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Endocannabinoid Signaling in Alzheimer's Disease: a Novel Target for Mechanistic Understanding and Potential Therapeutics

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1. ABSTRACT

Alzheimer's Disease (AD) is a leading neurodegenerative disorder and the primary cause of dementia among the elderly (Citron, 2010). Chronic neuroinflammation has emerged as a critical factor in neuronal loss in AD, with microglial cells playing a central role. This exacerbated pro-inflammatory environment may be influenced by microglial senescence, characterized by a persistent activation state and the adoption of a senescence-associated secretory phenotype (SASP), which amplifies neuroinflammation and oxidative stress, ultimately contributing to neuronal damage and cognitive decline (Streit et al., 2014; Y. Wang et al., 2022).

In this scenario, the endocannabinoid system (ECS) has gained attention as a critical modulator of immune and inflammatory responses, influencing microglial function and cellular senescence (Muccioli et al., 2007; Stella, 2009). Microglia express receptors and enzymes associated with the ECS, enabling the production of bioactive lipids like N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), which play key roles in promoting anti-inflammatory phenotypes during early neuropathology (Walter et al., 2003).

This study aims to investigate the role of the ECS in the processes of immunosenescence associated with AD. Neonatal microglia were isolated from both wild-type and Tg2576 mice—an AD-like model that overexpresses Aβ peptides—and exposed to chronic lipopolysaccharide (LPS) stimulation to model immunosenescence. Our results revealed that embryonic Aβ exposure in Tg2576 microglia alone was insufficient to induce a senescent phenotype. Instead, the combination of *in vivo* Aβ exposure during embryogenesis and *in vitro* LPS treatment was required to drive senescence, characterized by elevated β-galactosidase activity and increased expression of

senescence markers, including p16, CD11c, TREM2, and lipofuscin (González-Gualda et al., 2021).

Our research highlights the significant potential of fatty acid amide hydrolase (FAAH) inhibition in reversing microglial senescence. Treatment with 3′-carbamoyl[1,1′-biphenyl]-3-yl cyclohexyl-carbamate (URB597), a selective FAAH inhibitor, reduced key senescence markers and modulated NF-κB activity, with enhanced AEA signaling critically suppressing NF-κB activation, a core component of the SASP (Wang et al, 2022).

Furthermore, while elevated 2-AG levels in Tg2576 microglia were identified as a maladaptive response to chronic Aβ exposure, the findings indicate that this dysregulation, rather than 2-AG itself, exacerbates the pro-inflammatory and senescence-like phenotypes. In wild-type microglia, chronic exposure to 2-AG in combination with LPS did not induce a senescent phenotype, highlighting that the pathological effects are contingent upon the endogenous enzymatic imbalance in 2-AG synthesis and degradation. Cyclooxygenase-2 (COX-2) inhibition mitigated LPS-induced senescence markers, further supporting that 2-AG degradation through this pathway may drives microglial dysfunction in the context of AD.

2. INTRODUCTION

AD is the most prevalent form of dementia and a leading cause of cognitive decline in aging populations. Characterized by progressive neurodegeneration, AD is pathologically defined by the accumulation of beta-amyloid (Aβ) plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein, which disrupt neuronal integrity and function (Hardy & Selkoe, 2002). However, the presence of Aβ deposits alone does not fully account for disease onset, as not all individuals with amyloid plaques develop AD symptoms (Citron, 2010). This suggests the involvement of additional pathological mechanisms that contribute to amyloidosis and drive disease progression. Among these mechanisms, chronic neuroinflammation has emerged as a critical contributor to AD pathology. Microglial cells, the brain's resident immune cells, play a central role in this process. With age and chronic stressors, microglia can transition from a homeostatic state to a senescent phenotype. Senescent microglia are characterized by an irreversible loss of proliferative capacity and the adoption of a SASP, marked by the release of pro-inflammatory cytokines, chemokines, and reactive oxygen species (Streit et al., 2014). This chronic inflammatory state not only exacerbates neuronal damage and synaptic dysfunction but also contributes to the progression of amyloid and tau pathologies, reinforcing the neurodegenerative cycle (Y. Wang et al., 2022).

Unlike other microglial phenotypes observed in neurodegenerative diseases—such as disease-associated microglia (DAM) or neurodegenerative microglia (MGnD)—senescent microglia fail to provide neuroprotective benefits, such as efficient clearance of pathological proteins. Instead, their accumulation impairs tissue homeostasis and perpetuates a toxic environment for neurons (Angelova & Brown, 2019). Understanding the molecular pathways underlying microglial senescence, including its triggers and downstream effects, is crucial for identifying therapeutic targets.

In this context, the ECS has garnered attention for its role in modulating immune responses and inflammation. Microglia express receptors and enzymes associated with the ECS, enabling the production of bioactive lipids such as AEA and 2-AG. These molecules have been shown to influence microglial

activation states, promote anti-inflammatory effects, and potentially mitigate senescence-associated dysfunction (Muccioli et al., 2007; Stella, 2009). Dysregulation of the ECS has been reported in both preclinical AD models and human patients, suggesting its involvement in the pathogenesis of neuroinflammation and microglial senescence (C. Li et al., 2019).

2.1 ALZHEIMER'S DISEASE

AD, the most prevalent form of dementia, particularly impacts elderly populations, with a significant burden observed globally. This progressive neurodegenerative disease is estimated to account for almost two-thirds of dementia cases worldwide ((Fan et al., 2020; Karran et al., 2011). Pathologically, AD is marked by the accumulation of senile plaques resulting from the production and aggregation of neurotoxic beta-amyloid (Aβ) peptides, alongside neurofibrillary tangles (NFTs) formed through hyperphosphorylation of the tau protein (Citron, 2010; Hardy & Selkoe, 2002). Aβ oligomers and polymers, resultant of amyloid precursor protein (APP) proteolysis, build up in the brain, forming plaques that are considered a principal cause of neuronal toxicity (Karran et al., 2011). Concurrently, the tau protein, which normally stabilizes microtubules and regulates axonal trafficking, undergoes pathological hyperphosphorylation. This leads to tau aggregation and detachment from microtubules, further exacerbating neuronal dysfunction (Y. Wang & Mandelkow, 2016).

The etiology of AD is complex, involving both genetic and environmental factors. Although the amyloid cascade hypothesis has long dominated AD research, suggesting that Aβ accumulation triggers neurotoxic events leading to dementia, recent therapeutic strategies targeting Aβ have not met the expected outcomes, thereby challenging the completeness of this hypothesis. Additionally, there are elderly patients who exhibit amyloidogenic plaques but do not manifest AD symptoms (Ma et al., 2022). In recent years, neuroinflammation has played a central role in its etiopathogenesis, owing to its capacity to exacerbate Aβ and tau pathologies (Onyango et al., 2021).

2.1.1 Neuroinflammation in Alzheimer's Disease

Inflammation is a response produced towards any injury, trauma, or infection to cells and tissues. There is a recognized biochemical link between the brain and the immune system, and inflammation that occurs within the brain is termed neuroinflammation. Neuroinflammation in the central nervous system (CNS) can be triggered by pathological damage occurring in either the peripheral or central nervous systems, resulting in the release of proinflammatory cytokines like interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor (TNF), chemokines, complement proteins, and other inflammatory messengers, such as prostaglandins, nitric oxide (NO), and reactive oxygen species (ROS) (Leng & Edison, 2021; C. Wang et al., 2023). Acute neuroinflammation generally serves as a protective response against damage to the nervous system, infection, and other harmful stimuli. However, with prolonged inflammation, this acute response becomes chronic, leading to an amplified proinflammatory reaction that outstrips anti-inflammatory mechanisms, eventually causing neuronal damage and extensive pathological changes in the brain (Novoa et al., 2022; Shabab et al., 2017; Thakur et al., 2023).

In the context of AD, this chronic and dysregulated neuroinflammatory response plays a significant role in disease worsening rather than in protection. Persistent low-grade inflammation, triggered by abnormal protein accumulations, such as β-amyloid plaques and hyperphosphorylated tau, induces oxidative stress, disrupts cellular homeostasis, and promotes neuronal dysfunction and death. *In vivo*, positron emission tomography (PET) imaging has shown elevated microglial activation in the brains of AD patients, underscoring microglia's role as a primary source of neuroinflammation due to their higher production of inflammatory cytokines relative to other glial cells (Asby et al., 2021; Shen et al., 2018). Alongside microglia, other glial cells, including astrocytes, oligodendrocytes, and mast cells, contribute to the neuroinflammatory environment by

releasing cytokines, chemokines, and complement factors. These glial cells are increasingly linked to critical processes in AD, including tau phosphorylation, synaptic loss, and cognitive impairment, thus highlighting the integral role of neuroinflammation in the onset and progression of AD (X.-Q. Chen & Mobley, 2019; Heneka et al., 2015; Meraz-Ríos et al., 2013).

2.1.2 Immunosenescence and Alzheimer's Disease

Cellular senescence is a biological state in which cells irreversibly cease to divide but remain metabolically active. Initially characterized by Hayflick and Moorhead in 1961, this process involves cells reaching a limit in their division potential, commonly referred to as the Hayflick limit (Hayflick & Moorhead, 1961). Senescence plays a dual role in organisms; it is crucial for embryonic development, tissue repair, and acting as a tumor suppressant by preventing the proliferation of damaged cells. However, it also contributes to aging and age-related diseases by accumulating senescent cells that lose regenerative capacity and release pro-inflammatory factors and other molecules that disrupt local tissue environments (Storer et al., 2013).

Senescence is induced by a variety of physiological and pathological stimuli, such as DNA damage, oxidative stress, telomere shortening, oncogene activation, and chronic inflammation. The identification of senescent cells relies on several biomarkers, both *in vitro* and potentially *in vivo*, although these markers face challenges related to their sensitivity and specificity (Kudlova et al., 2022).

In addition to cellular senescence, the concept of immunosenescence significantly influences our understanding of aging, particularly in relation to neurodegenerative diseases like

AD. Immunosenescence refers to the age-related deterioration of the immune system, characterized by a decline in the production of new immune cells and a reduced capability to respond to infections and vaccines (Liu, 2022). This decline leads to an increase in memory cells and a decreased response to new antigens, coupled with a shift towards a chronic inflammatory state known as "inflammaging" (Ajoolabady et al., 2024). This state of persistent, low-grade inflammation exacerbates cellular stress and contributes to the progression of AD by promoting the deposition of amyloid-beta plaques and tau tangles, thereby accelerating cognitive decline and neuronal damage (Gaikwad et al., 2023).

Understanding both cellular senescence and immunosenescence is crucial for developing interventions that may delay or modify the progression of age-related diseases, including AD. By targeting the mechanisms of senescence and the aging immune system, researchers hope to devise strategies that can mitigate the effects of these aging processes and improve health outcomes in the elderly population.

In the context of AD, both cellular senescence and immunosenescence gain particular significance. Immunosenescence of microglia plays a crucial role in exacerbating the inflammatory environment associated with AD (Liu, 2022). These cells exacerbate neuroinflammation through the SASP, which includes the release of inflammatory cytokines, chemokines, and other toxic substances (Lee et al., 2022). The added layer of immunosenescence contributes to this inflammatory environment, promoting the spread of amyloid-beta and tau pathologies, leading to neuronal damage and cognitive decline, hallmarks of AD progression (Bowirrat, 2022). Studies suggest that targeting these senescent cells with senolytic drugs, which selectively induce their death, or senomorphic drugs that modify the SASP, could mitigate these effects and potentially reverse disease progression (Zhang et al., 2023). Further insights into senescence's role in AD

highlight that senescent cells can disrupt neuronal communication and metabolic processes, contributing to the synaptic dysfunction observed in AD. Moreover, the accumulation of senescent cells in the brain is thought to impair clearance mechanisms, leading to increased Aβ deposition and tau aggregation (Zhu et al., 2024).

Understanding both cellular senescence and immunosenescence is crucial for developing interventions that may delay or modify the progression of age-related diseases, including AD. By targeting the mechanisms of senescence and the aging immune system, researchers hope to devise strategies that can mitigate the effects of these aging processes and improve health outcomes in the elderly population.

