKNOWLEDGMENTS

Rashmi Das acknowledges the fellowship from University Grant Commission (UGC) India. We are grateful to Chinnathambi's lab members for their scientific discussions, helpful suggestions and critical reading of the manuscript. We are thankful to Mr. Prabhanjan Giram for providing Clopidogrel bisulfate (IP grade) as a kind gift and Dr. Dhanasekaran Shanmugam for his critical comments and valuable suggestion. The authors gratefully acknowledge Professor H. V. Thulasiram (Chemical Biology group at the Division of Organic Chemistry, CSIR-National Chemical Laboratory) for his excellent Molecular Biology Lab facilities and his constant support. This project is supported in part by grant from in-house CSIR-National Chemical Laboratory grant MLP029526 and under the Healthcare, fundamental basic research grant from CSIR MLP101726.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rashmi Das and Subashchandrabose Chinnathambi conducted most of the experiments, analyzed the results, and wrote the article. Subashchandrabose Chinnathambi conceived, designed, resource provided, supervised and wrote the article. All the authors approved the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/tra.12784.

DATA AVAILABILITY STATEMENT

All the necessary data has been includes in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Das R, Chinnathambi S. Microglial remodeling of actin network by Tau oligomers, via G protein-coupled purinergic receptor, P2Y12R-driven chemotaxis. *Traffic*. 2021;22:153–170. https://doi.org/10.1111/tra.12784

European Journal of Cell Biology 101 (2022) 151201

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

G-protein coupled purinergic P2Y12 receptor interacts and internalizes TauRD-mediated by membrane-associated actin cytoskeleton remodeling in microglia

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ARTICLE INFO

Keywords:
Alzheimer's disease
TauRD
Microglia
P2Y12R
Internalization
Phagocytosis

ABSTRACT

In Alzheimer's disease, the microtubule-associated protein, Tau misfolds to form aggregates and filaments in the intra- and extracellular region of neuronal cells. Microglial cells are the resident brain macrophage cells involved in constant surveillance and activated by the extracellular deposits. Purinergic receptors are involved in the chemotactic migration of microglial cells towards the site of inflammation. From our recent study, we have observed that the microglial P2Y12 receptor is involved in phagocytosis of full-length Tau species such as monomers, oligomers and aggregates by actin-driven chemotaxis. This study shows the interaction of repeat-domain of Tau (TauRD) with the microglial P2Y12 receptor and the corresponding residues for interaction have been analysed by various *in-silico* approaches. In the cellular studies, TauRD was found to interact with microglial P2Y12R and induces its cellular expression confirmed by co-immunoprecipitation and western blot analysis. Furthermore, the P2Y12R-mediated TauRD internalization has demonstrated activation of microglia with an increase in the Iba1 level, and TauRD becomes accumulated at the peri-nuclear region for the degradation. Similarly, immunofluorescence microscopic studies emphasized that TauRD is phagocytosed by microglial P2Y12R via the membrane-associated actin remodeling as filopodia extension. Upon internalization, we have demonstrated the P2Y12R signaling-mediated degradation of accumulated TauRD by lysosomal pathway. Altogether, microglial P2Y12R interacts with TauRD and mediates directed migration and activation for its internalization and degradation.

1. Introduction

Alzheimer's disease is characterized by aggregates of Tau and β-amyloid proteins in the intra- and extracellular regions of neuronal cells, respectively (Braak and Braak, 1991). Amyloid-β, the cleavage product of amyloid precursor protein (APP), accumulates in the extracellular region as senile plaques. Tau is a microtubule-associated protein that aggregates as oligomers and filaments in the AD brain (Sajjad et al., 2018; Binder et al., 1985). Tau is a soluble, cytoplasmic protein extensively expressed in neuronal cells for microtubule-binding and stability (Binder et al., 1985; Conde and Cáceres, 2009). Tau is expressed in six different isoforms, with the longest isoform comprising 441 amino acids with two N-terminal inserts, proline-rich domains, and a repeat domain (Andreadis et al., 1992; Neve et al., 1986). The repeat domain forms the stable β-sheet structure and rapidly aggregates to form stable filaments (Mukrasch et al., 2005).

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β; APOER, Apolipoprotein E receptor; CMKLR1, chemokine-like receptor 1; DAP12, DNAX-activating protein of 12 kDa; FPR2, formyl peptide receptor 2; GPCR, G-protein coupled receptor; GSK-3β, Glycogen synthase kinase – 3β; HEK, human embryonic kidney; HSPGs, heparan sulfate proteoglycan; LAMP-2A, Lysosome associated membrane protein- 2A; MTOC, microtubule-organizing center; MAPK, mitogen-activated protein kinase; POPC, Palmitoyl oleoyl phosphatidylcholine; ROCK, Rho-associated coiled-coil kinase; RAGE, receptor for advanced glycation end products; SCARA1, Scavenger receptor class A member 1; SCARB1, Scavenger receptor class B member 1; SYK, spleen tyrosine kinase; TauRD, Tau-repeat domain; TLR, Toll-like receptor; TREM-2, triggering receptor expressed in myeloid cells.

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https://doi.org/10.1016/j.ejcb.2022.151201
Received 6 August 2021; Received in revised form 20 January 2022; Accepted 20 January 2022
Available online 25 January 2022

Microglia are the resident immune cells that maintain constant surveillance in the central nervous system. Microglia are activated by molecules from damaged neurons, cell debris, aggregated proteins, etc., (Cowan and Petri, 2018). Several GPCRs and other membrane receptors are involved in the activation of microglia, which ultimately leads to pro-and anti-inflammatory responses (Hardy and Higgins, 1992; Karran et al., 2011; McGeer and McGeer, 2013). G-protein coupled receptors (GPCRs) such as formyl peptide receptor 2 (FPR2) and chemokine-like receptor 1 (CMKLR1) also act as receptors for microglial activation and phagocytosis (Yu and Richard, 2015; Zhao et al., 2018). The accumulated amyloid-β peptides and Tau species activates microglia through a wide variety of membrane receptors. Amyloid-β receptors in microglia include scavenger receptors such as SCARA-1, SCARB-1, MACRO & RAGE, toll-like receptors such as TLR-2, TLR-4, and Triggering receptor expressed in myeloid cells (TREM-2) (Yu and Richard, 2015; Zhao et al., 2018). Though there are not many evidence for Tau receptors, microglial internalization of Tau species such as monomers and oligomers has been well studied (Bolós et al., 2017; Gómez-Ramos et al., 2009; Gomez-Ramos et al., 2008). In addition to microglia, astrocytes are also reported to internalize monomeric Tau to form intracellular aggregates (Perea et al., 2019). A recent report emphasized that extracellular Tau are internalized by microglia via interacting with heparan sulfate proteoglycan (HSPGs) while astrocytes phagocyted Tau via HSPGs independent mechanism (Perea et al., 2019). The mechanism of internalization for monomeric vs aggregated Tau follows two distinct but inter-connected pathways. Aggregated Tau is internalized via a dynamin-dependant pathway related to the endocytic mechanism. While the internalization of monomeric Tau follows through slow actin-dependant micropinocytosis and endocytic pathway (Evans et al., 2018). In addition, Tau is reported to interact with a microglial GPCR, chemokine CX3C receptor-1 (CX3CR1) for its activation and internalization (Bolós et al., 2017; Chidambaram et al., 2020).

Purinergic receptors are membrane receptors that are expressed in microglia for their chemotactic migration towards purinergic molecules such as ADP, ATP, UDP, AMP, etc. P2Y12R is a purinergic GPCR that plays a vital role in platelet functions and hemostasis. In CNS, P2Y12R is abundantly expressed in microglial cells and involved in constant surveillance and activation (Yu et al., 2019). It has been recently reported that microglia form P2Y12R-mediated somatic synapses with neurons to survey neuronal health (Cserép et al., 2020). In physiological conditions, microglia maintain the 'ramified' state with long processes and no net displacement to survey constantly the microenvironment. Upon pathogenic attack or neuronal injury, protein aggregation activates microglia to retain the extensions and transforms them into an 'ameboid' state (Leyns and Holtzman, 2017). Activated microglia follow the chemical gradient to migrate to the site of neuronal injury. Previously, our group has reported that microglia can phagocytose extracellular Tau oligomers and monomers via membrane-associated actin remodeling (Das et al., 2020). Moreover, extracellular Tau exposure activates the microglia with increased Iba1 level and its colocalization with a modified actin network (Das et al., 2020). P2Y12 receptor involves p38 mitogen-activated protein kinase (MAPK) and ROCK2 kinase pathway for inflammatory and neuropathic pain (Yu et al., 2019; Horváth et al., 2014; Kobayashi et al., 2008). Moreover, P2Y12R mediates multi-faced cellular signaling cascades, involving chemotactic migration, actin remodeling, inflammasomes, microglial activation, and the maintenance of neuronal health (Das and Chinnathambi, 2020). In our previous work, the microglial P2Y12 receptor has shown interaction with extracellular full-length Tau species such as monomers and oligomers. The interaction of full-length Tau species with P2Y12 receptor associates with actin remodeling via the formation of lamellipodia, filopodia and the microtubule-organizing center (MTOC) localization for directed-microglial migration in ATP-dependant manner (Das and Chinnathambi, 2021).

In our present study, we report the domain responsible for direct P2Y12 receptor interaction and activation. The repeat-domain of Tau has been studied for its role in P2Y12R interaction, receptor-associated Tau^{RD} internalization, accumulation, and microglial activation via the involvement of Iba1 expression. Similarly, the presence of P2Y12R in the formation of filopodia and podosome-like structures as components of membrane-associated actin remodeling was also emphasized upon Tau^{RD} exposure during microglial migration. We have also studied the P2Y12R-mediated degradation of internalized Tau^{RD} in microglial cells. Tau^{RD} undergoes lysosomal degradation which decreases upon a potent P2Y12R inhibitor, Clopidogrel.

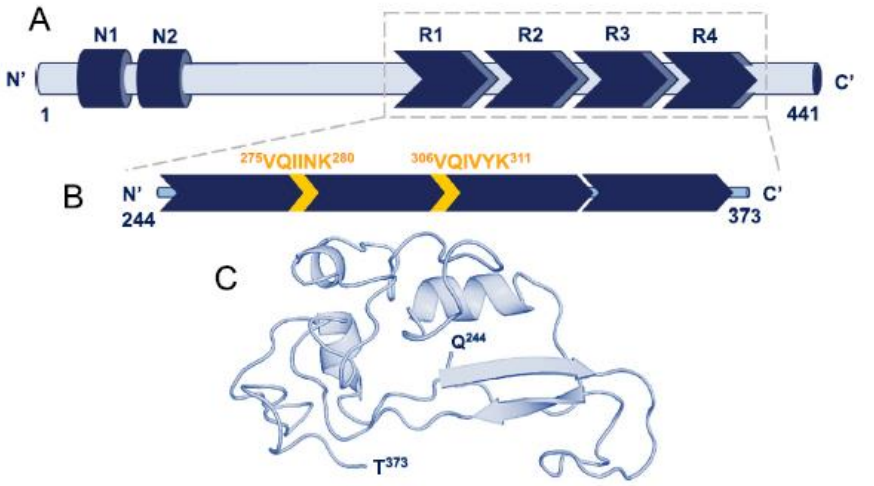
2. Results

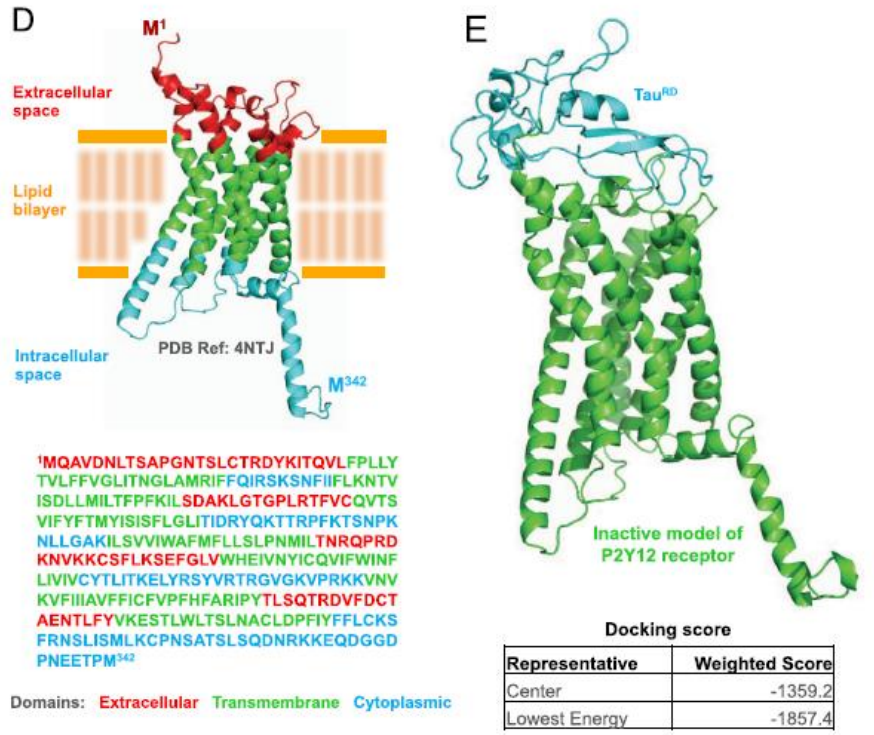
2.1. Molecular modeling and docking of repeat-domain of Tau with the purinergic receptor, P2Y12R

The model of the full-length Tau is unpredictable due to its high solubility and structural instability (Fig. 1A). Repeat-Tau model was adopted from Sonawane et al. (2019) with a stable secondary structure for hexapeptide regions ^{275}VQIINK^{280} and ^{306}VQIVYK^{311}and has a key role in aggregation and filament formation (Fig. 1A, B). The model is built for Tau residues 244–373, followed by structural validation and energy minimization (Fig. 1C). The antagonist-bound structure (inactive form) of P2Y12 receptor (PDB ID. 4NTJ), was used as a template for the modeling, and the best model has been used for further analysis. The pre-docking refinement includes adding hydrogens, building the missing residues, loops and the GPCR refinement to attain the stable structural conformation. The final refined model had an RMSD value of 0.4 Å and Ramachandran favored residues of 98.2%. The extracellular, cytosolic, and transmembrane domains were segregated based on the UniProt database (Entry no. Q9H244) (Fig. 1D). Tau^{RD} model was docked to the extracellular domain of P2Y12 receptor (highlighted in red, Fig. 1D) and the best model with a center score of − 1359.2 and lowest energy score of − 1857.4 is carried forward for the molecular dynamics simulation (Fig. 1E). Similarly, the P2Y12R model for active form (4PXZ), inactive form bound to the antagonist (4NTJ with antithrombic drug) were also built, and Tau^{RD} was docked to the respective extracellular domain of P2Y12 (data not shown).

2.2. Interaction studies of P2Y12R-Tau^{RD} by molecular dynamics simulation

The Tau^{RD} complex with different models of P2Y12 receptor (active, inactive, and inactive with antagonist) was individually subjected to 200 nanoseconds simulation and analyzed for the Lennard Jones potential and Coulombic energy between the interacting proteins (SI Fig. 1). 4NTJ model of P2Y12R was chosen for further MD-simulation analysis. The P2Y12R-Tau^{RD} complex was subjected to a molecular dynamics simulation of 500 nanoseconds to interpret the stability and the type of interaction to propose the corresponding amino acid residues involved in this interaction (Fig. 2A). Snapshot of the interacting complex at every 100 nanoseconds interval is shown in SI Fig. 2. The stability of this interaction is predicted using root mean square deviation (RMSD) analysis with timescale (ns) and RMSD values (nm) along the X- and Y-axis, respectively. The complex (black) attained stability over the initial 100 ns and retained at 1 nm throughout the 500 ns simulation with a minimum deviation of 0.22 nm (Fig. 2B). Similarly, individual RMSD values of Tau^{RD} (green) and P2Y12R (red) were plotted over the 500 ns timescale (Fig. 2B). Since Tau is highly unstable, we checked for the fluctuating residues during simulation through the root mean square fluctuation (RMSF) analysis. The lysine residues such as K274, K290, K294, K298, K369 and K370 show peak fluctuations, whereas the hexapeptide regions show a least throughout the simulation (Fig. 2C). The RMSF graph shows the Tau^{RD} residual instability at different time intervals, i.e., 0–100 ns (black), 200–300 ns (green), and 400–500 ns (magenta). The residual fluctuation reduced and attained stability during the latter part of the simulation (400–500 ns). We next plotted the





Docking score

Representative	Weighted Score
Center	-1359.2
Lowest Energy	-1857.4

Fig. 1. Structural characterization of Tau^{RD} and P2Y12 receptor. A. Bar graph representing the full-length Tau (hTau40wt) structure of 441 amino acids with two N-terminal inserts (N1 and N2), and a repeat-domain comprising of four repeats (R1, R2, R3, and R4). B. Tau^{RD} comprising of two hexapeptide regions ^{275}VQIINK^{280} and ^{306}VQIVYK^{311} which are key residues in Tau aggregation. C. The corresponding amino acid sequence of Tau^{RD} used for modeling studies. D. Diagrammatic representation of the inactive form of P2Y12 receptor (PDB ID: 4NTJ) with phospholipid bilayer. Red denotes residues of extracellular domain; green denotes transmembrane residues, and cyan denotes residues of the cytoplasmic domain. The corresponding amino acid sequence of the human P2Y12 receptor. E. Molecular docking model of P2Y12 receptor and Tau^{RD}, with Tau^{RD}, docked to the extracellular domain of P2Y12 receptor (Green-P2Y12 receptor, cyan-Tau^{RD}). Tau model for the repeat domain, built using modeler with NMR structure, 2MZ7 as a template (Structure adopted from Sonawane et al., 2019).