2.2 MICROGLIA

Microglia, the resident macrophages of CNS, are derived from mesodermal marrow precursor cells and migrate to the CNS during development. These cells serve as the major immunological effectors in the CNS, playing a pivotal role in both physiological processes and immune responses. Under healthy conditions, microglia maintain a small body with slender branches and typically do not engage in phagocytosis (Nimmerjahn et al., 2005). However, in response to various pathological conditions, they rapidly transform into an amoeba-like morphology, capable of strong phagocytic activities, synaptic pruning, and migrating to lesion regions (Nakamura et al., 1999). Microglia are essential for regulating neuronal numbers and promoting neuronal survival, differentiation, and synaptic formation through mechanisms such as brain-derived neurotrophic factor signaling (Q. Li & Barres, 2018; Scipioni et al., 2022). Depending on the microenvironment, they can adopt different activation states or phenotypes, engaging in functions ranging from neuroinflammation and tissue repair to pro-resolutive inflammatory processes (Leng & Edison, 2021).

2.2.1 The dynamic role of microglia in CNS

Microglia, the most abundant innate immune sentinels in the CNS, reside within the parenchyma, exhibiting remarkable plasticity that allows them to adopt various phenotypes for diverse functions in both health and disease (Heneka et al., 2015). In a healthy, mature CNS, microglia maintain a highly ramified morphology termed the 'resting phenotype,' with a small soma and fine processes, actively monitoring the surrounding microenvironment without disrupting neurovascular coupling (Kettenmann et al., 2011). This resting state enables microglia to constantly survey their

environment, looking for the presence of danger-, pathogen-, or resolution-associated molecular patterns (DAMP, PAMP, and RAMP, respectively) through an array of recognition systems including Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-like receptors (RLRs), collectively known as pattern recognition receptors (PRRs) (Kigerl et al., 2014; Solé-Domènech et al., 2016). Specifically, PRRs on microglia surfaces detect molecular signals indicating damage or cell stress such as ATP, nucleic acids, necrotic cells, and debris, including misfolded proteins like Aβ species (Kigerl et al., 2014; Leng & Edison, 2021). The recognition of these ligands triggers their internalization and initiates a phenomenon known as 'microglial activation'. Additionally, when brain homeostasis is disrupted by injury or disease, microglia undergo rapid and substantial alterations in shape, gene expression, and functionality (Kettenmann et al., 2011). This activation includes migration toward lesions, phagocytosis of debris, and orchestration of neuroinflammation through the release of inflammatory mediators, neurotrophic factors, and reactive oxygen species, which can have beneficial and detrimental effects on surrounding cells (Wolf et al., 2017). During different neuroinflammatory and neurodegenerative paradigms, although microglia can exhibit a variety of morphologies, their pathological activation generally results in a transition toward an amoeboid phenotype. This transformation triggers potent neuroinflammatory reactions characterized by the secretion of cytokines and chemokines (Guedes et al., 2018; Ji et al., 2019).

2.2.2 Microglial phenotypical heterogeneity: M1-like and M2-like

Microglial activation encompasses a spectrum of states and has traditionally been classified into M1 and M2 phenotypes, representing pro-inflammatory and anti-inflammatory roles, respectively. However, this binary classification oversimplifies the diversity observed in vivo, where microglia

demonstrate a range of dynamic states better described as M1-like and M2-like. These designations reflect the fluid nature of their functional roles, unlike the distinct states isolated in in vitro studies (Heneka et al., 2015; Ransohoff, 2016).

The pro-inflammatory, or M1-like microglia, are triggered by tissue injury and phlogistic factors, including bacterial components like LPS and cellular debris, along with cytokines such as IL-1β, IL-8, IFNγ, and TNFα. This environment stimulates inflammasome complexes like NOD-like receptor family pyrin 1 and 3 (NLRP1 and 3), leading to the release of factors such as pro-inflammatory cytokines (IL-1α/β, IL-6, IL-23, TNF), chemokines (CCL2, CXCL9, CXCL10, CCL20), co-stimulatory proteins (CD40, MHC-II), and enzymes inducing oxidative stress (NADPH oxidase, iNOS) (Jiang et al., 2020; Leng & Edison, 2021; Scipioni et al., 2022). These responses result in excitotoxicity, neurotoxicity, demyelination, and ultimately neuronal dysfunction and death. M1-like microglia exhibit upregulated markers such as CD11b, CD16/32, CD68, and CD86, and show a metabolic shift from oxidative phosphorylation to glycolytic/anaerobic metabolism during activation, increasing the production of lactate and reactive species (Jiang et al., 2020).

Conversely, M2-like microglia are influenced by anti-inflammatory or TH2-derived cytokines such as IL-4 and IL-13, and specialized pro-resolving lipids like resolvins and protectins, which bolster their homeostatic functions and suppress pro-inflammatory mediators (Cherry et al., 2014). These cells express phenotypic markers including CD163, CD206, Arg-1, Ym-1, and FIZZ-1, and play key roles in inflammatory dampening, debris scavenging, and tissue remodeling (Dobri et al., 2021; Leng & Edison, 2021; Xu et al., 2018). Their activity is essential for resolving inflammation and clearing debris like dead cells and misfolded proteins, preventing neuropathology. Dysfunctional phagocytosis in these cells is linked to conditions such as ischemic

stroke, multiple sclerosis, and AD. Metabolically, M2 microglia rely primarily on oxidative phosphorylation, supporting their roles in neuroinflammation resolution and CNS homeostasis (Scipioni et al., 2022).

This nuanced understanding of microglial activation, distinguishing *in vivo* complexity from *in vitro* simplicity, underscores the importance of context in studying neuroinflammatory responses.

2.2.3 Microglia in Alzheimer's Disease

Microglial cells undergo morphological and functional changes in AD brains, which could extend beyond the M1/M2 dichotomy. Despite their beneficial roles, persistent activation of microglia can contribute to the pathogenesis of neurodegenerative diseases by producing high levels of pro-inflammatory cytokines and chemokines, leading to neuronal dysfunction and cognitive decline (Mandrekar & Landreth, 2010).

Recent reports have indicated that during aging and age-related neurodegenerative diseases, microglia can change from homeostatic to other reactive states, including activated response microglia disease associated microglia, lipid droplet-accumulating microglia (LDAM), Neurodegenerative microglia and senescent microglia (Wei & Li, 2022).

2.2.3.1 Disease-associated microglia

Disease-associated microglia play a critical role in neuroinflammatory processes, particularly in neurodegenerative diseases like AD, frontotemporal dementia, and amyotrophic lateral sclerosis (ALS) (Deczkowska et al., 2018; Gao et al., 2023; Takahashi, 2023). Their involvement in clearing

amyloid-beta plaques is a key aspect of Alzheimer's pathology, yet this role is dual-faceted, impacting both protective and potentially harmful outcomes (Sobue et al., 2021).

In terms of inflammation, DAM is actively involved in both the promotion and regulation of the inflammatory response. They are known to upregulate specific genes such as Itgax, Apoe, and Clec7a, which are crucial for their phagocytic capabilities and inflammatory responses. These genes facilitate the recruitment and activation of other immune cells, promoting inflammation which is necessary for clearing pathogens and debris but can also lead to tissue damage if unchecked (Sobue et al., 2021). DAM enhance inflammation by releasing pro-inflammatory cytokines that attract other immune cells to the site of injury or disease, amplifying the inflammatory response. However, they also have the capacity to modulate and potentially resolve inflammation through the secretion of anti-inflammatory factors and the clearance of debris and dead cells, which helps in restoring tissue homeostasis (Gao et al., 2023).

The complexity of DAM's role in inflammation is underscored by their involvement in chronic inflammation, a common feature in neurodegenerative diseases. By modulating DAM activity, it may be possible to mitigate their harmful effects while enhancing their protective functions, providing a balanced approach to managing neurodegeneration (Wei & Li, 2022).

2.2.3.2 Lipid droplet-accumulating microglia

Lipid droplet-accumulating microglia play a noteworthy role in the progression of AD due to their unique characteristics and the environment they create within the aging brain. The accumulation of lipid droplets in LDAMs, primarily composed of glycerolipids with low levels of cholesteryl esters, signifies not only a cellular adaptation to metabolic and oxidative stresses but also highlights

a shift in microglial function from protective to potentially harmful. In AD, the presence of LDAMs correlates with an increase in pro-inflammatory cytokine production, such as IL-6, CCL3, and CXCL10, which are known to contribute to the inflammatory milieu associated with Alzheimer's pathology (Marschallinger et al., 2020; Praticò et al., 2001).

Furthermore, LDAMs exhibit impaired phagocytic capabilities, which is critical because effective phagocytosis by microglia is essential for clearing amyloid-beta plaques—a hallmark of AD. The reduced phagocytic activity in LDAMs leads to an accumulation of amyloid-beta, exacerbating neuronal damage and contributing to the cognitive decline observed in patients (Wei & Li, 2022). The link between LDAMs and enhanced oxidative stress also suggests that these microglia could further drive Alzheimer's pathology through mechanisms associated with oxidative damage to neurons and other cell types within the central nervous system.

Addressing the role of LDAMs in AD provides a clearer understanding of how aging-associated changes in microglial function can influence the progression of neurodegenerative diseases, potentially opening new avenues for therapeutic strategies aimed at modulating microglial activity to mitigate their detrimental effects in AD (Marschallinger et al., 2020).

2.2.3.3 Neurodegenerative microglia

The Neurodegenerative microglia phenotype, similar to the DAM phenotype, has been identified in various neurodegenerative models. It is triggered by the phagocytosis of apoptotic neurons, stimulated by amyloid-beta (Aβ) plaques and AAV-P301L-tau injections (Clayton et al., 2017). This transition from a homeostatic state to MGnD is marked by increased phagocytic activity

towards plaques, apoptotic neurons, and neuritic dystrophy, observed in both APP-PS1 mice and human AD(Clayton et al., 2017).

The MGnD phenotype shift is primarily driven by Triggering Receptor Expressed on Myeloid cells 2 (TREM2) his activation is primarily driven by its interaction with various ligands, including lipids, apoptotic cells, and other molecules associated with cellular damage and inflammation. When TREM2 is activated, leads to the upregulation of inflammatory genes (such as Axl, Itgax, Clec7a, and Apoe) and the suppression of genes associated with the homeostatic microglial phenotype (including Tgfb P2ry12, and Cx3cr1). This results in a partial overlap between the M1 and MGnD microglial phenotypes. However, it remains unclear whether MGnD microglia are ultimately beneficial or detrimental in the context of AD (Krasemann et al., 2017).

2.2.3.4 The senescent microglia

In the brain aging-related neurodegenerative diseases, the role of senescent microglia has come to the forefront as a pivotal player in disease progression. Similar to other specialized glial phenotypes observed in neurodegenerative conditions, senescent microglia emerge primarily due to the cellular aging process and the stressors associated with it, such as DNA damage and oxidative stress. These microglia are marked by several distinct characteristics including the activation of the senescence-associated secretory phenotype, which encompasses the release of pro-inflammatory cytokines and chemokines that exacerbate neuronal dysfunction and contribute to the synaptic abnormalities observed in diseases like AD (Rim et al., 2024).

Transitioning from a homeostatic state, senescent microglia show a heightened phagocytic activity but, unlike their neurodegenerative counterparts that might respond to plaque formation or

neuronal apoptosis, these cells contribute to chronic inflammation without clear benefits to tissue integrity or function. Their accumulation within the brain is thought to not only impede the clearance of pathological proteins such as amyloid-beta and tau but also to hinder the regenerative processes crucial for maintaining neuronal health (Angelova & Brown, 2019).

Critical to the understanding of these cells' role in neurodegenerative diseases is their unique origin and limited capacity for renewal, which underscores their vulnerability to aging and stress-related senescence triggers. This inherent characteristic makes them a promising target for therapeutic interventions aimed at either rejuvenating these cells or modulating their inflammatory outputs to mitigate their impact on disease progression (Chinta et al., 2015).

The complexity of senescent microglia, coupled with their significant contributions to the inflammatory milieu typical of aging brains, positions them as crucial targets for understanding and potentially intervening in the pathogenesis of age-related neurodegenerative diseases.

Morphologically, senescent cells, including microglia, typically appear enlarged and flattened, exhibiting enhanced lysosomal activity. Among the key markers is the increased activity of senescence-associated β-galactosidase (SA-β-gal), detectable through x-gal staining at an optimal pH of 6.0 (Noren Hooten & Evans, 2017; Piechota et al., 2016). Historically, SA-β-gal has been a principal marker for identifying senescent cells; however, its specificity has been questioned in recent research (Piechota 2016). As such, while SA-β-gal activity continues to be extensively used due to its correlation with aging and cellular proliferation, it is no longer considered sufficiently specific to identify senescent cells on its own (Kudlova et al., 2022). Another marker, SA-α-fucosidase, shows increased activity correlating with lysosomal activation during aging and may provide greater specificity than SA-β-gal (Hildebrand et al., 2013; Kudlova et al., 2022).

Lipofuscin, a yellowish-brown pigment composed of partially degraded lipids within lysosomes, accumulates in aging tissues and serves as another useful senescence marker. Its presence can be detected by light microscopy, exploiting its autofluorescence, or by Sudan Black B (SBB) staining (Terman & Brunk, 1998). Additional crucial markers for senescence include cell cycle regulators such as p53, p16, and p21, which are activated by DNA damage and stress responses. In the initial phase of senescence, the p53 protein and its primary effector p21 are crucial. Here, cells cease division but stay metabolically active (Rufini et al., 2013). P21 expression spikes early on, aiding in the G1/S and G2/M cell cycle arrest by modulating the activity of specific p53 targets (i.e. CDC25B, CDC25C, and survivin) (Al Bitar & Gali-Muhtasib, 2019). At this stage, senescence can still be reversed. As the process advances and passes a critical point where it becomes irreversible, cells transition into the senescence maintenance phase, governed by p16INK4a/pRb (Sharpless & Sherr, 2015). In this latter phase, p16 expression markedly rises, playing a pivotal role in maintaining senescence. Here, p16 alongside p21, helps inhibit the activity of cyclin-CDK complexes, thus influencing the phosphorylation of Rb family proteins and the regulation of E2F target gene expression (Fiorentino et al., 2009).

In addition, cytokine secretion associated with the SASP, characterized by the extensive secretion of pro-inflammatory compounds, is another marker of senescence (Cuollo et al., 2020). However, the reliance on these markers alone for confirming senescence is complex due to the potential for similar secretions from non-senescent cells under certain conditions (Basisty et al., 2020).

Premature senescence in human and murine cells is often triggered by oxidative stress from exposure to oxygen and hydroperoxides, leading to ROS generation and DNA damage response (Parrinello et al., 2003). Mitochondrial dysfunction significantly contributes to ROS production,

linking it to senescence induction. Notably, mitochondrial ROS are key senescence inducers, demonstrated by experiments where mitochondrial depletion in senescence models caused cell cycle arrest but did not increase SA-β-galactosidase activity, or upregulate p16 and p21, nor exhibit many SASP factors, highlighting mitochondrial ROS as potential markers for oxidative stress-induced senescence, identifiable through fluorimetry or flow cytometry (Pole et al., 2016). Additionally, ferritin, an iron storage protein, is recognized as a hallmark of aging and cellular stress. Its accumulation in microglia suggests a dysregulation of iron metabolism and further implicates oxidative stress in the progression of senescence (Rim et al., 2024) **(Figure 1).**

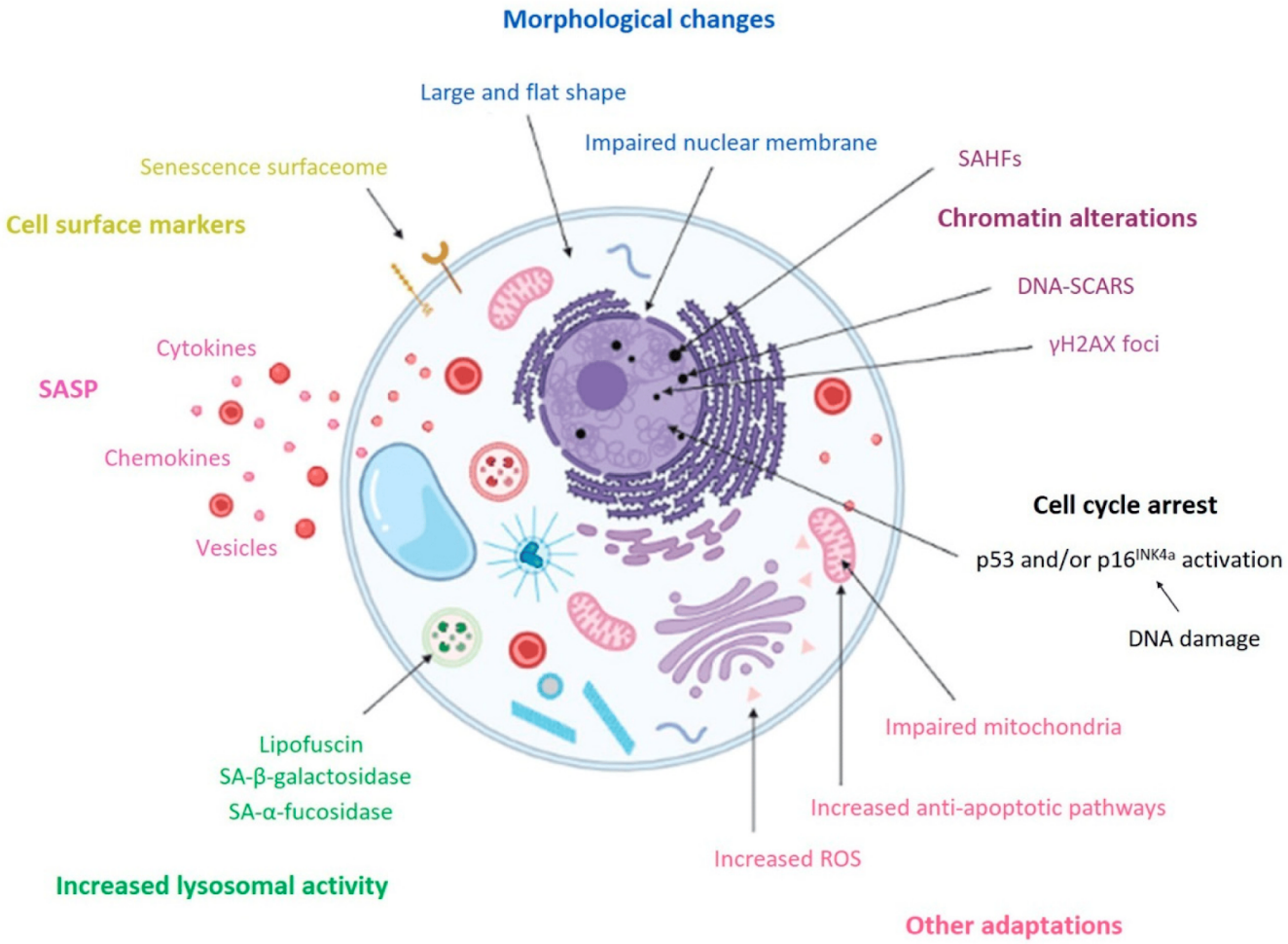

Figure 1: General hallmarks of cellular senescence. Taken from [Kudlova et al, 2022]: Markers that are strongly linked to senescence, commonly observed in most senescent cells, include an enlarged and flattened morphology. The additional features depicted in this figure, while not exclusive to senescent cells, are generally recognized as key indicators of senescence. These cells typically show activation of the p53 and/or p16 pathways, which leads to irreversible cessation of the cell cycle, alterations in chromatin, and disruptions in the nuclear envelope. There is often a noticeable increase in lysosomal mass that enhances SA-β-galactosidase activity, making this enzyme one of the most used markers for identifying senescence. Additionally, senescent cells may display significant paracrine secretion (SASP), substantial mitochondrial dysfunction coupled with the activation of anti-apoptotic pathways, and a variety of other distinct characteristics (Kudlova et al., 2022).

2.3 THE ENDOCANNABINOID SYSTEM

The ECS is primarily composed of N-arachidonoylethanolamine and 2-arachidonoylglycerol, the key endocannabinoids (eCBs) that are synthesized 'on-demand' (i.e., when and where they are needed) throughout the body. AEA, a member of the fatty acid amides, and 2-AG, a member of the monoacylglycerols, have emerged as critical mediators of many aspects of human health and disease (Cristino et al., 2020; Leuti et al., 2020a). These signaling lipids interact with at least two G protein-coupled cannabinoid receptors, cannabinoid receptor type 1 (CB_1) and cannabinoid receptor type 2 (CB2), and their production and degradation involve specific enzymes: N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL) for AEA and 2-AG synthesis respectively, along with FAAH and monoacylglycerol lipase (MAGL) for their degradation. The complexity of their synthesis, transport, and degradation underscores the significant role these eCBs play in physiological processes (Oddi et al., 2023; Scipioni et al., 2022).

2.3.1 Endocannabinoid synthesis and degradation

The ECS relies on AEA and 2-AG synthesized on-demand across various cells including those in the immune system. These eCBs are not stored but produced as needed through receptor-stimulated cleavage of precursor membrane phosphoglycerides by several hydrolases. The synthesis pathways for AEA and 2-AG are diverse and can coexist within the same cell, responding dynamically to temporal and spatial demands (Leuti et al., 2020a; Lutz et al., 2015). AEA is derived from N-arachidonoylphosphatidyl ethanolamine (*N*ArPE), formed from the N-arachidonoylation of phosphatidylethanolamine via both Ca2+-sensitive and Ca2+-insensitive N-acyltransferases

(NATs and iNATs) (Maccarrone & Finazzi-Agró, 2003) (**Figure 2**). This precursor can be converted into AEA through multiple pathways, including a direct conversion by N-Acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) or an alternative route involving phospholipase C (PLC) and subsequent dephosphorylation by protein tyrosine phosphate non-receptor type 22 (PTPN22) (Binte Mustafiz et al., 2019; Maccarrone & Finazzi-Agró, 2003). Similarly, 2-AG synthesis occurs through the hydrolysis of diacylglycerols (DAGs) by DAG lipases, DAGLα and DAGLβ, the last one is more expressed on macrophages, and, although its relative brain expression is sparse, it is highly expressed on microglia (Scipioni et al., 2022). Alternatively, 2-AG can also be synthesized by dephosphorylation of sn-2 arachidonoyl-lysophosphatidic acid (LPA) via 2-LPA phosphatase; or by the sequential action of PLA1 and a lysophospholipase C (lyso-PLC) on sn-2 arachidonate-containing phosphatidylinositol (PI) and its derivative 2-arachidonoyl-lysoPI, respectively (Bisogno et al., 2003; Fezza et al., 2014).

The biological effects of these eCBs terminate upon cellular uptake and degradation. AEA and 2-AG, being highly lipophilic, can diffuse across cell membranes, facilitated by a proposed but not yet identified membrane transporter (i.e., eCB membrane transporter, EMT) that is postulated to be expressed in both neurons and glial cells (Araujo et al., 2019; Ghosh et al., 2021). Once internalized, these lipids are hydrolyzed by specific enzymes. AEA is mainly degradated by FAAH which releases arachidonic acid and ethanolamine (Cravatt et al., 1996). While 2-AG is primarily hydrolyzed by MAGL and the αβ-hydrolases (ABH6) and 12 (ABH12) (Blankman et al., 2007). Ultimately, AEA and 2-AG can also be metabolized through oxidation by lipoxygenases and COX enzymes (Valdeolivas et al., 2013a).