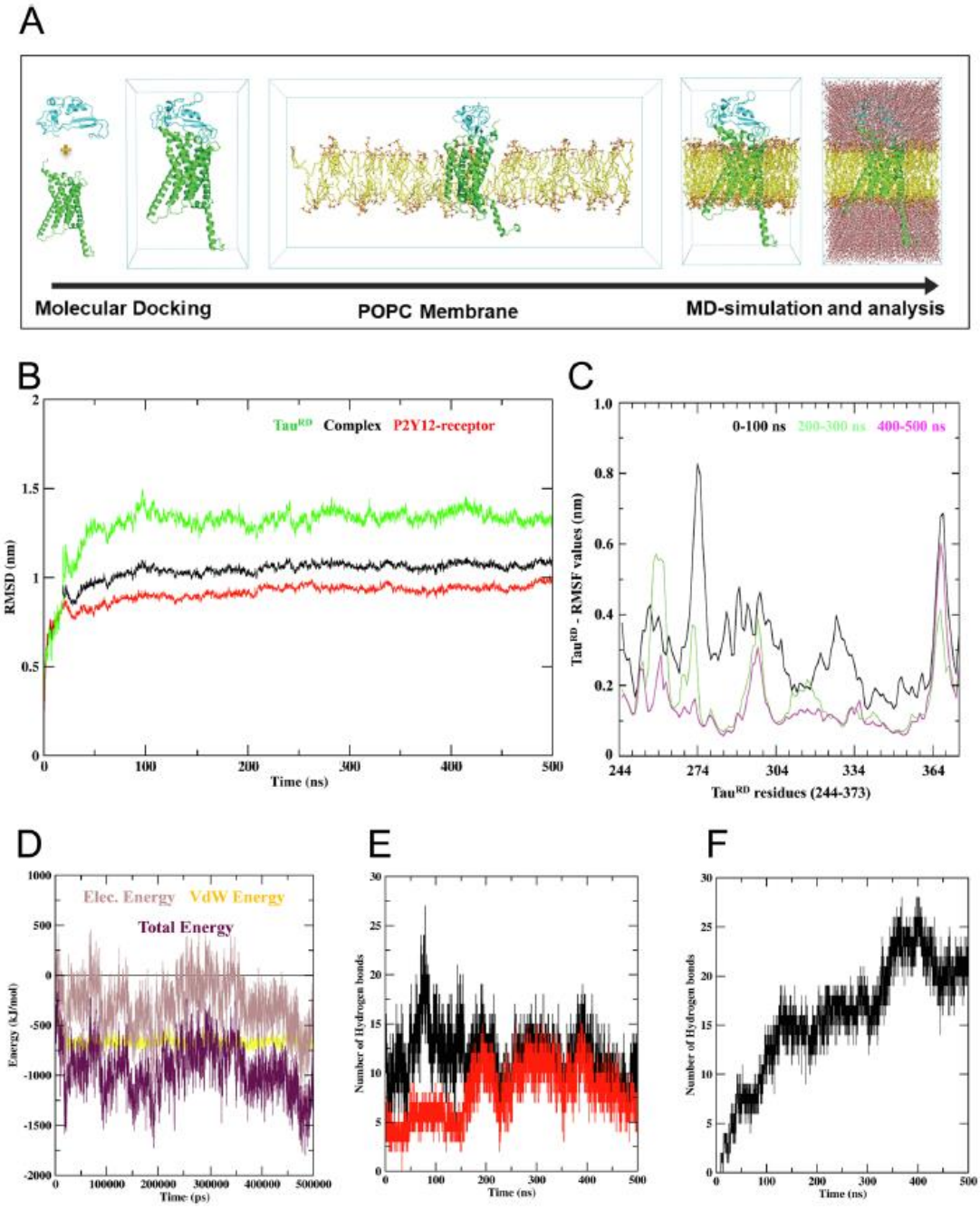


Fig. 2. Molecular-dynamics simulation of P2Y12R in complex with TauRD. A. Outline of molecular docking and MD-simulation studies performed for P2Y12R-TauRD interaction studies. Tau docked to the extracellular domain of P2Y12R, surrounded by a phospholipid bilayer and solvated with water, is subjected to molecular dynamics simulations. The overall simulation was performed for a time scale of 500 nanoseconds. B. The root-mean-square deviation (RMSD) graph shows time (ns) and RMSD values (nm) on the X- and Y-axis respectively. Green denotes RMSD values of TauRD, red denotes P2Y12 receptor and black denotes P2Y12R-TauRD complex. C. The root-mean-square fluctuation (RMSF) graph of TauRD shows RMSF values (nm) and amino acid residues on the Y- and X-axis, respectively. Black denotes RMSF values of TauRD over 0–100 ns, green denotes 200–300 ns, and magenta denotes 400–500 ns. D. Interaction energy graph (Extracellular domain of P2Y12R vs. TauRD) plotted with energy values (KJ/mol) and time-scale (ps) on Y- and X-axis, respectively. Brown represents electrostatic energy, yellow represents Vander Waal's energy and maroon represents the total energy between P2Y12R extracellular domain and TauRD. E. Hydrogen bond analysis with the number of hydrogen bonds plotted over time-scale (ns) on Y-and X-axis, respectively. Black denotes total hydrogen bonds formed between the P2Y12 receptor and TauRD, whereas red denotes hydrogen bonds formed between specific residues of Tau and P2Y12 receptor (mentioned in the h-bond analysis of Fig. 4) that were stable throughout the simulation. F. Hydrogen bond formed between TauRD and POPC membrane is plotted with the number of hydrogen bonds and time-scale (ns) on Y- and X-axis.

energy graph for analysing, electrostatic, Vander Waal's and total interaction energy between P2Y12R and TauRD complex. The total binding energy becomes more negative during the latter part of the simulation (350–500 ns) and reached around − 1250 KJ/mol during the simulation, which indicates a favorable interaction (Fig. 2D). We have calculated the number of hydrogen bonds formed, to check the hydrogen bond interactions within the complex. The number of hydrogen bonds (black) reached a maximum of 27 during the initial timescale and attained a stable count around 10–15 during the simulation of 500 ns (Fig. 2E). In earlier studies, Tau has already been reported to interact with cellular membrane components. In this study, the TauRD also interacts with palmitoyl oleoyl phosphatidylcholine (POPC) membrane, and the corresponding hydrogen bond analysis is mentioned in Fig. 2F with around 20–25 hydrogen bonds during the time of 350–500 ns of the simulation.

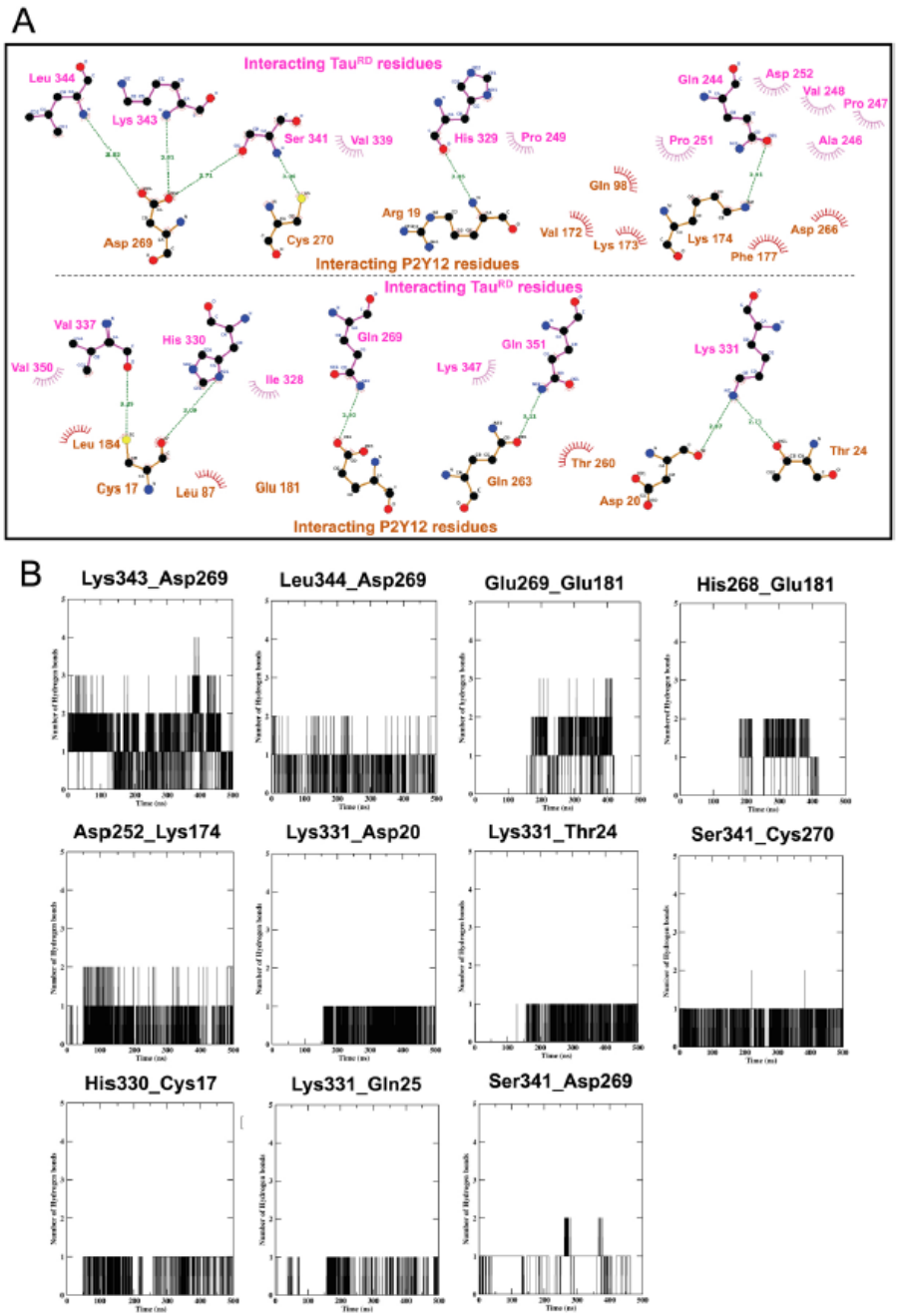


Fig. 3. Residue-specific interaction analysis of P2Y12R-TauRD complex. A. 2D interaction analysis of P2Y12-TauRD that includes hydrogen bonding and hydrophobic residues (Data collected from the last 350–500 ns of simulation). B. Residual hydrogen bond analysis of TauRD with extracellular P2Y12 receptor identified from the 2D interaction graph with the number of hydrogen bonds formed and time (ns) on Y- and X- axis respectively (Amino acid residue names on the left and right side of the individual graph title corresponds to TauRD and P2Y12 receptor respectively).

2.3. Residual interaction analysis of P2Y12R-TauRD complex

For this part of the study, the trajectory of 350–500 ns simulation was considered and the residues that have stable interaction (hydrogen bonding and hydrophobic interaction) have been plotted for residual interaction analysis (Fig. 3A). The charged amino acids of Tau are playing a pivotal role in P2Y12R interaction. The amino acid residues such as D252, E269, K331, K343, and H330 of TauRD contribute to the hydrogen bonding (plotted in Fig. 3B) with the extracellular residues of P2Y12R, K174, E181, D20, D269, and C17, respectively. Hence, the cysteine residues of P2Y12R, C17, and C270 could play a critical role in P2Y12R-TauRD interaction. The contribution from these specific residues to the overall hydrogen bonding is plotted as red in Fig. 2E. The valine and proline residues of Tau such as V248, V339, V350, P247, P249, and P251 are majorly contributing to the hydrophobic interaction between the complex (Fig. 3A). Similarly, the extracellular residues of P2Y12R such as L87, V172, K173, T260, F177, and L184 are majorly contributing to the hydrophobic interactions with TauRD.

We have also studied the surface electrostatic potential of the P2Y12R-TauRD complex obtained after 500 nanoseconds simulation (Fig. 4A-D). The protein color is based on the electrostatic potential of the protein surface where blue and red denote the positive and negative energies (KJ/mol), respectively. The side view of the TauRD protein and a 90° rotation (bottom view/picture) show its surface potential on its interacting region (Fig. 4A), which is predominantly positively charged (blue). The surface potential of the interacting P2Y12R-TauRD complex and the side and the top view is also visualized (Fig. 4B). To visualize the extracellular binding site of the P2Y12 receptor for TauRD, the top view of the P2Y12R-TauRD complex with a cartoon representation for TauRD (green) is generated (Fig. 4C). This clearly suggests that TauRD interacts with the negatively charged N-terminal region of the extracellular P2Y12 receptor. The side view of the P2Y12 receptor and a 90° rotation (top view) shows the TauRD-interacting surface along the negatively charged N-terminal region (red) of the extracellular P2Y12 receptor, which suggests a favorable interaction for the P2Y12R-TauRD complex. (Fig. 4D).

2.4. Activated microglial P2Y12R interacts with extracellular TauRD

Microglia is one of the most important brain-resident immune cells, which become activated by encountering extracellular protein deposits. Iba1 is a calcium receptor-binding adapter protein, which is involved in microglial activation and migration. To check microglial activation, N9 cells were exposed with TauRD for 24 h. Upon encountering TauRD, microglia have increased the level of Iba1 as observed by western blot and quantification (Fig. 5A, B). Microglial activation was also studied by colocalization of Iba1 with migratory actin structures. Upon exposure of TauRD, membrane-associated actin network (F-actin) remodeling was observed in microglia, which colocalized with Iba1, signifying microglial migration upon activation (Fig. 5C, D). Iba-1 colocalization increased with the actin-network at the lamellipodial structure and decreased at the cell body of TauRD-treated cells denoting the microglial activation and migration towards TauRD (Fig. 5D).

Previously our group showed that full-length Tau interacts with microglial P2Y12R and facilitates cellular expression. Here, we studied the repeat domain of Tau to emphasize the specific residues involved in P2Y12R interaction. TauRD co-precipitated with microglial P2Y12R, which ultimately helps in receptor desensitization-mediated Tau internalization (n = 3) (Fig. 5E, F). Similarly, when N9 microglia were exposed to Alexa 647-labeled TauRD, P2Y12R was found to be colocalized with extracellular TauRD at different time intervals which denotes P2Y12R-TauRD interaction (Fig. 5G, control data shown only for 6 h as there is no change in the morphology). The colocalization at initial time points (0.5 and 1.0 h) were observed to be at the membrane surface of microglial cells. Whereas at later stages (3.0 and 6.0 h), TauRD colocalized with P2Y12R more in cytosolic regions which denotes receptor-mediated internalization. P2Y12R and TauRD colocalization is quantified by positive Pearson's coefficient (R) values (n = 20) (Fig. 5H). Clopidogrel is an irreversible inhibitor of P2Y12 receptor which upon treatment inhibits P2Y12 signaling and promotes receptor desensitization. There is no significant changes in Pearson's co-eficient values upon Clopidogrel treatment as compared to TauRD treated cells (Fig. 5G, H). Further, on average, 70% of microglia were TauRD positive, ranging from 40% to 100% phagocytosis positive in the total population

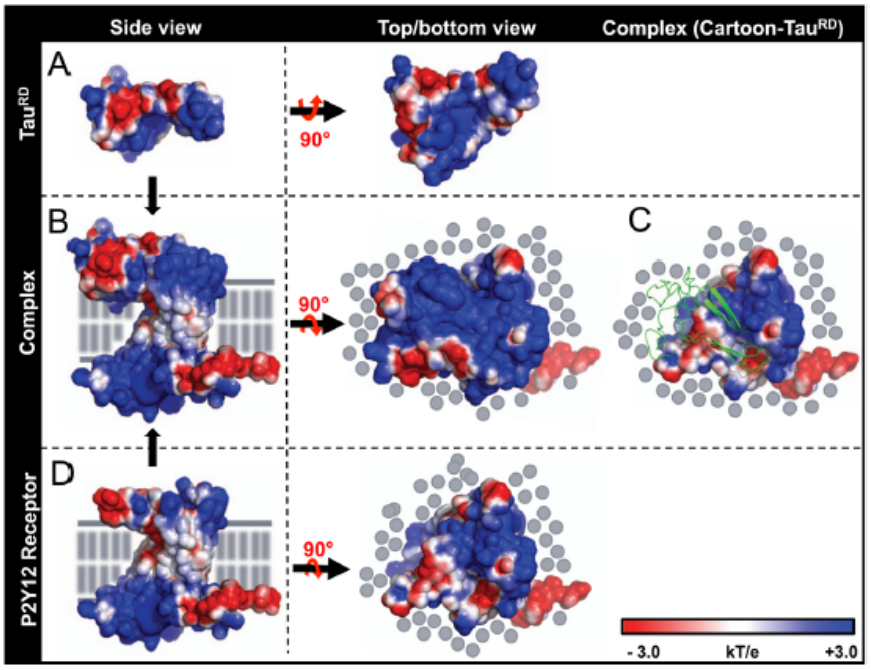


Fig. 4. Surface electrostatic potential analysis of TauRD (A), P2Y12R-TauRD complex (B), and P2Y12R (C). Studies were performed from the P2Y12R-TauRD complex structure obtained after 500 nanoseconds simulation. The protein's surface is colored based on electrostatic potential, where blue and red denote the positive and negative energies (KJ/mol), respectively. Gray lines/ dots represent the phospholipid membrane surrounding P2Y12R. A. Side view of the TauRD and a 90° rotation (bottom view) shows the P2Y12R-interacting surface of TauRD that is predominantly positive. B. Side view of the P2Y12R-TauRD complex and a 90° rotation shows the top view of the complex. C. Top view of P2Y12R-TauRD complex with cartoon representation for TauRD (green) that shows its binding region on the extracellular surface of P2Y12 receptor. D. Side view of the P2Y12 receptor and a 90° rotation (top view) shows the TauRD interacting surface, towards the negatively charged N-terminal region (red) of P2Y12 receptor.