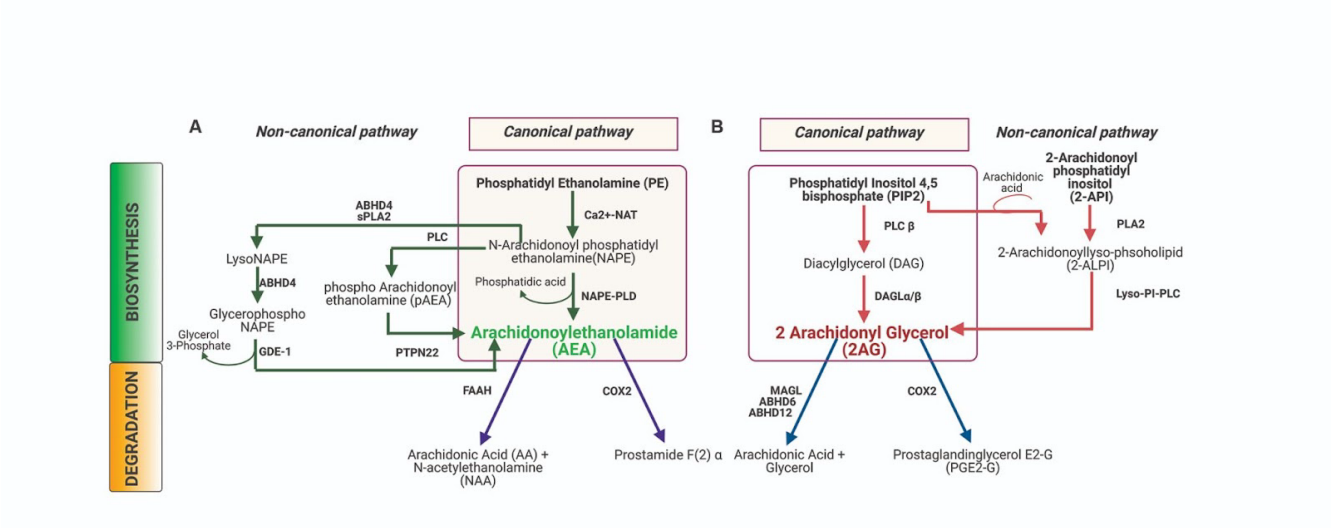

Figure 2: Biosynthesis and degradation of N-arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG). Taken from [Gosh et al, 2021]: (A) Synthesis and catabolism of AEA: In the canonical pathway, N-acetyltransferase (NAT) adds an N-acetyl group to phosphatidylethanolamine (PE), producing N-arachidonoyl PE (NAPE). NAPE is then hydrolyzed by N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) to generate AEA. In a non-canonical pathway, NAPE is hydrolyzed by phospholipase C (PLC) to form phosphoanandamide (pAEA), which is subsequently dephosphorylated by protein tyrosine phosphatase non-receptor type 22 (PTPN22) to yield AEA. Alternatively, NAPE can be converted to lysophosphatidylethanolamine (lyso-NAPE) through the catalytic activity of α/β-hydrolase domain-containing 4 (ABHD4) or secretory phospholipase A2 (sPLA2). Lyso-NAPE is then hydrolyzed by ABHD4 and glycerophosphodiester phosphodiesterase-1 (GDE-1) to produce AEA. AEA is primarily degraded by fatty acid amide hydrolase (FAAH) into arachidonic acid (AA) and N-acylethanolamine (NAA). Alternatively, AEA can be oxidized by cyclooxygenase-2 (COX2) to form prostamide F(2)a. (B) Synthesis and degradation of 2-AG: In the canonical pathway, phosphatidylinositol-4,5-bisphosphate (PIP2) is hydrolyzed by phospholipase C beta (PLCβ) to form diacylglycerol (DAG), which is subsequently converted to 2-AG by diacylglycerol lipase (DAGL). In the non-canonical pathway, 2-acyl-lysophosphatidylinositol (2-ALPI) is synthesized from either PIP2 or 2-acyl-phosphatidylinositol (2-API) and hydrolyzed by lyso phosphatidylinositol phospholipase C (lyso-PI-PLC) to generate 2-AG. The degradation of 2-AG involves hydrolysis by monoacylglycerol lipase (MAGL), ABHD6, or ABHD12, resulting in the production of AA and glycerol. Additionally, 2-AG can be oxidized by COX-2 to generate prostaglandin E2-glycerol ester (PGE2-G) (Ghosh et al., 2021).

2.3.2 Endocannabinoid receptors and molecular pathways

CB_1 and CB_2 receptors are part of the Class A family of seven-transmembrane G protein-coupled receptors (GPCRs) and are primarily associated with Gi/o proteins (Duffy et al., 2021; Oddi et al., 2023). These receptors are expressed in various immune cells, with CB_2 being predominant under both physiological conditions and during acute and chronic inflammation (Klein, 2005; Komorowska-Müller & Schmöle, 2020a). While CB_1 is mainly found in specific neuronal populations, where it exerts neuromodulatory effects, it also plays a role in modulating immune responses and inflammatory pathways (Ibsen et al., 2017).

Activation of CB receptors by eCBs influences several cellular pathways, including the inhibition of adenylate cyclase leading to reduced protein kinase A (PKA) activity, modulation of ionic currents (such as inhibition of voltage-gated L, N, and P/Q-type Ca^{2+} channels, and activation of K^+ channels), activation of focal adhesion kinases, mitogen-activated protein kinases (MAPKs) like p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K)/Akt pathway, cytosolic phospholipase A2, and differential regulation of inducible nitric oxide synthase (iNOS)—activation via CB_1 and inhibition via CB_2 (Scotter et al., 2010; Tanaka et al., 2020). Additionally, CB receptor activation can influence the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, a key regulator of immune and inflammatory responses. CB_1 activation has been associated with NF-κB activation, promoting pro-inflammatory gene expression, whereas CB_2 activation can inhibit NF-κB, leading to anti-inflammatory effects (Fezza et al., 2014; Tian et al., 2017).

Emerging evidence indicates that eCBs also exert their effects through non-cannabinoid receptors. For instance, the transient receptor potential vanilloid type-1 (TRPV1) ion channel, which is functionally expressed in microglia, can be activated by eCBs, influencing microglial activity (Marrone et al., 2017). Additionally, microglia express other non-canonical eCB receptors, including nuclear peroxisome proliferator-activated receptors (PPAR) α and γ, and orphan G protein-coupled receptors such as GPR55 and GPR119 (Friedman et al., 2019; Leuti et al., 2020a; Scipioni et al., 2022).These receptors contribute to the diverse roles of eCBs in modulating microglial behavior and the broader immune response

2.3.3 The Role of the Microglial Endocannabinoid System in Alzheimer's Disease

The ECS plays a key role in brain inflammation by regulating microglial activities such as proliferation, migration, phagocytosis, and the balance between pro- and anti-inflammatory mediator production (Stella et al., 2009). Dysregulation of the ECS in AD exacerbates inflammation, making it a promising therapeutic target for modulating microglial activity and slowing disease progression (Scipioni et al., 2022). (**Figure 3**)

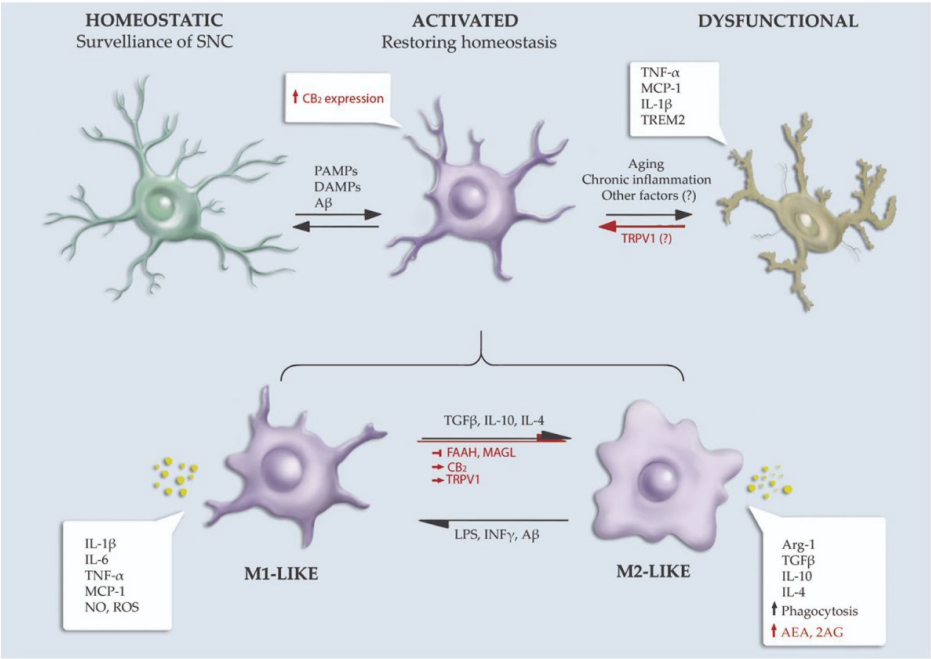

Figure 3: Schematic overview of endocannabinoid system (ECS) role in microglial phenotype. Taken from [Scipioni et al, 2022]: Abbreviation: CB2, Cannabinoid receptor 2; PAMPs, Pathogen-Associated Molecular Patterns, DAMPs, Damage-Associated Molecular Patterns; Aβ, Amyloid Beta peptide; TNF-α, Tumor Necrosis Factor-alpha; MCP-1, Monocyte Chemoattractant Protein-1; il1-β, interleukin 1-beta; TREM2, Triggering receptor expressed on myeloid cells 2; TRPV-1, Transient Receptor Potential Vanilloid 1; IL-6, interleukin 6; NO, Nitric Oxide; ROS, reactive oxygen species; FAAH, Fatty Acid Amide Hydrolase; LPS, lipopolysaccharide; INF-γ, Interferon gamma receptor; Arg1, Arginase 1; Il10, Interleukin 10; AEA, Anandamide; 2-AG, 2-Arachidonoylglycerol (Scipioni et al., 2022).

2.3.3.1 eCBs-binding receptors

CB_1 and CB_2 receptors play critical but distinct roles in microglial function and neuroinflammation, particularly in AD (Mecha et al., 2016). While CB_1 was initially considered neuron-specific, evidence shows it is expressed at low levels in microglia, primarily in intracellular compartments (Walter et al., 2003). Conditional knockout studies have linked CB_1 to the modulation of

inflammatory responses, such as reduced TNF-α expression after LPS stimulation (Hu et al., 2012). However, its role in microglial physiology remains unclear, with inconsistent findings across models regarding its expression and function (Sj et al., 2002). *In vitro* studies indicate that CB_1 activation produces context-dependent effects, such as modulating nitric oxide (NO) release and cytokine production, but these effects often require high agonist concentrations, raising questions about receptor specificity and culture-induced variability (Walter et al., 2003).

CB_2 receptors are widely associated with microglial function, particularly in the context of inflammation and neurodegeneration (Komorowska-Müller & Schmöle, 2020b). In healthy brain tissue, CB_2 immunoreactivity is primarily observed in astrocytes and microglia, with expression significantly upregulated following brain trauma or under pathological conditions such as AD (Benito et al., 2003), Parkinson's disease (Galve-Roperh et al., 2007), and multiple sclerosis (Y et al., 2006). Similarly, increased CB_2 expression has been reported in various mouse models of neurodegenerative conditions (Bisogno et al., 2016). In primary microglial cultures, CB_2 receptor expression is a well-documented phenomenon across human, rat, and mouse tissues, although these cells are considered "primed" due to the activation state induced by isolation procedures (Sj et al., 2002; Walter et al., 2003). CB_2 receptor levels in these cultures can be modulated by cytokines and pathogens, with agents such as GM-CSF, IFN-γ and LPS enhancing expression (Scipioni et al., 2022; Sj et al., 2002). CB_2 plays a crucial role in microglial polarization, particularly in transitioning from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. Genetic ablation of CB_2 results in reduced phagocytic capacity and morphological alterations in M2a microglia, including a loss of lamellipodia and diminished Arg1 expression both under basal conditions and upon M2a stimulation (Mecha et al., 2016). These findings suggest a pivotal role for CB_2 in promoting anti-inflammatory functions. Experimental data further support CB_2's involvement in modulating microglial activity. Activation of CB_2 by 2-AG has been shown to

enhance microglial proliferation and endocytosis, effects reversed by the CB_2 antagonist SR144528 and mimicked by the CB_2-specific agonist JWH133 (Wu et al., 2013). CB_2 also regulates the production of inflammatory mediators. For example, the CB_2 agonist AM1241 suppresses the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and iNOS while increasing anti-inflammatory markers like Arg1, IL-10, and neurotrophic factors BDNF and GDNF in LPS/IFN-γ-activated microglial cells (Lutz et al., 2015). Similar findings have been confirmed in various primary and immortalized microglial cell lines (Correa et al., 2010b; Ehrhart et al., 2005; N et al., 2015). In neurodegenerative diseases, CB_2 is highly expressed in activated microglia associated with pathological structures, such as amyloid plaques in AD, and lesions in multiple sclerosis and amyotrophic lateral sclerosis (ALS) (Benito et al., 2003, 2007; Y et al., 2006) . We demonstrated that primary microglia isolated from an AD mouse model showed increased basal CB_2 receptor expression compared to wild-type counterparts, with the receptor predominantly localized to intracellular compartments, including structures resembling the endoplasmic reticulum and Golgi apparatus (Scipioni et al., 2023). In AD models, CB_2 activation has been linked to reduced Aβ levels, neuroinflammatory marker suppression, and enhanced microglial proliferation and migration (G et al., 2012).

In addition to CB_1 and CB_2, other receptors like TRPV1 play critical roles in microglial function. TRPV1, primarily expressed in microglia, is involved in modulating inflammatory responses. Studies using TRPV1-/- mice exposed to LPS to induce systemic inflammation demonstrated improved survival rates, accompanied by reduced microglial activation (Miyake et al., 2015). TRPV1 activation has been linked to pro-inflammatory effects, including increased TNF-α production, while its deletion has been associated with reduced microglial activation and less severe inflammatory outcomes in models of neuroinflammation (Marrone et al., 2017). Interestingly, in vitro studies have shown conflicting results, with TRPV1 activation, rather than

its inhibition, leading to reduced cytotoxic factors such as microglial-derived ROS (Schilling & Kluge, 2009).

2.3.3.2 eCBs metabolic enzymes

Microglia express a complete set of endocannabinoid synthetic and catabolic enzymes, enabling them to metabolize both AEA and 2-AG (Leuti et al., 2020a; Scipioni et al., 2022). Notably, microglia produce significantly higher levels of eCBs—up to 20 times more than neurons and other glial cells—positioning them as the primary source of bioactive lipids under neuroinflammatory conditions (Walter et al., 2003).

The synthesis and degradation of AEA are closely linked to microglial activation states. In particular, the upregulation of biosynthetic enzymes, such as NAPE-PLD, is observed in the anti-inflammatory M2 phenotype, alongside downregulation of its degrading enzyme FAAH, resulting in increased AEA production (Stella, 2009). In AD, findings regarding AEA are complex and stage-dependent. For example, in 5×FAD mice, AEA levels remain unchanged (Vazquez et al., 2015), while in Aβ-induced neurotoxicity models, hippocampal AEA levels decrease during later disease stages (van der Stelt et al., 2006). This aligns partially with human studies, where reduced AEA levels were observed in the midfrontal and temporal cortex of AD patients, inversely correlating with Aβ accumulation (Jung et al., 2012). Increased FAAH expression and activity have been detected in astrocytes and microglia associated with neuritic plaques in AD brains (Benito et al., 2003). Genetic ablation of FAAH in 5×FAD mice reduced Aβ levels, plaque deposition, and gliosis, though neuroinflammation worsened due to interactions with PPARα, PPARγ, and TRPV1 (Benito et al., 2003; Vazquez et al., 2015). Pharmacological inhibition of FAAH has shown neuroprotective effects, potentially mediated by substrates such as

palmitoylethanolamide, which has been demonstrated to reduce toxicity and reverse memory deficits in PPARα-dependent mechanisms (Bronzuoli et al., 2018).

The metabolism of 2-AG is finely regulated by the expression of enzymes like DAGLα and MAGL (Muccioli et al., 2007; Walter et al., 2003). In the M2 phenotype, DAGLα is upregulated while MAGL is downregulated, leading to increased 2-AG production (C et al., 2003). Pharmacological inhibition of MAGL elevates 2-AG levels in the brain (Makara et al., 2005) and exerts immunomodulatory effects, notably in neuroinflammatory conditions. For instance, in a viral-induced neuroinflammation model, MAGL inhibition and 2-AG administration acted via CB2 receptors, reducing microglial activation and preserving their resting morphology (Mecha et al., 2016). In AD models, 2-AG dynamics are variable. In 5×FAD mice, hippocampal 2-AG levels remain unchanged (Vazquez et al., 2015), whereas in Aβ-induced neurotoxicity models, early stages are marked by elevated hippocampal 2-AG levels (van der Stelt et al., 2006). Post-mortem studies in AD patients indicate increased 2-AG signaling in the hippocampus, with elevated DAGLα expression near amyloid plaques (Mulder et al., 2011). Elevated plasma levels of 2-AG have also been reported, particularly in AD patients with ischemic heart disease or cerebral leukoaraiosis (Iorio et al., 2013). In APP/PS1 mice, genetic inactivation of MAGL reduced prostaglandin production, further mitigating inflammation (Piro et al., 2012a).

3. AIM

This study investigates the potential influence of the ECS on the senescence phenotype in microglial cells, particularly in the context of AD pathology.

Neonatal microglia were isolated from wild-type and Tg2576 mice, an AD-like model that overexpresses amyloid-beta (Aβ) peptides and develops symptomatology at 6 months of age. The

presymptomatic microglia thus obtained were subjected to chronic LPS stimulation to simulate immunosenescence conditions. The research focuses on characterizing the senescence phenotype through key biomarkers, including β-galactosidase activity, p16 and p21 expression, CD11c, and lipofuscin accumulation.

Specifically, the study aims to elucidate the role of the ECS in the process of immunosenescence by initially targeting FAAH inhibition. This method involves assessing the efficacy of URB597, a specific FAAH inhibitor, in reversing microglial senescence. A focused examination of changes in AEA signaling and its implications for the activation of NF-κB, a pivotal regulator of the SASP, is conducted.

Additionally, the impact of elevated 2-AG levels in Tg2576 microglia is addressed, characterizing this increase as a maladaptive response to chronic Aβ exposure that intensifies pro-inflammatory and senescence phenotypes, attributed more to enzymatic imbalances than to the direct actions of 2-AG itself. Given that chronic administration of 2-AG alongside LPS does not induce a senescent phenotype in wild-type microglia, it is hypothesized that such discrepancies may arise from its conversion into AA and subsequent bioactive COX-2 metabolites. To investigate this, COX-2 will be inhibited with indomethacin to determine if such treatment can mitigate senescence markers, including β-galactosidase activity, p16 expression, and lysosomal dysfunction. This assessment will elucidate the potential role of prostaglandin signaling, facilitated by the degradation of 2-AG-derived AA and pro-inflammatory prostaglandins, in driving microglial senescence in the context of AD.

The expected outcomes of this thesis are to deepen the understanding of the ECS's role in neuroinflammation and cellular aging in AD, elucidating mechanisms through which ECS modulation can influence microglial senescence and highlight novel therapeutic targets.

4. RESULTS

4.1 Microglia isolation and purity assessment

To explore the functional role of the ECS in amyloidosis-associated immunosenescence, we isolated and characterized neonatal microglia from both wild-type and Tg2576 mice. Microglia were isolated from the brains of neonatal mice (P0–P2), representing a presymptomatic phase prior to amyloid plaque formation and cognitive deficits in the Tg2576 murine model of amyloidosis. This model is characterized by the overproduction of Aβ42 peptides from the earliest developmental stages (Heiland et al., 2019).

The isolation protocol, depicted in Figure 4, Panel A, involved cortical dissection followed by the establishment of mixed glial cultures. After 10 days in culture, microglia were separated using either mechanical or chemical methods. Successful isolation was confirmed through immunofluorescence staining for Iba1, a microglial-specific marker (Figure 4, Panel B).

To further validate the isolation procedure, flow cytometry analysis was performed to assess the purity and viability of the microglial population. The gating strategy demonstrated that 99.8% of the isolated cells expressed the microglial-specific markers CD11b and CD45, indicating a highly pure population. Additionally, 92.6% of the cells were viable, and 63.3% were identified as single-cell events, further confirming the reliability and robustness of the isolation protocol (Figure 4, Panel C).

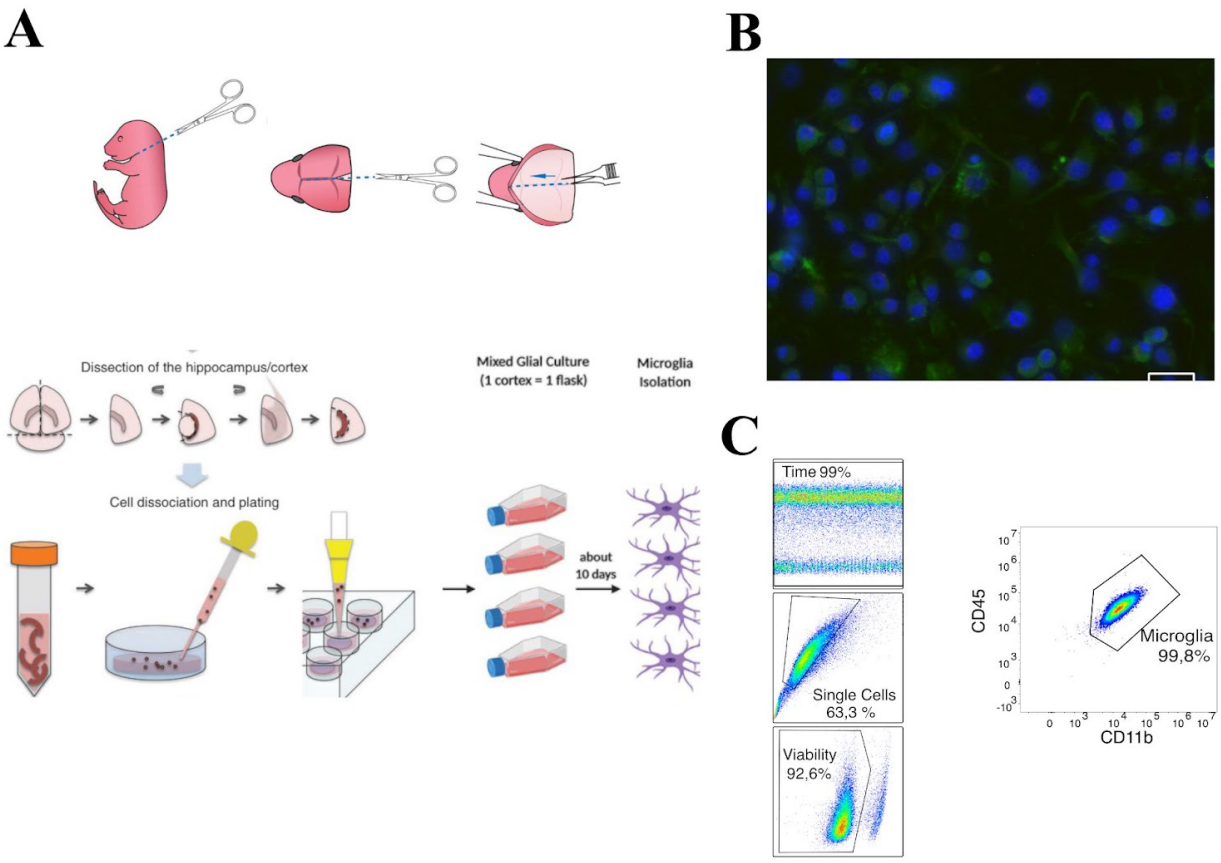

Figure 4: Validation of Microglial Purity Extracted from Neonatal Mouse Brains (P0–P2). (A) Schematic representation of the microglial extraction protocol, starting from cortical dissection, followed by the establishment of mixed glial cultures and subsequent isolation of microglia after 10 days using mechanical or chemical separation methods (adapted from Beaudoin, 2012). (B) Fluorescence microscopy image showing microglial cells stained with Hoechst 33342 (blue) for nuclear counterstaining and IBA1 (green) as a specific marker for microglia. Scale bar = 30 µm. (C) Assessment of microglial purity using polychromatic flow cytometry. Microglia were identified based on physical parameters and further gated as CD11b+ and CD45+, confirming a highly pure population.

4.2 Altered Endocannabinoid System and CB_2 Upregulation in Tg2576 Microglia: A Response to Chronic Aβ Exposure

Microglia express a comprehensive array of synthetic and catabolic enzymes for eCBs, enabling them to metabolize both 2-AG and AEA (Stella, 2009; Walter et al., 2003). *In vitro*, microglia produce eCBs at levels approximately 20 times higher than those of neurons or other glial cells, suggesting they are a primary cellular source of these bioactive lipids under neuroinflammatory conditions (Muccioli et al., 2007). To assess whether chronic exposure to amyloid-beta (Aβ) peptides during embryogenesis alters eCB metabolism in microglia, we evaluated the basal synthesis of eCBs in Tg2576 microglia compared to wild-type microglia.

As illustrated in Figure 5, panel A, Tg2576 microglia exhibited a ~2-fold increase in 2-AG levels compared to wild-type microglia (WT vs. Tg, $P = 0.010$), while AEA levels remained unchanged. To elucidate the underlying mechanisms, we examined the expression of enzymes involved in 2-AG synthesis and degradation, as its levels were markedly altered (Figure 5, Panel B). Analysis revealed a significant upregulation of DAGLα, the key enzyme for 2-AG synthesis, at both mRNA and protein levels (~4-fold increase; WT vs. Tg, $P = 0.013$), while DAGLβ levels remained unchanged. Conversely, MAGL, responsible for 2-AG degradation, was significantly downregulated (WT vs. Tg, $P = 0.012$), further supporting the elevated 2-AG levels in Tg2576 microglia.