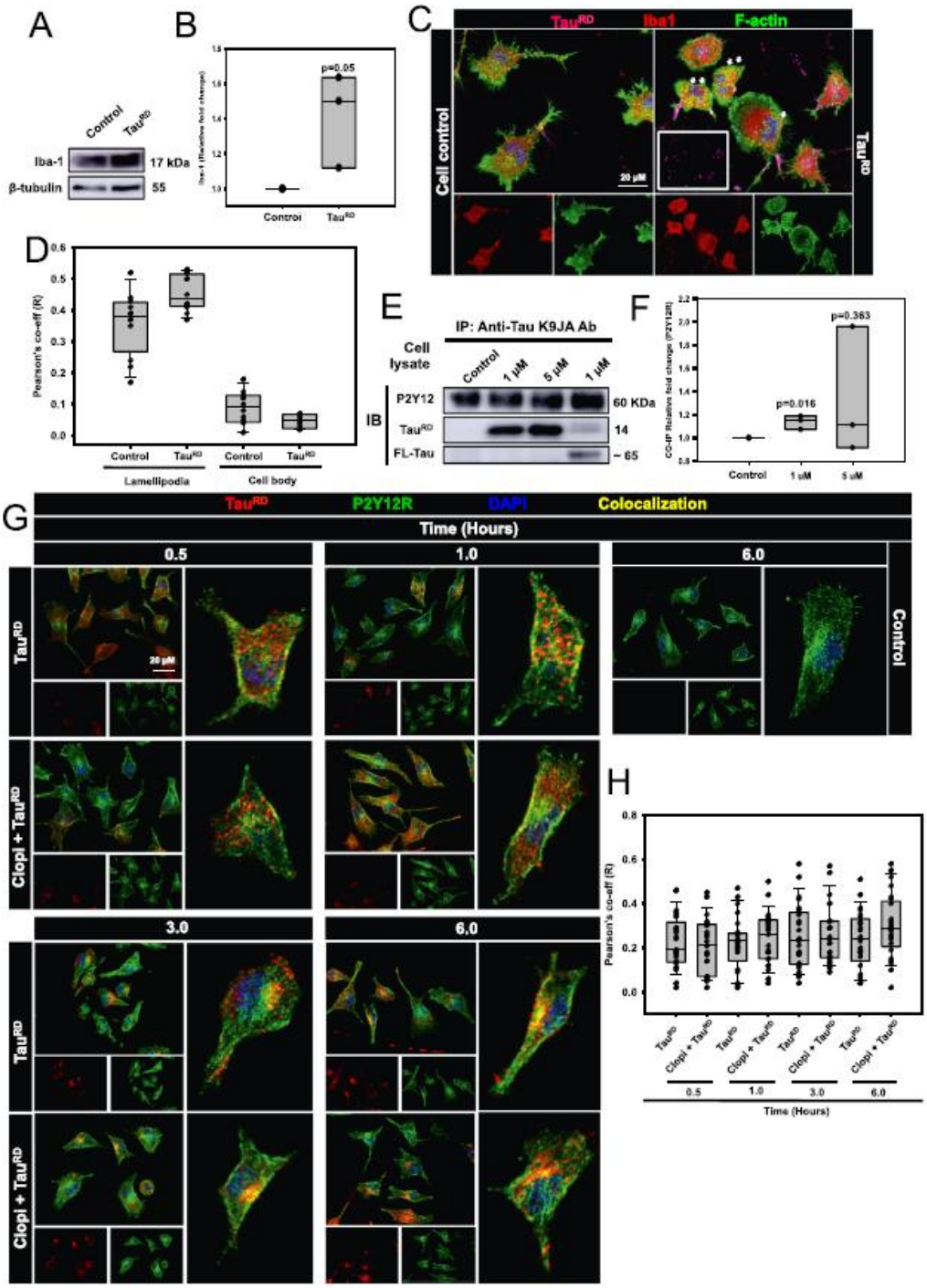


Fig. 5. Activated microglial P2Y12R interacts with extracellular Tau^{RD}. A. Upon exposure of Tau^{RD}, microglia become activated with an increase in the level of Iba1 by western blot analysis. B. The Iba1 level was quantified from the western blot upon Tau^{RD} exposure as compared to cell control. C. Iba-1 colocalized with F-actin structures more at the lamellipodial structures as compared to cell body in TauRD treated cells. D. Colocalization quantified by positive Pearson's co-efficient of colocalization (R) values (n=12) with increase and decrease in the lamellipodia and cell body, respectively in TauRD treated cells. E. Co-immunoprecipitation was performed for extracellular Tau^{RD} and microglial cell lysate from N9 cells against K9JA antibody. Full-length Tau was used as a positive control. Blots were developed against the P2Y12 receptor and K9JA. F. 1 μM Tau^{RD} shows a 1.2-fold increase in the band intensity and 5 μM shows a 2-folds increase, compared to the cell control. G. Microglial P2Y12 receptor colocalized with extracellular Tau^{RD} at different time intervals (0.5, 1.0, 3.0, and 6.0 h). H. Colocalization quantified by positive Pearson's co-efficient of correlation (R) values (n = 20) with no significant changes within Tau^{RD} and Clopidogrel treatment groups at different time intervals.

as observed by microscopic fields quantification (n = 12) (SI Fig. 4B).

2.5. Microglia internalize TauRD at substratum layer and accumulates at internal cytosolic location

Next, we have observed the microglial internalization of TauRD mediated by P2Y12R at different time intervals i.e. 0.5, 1.0, 3.0 and 6 h (Fig. 6A). The intensity of TauRD upon extracellular treatment increased upto 3 h, whereas 2 μM Clopidogrel significantly reduced TauRD internalization (Fig. 6A, B and SI Fig. 3). Conversely, TauRD intensity in Clopidogrel treated cell groups increased linearly and reached a maximum during the later time points. While, the intensity reduced after

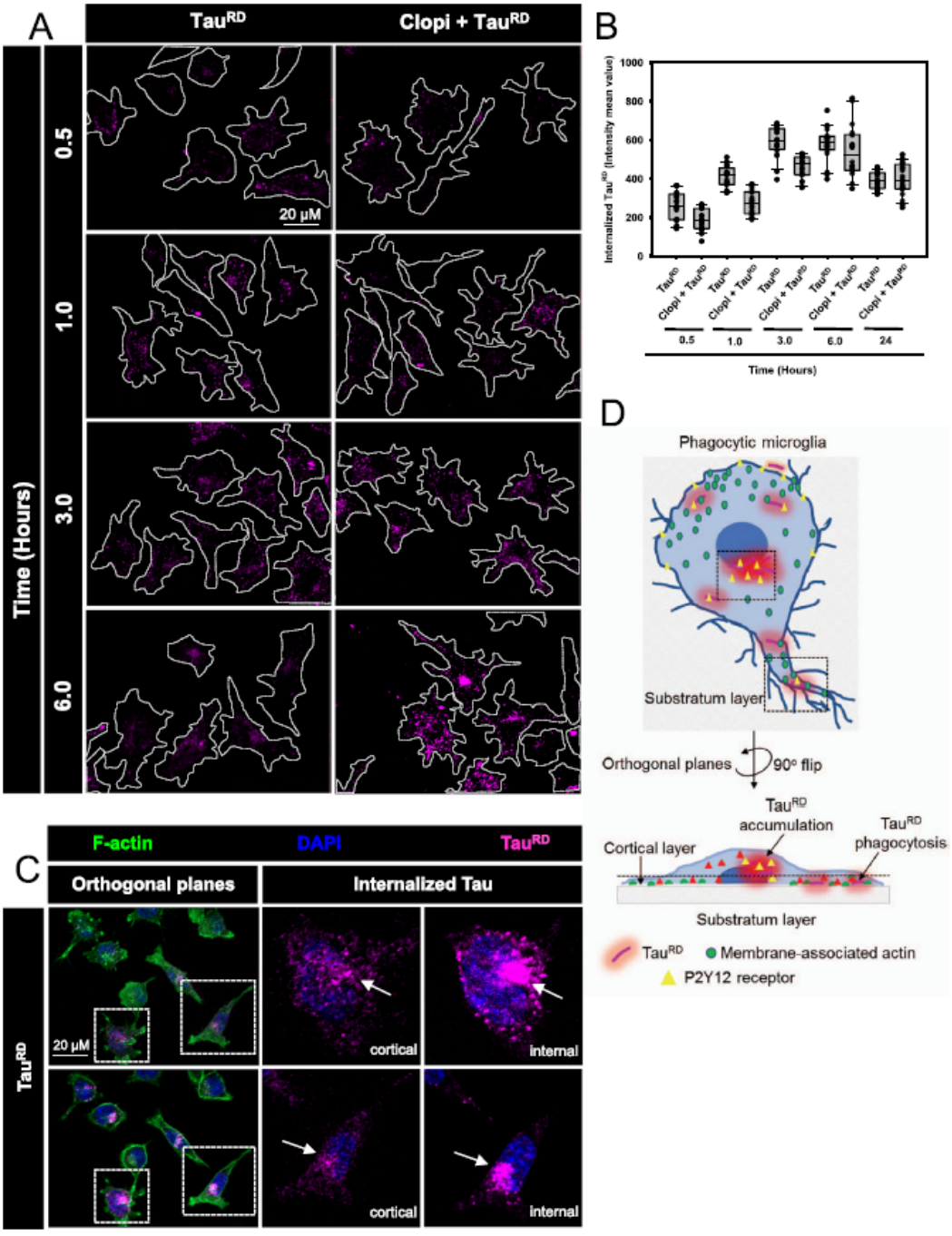


Fig. 6. Microglia internalize TauRD at the substratum layer and accumulates at the internal cytosolic location. A. Time-dependent internalization of TauRD upon extracellular Tau and Clopidogrel treatment. B. The corresponding intensity of internalized TauRD was quantified at different time intervals. C. Phagocytic microglia internalizes TauRD and accumulates at a different cytosolic location. D. Phagocytosis of TauRD was occurring from the substratum layer through actin remodeling at uropod, while the TauRD become accumulated into internal cytosolic location, near to the nucleus.

6 h in both Clopidogrel and TauRD treated groups which suggests TauRD degradation by microglial cells at later stages (Fig. 6A, B and SI Fig. 3). Orthogonal microscopic projections of phagocytic microglia have emphasized that TauRD is internalized at the cortical plane and accumulated at different cytosolic locations. Phagocytosis of TauRD was found to be mediated from the substratum layer through the remodeling of the cortical layer actin network. While, the accumulation of TauRD was observed above the plane of the cortical layer, at peri-nuclear region (Fig. 6C, D).

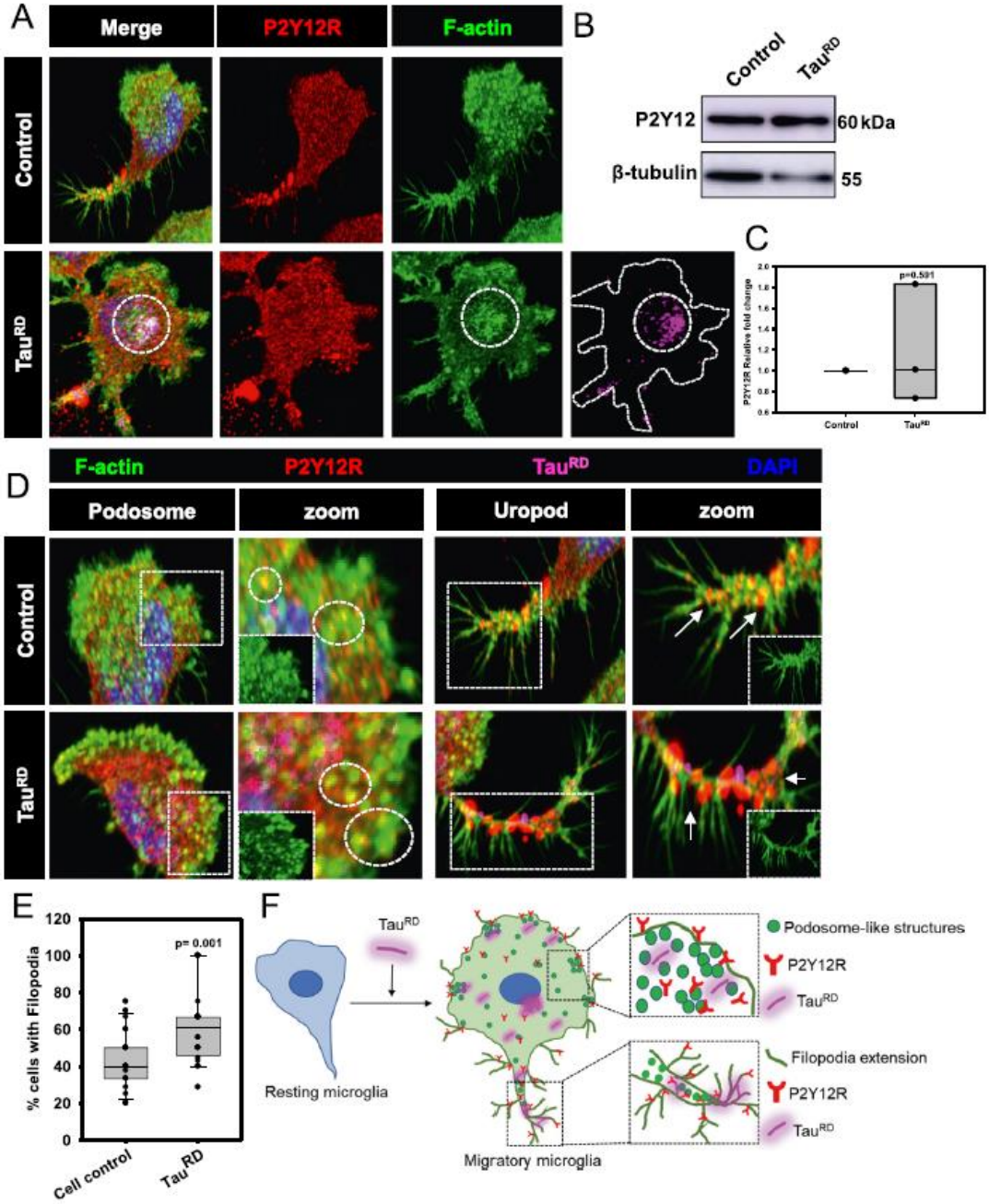


Fig. 7. Microglia remodel P2Y12R-localized membrane-associated actin as filopodia by phagocyting TauRD. A. Microglia phagocytose TauRD, which were found to be colocalized with podosome-like structures in migratory microglia. B, C. There is no significant change in the levels of P2Y12R expression upon extracellular TauRD exposure as compared to control cells. D. Microglia induces the accumulation of P2Y12R-associated filopodia and podosome-like structures in lamellipodia after phagocyting TauRD as a component of active migration. E. The % of cells showing filopodia has been increased from 40% to 70% in TauRD treated group from untreated control. F. Tau^{RD+} phagocyting microglia develop branching filopodia and podosome-like actin structures in lamellipodia for P2Y12R-associated migration.

2.6. Tau^{RD+} phagocytic microglia remodel P2Y12R-associated filopodia

P2Y12R is a chemotaxis-related purinoceptor, which is directly linked with membrane-associated actin-remodeling, adhesion, and migration. Our study showed the microglia-mediated P2Y12R-driven TauRD phagocytosis where membrane-associated remodeled actin colocalized with TauRD (Fig. 7A; SI Fig. 4A). On the other side, TauRD exposure has no significant change in the cellular P2Y12R levels in microglia cells compared to cell control (n = 3) (Fig. 7B, C). Migratory microglia are known to orchestrate lamellipodia and filopodia during active migration. Moreover, the involvement of podosome is important for matrix adhesion and generation of tensile forces during forwarding movement. In our study, migratory microglia were observed to form podosome-like structures in frontal lamella and filopodia at uropod during the phagocytosis of TauRD (Fig. 7D). Also, the filopodia positive cells were increased to 70% (some cells 100%) in TauRD exposed group as compared to untreated control as 40% filopodia^{+} cells (n = 12) (Fig. 7E). Altogether, resting microglia upon Tau exposure transformed into migratory one with increased filopodia structures and phagocytic extracellular protein species (Fig. 7F).

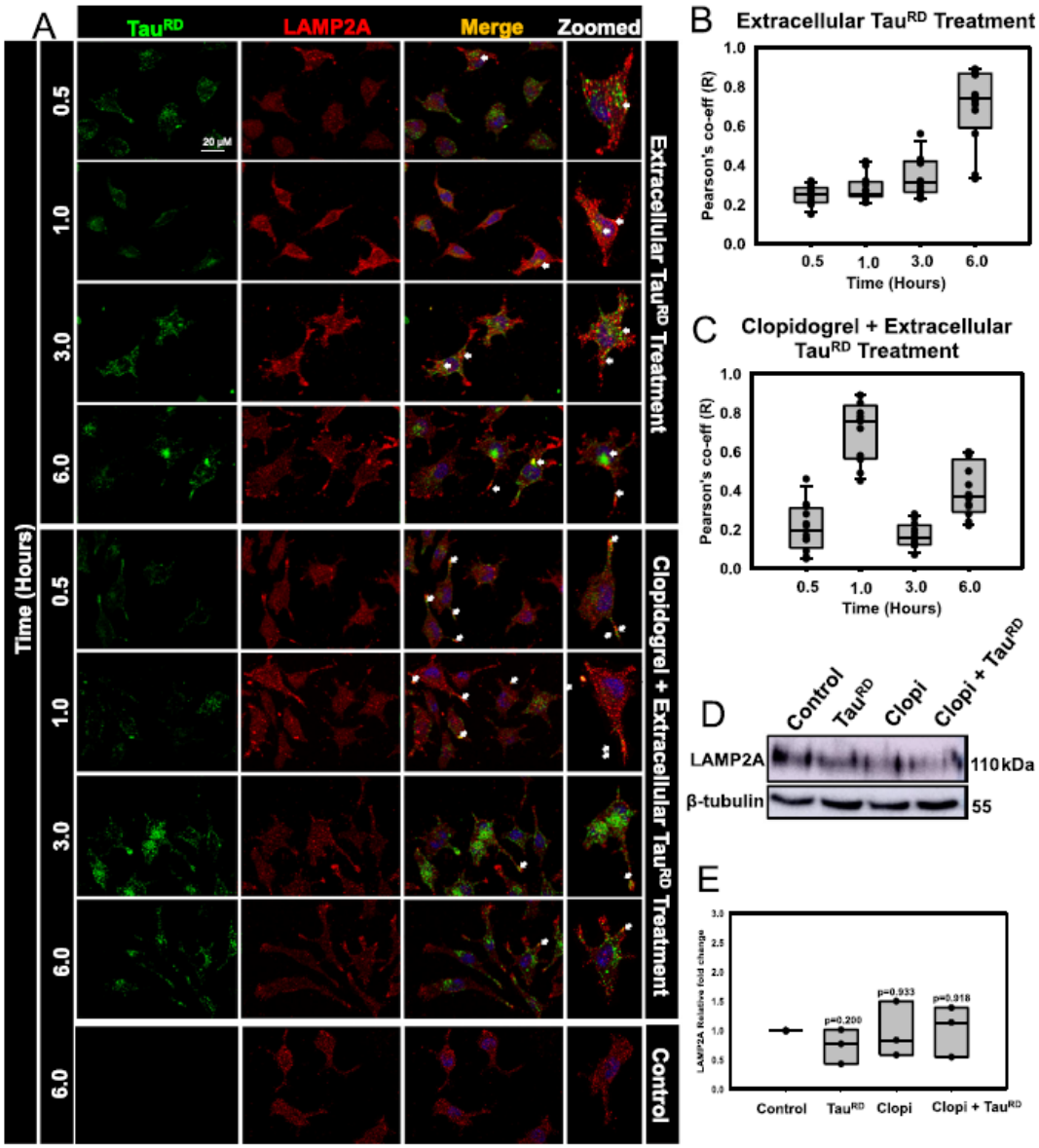


Fig. 8. Lysosomal degradation of internalized TauRD mediated by microglial P2Y12R. Microglia phagocytosed TauRD colocalizes with LAMP2A, a marker for lysosomal degradation. A. TauRD colocalization with LAMP2A upon extracellular TauRD and with Clopidogrel treatment to microglia cells. B, C. Quantification of internalized TauRD by Pearson's coefficient analysis shows significant changes in the level of colocalization upon extracellular TauRD and treated with Clopidogrel, respectively. D, E. LAMP-2A expression upon extracellular TauRD treatment and the corresponding quantification respectively.