We also evaluated the expression of cannabinoid receptors CB_1 and CB_2 in wild-type and Tg2576 microglia. CB_1 expression was undetectable at the mRNA level in both groups (data not shown).

In contrast, CB_2 receptor expression was significantly upregulated at the protein level in Tg2576 microglia compared to wild-type microglia (WT vs. Tg, P = 0.004; Figure 5, Panel C). This observation was corroborated by immunofluorescence staining, which showed stronger CB_2 labeling in Tg2576 microglia. Live-cell imaging was performed using the fluorescent probe 8-SiR that is a highly potent, cell-permeable fluorescent probe designed as an agonist for the CB_2. It incorporates a silicon-rhodamine fluorophore, making it suitable for live-cell imaging and super-resolution microscopy. This probe preserves high affinity and selectivity for both human and mouse CB2R, allowing researchers to visualize receptor expression and dynamics in real-time with high specificity and sensitivity (Gazzi et al., 2022).

These findings demonstrate a dysregulated ECS in Tg2576 microglia, characterized by increased 2-AG production, altered enzymatic activity, and upregulated CB_2 expression, likely reflecting an adaptive response to chronic Aβ-exposure.

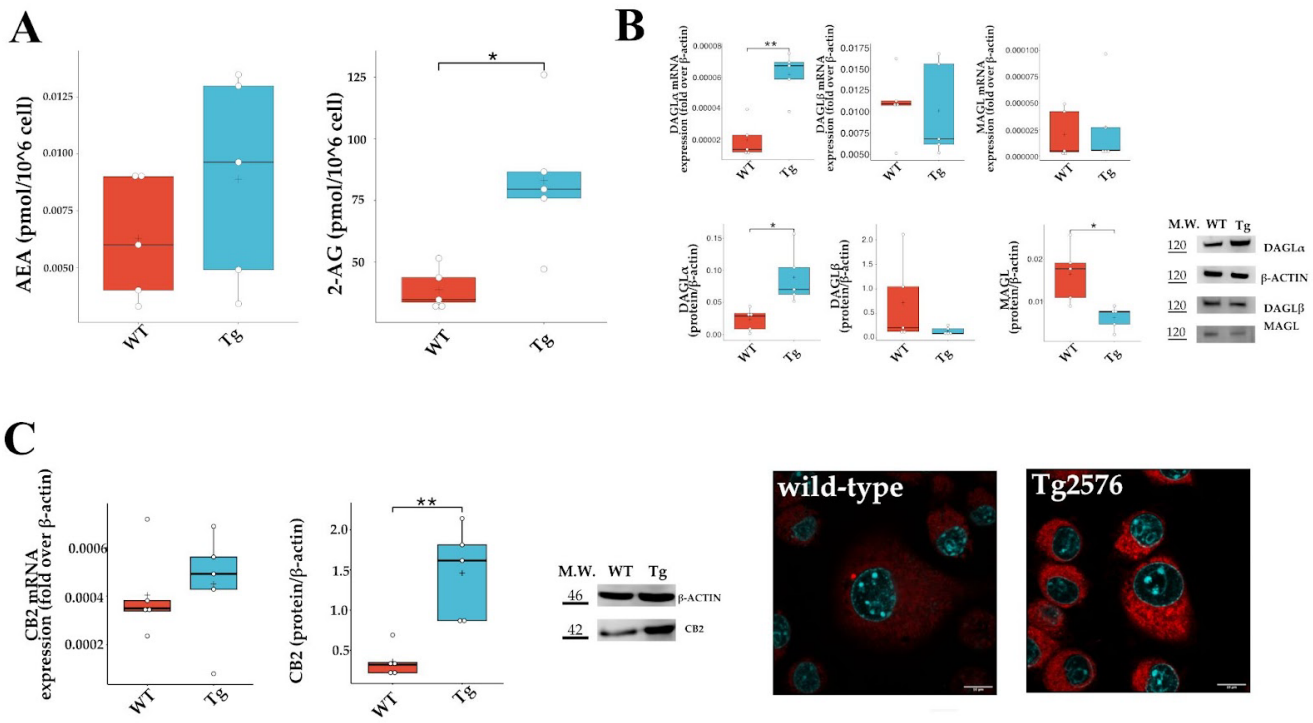

Figure 5: Endocannabinoid Levels, 2-AG Metabolism, and CB2 Expression in Wild-type (WT) and Tg2576 (Tg) Microglia. (A) Quantification of basal levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in Wild-type (WT) and Tg2576 (Tg) microglia using liquid chromatography-mass spectrometry. (B) Characterisation of 2-AG metabolic enzymes in wild-type microglia (WT) and Tg2576 microglia (Tg). Enzymes of synthesis (i.e., DAGLα and DAGLβ) and degradation (i.e., MAGL) were quantified for their mRNA content by quantitative real-time polymerase chain reaction, upper panel, and for their protein expression by Western blot analysis, lower panel. (C) CB2 was quantified for its mRNA content by quantitative real-time polymerase chain reaction and for its protein expression by Western blot analysis, left panel. Super-resolution fluorescence microscopy in microglial cells using probe 8-SiR. Cells were pre-stained with Hoechst 33342 (cyan) to counterstain the nuclei and incubated for 20 min with 0.25 µM 8-SiR (red). Scale bar, 10 µm. Values are means ± S.D. of 5 independent experiments. Significance is shown as P value, calculated using an unpaired t-test. *P<0.05, **P<0.01.

4.3 Induction of a Senescence-Like Phenotype in Microglia Tg2576 Through Chronic Inflammatory Stimulation

Microglia were isolated from P0–P2 wild-type (WT) and Tg2576 mice to investigate the interplay between amyloid-beta (Aβ) exposure and chronic inflammatory stimulation in the induction of cellular senescence. At this presymptomatic developmental stage, chronic exposure to Aβ alone is insufficient to induce a senescent morphology in microglia. To model immunosenescence, microglial cultures were exposed to chronic inflammatory stimuli using LPS, 50 ng/mL, administered three times on alternate days over the course of seven days (Figure 6A; see Materials and Methods). Following treatment, various senescence markers were analyzed to evaluate the cellular response to chronic stimulation (Figure 6B–D).

The activity of β-galactosidase (β-gal), a hallmark marker of cellular senescence, was assessed via flow cytometry. Tg2576 microglia exhibited a statistically significant increase in β-gal activity, both in the percentage of β-gal-positive cells (Tg vs. Tg_LPS, $P = 0.005$) and in mean fluorescence intensity (MFI; Tg vs. Tg_LPS, $P = 0.001$), while no significant changes were observed in WT microglia. These results were corroborated by a colorimetric X-Gal assay, which demonstrated increased staining intensity exclusively in Tg2576 microglia treated with LPS (Figure 6B).

Senescence-associated markers p16 and p21 were further analyzed. Tg2576 microglia exhibited a significant increase in p16 expression following LPS treatment (Tg vs. Tg_LPS, $P = 0.005$), while p21 levels remained unchanged. In WT microglia, neither p16 nor p21 showed significant changes

(Figure 6C). Additionally, analyzed CD11c, a DAM-specific marker and integrin prominently expressed on antigen-presenting cells. CD11c is predominantly observed in microglia with high p16 expression, establishing a link between cellular senescence and DAM identity. In the CNS, particularly under conditions of aging and neurodegeneration, CD11c marks microglia undergoing transcriptional reprogramming associated with DAM (Matsuraida et al., 2023). CD11c was significantly upregulated in LPS-treated Tg2576 microglia ($P = 0.0036$). Increased ferritin-positive cells in Tg2576 microglia following LPS exposure (Tg vs. Tg_LPS, $P = 0.043$) further support the induction of a senescence-like phenotype (Figure 6C).

The accumulation of lipofuscin, a marker of lysosomal activity and cellular aging, was also analyzed in WT and Tg2576 microglia (Figure 6D). Under untreated conditions (Ctrl), both WT and Tg2576 microglia exhibited minimal lipofuscin granules, reflecting baseline lysosomal activity. However, following LPS treatment, a marked increase in lipofuscin accumulation was observed exclusively in Tg2576 microglia, aligning with the induction of a senescence-like phenotype in these cells. WT microglia did not exhibit significant changes in lipofuscin granule accumulation, further supporting the hypothesis that Aβ exposure primes Tg2576 microglia for senescence upon inflammatory stimulation.

The collective upregulation of β-galactosidase activity, p16 expression, CD11c, ferritin, and lipofuscin accumulation in LPS-treated Tg2576 microglia strongly indicates the development of a senescence-like state. These findings highlight the interplay between Aβ exposure and chronic inflammatory stimuli in driving microglial immunosenescence. Importantly, only Tg2576 microglia exhibited a shift toward a senescent phenotype across all markers analyzed, suggesting that both *in vivo* Aβ peptide exposure during embryogenesis and *in vitro* LPS treatment over seven days are necessary to induce a senescence-like profile.

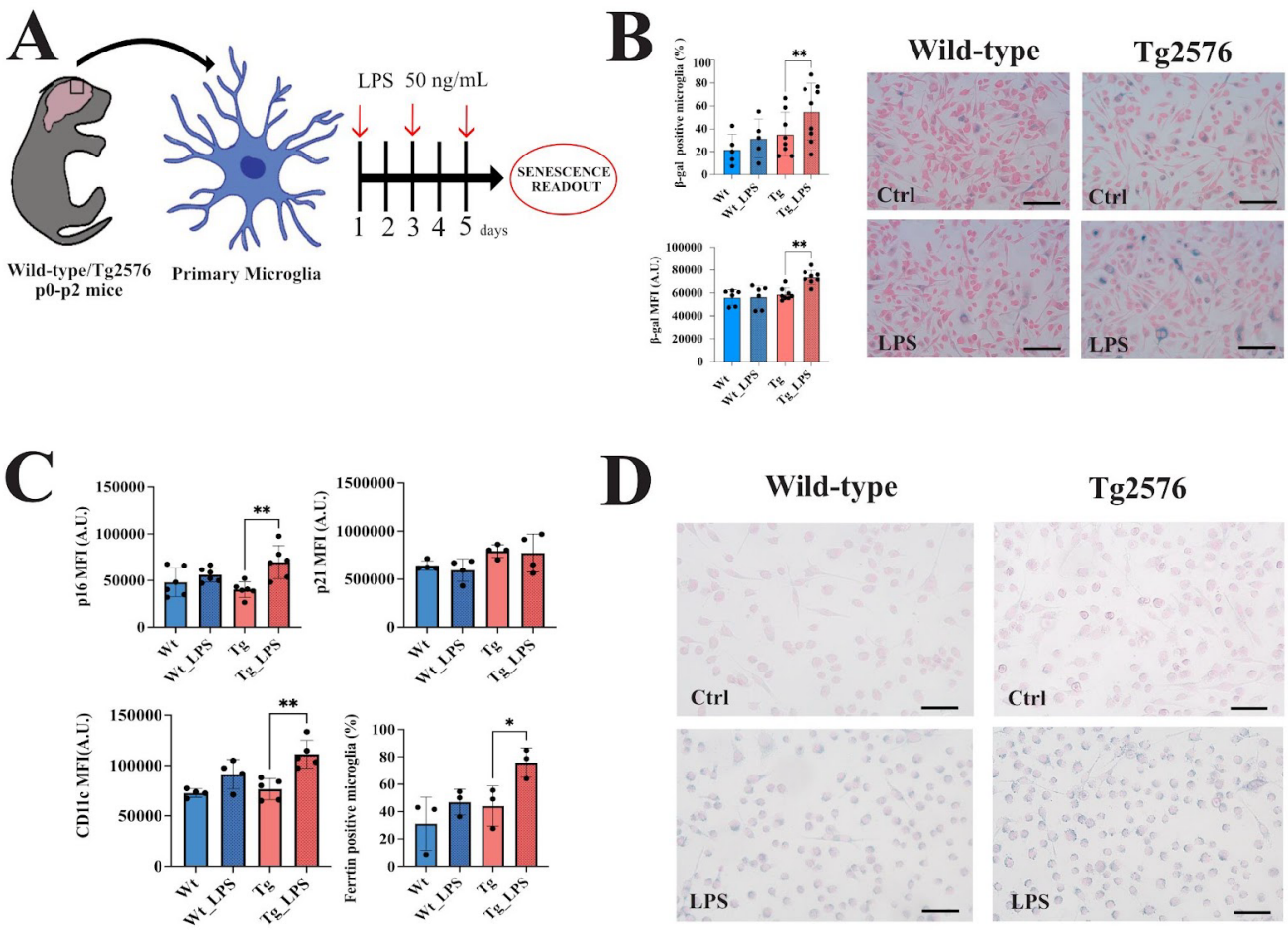

Figure 6: Senescence Markers in Wild-type (WT) and Tg2576 (Tg) Microglia Following lipopolysaccharides (LPS) Treatment. (A) Experimental design for microglial isolation and treatment. Primary microglia were isolated from P0-P2 Wild-type WT and Tg2576 mice and treated with lipopolysaccharides (LPS, 50 ng/mL) three times at alternate days. Senescence markers were evaluated at the end of the treatment. (B) Histograms represent quantification of β-galactosidase (β-gal) activity using flow cytometry and representative images for X-Gal staining. (C) Histograms represent Analysis of senescence-associated markers p16, p21, CD11c and ferritin using flow cytometry. (D) Representative images of lipofuscin granule accumulation. Scale bar, 30 µm. Values are means ± S.D. of 3-8 independent experiments, Statistical analysis was performed using a one-way ANOVA followed by post hoc multiple t-tests with Tukey's correction for multiple comparisons (*$P < 0.05$, **$P < 0.01$).

4.4 FAAH Inhibition with URB597 Reduces LPS-Induced Senescence Markers in Transgenic Microglia

Recent studies have demonstrated that FAAH inhibition using URB597, a selective FAAH inhibitor, effectively increases AEA levels, mitigating chronic inflammation, endoplasmic reticulum stress, and mitochondrial dysfunction in aging-related models (Murphy et al., 2012; Su et al., 2019). Additionally, FAAH inhibition has been shown to enhance AEA signaling, resulting in reduced cognitive deficits, amyloid pathology, and neuroinflammation in AD models (Oddi et al., 2024, *accepted for publication, FEBS Journal*). Collectively, these findings underscore the potential of FAAH inhibitors as therapeutic strategies to address neuroinflammatory processes in conditions such as AD.

To investigate whether FAAH inhibition could influence immunosenescence in microglia from Tg2576 mice, we administered chronic treatments of URB597 (5 μM) three times on alternate days over the course of a week, concurrently with LPS exposure. The analysis of senescence markers, which are typically elevated following chronic LPS treatment, revealed notable effects of URB597. As shown in Figure 7, Panel A, URB597 significantly reduced β-galactosidase (β-gal) expression (LPS vs. LPS_URB, $P = 0.001$). While the percentage of β-gal-positive cells did not significantly decrease, a positive trend toward reduction was observed.

In addition, other senescence-associated markers, including p16, CD11c, and ferritin, were significantly reduced by URB597 treatment (LPS vs. LPS_URB: p16, $P = 0.007$; CD11c, $P = 0.02$; ferritin, $P = 0.02$). These findings further support the ability of FAAH inhibition to attenuate microglial senescence markers under inflammatory conditions.

We also analyzed TREM2 expression, a critical marker associated with the anti-inflammatory capacity of microglia, which is typically downregulated in response to pro-inflammatory stimuli such as LPS (Liu, 2022; Zhu et al., 2024). Consistent with previous studies, LPS exposure significantly suppressed TREM2 expression (Ctrl vs. LPS, P = 0.004). Remarkably, treatment with URB597 reversed this suppression, restoring TREM2 levels (LPS vs. LPS_URB, P = 0.005), indicating its potential role in modulating microglial function under inflammatory stress.

Lastly, we evaluated the accumulation of lipofuscin deposits, as shown in Figure 7, Panel B, URB597 significantly reduced lipofuscin accumulation compared to the LPS-only condition. This reduction further supports the role of FAAH inhibition in counteracting age-related microglial dysfunction.

These findings collectively indicate that FAAH inhibition with URB597 can effectively mitigate LPS-induced immunosenescence. By reducing β-gal activity, senescence markers (p16, CD11c, ferritin), and lipofuscin deposits, while restoring TREM2 expression, URB597 demonstrates therapeutic potential in modulating age-related inflammation and microglial dysfunction, particularly in the context of neurodegenerative diseases such as AD.

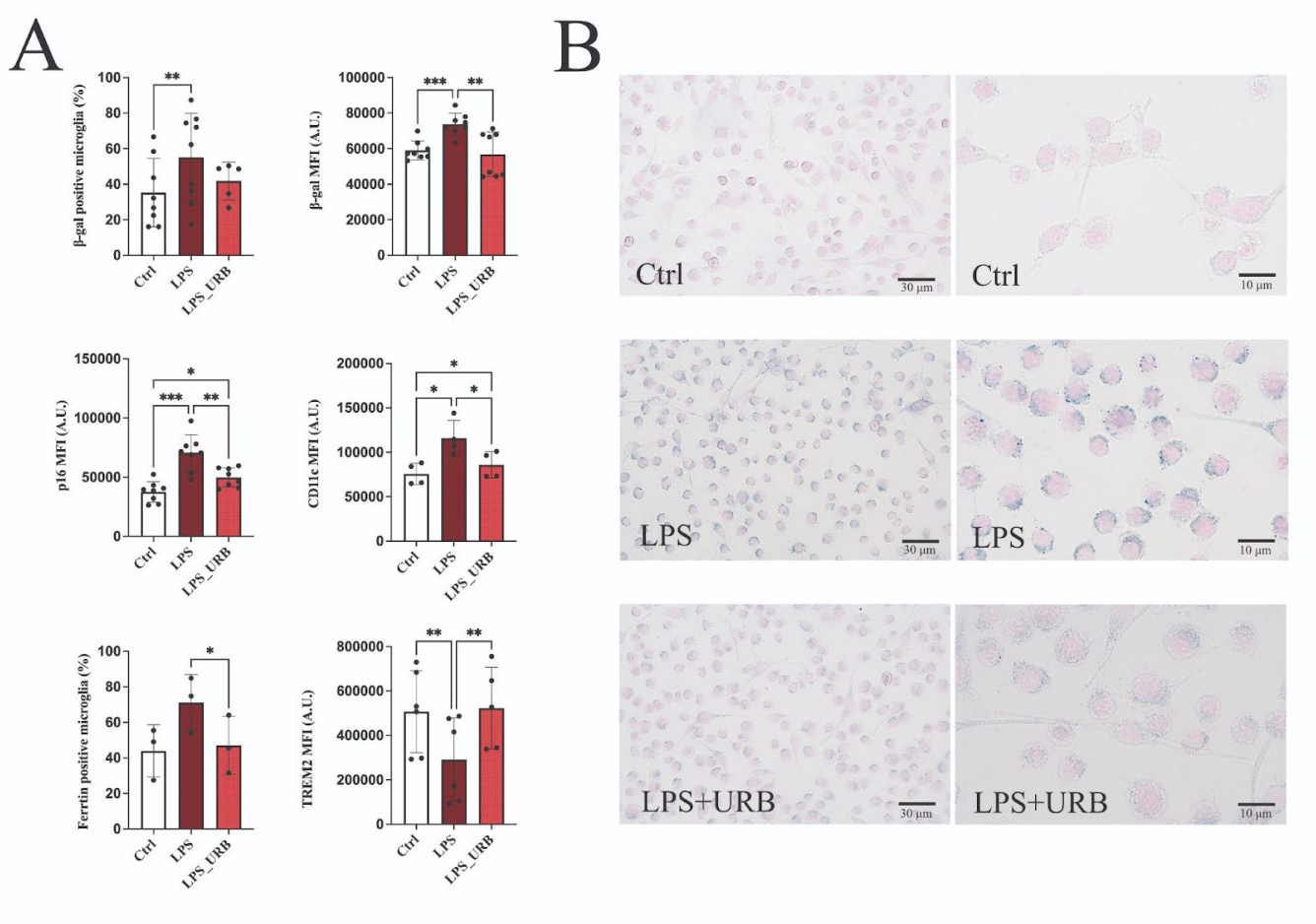

Figure 7: Analysis of Senescence Markers in Tg2576 Microglia After lipopolysaccharides (LPS and URB597 (URB) Treatment. Transgenic microglia (Tg2576) were chronically treated simultaneously with lipopolysaccharides (LPS, 50ng/mL) and URB597 (URB, 5 µM). (A) Histograms represent senescence markers including β-gal, p16, CD11c, and ferritin, measured by flow cytometry. (B) Representative images showing microglial cells stained for lipofuscin accumulation, across different treatment groups: Control (Ctrl), LPS only, and LPS with URB597. Scale bars are set at 30 µm in the left panel and 10 µm in the right panel. Data are presented as means ± SD from 3-8 independent experiments. Statistical analysis utilized one-way ANOVA followed by Tukey's post hoc tests for multiple comparisons (*P < 0.05, **P < 0.01, ***P<0.001).

4.5 FAAH Pharmacological Inhibition Mitigates NF-kB Activation in Immunosenescent-like Tg2576 Microglia

Immunosenescence-related neuroinflammation represents a critical pathological process driving the senescent state of microglial cells, with a central role attributed to the activation of nuclear factor-kappa B (NF-kB), a key regulator of immune responses and inflammation and is a central signaling pathway responsible for the activation of SASP (Kumari & Jat, 2021; Y. Wang et al., 2022). To investigate whether enhanced AEA signaling via FAAH inhibition could modulate NF-kB expression levels and influence microglial aging, we analyzed NF-kB activation in Tg2576 microglia under pathological conditions.

As shown in Figure 8, chronic exposure to LPS significantly increased the percentage of cells positive for phospho-NF-kB, a marker of NF-kB activation, by at least two-fold compared to untreated controls (Ctrl vs. LPS, $P = 0.014$). This finding confirms the pro-inflammatory effects of chronic LPS stimulation, leading to heightened NF-kB signaling in microglial cells.

Notably, treatment with URB597, a selective FAAH inhibitor, reversed the LPS-induced increase in phospho-NF-kB expression (LPS vs. LPS_URB, $P = 0.026$). These results demonstrate the potent ability of URB597 to modulate NF-kB activity, reducing its pathological activation in the context of chronic inflammatory stress.

Overall, these findings highlight the therapeutic potential of URB597 in counteracting the detrimental effects of immunosenescence-driven neuroinflammation in microglial cells. By attenuating NF-kB activation, URB597 emerges as a promising strategy to mitigate age-related microglial dysfunction and its contribution to neurodegenerative processes.

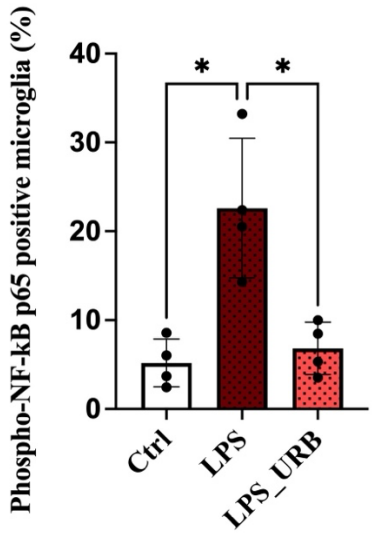

Figure 8: Effects of URB597 (URB) and lipopolysaccharides (LPS) treatment on Phospho-NF-kB in Tg2576 microglia. Transgenic microglia (Tg2576) were chronically treated simultaneously with lipopolysaccharides (LPS, 50ng/mL) and URB597 (URB, 5 μM). Histograms represent the percentage of phospho-NF-kB p65 positive cells measured by flow cytometry. Data are presented as means ± SD from 5 independent experiments. Statistical analysis utilized one-way ANOVA followed by Tukey's post hoc tests for multiple comparisons (*P < 0.05).

4.6 DAGL Inhibition with KT172 Reduces LPS-Induced Senescence Markers in Transgenic Microglia

Previous studies have established that Tg2576 microglia exhibit elevated basal levels of 2-AG and increased nitric oxide (NO) production when primed with LPS, effects that can be mitigated by inhibiting diacylglycerol lipase alpha (DAGLα). Given the well-documented role of 2-AG signaling in various inflammatory processes (Chang et al., 2001a, p. 200; Chiurchiù et al., 2015; Scipioni et al., 2023), we hypothesized that modulating 2-AG signaling could influence age-related changes in microglial function and thereby affect their contribution to neurodegenerative diseases such as AD.