2.7. Microglial P2Y12R mediates lysosomal degradation of internalized TauRD

In addition to its role in TauRD internalization, microglial P2Y12R also mediates lysosomal degradation of internalized TauRD. Upon extracellular TauRD treatment, the internalized TauRD undergoes endosomal trafficking for degradation and colocalized with lysosome associated membrane protein – 2 A (LAMP2A), which is a marker for lysosomes and late endosomes. (Fig. 8A, control data shown only for 6 h as there is no change in the morphology). Pearson's co-efficient of

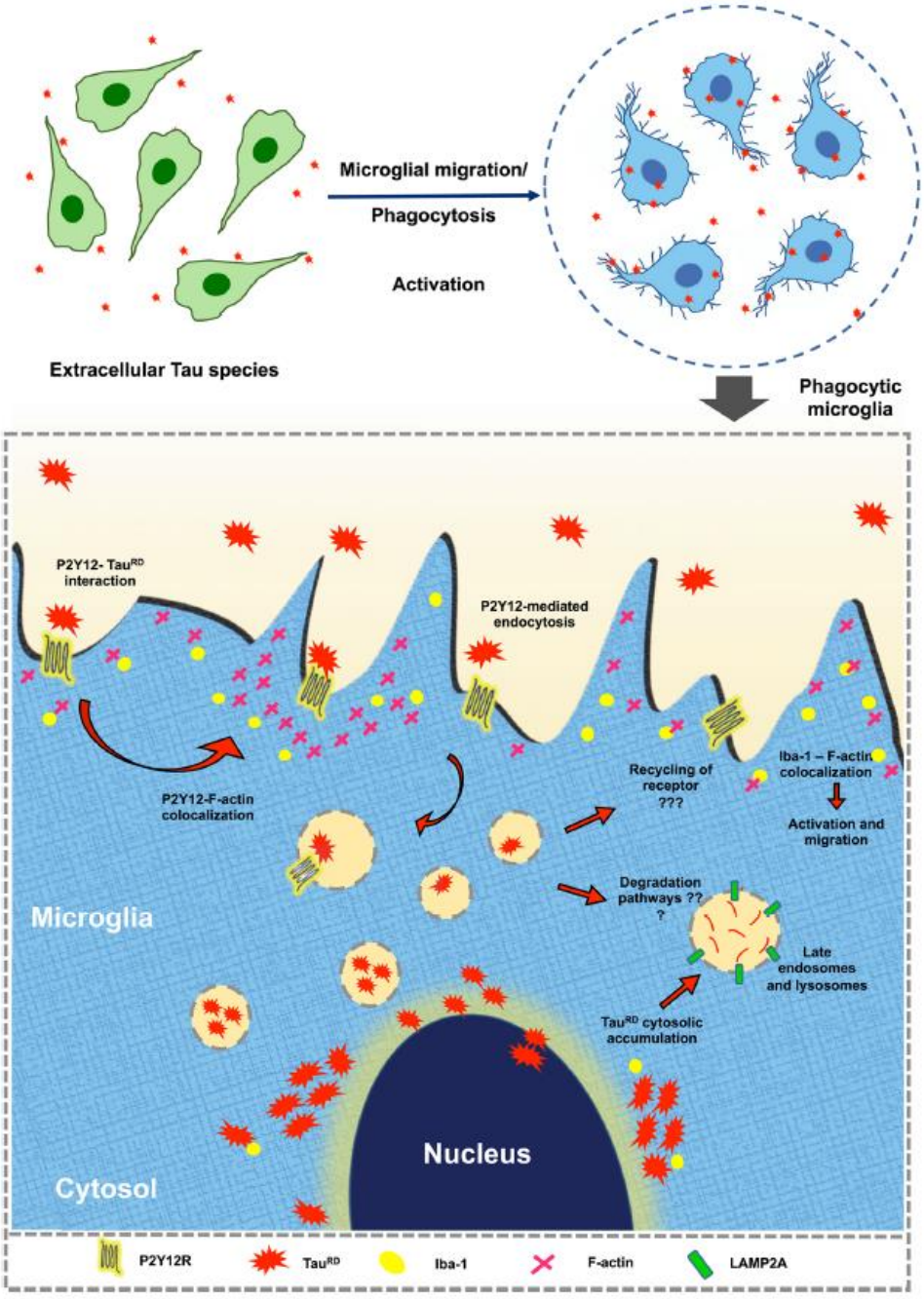


Fig. 9. Interaction of TauRD with microglial P2Y12R for activation and Tau internalization. Extracellular Tau species activate microglia for their active migration and phagocytosis. TauRD interacts with the extracellular P2Y12 receptor for its internalization mediated by receptor desensitization. The P2Y12 receptor co-localize with filamentous actin to mediate Tau internalization. The internalized Tau is observed to be co-localized with the Iba-1 marker and accumulated in the internal layer near the nuclear region of microglial cells. Hence the microglia are involved in P2Y12R-localized membrane-associated actin remodeling to form filopodial structures in phagocyting TauRD.

correlation (R) calculated at different time intervals i.e. 0.5, 1.0, 3.0 and 6 h indicated a significant increase in the values and highly colocalized with increase in time (Fig. 8B). This clearly suggests that Tau^{RD} undergoes degradation upon internalization. In case of cells treated with Clopidogrel, Pearson's coefficient values keep fluctuating and reduced as compared to Tau^{RD} which denotes the role of Clopidogrel in the inhibition of P2Y12R-mediated Tau^{RD} degradation (Fig. 8C). We have also analysed the expression levels of LAMP2A upon extracellular Tau^{RD} treatment at 24 h. There is no significant change in the expression in Tau^{RD} treated groups as compared to cell control (n = 3) (Fig. 8D, E).

3. Discussion

The Tau and amyloid-β concentration are highly elevated in the AD brain. These intra- and extra-cellular proteins are involved in transmitting intracellular signaling of neuronal and glial cells by interacting with cell-surface membrane receptors. Several GPCRs and other membrane receptors interact directly with Tau and amyloid-β proteins (Zhao et al., 2018; Bolós et al., 2017; Gómez-Ramos et al., 2009; Gomez-Ramos et al., 2008; Chidambaram and Chinnathambi, 2020a). In neurons, extracellular Tau has more affinity than acetylcholine towards muscarinic acetylcholine receptors M1 and M3 (Gómez-Ramos et al., 2009). Hence, extracellular Tau directly interacts with M1 and M3 acetylcholine receptors and increases intracellular calcium levels that are toxic to neuronal cells and promotes cell death (Gómez-Ramos et al., 2009, 2006; Gomez-Ramos et al., 2008). In microglia, TREM-2 is a receptor for amyloid-β oligomers, which on its interaction promotes amyloid-β clearance, cytokine synthesis, depolarization, migration, and apoptosis in microglial cells (Zhao et al., 2018). Microglial chemokine receptor, CX3CR1 has been recently reported to interact with extracellular Tau and promotes microglial activation and Tau internalization. Tau competes with the CX3CR1 ligand, fractalkine, to bind this receptor, and the absence of this receptor results in impaired microglial activation and Tau internalization (Bolós et al., 2017; Chidambaram et al., 2020; Chidambaram and Chinnathambi, 2020a). Our previous study reported that the purinergic P2Y12 receptor can directly interact with full-length Tau, promote microglial activation, and mediate actin remodeling for migration and chemotaxis (Das and Chinnathambi, 2021). In order to determine whether Tau^{RD} domain is involved in P2Y12R interaction, we carried out molecular docking and MD-simulation studies with Tau^{RD} and P2Y12R model. Here, we have showed the region-specific interaction of Tau^{RD} and P2Y12R and its subsequent internalization via receptor-mediated endocytosis by various computational, biochemical and cellular studies (Fig. 9). It is also reported that Tau strongly binds to lipid membrane through helices from microtubule-binding repeat regions that lead to membrane disruption and membrane-mediated aggregates formation in AD (Georgieva et al., 2014; Jones et al., 2012). Here, we propose that the Tau^{RD}-P2Y12R interaction may be supported by cell membrane interactions i.e., phospholipids, as evident from our analysis.

In recent years, the Tau^{RD} domain is attaining more attention in Alzheimer's disease as it can self-aggregate to form filaments. Moreover, cross seeding of Tau^{RD} fragments with full-length Tau monomers enhances aggregation rate into filamentous structures (Nizynski et al., 2018). Aggregated Tau species can propagate in a prion-like fashion among which Tau^{RD} aggregates have been widely studied (Sonawane and Chinnathambi, 2018; Sydow and Mandelkow, 2010). Tau^{RD} transfected HEK cell lines developed pathological features and amyloid conformations of Tau, which propagated for three generations in mouse models (Kaufman et al., 2016; Sanders et al., 2014). Hence the propagation of Tau species from affected to healthy neurons is the leading cause of spreading Tauopathy.

But, the glial contribution during Tau propagation needs to be considered thoroughly due to disease progression. Recently, we found that Tau^{RD} assemblies ($n \geq 3$) are the minimum units that are internalized readily by HEK293T cells via heparan sulfate proteoglycans (HSPGs) (Mirbaha et al., 2015). Glial cells i.e., microglia and astrocytes, do not express Tau, so the presence of Tau in glia signifies only the internalization of extracellular protein and its propagation as seed species (Leyns and Holtzman, 2017). In the AD condition, microglia gets converted from its surveilling state into the inflammatory state with active migration, phagocytosis, and cytokine production upon encountering extracellular protein deposits (Das and Chinnathambi, 2019). During the later stage, excessive extracellular Tau and amyloid-β aggregates may hamper the phagocytic activity of microglia. The dietary fatty acids are playing a significant role in microglial activation and polarization (Desale and Chinnathambi, 2020). α-linolenic acid treatment is observed to promote the clearance of Tau seeds by enhanced microglial activation, phagocytosis via actin remodeling, and degradation of internalized Tau by endosome-lysosome mediated pathway (Desale and Chinnathambi, 2021a, 2021b). Also, a novel antibody targeting Tau^{RD} oligomers can block the intracellular Tau aggregation and induce cellular uptake-clearance via the lysosomal pathway in the N2A cell model (Chandupatla et al., 2020). In recent advancements, real-time monitoring of Tau^{RD} internalization and its conversion of endogenous deposits are performed in neuronal cell models. These endogenous deposits colocalized with P62 and ubiquitin by which Tau^{RD} escapes the macro-autophagosome pathway and gets concomitantly propagated to neighboring neurons and astrocytes via contact-mediated tunneling nanotube (Chastagner et al., 2020).

Moreover, in this study, the extracellular Tau^{RD} induces the filopodia extension, colocalized with P2Y12R for dual functioning-directed chemotaxis and receptor-mediated phagocytosis. Tau^{RD} phagocytosed by activated microglia accumulates at a different cytosolic location. In particular, the phagocytosis of Tau^{RD} was evident at uropod, which is associated with branched filopodia. But, the accumulation of Tau^{RD} has been observed at the peri-nuclear region of microglial cells (Fig. 9). Previously, our group emphasized that extracellular Tau are phagocytosed via membrane-associated actin remodeling, such as lamellipodia and filopodia extension by $Iba1^{high}$ activated microglia (Das et al., 2020). Hence, these may signify that membrane-associated actin remodeling, especially filopodia formation is necessary for Tau^{RD} engulfment while the downstream processing may include either degradation or cytosolic accumulation (Fig. 9).

4. Experimental procedures

4.1. Chemicals and reagents

For biochemical studies, Luria-Bertani broth from Himedia; ampicillin, KCl, NaCl, Na_2HPO_4, KH_2PO_4, $MgCl_2$, ethylene glycol-bis (b-aminoethyl ether)- N,N,N′,N′-tetraacetic acid (EGTA), phenylmethylsulfonylfluoride (PMSF), ammonium persulfate (APS), ammonium acetate, sodium azide, Dimethyl Sulfoxide Solvent (DMSO) and methanol from MP Biomedicals; isopropyl β-d-1-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) from Calbiochem. $CuSO_4$, glutaraldehyde, bicinchoninic acid (BCA), bovine serum albumin (BSA), Thioflavin S (ThS), MES, BES, TritonX-100 and SDS from Sigma; and Tris base, acrylamide, paraformaldehyde, N,N,N′,N′-Tetramethyl ethylenediamine (TEMED), Pierce™ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), Pierce™ RIPA buffer, Alexa 647-C2 maleimide purchased from Invitrogen. Immobilon PVDF membrane from Merck. Glycine and Protein Assay Dye Reagent from Bio-Rad. Protease inhibitor cocktail was from Roche cOmplete™. For cell culture, RPMI 1640 media, Fetal Bovine Serum (FBS), trypsin-EDTA, Penicillin-streptomycin, Horse serum were purchased from Invitrogen. In immunofluorescence and western blot studies, we used the following antibodies: total pan-Tau antibody K9JA (Dako, A0024), β-Actin loading control monoclonal antibody (BA3R) (Thermo, MA5–15739), P2Y12R antibody (4H5L19) (Thermo, 702516), P2Y12 (extracellular) antibody (Thermo, PA5111827), LAMP-2A antibody (Thermo, 51–2200), Phalloidin-Alexa 488 for F-actin (Thermo, A12379), Goat anti-rabbit IgG (H+L) Cross-

adsorbed secondary antibody HRP (Invitrogen, cat no. A16110), anti-mouse secondary antibody conjugated with Alexa flour-488 (Invitrogen, cat no A-11001), Goat anti-Guinea Pig IgG (H+L) Cross adsorbed secondary Antibody, Alexa 488 (Thermo, A11073), Goat anti-rabbit IgG (H+L) Cross-adsorbed secondary antibody with Alexa Fluor 555 (Invitrogen, cat no. A-21428), DAPI (Invitrogen, cat no. D1306), Pierce Co-Immunoprecipitation Kit (Thermo 23600), Precision Plus Protein Standards (Bio-Rad, cat no. 161–0374) and ECL reagent (Bio-Rad, cat no. 1705060). The N9 microglial cell line no. is CVCL_0452.

4.2. Molecular modeling of P2Y12 receptor and TauRD

The crystal structures of the P2Y12 receptor from the RCSB-PDB database (https://www.rcsb.org/) (PDB. ID: 4PXZ and 4NTJ) are used as templates to build the receptor model for active and inactive structures, respectively (Berman et al., 2000; J. Zhang et al., 2014; K. Zhang et al., 2014). SWISS-MODEL-server is used to build P2Y12 receptor models (https://swissmodel.expasy.org/) (Waterhouse et al., 2018). The models with a global model quality estimation (GMQE) of 0.66 is used for further studies. The loops and terminal residues were then built using the GalaxyGPCRloop module of the Galaxy WEB server (Won et al., 2018). The model is then refined using GalaxyRefine module of the Galaxy WEB server, and the best model is taken for the docking studies (Heo et al., 2013; Lee et al., 2016). Model of repeat domain of Tau built by our group in previous studies by Sonawane et al. (2019) is adopted for the interaction study (Sonawane et al., 2019). TauRD is docked to the P2Y12 receptor on the extracellular region that includes the extracellular loops and the N-terminal domain for the molecular docking studies. Molecular docking is performed in the ClusPro server where extracellular residues of P2Y12 receptor (referred to UniProt database, Q9H244) are provided for the binding site (Consortium, 2015; Desta et al., 2020; Kozakov et al., 2013, 2017; Porter et al., 2017; Vajda et al., 2017). The best model is visualized using PyMol (Schrodinger, 2010) and taken forward for the MD-simulation analysis.

4.3. Molecular dynamics simulation of P2Y12R - TauRD complex

Molecular dynamics simulation of the P2Y12 receptor in complex with TauRD was performed in GROMACS 2019.3 (https://doi.org/10.5281/zenodo.3243833). Tip3p water model was used for solvation (Berendsen et al., 1995; Duan et al., 2003; Van Der Spoel et al., 2005). GROMOS96 53A6 force field, extended to include Berger lipid parameters, was used throughout the simulation (Pol-Fachin et al., 2012). Palmitoyl oleoyl phosphatidyl choline (POPC) lipid bilayer model with 128 molecules and their topologies were downloaded from the site of D. Peter Tieleman, Biocomputing Group, University of Calgary (http://wcm.ucalgary.ca/tieleman/downloads). The protein-complex was oriented, and lipid molecules were packed around the protein using inflateGRO methodology with a scaling factor of 0.95. Energy minimization was performed after every shrinking step with strong position restraints applied to the protein complex. The final membrane density was maintained at -70 A^2. The system was then solvated on both hydrophilic sides of the lipid bilayer, and Cl^- ions were added to neutralize the system using genion command of gromacs. The system was then energy minimized using a steep descent minimization algorithm. The maximum force was minimized to < 1000.0 KJ/mol/nm. Leap-frog integrator was applied to equilibrate the system using NVT and NPT ensembles for 0.1 and 1 ns, respectively. Verlet cutoff scheme and PME (particle mesh Ewald) algorithm were used for nonbonded interactions and long-range electrostatics, respectively. Temperature coupling was applied on protein-complex and POPC groups, and the system had been equilibrated to 310 K using a V-rescale thermostat (modified Berendsen thermostat). Random velocities were assigned and generated from Maxwell distribution across the periodic boundaries. The pressure is equilibrated at 1.0 bar using Parrinello-Rahman barostat. The production MD was performed for 500 ns (250,000,000 steps) and observed for the interaction and structural changes in the P2Y12R - TauRD complex by various methods. RMSD and RMSF graphs were plotted to determine the stability and residual fluctuation during the simulation. The interaction energy between the complex is calculated using the g_mmpbsa package of gromacs (Kumari et al., 2014). The 2-dimensional graph of interacting residues is plotted using LigPlot+ (version:2.2) (Laskowski and Swindells, 2011). APBS software was used to determine the electrostatic surface potential of the P2Y12R-TauRD complex as performed by Pontarollo et al., 2021 (Pontarollo et al., 2021). The complex structure obtained after the 500 ns simulation was used for this surface potential analysis. The pqr file was generated for the complex, TauRD and P2Y12R individually from the APBS-PDB2PQR web server using CHARMM forcefield (Dolinsky et al., 2004). The surface potential was then visualized using PyMol software plugged in with APBS (Baker et al., 2001).

4.4. Preparation of TauRD

TauRD is recombinantly expressed in *E. coli* cells and purified by various chromatographic techniques (Chidambaram and Chinnathambi, 2020b). TauRD protein is expressed by inducing with 0.5 mM IPTG once the OD (absorbance @ 600 nm) reaches 0.5–0.6. The cells were then harvested following 4 h of incubation and pelleted down at 4000 rpm for 10 min (4 °C). Resuspended in cell lysis buffer containing 5 mM DTT, 1 mM PMSF and protease inhibitor cocktail, and homogenized at 15,000 psi using cell disruption system. The lysate is then boiled at 90 °C for 15 min by adding 0.5 M NaCl and 5 mM DTT. The lysate is cooled and centrifuged at 40,000 rpm for 45 min at 4 °C to remove the precipitated proteins and other cellular components. The supernatant is dialyzed overnight with dialysis buffer (added with 50 mM NaCl) and centrifuged again. The supernatant is then loaded for cation exchange chromatography and eluted using a buffer containing 1000 mM NaCl. The eluted protein is then loaded for size-exclusion chromatography with 1X PBS buffer to get rid of other high and low-molecular-weight proteins. TauRD concentration is then estimated by the BCA method.

4.5. Western blot

N9 microglial cells were treated with 1 μM TauRD and the expression levels of P2Y12R, Iba-1 and LAMP2A are determined using western blot analysis. 2 μM Clopidogrel treatment was given for P2Y12 inhibition, separately and together with TauRD. Three lakh cells were seeded and incubated at 37 °C (5% CO_2) for 24 h before the treatment. The treatment was provided for 24 h and harvested by washing with PBS buffer (pH. 7.4) followed by trypsinization and centrifugation. The cells are lysed with RIPA buffer, and the total protein concentration is estimated by Bradford assay. An equal protein volume was used for western blot with rabbit P2Y12R polyclonal antibody (1:1000), Rabbit Iba-1 polyclonal antibody (1:1000), Rabbit LAMP-2A antibody (1:1000) and loading control as mouse β-tubulin monoclonal antibody (1:2000). The quantification of the band intensity was performed using ImageJ 1.53e software. Band intensities are then normalized using house-keeping genes used (β-tubulin) ($n = 3$), and the change in the expression of the protein is compared with the control groups (Das and Chinnathambi, 2021).

4.6. Co-immunoprecipitation

N9 microglial cells are seeded in 100 mm petridishes and incubated for 24 h at 37 °C (5% CO_2). The cells are then harvested, and co-immunoprecipitation was performed using manufacturer protocol with few modifications. Co-IP lysis buffer was used to lyse the cells and incubated for 20 min with pipetting at regular intervals. An equal volume of lysate was added with different concentrations of TauRD (1 μM and 5 μM) and one group without TauRD as a negative control. The mixture is then incubated by constant shaking for 1 h (RT). Pan-Tau K9JA primary antibody was coupled with the amino-linked resins by a

reductive amination reaction. Appropriate controls for non-specific binding and isotype IgG were maintained. Tau-lysate mixture is then added to the antibody-coupled resin columns and incubated at 4 °C rotor for overnight binding. The proteins that precipitated along Tau were eluted using the elution buffer according to the manufacturer's protocol. The eluted proteins are analysed by SDS-PAGE and western blot by P2Y12R and K9JA antibody (1:1000 and 1:8000 dilution, respectively).

4.7. Alexa Fluor 647 labeling of Tau^{RD} for internalization

100 μM of Tau^{RD} was incubated with 2 μM of Tris(2-carboxyethyl) phosphine (TCEP) to maintain a reduced state of sulfur group in Tau cysteine residues. 200 μM of Alexa Fluor™ 647 C_2 Maleimide is then slowly added to the protein and incubated overnight at 4 °C shaker. The untagged molecules are removed by washing with excess PBS buffer using 3 kDa centricons. The final concentration after labeling is measured by BCA assay.

4.8. Immunofluorescence microscopy

The localization of P2Y12R and Iba1 with actin remodeling on microglia upon Alexa647-Tau^{RD} exposure (1 μM) were studied by Immunofluorescence microscopy. 2 μM Clopidogrel treatment was given for P2Y12 inhibition, separately and together with Tau^{RD}. N9 cells (10,000 cells) were treated with a Tau^{RD} monomer for 24 h. The time-dependent assays are treated for several time points such as 0.5, 1, 3 and 6 h. Then, the cells were washed with PBS and fixed with 4% paraformaldehyde solution for 15 min, and permeabilized with 0.2% TritonX-100. The cells were stained with P2Y12R (1:100), Extracellular P2Y12 (1:250), LAMP-2A (1:500), Iba1 (1:100), and phalloidin-alexa488 (1:40) antibody overnight at 4 °C. Then, Alexa flour-secondary antibodies were allowed to bind for 1 h along with nuclear stain-DAPI (300 nM). The microscopic images were captured in Zeiss Axio observer Apotome2 fluorescence microscope at 63X oil immersion objective. The quantifications were carried out using ZEN 2.3 software. The numbers of filopodia^{+} microglial cells were counted in multiple fields (n = 12). The colocalization analysis of Pearson's coefficient was done by ImageJ software in Tau^{RD} treated group in multiple fields (n = 12).

4.9. Statistical analysis

All experiments were performed in two or three biological replicates and triplicate measurements for each experiment. One-way ANOVA is used for performing all statistical analyses. The statistical significance of multiple groups has been calculated by Tukey-Kramer's post-hoc analysis for multiple comparisons at 5% level of significance. The p-values are calculated in comparison with control groups, and values are mentioned within the graph. The results are considered significant if the mean difference between treatment groups is greater than calculated Tukey's criterion ($X\text{-}X^{`} > T$).

Funding and additional information

This project is supported by in-house Council of Scientific and Industrial Research-National Chemical Laboratory grant MLP101726. The authors also thank Department of Biotechnology and University Grants commission for the fellowship.

Declaration of Competing Interests

The authors declare that they have no conflicts of interest with the contents of this article.

Data availability

All the data is contained in the manuscript.

Acknowledgments

We thank Shivashankar S and Amit Naglekar for their valuable comments and suggestions in performing the *in-silico* experiments. We thank Tazeen Qureshi for proof-reading the manuscript. We are grateful to Chinnathambi's lab members for their scientific discussions, helpful suggestions, and critical reading of the manuscript. Clopidogrel bisulfate (IP grade) is a generous gift from Mr. Prabhanjan S Giram (Shitole et al., 2019).

Author contributions

Hariharakrishnan Chidambaram and Subashchandrabose Chinnathambi performed the literature search and wrote the manuscript. Hariharakrishnan Chidambaram and Subashchandrabose Chinnathambi performed the *in-silico* and biochemistry experiments. Hariharakrishnan Chidambaram, Rashmi Das and Subashchandrabose Chinnathambi performed the cell biology experiments and wrote the manuscript. Subashchandrabose Chinnathambi conceived the idea of the work and supervised the project. All authors read and approved the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2022.151201.

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Microglia degrade extracellular Tau oligomers deposits via purinergic P2Y12-driven podosomes, filopodia formation and induce chemotaxis

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Abstract

Tau is a microtubule-associated protein, which accumulates as neurofibrillary tangles and becomes deposited as plaques in the brain during Alzheimer's disease (AD). Tau undergoes various post-translational modifications that lead to its self-oligomerization, aggregation, and subsequent escape from affected neurons. Tau oligomers are the most reactive species associated with neurotoxic and inflammatory activity. Microglia are the immune cells in the central nervous system, sense the extracellular presence of Tau *via* various cell surface receptors among which purinergic P2Y12 receptor can directly interact with Tau oligomers and mediates microglial chemotaxis *via* actin remodeling. The disease-associated microglia leads to impaired migration and expresses a reduced level of homeostatic genes such as P2Y12 while elevates the expression of phagocytic, reactive oxygen species, and pro-inflammatory cytokines. Extracellular Tau oligomers facilitated the microglial migration *via* Arp2-associated podosomes and filopodia formation through the activation of P2Y12 signaling. The Tau oligomers-induced the decoration of TKS5-associated podosomes clusters at microglial lamella in a time-dependent manner. Moreover, the P2Y12 receptor was evidenced to localize with the actin core of podosomes and in filopodia for Tau-deposits degradation. While the blockage of P2Y12 signaling resulted in decreased microglial migration and reduced Tau-deposits degradation. Hence, the P2Y12 signaling mediated the formation of migratory actin structures like-podosomes and filopodia to exhibit chemotaxis and degrade Tau deposits. These beneficial roles of P2Y12 signaling in microglial chemotaxis, migratory actin network remodeling, and Tau clearance can be intervened as a therapeutic targets in AD.

Keywords

P2Y12, migration, filopodia, podosomes, microglia, Tau oligomers.

Introduction

Alzheimer's Disease (AD) is an age-associated progressive neurodegenerative disease, characterized by extracellular depositions of amyloid-β (Aβ) plaques, intracellular neuro-fibrillary tangles (NFTs) of Tau, and overactivation of central nervous system (CNS) inflammation which ultimately leads to excessive synaptic loss and neuronal death (Das and Chinnathambi, 2019; Gorantla and Chinnathambi, 2018). In progressive stages of AD, the microtubule-associated protein Tau becomes post-translationally modified that subsequently leads to disassembly from the microtubule, oligomerization, and aggregation (Castillo-Carranza et al., 2014; Iqbal et al., 2013; Sonawane and Chinnathambi, 2018). Disease-associated Tau species can propagate from damaged neurons to healthy neurons through various processes such as exosomes, membrane leakage, cell-to-cell junctions and neurotransmitter release *etc.* (Asai et al., 2015; Croft et al., 2017; Zhou et al., 2017). Microglia are the prime immune cells in the brain, which essentially sense the presence of extracellular Tau *via* death-associated molecular pattern (DAMPs) receptors and become activated in order to clear 'non-self' species from parenchyma (Desale et al., 2021; Perea et al., 2018; Pooler et al., 2014; Vogels et al., 2019). Microglia migrate at the site of neuronal damage and plaque deposits *via* sensing extracellular chemical gradient and mediate immune response (Das and Chinnathambi, 2020; Maeda et al., 2021). But, the senescent microglia or disease-associated microglia (DAMs) exacerbate the hyper-inflammation, Reactive oxygen species (ROS) production, and complement-mediated faulty engulfment of synapses, which leads to neuronal death and cognitive loss (Brown and Neher, 2014; Deczkowska et al., 2018; Rangaraju et al., 2018; Werneburg et al., 2020).

Microglia alternate between two distinct phenotypes to mediate cellular functions, which include synaptic surveillance, neuronal health, neurotransmitter recycling, tissue homeostasis, migration, and pathogen recognition (Colonna and Butovsky, 2017; Koellhoffer et al., 2017). The 'ramified' microglia are associated with homeostatic conditions with long cellular processes and without net displacement. In contrast, 'ameboid' microglia retracted their cellular extensions to migrate a long distance by following chemical gradient for immune activation (Au and Ma, 2017; Savage et al., 2019). Microglia remodel its membrane-associated actin network for chemotaxis, phagocytosis, endocytosis, and vesicular trafficking (Das and Chinnathambi, 2021; Desale and Chinnathambi, 2021; Stuart et al., 2007). Actin remodeling consists of various microstructures such as lamellipodia, filopodia, podosomes, invadopodia, focal adhesion-stress fiber, and cortical actin sheet. All of these actin structures can be distinguished from others by depending on actin organization, polymerization, three-dimensional cellular location, function,

and specific molecular signature (Blanchoin et al., 2014; Puleo et al., 2019). Lamellipodia are the membrane protrusions with crosslinked actin network at the cell front, while the filopodia are finger-like projections with bundled actin polymers (Mattila and Lappalainen, 2008; Vitriol et al., 2015). Lamellipodia produces firm anchorage onto the substratum and generates tensile mechanical force to move forward, but filopodia involve mechano-sensing, adhering, object trapping, and polarization (Wu et al., 2017). Podosomes are the short-lived, complex, protrusive ventral actin network, which is composed of cross-linked actin core, surrounded by vinculin and adhesion receptor's ring with bundled actin(Block et al., 2008). Podosomes are dynamically formed at the cell periphery in contact with focal adhesion and then eventually relocate throughout the ventral surface of lamellipodia (Zhu et al., 2016). Podosomes mediate various physiological functions, including matrix adhesion, degradation, migration, invasion, and many more that need to explore further (Juin et al., 2013; van den Dries et al., 2019). The growth factor activation induces podosomes formation and subsequent Src kinase and PKC activation, which leads to actin nucleation, elongation, and complex network formation (Tatin et al., 2006). The signaling through Src kinase and TKS5 scaffold regulates the actin flux, nucleation, and podosomes formation for physiological cell migration (Schachtner et al., 2013). Recent reports emphasize microglial migration in response to plaques accumulation, neuronal damage, and inflammation in many neurodegenerative diseases (Schachtner *et al.*, 2013). Hence, the structural and functional organization of podosomes and other actin protrusions need to explore in extracellular Tau-induced microglial migration.

Purinergic signaling plays a vital role in microglial chemotaxis, neuronal health maintenance, and tissue homeostasis (Illes et al., 2021). Microglia form P2Y12-mediated somatic junction with neurons to surveil neuronal health (Cserép et al., 2019). Similarly, the induction of P2Y12 signaling leads to microglial process extension, but the loss of P2Y12 resulted in reduced brain surveillance, immune activation, IL1β secretion, and deleterious synaptic elimination (Liu et al., 2017; Sipe et al., 2016; Walker et al., 2020). P2Y12 pathway mediates through VASP (Vasodilator-stimulated phosphoprotein) phosphorylation and Ca^{2+} signaling in migratory microglia where migration-associated actin structure-podosomes contains Ca^{2+} ion channels, Iba1 and calmodulin for directional movements (Lee and Chung, 2009; Siddiqui et al., 2012a). Previously, the P2Y12 signaling and actin remodeling-associated microglial migration become hampered due to aging (Mildner et al., 2017). Therefore, the exact function and occurrence of this purinoceptor- P2Y12 in migratory actin structures like-podosomes, filopodia, and lamellipodia need to be explored in Tauopathy-associated migratory microglia.

Thus, we are interested in studying the role of P2Y12 in remodeled actin structures such as podosomes, filopodia, and uropod in Tau-induced microglial migration. Moreover, the actin nucleation and podosomes formation will be depicted in terms of Arp2 and TKS5 colocalization within F-actin-rich

region of microglia. Thereafter, the effect of P2Y12 activation by ADP and blockage by Clopidogrel will elucidate the microglial migration, podosomes, and filopodia-associated Tau deposits degradation.

Materials and Methods

Key resource table

Reagent or Resource	Source	Identifier
Antibody		
Total pan-Tau antibody K9JA	Dako	A0024
A11 Oligomers specific antibody	Thermo	AHB0052
β-Actin loading control monoclonal antibody (BA3R)	Thermo	MA5-15739
P2Y12R antibody (4H5L19)	Thermo	702516
Phalloidin-Alexa 488	Thermo	A12379
β-tubulin monoclonal antibody	Thermo	MA5-16308
Arp2 antibody	Thermo	PA5-27879
TKS5 antibody	Thermo	PA5-58169
Goat anti-rabbit IgG (H+L) Cross-adsorbed secondary antibody HRP	Thermo	A16110
Goat anti-mouse IgG (H+L) secondary antibody HRP	Thermo	32430
Goat anti-rabbit IgG (H+L) Cross-adsorbed secondary antibody with Alexa Fluor 555	Thermo	A-21428
Alexa647- C2 maleimide	Thermo	A20347
DAPI	Thermo	D1306
RPMI 1640 media	Invitrogen	61870127
Fetal Bovine Serum (FBS)	Invitrogen	26140079
Horse serum	Invitrogen	26050088
Phosphate buffer saline (PBS, cell biology grade)	Invitrogen	10010031
Trypsin-EDTA	Invitrogen	15400054
Penicillin-streptomycin	Invitrogen	15070063
RIPA buffer	Thermo	89900
Cell culture 24-well inserts	Invitrogen	140629
EGTA	Invitrogen	194823
Protease inhibitor cocktail	Invitrogen	A32955
Tris-Cl	Invitrogen	15506-017

Acrylamide	Invitrogen	AM9022
TEMED	Invitrogen	17919
Glutaraldehyde	Sigma	G5882
BCA reagent	Sigma	BCA1
Copper sulphate	Sigma	209198
TCEP	Sigma	75259
SDS	Sigma	L3771
Thioflavin-S	Sigma	T1892-25G
ANS	Sigma	10417
TritonX-100	Sigma	T8787
MES hydrate	Sigma	M8250
PVDF membrane	Merck-Sigma	IPVH00010
Ampicillin	MP Biomedicals	02190148-CF
NaCl	MP Biomedicals	210289205
$MgCl_2$	MP Biomedicals	2191421.5
Ammonium persulphate (APS)	MP Biomedicals	4802811
Phenylmethylsulfonylfluoride (PMSF)	MP Biomedicals	04800263
Methyl alcohol	MP Biomedicals	02300141-CF
Heparin (17500 Da)	MP Biomedicals	101931
Paraformaldehyde	MP Biomedicals	MFCD00133991
Polysorbate 20	MP Biomedicals	103168
IPTG	Calbiochem	5815
Dithiothreitol (DTT)	Calbiochem	12/3/3483
400 mesh copper coated carbon grids	Ted Pella, Inc.	01814-F
Bradford reagent	Bio-Rad	5000002
ECL reagent	Bio-Rad	1705060
Glycine	Bio-Rad	161-0718
Luria-Bertani broth	Himedia	M1245
Software		
Microsoft excel	MS office	NA
Sigma plot 2.0	Sigmaplot	NA
ZEN 2.3	Zen	NA
ImageJ2.0	https://imagej.nih.	NA

	gov	
BIORAD Quality one 4.6.6	BIORAD	NA
N9 microglial cell line	CVCL_0452	

Preparation of Tau monomer and oligomers and its characterization

Tau protein was expressed in *E. coli* BL21* and purified by cation exchange and followed by size-exclusion chromatography, as described previously (Gorantla et al., 2017). Tau oligomers was prepared by inducing with polyanionic factor heparin (17.5 kDa) in PBS (pH 7.4) for 12 hours, as described earlier (Das et al., 2020). Briefly, Tau oligomers was stabilized using 0.01% glutaraldehyde for 10 minutes. The oligomers were then buffer exchanged twice with PBS by centrifuging at 3200 rpm for 2 hours with 10 kDa molecular cut-off filters. The concentrated oligomers were collected and the concentration was measured by BCA assay. Further, the quality of the oligomers and monomers were checked by 10% SDS-PAGE and stored at -80°C. The core-β sheet structures of Tau oligomers were characterized by Thioflavin-S (440/521 nm), and the exposed surface hydrophobic patches on Tau species were quantified by ANS fluorescence (375/490 nm) in fluorescence spectrophotometer (Infinite® 200 M PRO, Tecan). Tau oligomers were characterized by transmission electron microscopy (TEM) by spotting onto 400-mesh coated copper grids and stained with 2% uranyl acetate. The grids were dried and analyzed by Tecnai T20 at 120 kV for TEM. Tau oligomers and monomers were spotted onto the nitrocellulose membrane at equal concentrations and allowed for complete drying. The blots were blocked with 5% skimmed milk in PBS and probed with A11 antibody (1:1000 dilution) and K9JA antibody (1:8000 dilution) overnight and 1 hour, respectively. The blots were probed with anti-rabbit secondary antibody, developed using ECL reagent in Amersham Imager 600.

Tagging of Tau monomer and oligomers by Alexa647- C2 maleimide

The 100 µM of Tau monomer was diluted in PBS and incubated with 10 molar excess TCEP (tris(2-carboxyethyl)phosphine) for 10 minutes. But, Tau oligomers were diluted only in PBS. Then, Tau monomer and oligomers were mixed with 2 molar excess Alexa 647-C2 maleimide drop-wise and incubated overnight at 4°C in shaking condition. After incubation, the unbound Alexa 647 were removed by buffer-exchange twice with PBS in 3 KDa molecular cut-off filters by centrifuging at 12000 rpm for 5 minutes and the Alexa^{647} tagged Tau monomer, and oligomers were collected separately. The concentration of Alexa^{647} tagged Tau monomer and oligomers were checked by BCA assay and characterized by SDS-PAGE, Thioflavin-S, ANS fluorescence, and TEM study.

Western Blot

To study the expression level of Arp2, TKS5 upon Tau exposure, the N9 cells ($3X10^6$ cells/ treatment group) were treated with Tau monomer and oligomers at a concentration of 45 µg/ml along with ADP at 50 µM concentration, as a positive control of P2Y12 signaling activation, for 24 hours. The cells were washed with PBS and lysed with RIPA buffer. The cell lysates were subjected to Bradford assay, and an equal amount of cell lysate of 75 µg was processed for western blot with anti Arp2 and TKS5 antibody (1:1000 dilution) with anti-β-tubulin antibody (1:5000 dilution) as an internal control. Then, the band intensity for target protein and loading control gene among various groups was quantified by using BIORAD Quality one 4.6.6 software. The band-density of the treated group was compared with cell control group and normalized with the loading control gene (β-tubulin) (n=3). Then, the relative fold changes for the target proteins were plotted in comparison with house-keeping control.

Immunofluorescence Study

The Arp2, TKS5-localized and P2Y12-associated actin remodeling on microglia upon of Tau exposure (45 µg/ml) were checked by Immunofluorescence study. The N9 cells (25000 cells) were treated with Tau monomer, oligomers and ADP (50 µM) for 24 hours. For the time-dependent TKS5-localized podosomes accumulation experiment, N9 microglia were treated with Tau oligomers at 1 µM exposure (45 µg/ml) concentration along with cell control from 1 hour to 12 hours' time points. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.2% TritonX-100 and blocked with 2% horse serum in PBS buffer. The cells were stained with P2Y12 (1:100), Arp2 (1:100), TKS5 (1:100) antibody, and phalloidin-alexa488 (1:40) for overnight at 4°C. Then, Alexa flour-tagged secondary antibodies were allowed to bind P2Y12, Arp2 and TKS5 for 1 hour along with nuclear stain-DAPI (300 nM). The microscopic images were taken in Zeiss Axio observer with Apotome2 fluorescence microscope at 63X oil immersion objective. The quantification for mean fluorescence intensity (n=50) and area of cells (n=40) were done using ZEN 2.3 software and plotted for different test groups. The numbers of podosomes (n=13 fields) and filopodia (n=22 fields) positive N9 cells, %cells with different podosomes rearrangements (n=28), time kinetics podosomes formation (n=10 fields) were counted in multiple fields.

Wound scratch assay

For the determination of 2D microglial migration upon P2Y12 activation and blockage, the N9 cells ($5X10^6$ cells) were treated with 45 µg/ml concentration of Tau monomer, oligomers, along with ADP (50 µM) and Clopidogrel (2 µM). At first, 3 scratches per group were made with 200 µl tips, washed, and then, Tau monomer and oligomers were added separately along with ADP and Clopidogrel and incubated for 24 hours. The phase-contrast images were taken at a various time intervals from 0 to 24 hours in Zen

Axio observer 7 microscope at 20X magnification. The wound lengths were measured in 4 different positions at 24 hours time intervals and the % of wound closure was calculated in comparison with cell control. The experiment was performed thrice, and multiple values were taken from single groups for wound closure (n=12).

Trans-well migration assay

N9 microglia were subjected to trans-well migration assay in response to extracellular Tau species along with ADP and Clopidogrel as a determinant of P2Y12-related chemotaxis. N9 cells (50,000 cells/inserts), treated with ADP and Clopidogrel, were seeded in the upper chamber of 24-well plate format. While, the Tau monomer, oligomers were added at a concentration of 45 µg/ml in lower chamber for 24 hours. The lower surface of the inserts containing migrated microglia was fixed with 4% paraformaldehyde and stained with 0.2% crystal violet solution, and dried. The upper surface of the inserts containing non-migrated cells was removed by using cotton swab and the lower surfaces were imaged at 20X objective under bright-field microscope. The experiment was performed twice and the number of migrated cells per field were counted (n=16 fields) in different treatment groups.

Preparation of Tau-coated coverslips for ECM degradation by immunofluorescence assay

For the ECM degradation assay, the 18 mm coverslips were coated with 10 µg/cm^2 Alexa^{647} tagged-Tau protein (monomer and oligomers) in PBS and incubated overnight at 4°C. After incubation, the excess solution was aspirated by vacuum, and the coverslips were dried at 37°C for 30 minutes. The coated coverslips were sterilized by UV exposure for 30 minutes in a laminar hood and washed with PBS twice. The Alexa^{647}-tagged Tau (monomer and oligomers) coated coverslips were neutralized with 10% FBS containing RPMI media for 15 minutes. The N9 cells were seeded at 50,000 cells/well for ECM degradation and were incubated for 8 hours for monomer-ECM deposits and 24 hours for oligomers-ECM deposits. After incubation, the cells were fixed with 4% paraformaldehyde and directly blocked with 2% horse serum, 0.2% TritonX-100 containing PBS for 1 hour. The cells were stained with phalloidin (1:40), TKS5 (1:100), Arp2 (1:100), and P2Y12 (1:100) for overnight at 4°C. The anti-rabbit secondary antibody-Alexa 555 was used to bind Arp2, TKS5, and P2Y12 for 1 hour along with nuclear stain-DAPI (300 nM). The microscopic images were taken in Zeiss Axio observer with Apotome2 fluorescence microscope at 63X oil immersion objective. The quantification for mean fluorescence intensity was done using ZEN 2.3 software and plotted for different test groups. The % cells with ECM degradation (n=40) and P2Y12 activation/blockage-related ECM degradation (n=30) were counted in multiple fields. The colocalization analysis in podosomes and filopodia-associated Tau ECM degradation (n= 25) in treated groups in multiple fields.

Statistical analysis and significance

All experiments were performed in three biological replicates and each measurement for every experiment was taken in triplicate. Statistical analyses were performed for fluorometric assay and microscopic quantification by using one-way ANOVA. The statistical significance among various groups have been calculated by Tukey-Kramer's analysis at 5% level of significance. In microscopic analysis, several data points from multiple fields were plotted. The test groups were compared with untreated cell control and the p-values were mentioned within the figures. In ADP or Clopidogrel-mediated actin remodeling and ECM degradation experiment, the ADP+Tau species treated groups were compared to only ADP/Clopidogrel treated group (For *eg.* ADP+ Tau monomer vs. ADP, Clopidogrel+ oligomers vs. Clopidogrel *etc.*). Hence the p-values were quantified and depicted within the figures.

Results

Extracellular Tau oligomers induce Arp2-associated podosomes and filopodia formation but reduce Arp2 from uropod.

Tau is a microtubule-associated protein that becomes oligomerized and further aggregated as neurofibrillary tangles during the progression of AD (Gorantla and Chinnathambi, 2020). The subsequent release of reactive Tau oligomers in brain parenchyma activates the surveilling microglia to mediate migration, phagocytosis and immune response (Guerriero et al., 2017). To understand the effect of Tau oligomers on microglial migration, we have prepared and characterized a stable globular Tau oligomer, which contained more β-sheet structures and surface hydrophobicity by ThS and ANS fluorescence, respectively, as compared to its monomeric species (Suppl. Fig 1A-E). Tau monomer and oligomers were tagged with Alexa^{647}-C2 maleimide. The Alexa^{647} tagged Tau oligomers were found to retain globular structures, as observed by TEM and similar β-sheet content by ThS fluorescence (Suppl. Fig 1F-I).

Previously, we showed that microglia remodel their membrane-associated actin network in response to extracellular toxic Tau species (Das *et al.*, 2020). Here, we further showed that microglia displayed a specialized actin structure called podosomes, which were colocalized with actin-nucleator protein-Arp2, in response to extracellular Tau oligomers. The podosomes were observed to accumulate more at the frontal lamellipodia upon Tau oligomers exposure than its monomeric counterpart (Fig 1A). In particular, the podosomes contains the core of branched actin networks, surrounded by a vinculin ring where each branching is connected by the Arp2/3 complex. The TKS5 scaffolding protein binds the actin network with adhesive membrane receptors such as integrins (Fig 1B). The extracellular Tau exposure induced the number of podosome-bearing microglia by 20% as compared to cell control (Fig 1C). Also, the

podosomes-associated area was increased by Tau oligomers exposure in migratory microglia as similar to ADP, positive regulator (Fig 1D, Suppl. Fig 2A).

Extracellular Tau has induced the Arp2-decorated filopodia formation during microglial migration as compared to untreated control. Moreover, the number of filopodia was increased by two times in Tau monomer exposure and three times in oligomers exposure compared to untreated control (Fig 1E, F). The contractile rear end of the migratory cells is called uropod (Kuras et al., 2012). The exposure of extracellular Tau oligomers was associated with branched uropod formation in microglia, emphasizing more cortical adhesions during directed migration (Fig 1E, Suppl. Fig 2B). The microscopic quantification depicted that the reduced level of Arp2 in the uropod of oligomer-induced migratory microglia, which probably emphasizes on the rapid turnover of actin nucleation at the lamella from uropod (Fig 1G). The western blot analysis showed that the Arp2 level remained unaltered during Tau-induced and ADP-induced microglial populations (Fig 1H, I). Together, extracellular Tau oligomers facilitated the podosomes and filopodia-associated actin polymerization by Arp2 *via* altering the actin turnover from uropod to lamella during migration (Fig 1J).

Tau oligomers trigger the accumulation of TKS5-localized podosomes clusters, not belts at lamellipodia in a time-dependent manner

Podosomes are categorized as one of the migratory structures of the actin network, involved in cortical adhesion, matrix anchorage, and extracellular matrix (ECM) degradation (Blanchoin *et al.*, 2014). Several podosome rearrangements occurred in cells, such as podosome clusters, podosome belts, podosome rosettes, and single podosomes (Fig 2A). But, the actual function of these particular rearrangements is not known (Schachtner *et al.*, 2013). TKS5 scaffold plays an important role in initiating and forming podosomes and positioning metalloproteases, while the loss of TKS5 leads to the reduced podosome formation (Burger et al., 2011). In our study, microglia showed the unaltered expression level of TKS5 in various treatment groups, although the formation and area of podosomes have been increased significantly in Tau/ADP-exposed microglia (Fig 2B, C). We observed that microglia formed various arrangements of podosomes such as clusters, belts, and single upon extracellular stimuli like-Tau oligomers or ADP (Fig 2D, Suppl. Fig 3). The quantification of microscopic images depicted that percentage of cells showing podosomes clusters have significantly increased by 20% upon Tau oligomers and ADP exposure (Suppl. Fig 4A). While the other podosomes arrangements like-single and podosomes belts were elusive in Tau/ADP-induced microglia (Fig. 2E-G). Similarly, the TKS5 intensity was reduced in uropod, while the F-actin intensity remains unaltered in both podosomes and filopodia of Tau oligomers and ADP-exposed microglia (Fig 2H, Suppl. Fig 4B, C). These might emphasize the skewed podosomes turnover towards lamellae from uropod during Tau-induced microglial migration. Similarly,

the TKS5 intensity and the podosomes cluster formation were found to be elevated time-dependently, after 4 hours exposure, and from the first hour of exposure of Tau oligomers, respectively (Fig 2I-K, Suppl. Fig 5). Hence, microglia preferentially organizes the TKS5-associated podosomes clusters at lamellipodia in adhere to nascent sites and move forward during Tau-indued migration.

Tau oligomers orchestrate P2Y12 in podosomes

P2Y12 is a metabotropic purinergic receptor involved in ADP-mediated chemotaxis in microglia towards the site of neuronal damage and amyloidogenic plaques deposition (Domercq et al., 2019; Wendt et al., 2017). Here, we observed that during Tau oligomers-induced microglial migration, P2Y12 particularly localized with actin core in podosomes at frontal lamellipodia (Fig 3A). The quantification of microscopic images depicted that the colocalization of F-actin and P2Y12 in podosomes clusters of Tau-exposed microglia (Fig 3B). Moreover, the extracellular Tau oligomers exposure-induced the number of microglia, which contained $P2Y12^{+}$ podosomes-orchestrated lamella in the population as compared to positive regulator ADP (Suppl. Fig 6). This might suggest that the existence of this purinoceptor to dictate the cellular directionality through podosome-mediated adhesion and migration.

The blockage of P2Y12 signaling reduces cell migration in Tau-induced microglia.

P2Y12 signaling plays an important role in microglial migration in response to ischemic brain injury, and the blockage of P2Y12 results in reduced immune activation (Kluge et al., 2019; Li et al., 2020). Our previous study showed that extracellular Tau oligomers could act as chemoattractant by interacting with microglial P2Y12 with the elevated rate of wound closure and trans-migration (Das and Chinnathambi, 2021). To identify the importance of P2Y12 signaling in Tau oligomers-induced migration, P2Y12 was activated and blocked by ADP and Clopidogrel, respectively, then the migration was measured in microglial culture. We observed that P2Y12 blockage has reduced the percentage of wound closure in Tau oligomers-induced microglia (Fig 3C, D). While the Tau monomer-induced wound closure remains unaltered in both P2Y12 activated and blockage conditions (Suppl. Fig 7A). In comparison, the ADP-induced P2Y12 activation resulted in increased microglial trans-migration, which was also evident in ADP+ Tau oligomers-induced state (Fig 3E, Suppl. Fig 7B). Similarly, the P2Y12-blockage led to the reduced level of microglial trans-migration, but the Tau oligomers could reverse the event of trans-migration, even in the P2Y12-blocked conditions (Fig 3F). Hence, it is evident that P2Y12 signaling influences microglial migration while the Tau oligomers can intervene in the P2Y12-mediated chemotaxis to invade during disease conditions.

Microglia display P2Y12 in filopodia and uropod for directed migration

Microglia displays P2Y12-associated filopodia to migrate at the site of injury, while the P2Y12 mutation results in fewer filopodia formation and induced engulfment of damaged neurons in epileptic brain (Bernier et al., 2019; Mo et al., 2019). In our study, we found that extracellular Tau-induced the P2Y12-associated filopodia formation in migratory microglia. Similarly, the P2Y12 localization was observed to increase in branched uropod structures during Tau oligomers-induced migration (Fig 4A). Microscopic quantification showed that extracellular Tau oligomers-induced the number of cells, which contained the $P2Y12^{+}$ filopodia (Fig 4B). Moreover, the localization of P2Y12 was increased in microglial rear ends uropod (Fig 4C), suggesting that the P2Y12 localizes in migration associated structures- filopodia for increased cell adhesion and matrix sensing.

Microglia orchestrate P2Y12-associated filopodia to degrade Tau monomer more than oligomers as deposits

Podosomes are the short-lived actin protrusions at the leading edge and ventral surface of the cells for mediating matrix anchorage, ECM degradation, and migration (Ito et al., 2007; Lively and Schlichter, 2013). The activation of the ECM-sensing integrin receptors and growth factor receptors (PDGF, EGF) results in the induction of the podosomes formation by Src kinase and PKC in various migratory cells (Murphy and Courtneidge, 2011; Yu et al., 2013). But, the occurrence of the chemotactic receptor like-purinergic P2Y12 receptor in podosomes is not yet described in cellular migration. Here, we observed the presence of P2Y12 receptors in microglial podosomes, which were also found to degrade Tau monomer and oligomers as ECM depositions (Fig 4D, Suppl. Fig 8). Quantitatively, microglia preferred to degrade Tau monomer than Tau oligomers as ECM deposits where 41% and 33% cells were degrading Tau monomer and oligomers, respectively (Fig 4E). Further, 72% of cells were associated with Tau monomer-ECM degradation, while only 26% microglia could degrade Tau oligomers-ECM through the formation of podosomes (Fig 4F).

Filopodia are the important bundled actin structures that functions in adhesion, mechano-sensing, directional migration and phagocytosis (Gallop, 2020). But, the functions of filopodia in the degradation of ECM or plaques deposits were not yet studied. Like podosomes, microglial filopodia was found to degrade Tau ECM, which was also decorated with P2Y12 (Fig 4G). The microscopic quantification showed that filopodia-associated Tau-ECM degradation was mediated by 70% microglia in the case of monomer deposits, while 50% of cells were positive for oligomers deposits degradation (Fig 4H). Hence, these results signify that P2Y12-driven chemotaxis actively takes part in podosomes and filopodia-associated matrix adhesion and microglial migration to degrade Tau-ECM deposits (Fig 4I).

Microglia degrade Tau deposits by Arp2-decorated podosomes and filopodia

The previous report has shown that P2Y12 activation is associated with alternatively activated microglia (M2) with a reduced level of pro-inflammatory cytokines, and the P2Y12^{+} microglia were present only adjacent to multiple sclerosis plaques areas (Moore et al., 2015). But, within the plaques, the P2Y12 expression becomes downregulated, which elicits the potential role of this purinoceptor as a therapeutic target (Zrzavy et al., 2017). In our study, microglial podosomes and filopodia, which were associated with rapid actin nucleating events by Arp2, were evidenced with Tau-ECM degradation (Fig. 5A, Suppl. Fig. 9A). Colocalization analysis depicted that filopodia were more orchestrated with F-actin and Arp2 than podosomes during microglia-mediated Tau-ECM degradation (Fig 5B). Similarly, microglial podosomes and filopodia were observed to degrade Tau deposits, colocalized with TKS5, responsible for podosome formation and matrix degradation (Fig. 5C, Suppl. Fig 9B). But, the TKS5 association with F-actin in podosomes and filopodia remain unchanged during Tau degradation (Fig. 5D). Thus, it is evident that filopodia-mediated Tau degradation requires faster actin nucleation/polymerization than podosome-mediated degradation by migratory microglia.

The blockage of P2Y12 signaling reduces Tau-ECM degradation

Microglial P2Y12 dictates the directional migration *via* actin remodeling, filopodia formation for matrix degradation by secreting various proteases at the site of neuronal damage (Bernier *et al.*, 2019; Lively and Schlichter, 2013; Seizer and May, 2013). But once reached, microglia transformed into phagocytic and inflammatory phenotype(Méndez et al., 2019). In our study, the situation of the Tau deposited area was mimicked by coating the coverslips with Tau, and then the function of P2Y12 was explored in matrix degradation and Tau clearance. The ADP-induced microglia showed increased filopodia formation during Tau-ECM degradation, but the blockage of P2Y12 signaling did not alter the podosomes formation (Fig. 6A, Suppl. Fig 10A, B). Microscopic quantification elucidated that the blockage of P2Y12 signaling by clopidogrel has reduced the level of Tau-ECM degradation while ADP induction did not alter extracellular Tau degradation (Fig. 6B). Similarly, the podosomes and filopodia-associated ECM degradation area were colocalized with Arp2 and TKS5, which signify rapid actin nucleation and podosomes turnover in microglia (Fig. 6C, Suppl. Fig. 11A, B). The colocalization analysis depicted that P2Y12 blockage lead to more accumulation of Arp2 with F-actin, at the same time the ADP-induced activation resulted in less TKS5 localization in podosomes and filopodia of Tau-degrading microglia (Fig 6E, F). Hence, the influential function of microglial purinergic receptor P2Y12 can be decoded in directional chemotaxis and to degrade extracellular Tau deposits for therapeutic intervention.

Discussion

Podosomes are the specialized actin structures which are remarkably different from other protrusive structures such as lamellipodia and filopodia based on their structural and functional organization (Blanchoin *et al.*, 2014; Murphy and Courtneidge, 2011). The F-actin resides at the podosomes core with actin nucleator Arp 2/3 complex and cortactin, while, the TKS5 localizes with podosomes at the ventral surfaces as a critical component of ECM degradation (Seals et al., 2005; Yamaguchi et al., 2005). In AD, the altered activation of ADF/cofilin leads to abnormal actin polymerization and form actin rods, which block the vesicular transport, neuritic outgrowth, and growth-cone formation in neurons. (Bamburg and Bernstein, 2016; Maloney et al., 2005; Rush et al., 2018). The podosomes, lamellipodia, and filopodia in immune cells function in the surveillance, tissue remodeling, ECM degradation, cytokine release, and in antigen recognition (Calle et al., 2008; Mersich et al., 2010; Sage et al., 2012). Here, we emphasized that microglia sense the extracellular soluble Tau oligomers and induces actin nucleation by incorporating Arp2 in migration-associated structures such as podosomes and filopodia. Moreover, the microglia mediate the turnover of podosomes and filopodia at the lamellipodia from the rear uropod as a consequence of directional migration and polarization in Tau-induced conditions. Migratory cells form various rearrangements of podosomes, while the exact functions of these structures are not clearly known (Murphy and Courtneidge, 2011). For *e.g.*, osteoclasts formed single podosome-rich structures called 'sealing zone', which become matured into podosome belts for bone remodeling (Jurdic et al., 2006). The podosomes clusters are constantly formed by fusion and fission of parent podosomes for mechano-transduction during macrophage migration (Evans et al., 2003). Similarly, podosome rosettes in various cells are formed by PKC, Rho-GTPase, and integrin signaling which is induced by the accumulation of phosphoinositide-(3,4)-P2 and N-WASP, that subsequently interacts with TKS5 and Grb2 for migration and ECM degradation (Desale *et al.*, 2021; Kuo et al., 2018; Oikawa et al., 2008; Seano et al., 2014; Tatin *et al.*, 2006) . In our study, we investigated the appearance of different podosome arrangements in Tau-induced microglia. Extracellular Tau enhanced the formation of various podosome structures such as podosome belts, clusters, and well-connected single podosomes, among which the formation of podosomes clusters were induced with increased TKS5 localization within the F-actin core. Hence, it is emphasized that extracellular Tau influences TKS5-associated podosomes formation for matrix adhesion and degradation in migratory microglia.

Cell migration and invasion play a vital role in embryonic development, combating infection, and repairing injury. But the abnormal cellular migration resulted in carcinogenesis, immune disease, genetic disorders (Wiskott Aldrich Syndrome, Frank-Ter Harr Syndrome) and neurodegenerative disease- AD (Murphy and Courtneidge, 2011). Microglia respond to extracellular plaques and nucleotides from damaged neurons through the activation of purinergic P2Y and P2X receptors (Burnstock, 2017). The metabotropic P2Y12 signaling is mainly associated with homeostatic microglia, which involves in

filopodia formation, maintaining neuronal health and chemotaxis. But, the DAMs showed a reduced expression of various homeostasis gene, such as- P2Y12 and CX3CR1 which are evident in AD and Tauopathy mice model (Keren-Shaul et al., 2017). Similarly, the P2Y12-activated microglia induce the formation of neuronal projection in seizure-induced neurogenesis mice model (Mo *et al.*, 2019). On the contrary, the activation of P2Y12 signaling collapses filopodia and induces large process extensions with bulbous tips, which signifies the distinct morphological features of homeostatic and immunosurveillance microglia (Bernier *et al.*, 2019). The $P2Y12^{+}$ microglia were observed to surround the Aβ and Tau plaques in the Tauopathy mice brain (Maeda *et al.*, 2021). But, the presence and function of the purinoceptor, specifically P2Y12 in the migratory actin network, need to be explored in neurodegenerative microglia (Siddiqui et al., 2012b). (Szabo et al., 2016). We hypothesized that the chemotactic microglia remodel its lamellipodia and filopodia for migration and podosomes for ECM degradation to mediate Ca^{2+} signaling, phagocytosis, and inflammation (Das and Chinnathambi, 2020). Therefore, our group showed that microglial P2Y12 directly interacts with extracellular Tau oligomers and induces the formation of membrane-associated actin structures (Das *et al.*, 2020; Das and Chinnathambi, 2021). Here, we particularly elucidated that the P2Y12 receptor localized with F-actin-containing migratory structures such as podosomes and filopodia in response to extracellular Tau oligomers. These may emphasize the coupling of the microglial chemotaxis with podosomes formation to chase and eliminate soluble Tau oligomers from the brain microenvironment. The inflammatory state affects microglial migration and podosomes formation in CNS development and repair injury. The LPS-activated microglia migrate poorly while IL-4 and IL-10-treated microglia have induced adhesion and podosomes ring formation (Vincent et al., 2012). Similarly, the P2Y12 interacts with β1-integrin to mediate chemotaxis and tissue invasion, while the blockage of P2Y12 leads to reduced microglial activation, migration, pro-inflammatory cytokine production and neuroprotection in ischemic stroke (Li *et al.*, 2020; Ohsawa et al., 2010). In our study, we evidenced that soluble Tau oligomers increased microglial migration, which is even more pronounced in ADP-induced microglia. But, the trans-well migration was reduced upon the blockage of P2Y12 signaling, which becomes restored moderately with Tau oligomers. Hence, it signifies that extracellular Tau oligomers mediate the microglial migration and invasion by influencing P2Y12-mediated chemotaxis.

Similar to cancer cells, the smooth muscle cell, endothelial cells, and immune cells showed podosome-mediated degradation by depending on ECM substrate and cell migration (Burgstaller and Gimona, 2005; Gawden-Bone et al., 2010; Varon et al., 2006). Similarly, human macrophages, dendritic cells and lymphocytes form matrix degrading long podosome protrusions when cultured in fibrillar collagen gel (Burgstaller and Gimona, 2005; Van Goethem et al., 2010). The genetic knockdown of TKS5 inhibits the podosomes formation while the loss of TKS4 affects both podosomes formation and MT1-MMP9-

associated ECM degradation (Buschman et al., 2009; Seals *et al.*, 2005). In our study, we showed that microglia degrade the extracellular Tau-deposits by Arp2- and TKS5-associated podosomes and filopodia formation. In the active lesion of Multiple Sclerosis, microglia transformed into phagocytic phenotype with oxidative injury, antigen presentation, and T-cell activation. While the later stages, microglia changed back to $P2Y12^{+}$ $TMEM119^{+}$ phenotype for the restoration of inactive plaque region (Zrzavy *et al.*, 2017). Similarly, we mimicked the plaques deposition in brain by immobilizing Tau onto the coverslip and then allowing the microglia for Tau-ECM degradation. The microglia degrade Tau deposits through the formation of podosomes and filopodia orchestrated with P2Y12 receptors, while the blockage of P2Y12 signaling resulted in the reduced Tau degradation. Hence, it can be stated that microglial P2Y12 mediates the directed migration and involves Tau deposits degradation by remodeling actin network.

In conclusion, extracellular Tau oligomers influence the formation of Arp2 and TKS5-associated migratory actin structure such as-podosomes and filopodia through the activation of P2Y12 signaling for directed microglial migration. The formation of podosomes clusters and filopodia may dictate the microglial degradation of Tau-ECM deposits *via* the activation of P2Y12 chemotaxis. Therefore, the P2Y12 signaling contributes a dual function in extracellular Tau oligomers-induced chemotaxis and the clearance of Tau-ECM deposits *via* podosomes and filopodia-associated actin remodeling (Fig 7).

Acknowledgements

Rashmi Das acknowledges the fellowship from University Grant Commission (UGC) India. We are thankful to Dr. Prabhanjan Giram for providing Clopidogrel bisulfate (IP grade) as a kind gift. This project is supported in part by grant from in-house CSIR-National Chemical Laboratory grant MLP029526 and under the Healthcare, fundamental basic research grant from CSIR MLP101726 .

Figure legends

Figure 1. Tau oligomers induce podosomes and filopodia formation, orchestrated with Arp2.

A. Extracellular Tau oligomers induced the podosomes accumulation in microglial lamellipodia, which are colocalized with actin-binding protein Arp2, compared to Tau monomer. ADP induces the P2Y12 signaling with increased podosomes formation in migratory microglia.

B. A simplistic illustration of podosomes-associated actin polymerization where actin cross-linking is mediated by Arp2/3 complex and membrane attachment of actin core-fibers are driven by adaptor protein TKS5. The actin cores are surrounded by the vinculin ring and adhesion protein receptors.

C. The exposure of Tau oligomers has significantly induced the percentage of $podosomes^{+}$ cells in the microglial population.

D. Moreover, the podosomes-associated area has increased in Tau-exposed in microglial cells as compared to cell control.

E. Tau oligomers exposure has increased the filopodia numbers, extending out from migratory microglia compared to monomer and ADP exposure.

F. As similar to podosomes, Tau oligomers facilitated the Arp2-associated filopodia formation and branched uropod decoration in microglia.

G, H. Western blot analysis revealed that Arp2 expression was unchanged in various Tau exposure.

I. While, the microscopic quantification of Arp2 level was found to be reduced in uropod, relating rapid actin dynamics upon Tau oligomers exposure.

J. Hence, extracellular Tau oligomers induced directed microglial migration by podosomes-associated lamellipodia and filopodia formation.

Figure 2. Extracellular Tau modulates TKS5-mediated podosomes clusters formation in migratory microglia.

A. Upon extracellular Tau exposure, the migratory microglia rearranges podosomes at frontal lamellipodia as podosomes belts, clustered podosomes, and single podosomes.

B, C. Western blot analysis showed no change in the expression of TKS5 adaptor protein upon various Tau exposure in migratory microglia.

D. Extracellular Tau exposure has induced various rearrangements of podosomes in lamellipodia where podosomes clusters formation was more evident in migratory microglia.

E. The amount of single podosomes arrangement in microglia was unaltered upon various Tau species exposure.

F. While, the podosomes clusters were accumulated in microglial lamellipodia in the exposure of Tau oligomer for rapid migration as compared with ADP exposure.

G. The podosomes belts rearrangements remain unaltered in Tau-induced migration.

H. Moreover, the TKS5 intensity becomes decreased from the uropod during Tau oligomer-induced migration, which may signify the rapid depolymerization of actin from the rear end in response to Tau oligomers.

I, J. Time-dependent exposure of Tau oligomers has induced podosomes formation in lamellipodia where the TKS5 intensity has increased after 4 hours of oligomers treatment.

K. Further, the percentage of microglia containing podosomes clusters has increased exponentially upon the addition of Tau oligomers, even after the first hour.

Figure 3. P2Y12-associated podosomes modulates microglial migration and chemotaxis.

A. P2Y12 has colocalized with podosomes and membrane-associated actin network in lamellipodia during Tau-induced microglial migration.

B. Fluorescence quantification in podosomes-associated lamellipodia showed that the colocalization of P2Y12 and F-actin was more in Tau oligomers-exposed microglia than monomer.

C. The activation and blockage of the P2Y12 signaling by ADP and Clopidogrel, respectively, induced the microglial migration in combination with extracellular Tau species.

D. ADP did not induce wound closure by microglia in combination with Tau monomer and oligomers. While, Clopidogrel exposure has significantly increased the wound closure in combination with only Tau monomer, but not with Tau oligomers.

E. In trans-well migration assay, microglia become invaded by ADP-induced migration and also in combination with Tau oligomers.

F. While, Clopidogrel exposure has significantly reduced the microglial invasion, which was eventually restored by Tau oligomers exposure.

Figure 4. Extracellular Tau facilitates P2Y12 localization in filopodia and uropod, relating ECM degradation.

A. In similar to P2Y12-associated podosomes accumulation, P2Y12 was colocalized with membrane-associated actin in filopodia and branched uropod as migratory microglial structures.

B. The percentage of microglia containing $P2Y12^{+}$ filopodia has significantly increased upon Tau oligomers exposure,

C. While, the P2Y12 intensity has increased significantly in uropod by Tau oligomers exposure and in ADP-induced microglia.

D. Microglia degrades Tau monomers by 8 hours and Tau oligomers by 24 hours as ECM deposits through the formation of the podosomes.

E. The percentage of microglia, which degraded Tau monomer-ECM, was 38%, while 30% of microglia degraded Tau oligomers deposits.

F. The P2Y12-associated podosomes can degrade ECM more where Tau monomer present as ECM, than Tau oligomers ECM.

G. Microglia was also found to degrade Tau containing ECM through the formation of filopodia, which was orchestrated with P2Y12.

H. Microglia degraded the ECM more in the case of the Tau monomer matrix than the Tau oligomers matrix.

I. Extracellular Tau oligomers have induced the P2Y12-driven chemotaxis, which leads to the substratum adhesion and Tau deposits degradation through the formation of podosomes and filopodia in migratory microglia.

Figure 5. Microglia degrades Tau ECM by actin remodeling, localized with TKS5 and Arp2.

A. Microglia degraded Tau as ECM through the accumulation of podosomes and filopodia at the site of degradation.

B. The actin remodeling is mediated by Arp2, where filopodia contained more Arp2 than podosomes, relating rapid actin polymerization.

C. Similarly, TKS5 became colocalized with podosomes and filopodia at the site of Tau ECM degradation.

D. But, the colocalization of F-actin and TKS5 did not alter between podosomes and filopodia-associated Tau ECM degradation.

Figure 6. P2Y12-activation and blockage influence Tau ECM degradation through the Arp2-mediated podosomes and filopodia formation.

A. P2Y12 activation by ADP-induced the filopodia formation for Tau ECM degradation, while, P2Y12-associated podosomes formation remained unaltered upon P2Y12 activation/blockage.

B. Therefore, the percentage of cells with Tau deposits degradation has been reduced by P2Y12-blockage-Clopidogrel exposure. While, the ADP-mediated P2Y12 activation showed unaltered Tau deposits degradation.

C. P2Y12 activation leads to more Arp2-localized filopodia formation for Tau ECM degradation, while Clopidogrel treatment-induced membrane-associated actin remodeling in microglial for ECM degradation.

D. Similarly, TKS5 localization was reduced in podosomes and filopodia for Tau ECM degradation upon P2Y12 activation.

E. P2Y12 activation leads to the Arp2-induced filopodia formation at the site of Tau ECM degradation, at the same time the blockage of P2Y12 signaling by Clopidogrel resulted in more accumulation of Arp2-orchestrated podosomes and filopodia at the ECM degradation site.

F. ADP-induced P2Y12 activation leads to reduce TKS5 colocalization at podosomes and filopodia while P2Y12 blockage remained unaltered for TKS5 localization in remodeled actin network.

Figure 7. Microglia degrade extracellular Tau deposits by P2Y12-mediated podosomes and filopodia formation.

Extracellular Tau oligomers have -induced Arp2 and TKS5-associated podosomes clusters and filopodia formation for P2Y12-mediated microglial chemotaxis and migration. Moreover, Tau monomer and oligomers can be degraded as ECM by microglial filopodia and podosomes induction. While the blockage of purinergic P2Y12 signaling have reduced the P2Y12-associated filopodia accumulation and reduced P2Y12-mediated Tau-ECM degradation. Hence, P2Y12 signaling plays a dual role in extracellular Tau-induced microglial chemotaxis and the clearance of Tau deposits *via* podosomes-filopodia-associated actin remodeling.

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Figure 1: Tau oligomers induce podosome and filopodia formation, orchestrated with Arp2

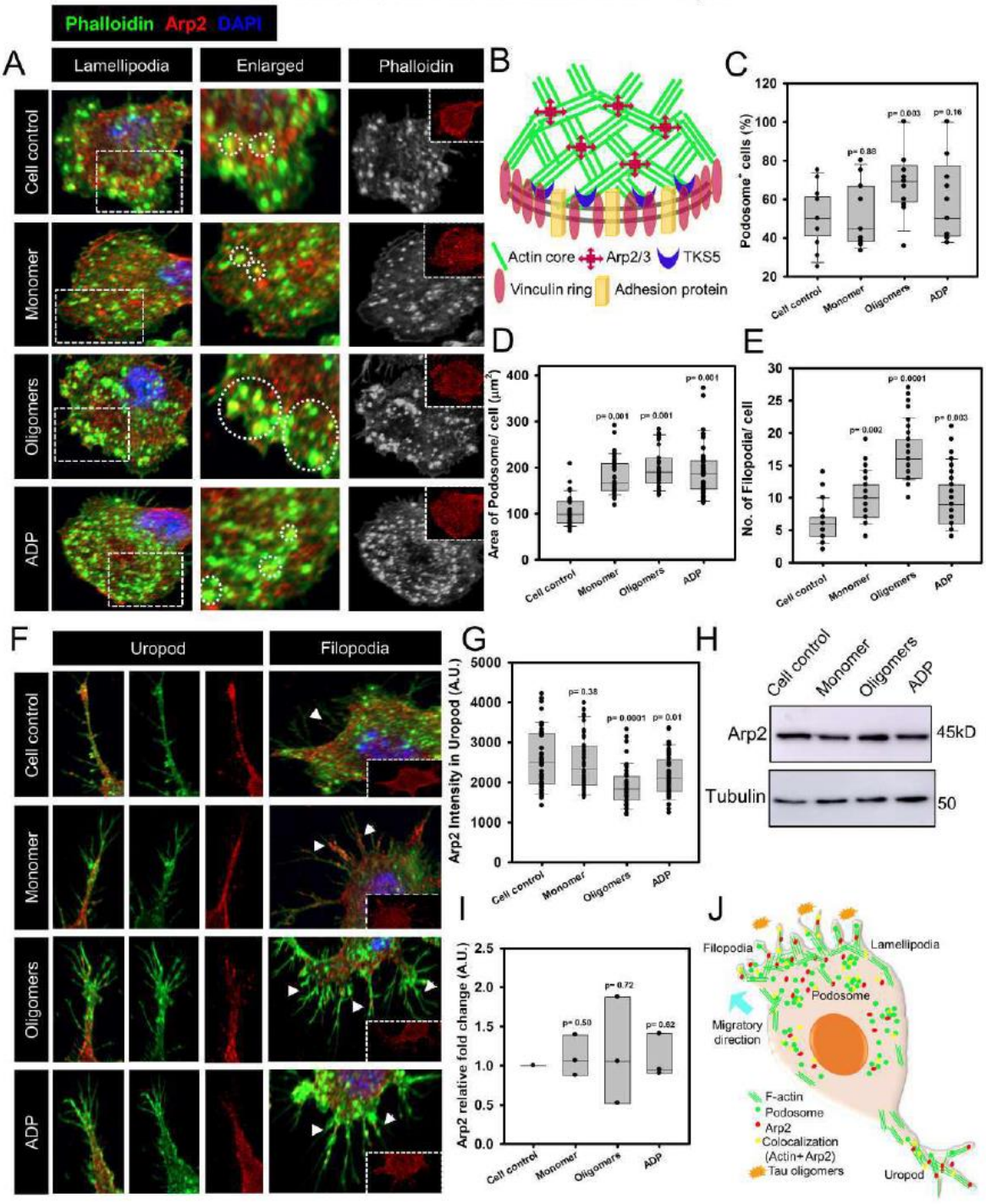

Figure 2: Extracellular Tau modulates TKS5-mediated podosome clusters in migratory microglia

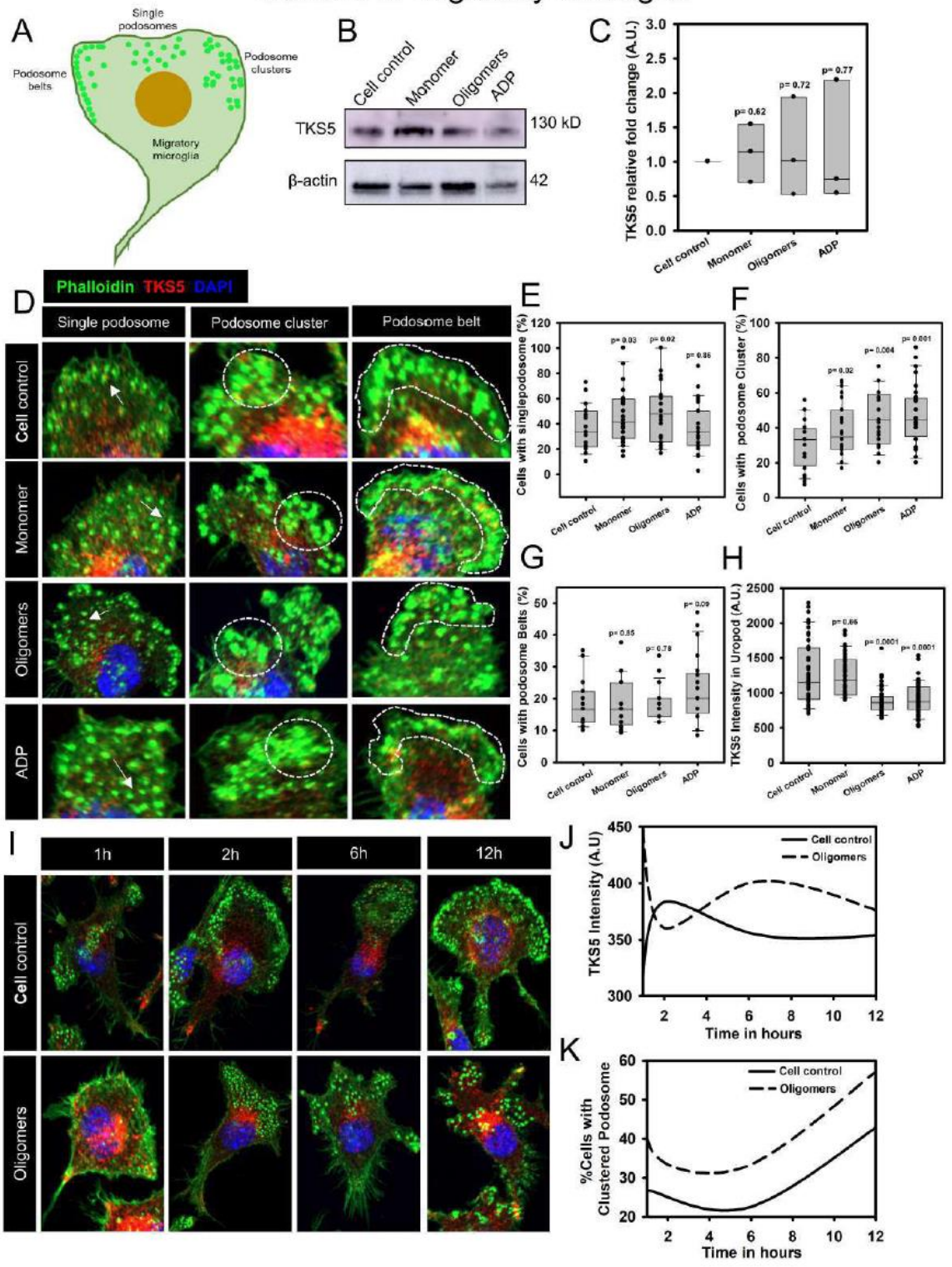

Figure 3: P2Y12-associated podosome modulates microglial migration and chemotaxis.

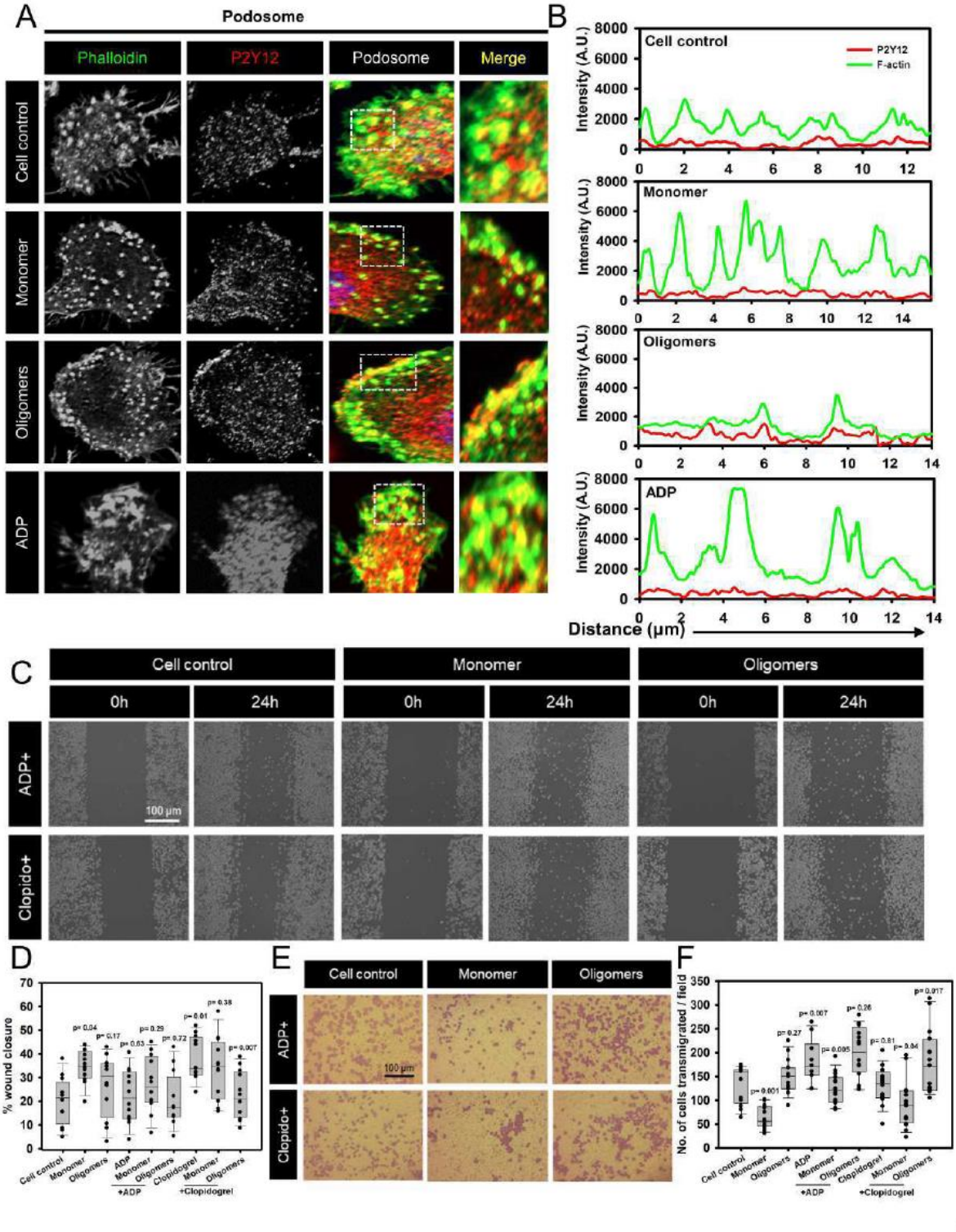

Figure 4: Extracellular Tau facilitates P2Y12 localization in filopodia and uropod, relating ECM degradation

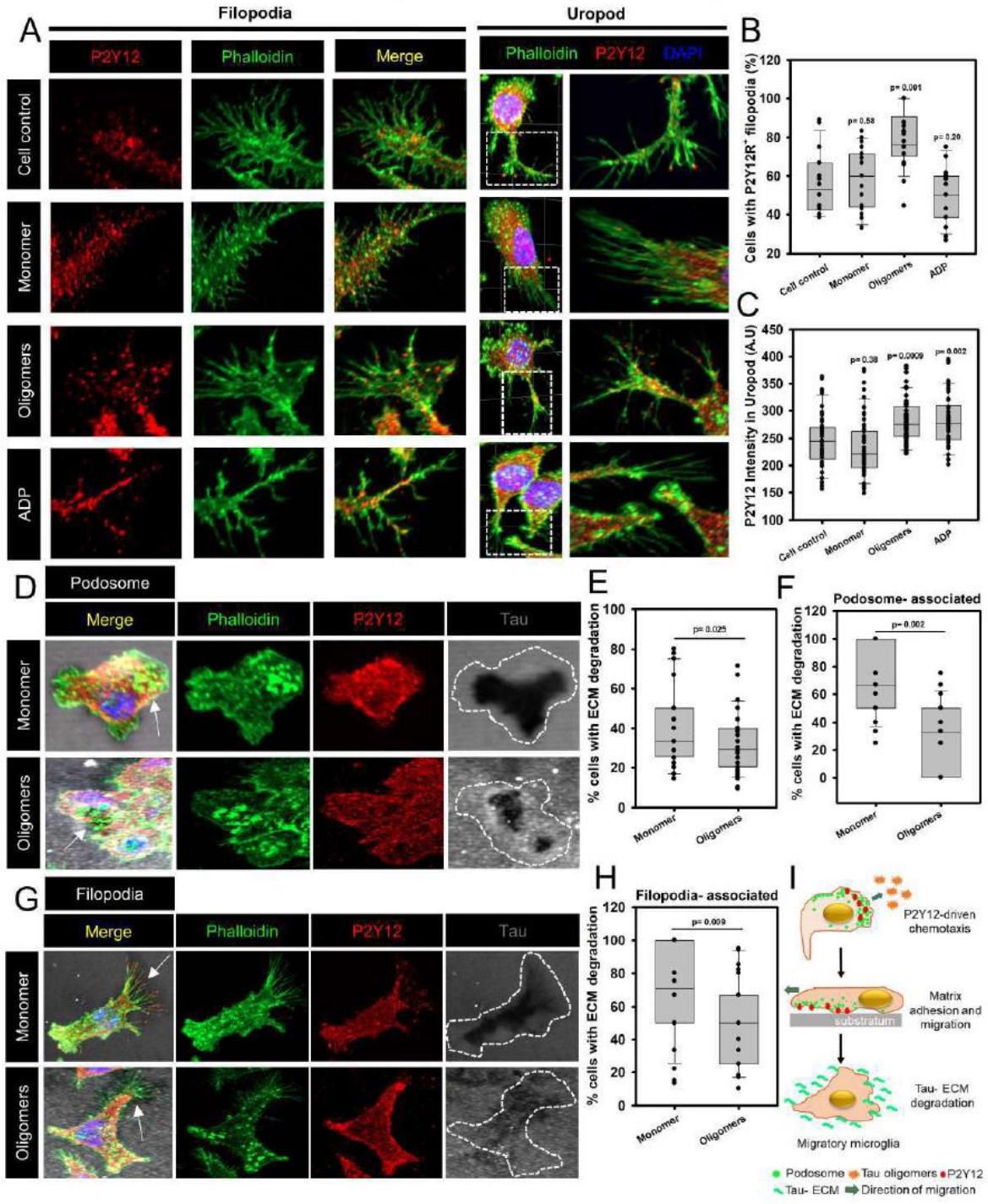

Figure 5: Tau ECM degradation, localized with TKS5/Arp2

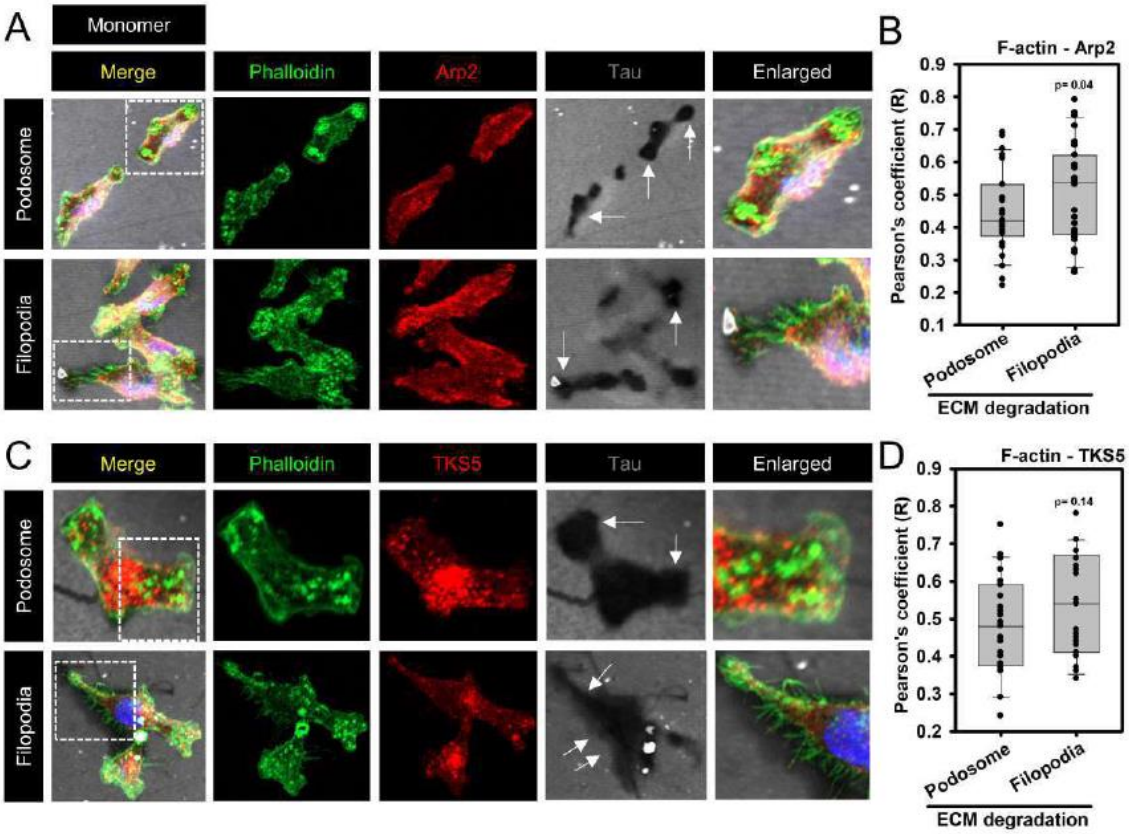

Figure 6: P2Y12-activation/blockage influence Tau ECM degradation, localized with TKS5/Arp2

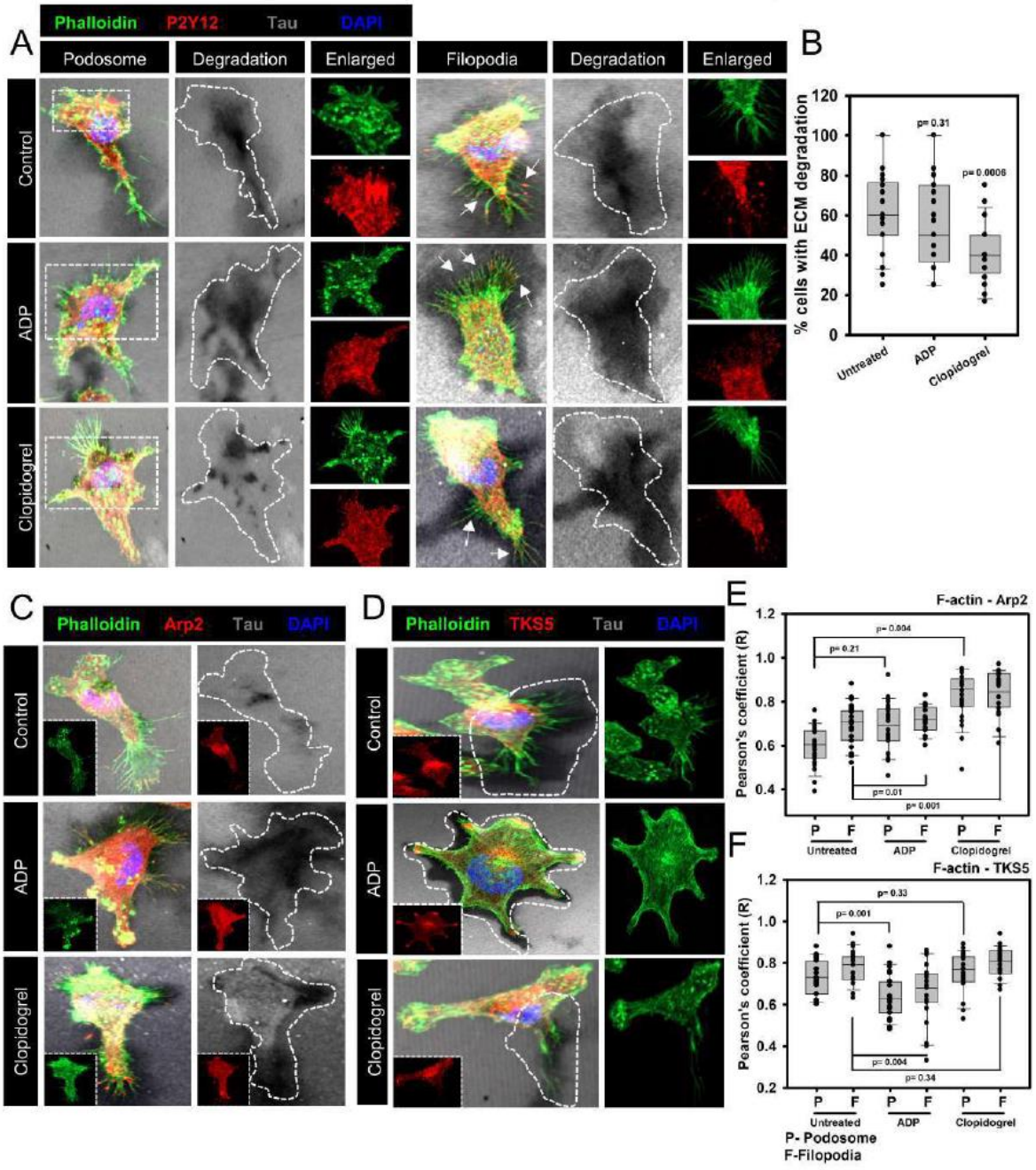

Figure 7: Microglia degrade extracellular Tau deposits by P2Y12-mediated podosome and filopodia formation