To evaluate whether inhibiting DAGLα could modulate immunosenescence *in vitro*, Tg2576 microglia were chronically treated with KT172 (0.1 μM), a selective DAGLα inhibitor, administered three times on alternate days over a week, concurrently with LPS exposure. As shown in Figure 9, Panel A, KT172 treatment significantly reduced β-galactosidase (β-gal) expression, both in overall expression (LPS vs. LPS_KT, P = 0.0003) and in the percentage of β-gal-positive cells (LPS vs. LPS_KT, P = 0.04). These findings indicate that DAGLα inhibition mitigates one of the hallmark markers of cellular senescence in Tg2576 microglia under inflammatory conditions.

We further analyzed additional senescence-associated markers that were previously found to be upregulated following chronic LPS exposure. Consistent with the effects observed for β-gal, treatment with KT172 significantly reduced the expression of p16 (LPS vs. LPS_KT, P = 0.009), CD11c (LPS vs. LPS_KT, P = 0.018), and ferritin (LPS vs. LPS_KT, P = 0.019). In addition, KT172 treatment restored the levels of TREM2, a critical marker for microglial anti-inflammatory capacity, which were suppressed by LPS (LPS vs. LPS_KT, P = 0.021). These results strongly suggest that 2-AG signaling plays a central role in the immunosenescence process induced by chronic inflammatory stimulation.

Lastly, we evaluated the accumulation of lipofuscin deposits, a hallmark of cellular aging and impaired lysosomal function. As shown in Figure 9, Panel B, KT172 treatment significantly reduced the accumulation of lipofuscin deposits compared to the LPS-only condition, further supporting the role of DAGLα inhibition in mitigating cellular senescence.

Overall, these findings highlight the contribution of 2-AG signaling to LPS-induced immunosenescence in Tg2576 microglia and demonstrate that targeting DAGLα with KT172 can attenuate key senescence markers, offering potential therapeutic strategies for neurodegenerative diseases characterized by chronic inflammation and microglial dysfunction.

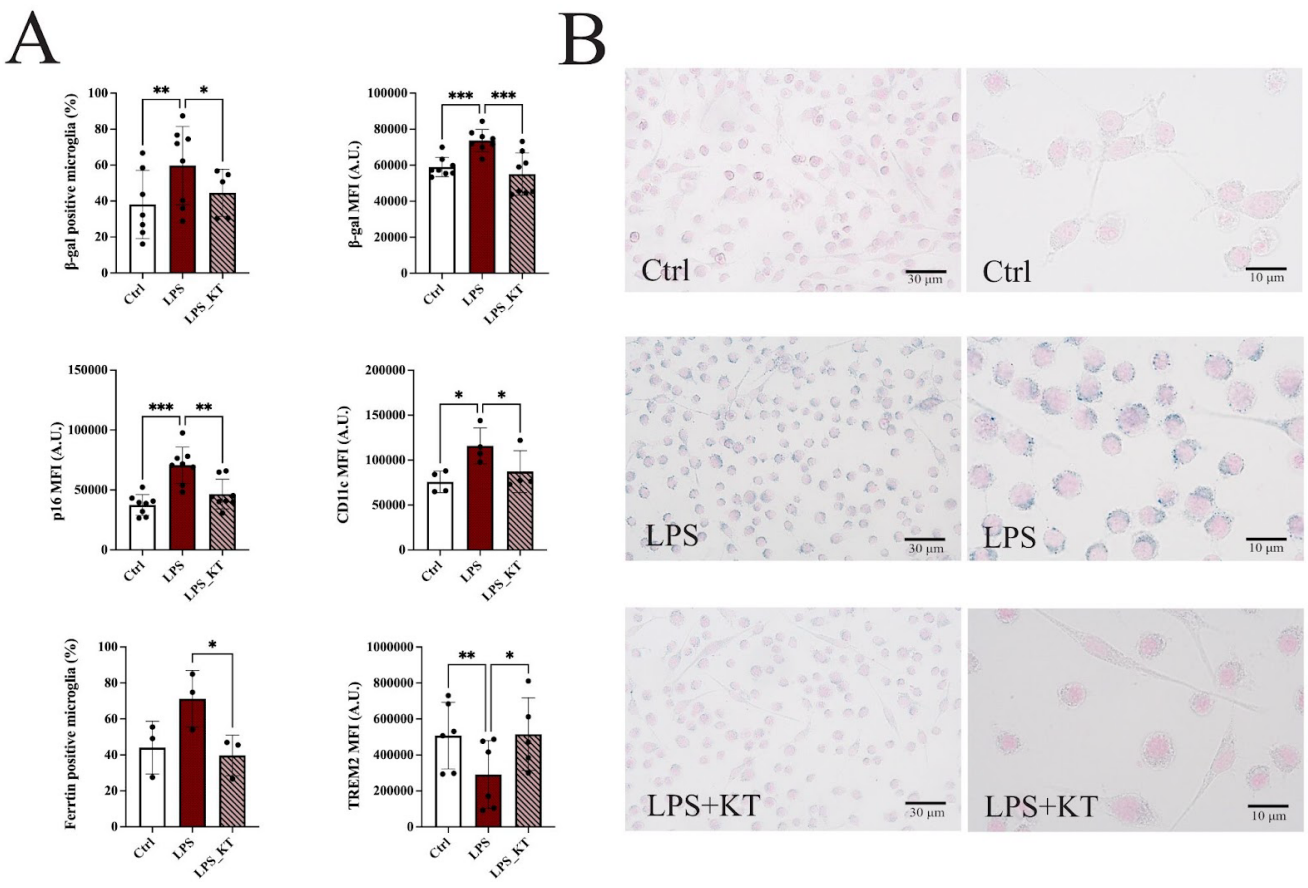

Figure 9: Analysis of Senescence Markers in Tg2576 Microglia After lipopolysaccharides (LPS) and KT172 (KT) Treatment.

Transgenic microglia (Tg2576) were chronically treated simultaneously with lipopolysaccharides (LPS, 50 ng/mL) and KT172 (KT, 0.1µM) (A) Histograms represent senescence markers including β-gal, p16, CD11c, and ferritin, measured by flow cytometry. (B) Representative images showing microglial cells stained for lipofuscin accumulation, across different treatment groups: Control (Ctrl), LPS only, and LPS with kt. Scale bars are set at 30 µm in the left panel and 10 µm in the right panel. Data are presented as means ± SD from 3-8 independent experiments. Statistical analysis utilized one-way ANOVA followed by Tukey's post hoc tests for multiple comparisons (*P < 0.05, **P < 0.01, ***P<0.001).

4.7 Exploring the impact of chronic LPS and 2-AG exposure on wild-type microglia

Previous findings demonstrated that Tg2576 microglia exhibit elevated basal levels of 2-AG compared to wild-type microglia (Figure 5A). Pharmacological inhibition of DAGL, the enzyme responsible for 2-AG synthesis, effectively reversed the senescence-like phenotype induced by chronic LPS exposure (Figure 9). Based on these results, we investigated whether the combined effect of chronic LPS exposure and exogenous 2-AG administration could induce a similar senescence-like phenotype in WT microglia.

To achieve this, we administered chronic treatments of 2-AG (20 nM) in combination with LPS (50 ng/mL) to wild-type microglia. These treatments were delivered three times on alternate days over the course of a week to simulate prolonged exposure and assess the potential effects of elevated 2-AG levels.

The analysis of β-galactosidase activity revealed a modest increase in both the percentage of positive cells and mean fluorescence intensity following LPS treatment. Notably, the addition of 2-AG attenuated these increases, suggesting a modulatory rather than an exacerbatory role for 2-AG in this context. Furthermore, no significant changes were observed in the expression levels of critical senescence markers such as p16 and CD11c (Figure 10).

These findings indicate that, under these experimental conditions, exogenously supplied 2-AG does not promote immunosenescence in wild-type microglia, even in the presence of chronic LPS exposure. This suggests a more complex and potentially protective role for 2-AG in the regulation of microglial senescence.

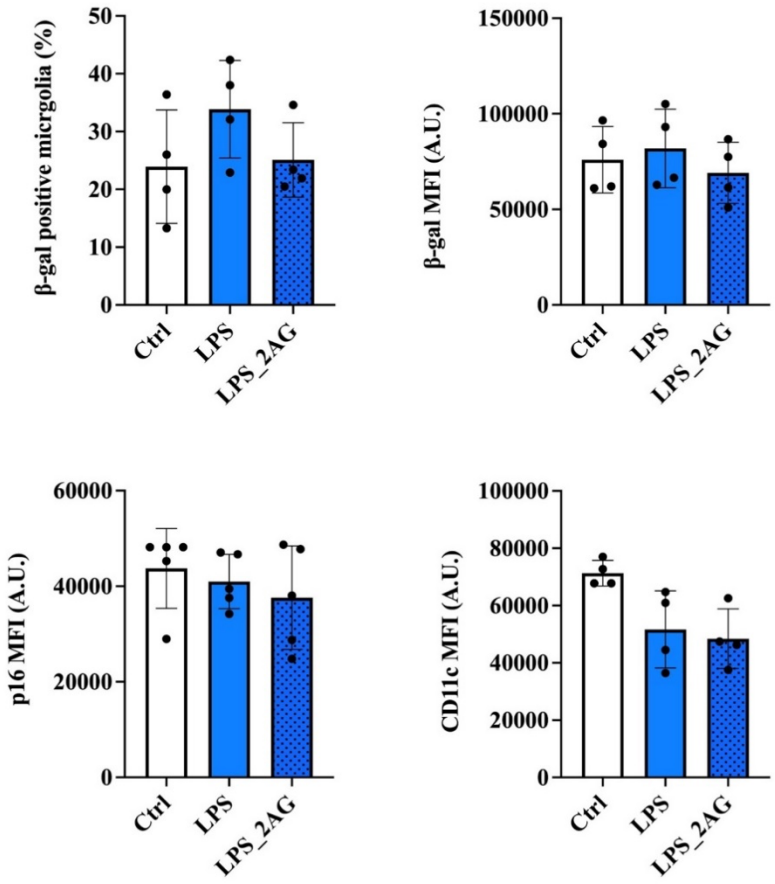

Figure 10: Analysis of Senescence Markers in wild-type microglia after lipopolysaccharides (LPS) and 2-arachidonoylglycerol (2-AG) Treatment. Wild-type microglia were chronically treated simultaneously with lipopolysaccharides (LPS, 50 ng/mL) and 2-arachidonoylglycerol (2-AG, 20nM). Histograms represent senescence markers including β-gal, p16, CD11c, measured by flow cytometry. Control (Ctrl), LPS only, and LPS with 2-AG. Data are presented as means ± SD from 4-5 independent experiments. Statistical analysis utilized one-way ANOVA followed by Tukey's post hoc tests for multiple comparisons.

4.8 COX-2 Inhibition Attenuates LPS-Induced Senescence-Like Phenotype in microglia Tg2576

We found that the exogenous administration of 2-AG, combined with concurrent LPS treatment, does not induce phenotypic alterations in microglia characteristic of a senescence-like state. Consequently, we investigated the downstream pathways of its signaling. It is well-established that this eCB can be degraded by COX-2, leading to the formation of prostaglandins. Prostaglandins,

derived from arachidonic acid via COX enzymes, play a critical role in resolving physiological inflammation; however, under pathological conditions, their excessive production exacerbates inflammatory signaling and promotes microglial senescence through increased cytokine production, reactive oxygen species generation, and the upregulation of markers such as β-galactosidase and p16 (Y. Wang et al., 2022).

To investigate the impact of COX-2 inhibition on LPS-induced responses, Tg2576 microglial cultures were treated with indomethacin (5 μM) in conjunction with LPS (50 ng/mL). The treatments were administered three times on alternate days over a one-week period to simulate prolonged inflammatory exposure and assess the modulatory effects of COX-2 inhibition on microglial responses within this AD model.

Consistent with previous findings, LPS treatment significantly increased both the percentage of β-galactosidase-positive microglia and the mean fluorescence intensity (MFI) of β-galactosidase activity, indicative of a pronounced senescence-like phenotype. Co-treatment with indomethacin significantly attenuated these markers, reducing the percentage of β-galactosidase-positive microglia (% β-gal positive microglia, LPS vs. LPS_INDO, $P = 0.012$) and MFI β-galactosidase activity (MFI β-gal, LPS vs. LPS_INDO, $P = 0.004$).

In addition, LPS exposure induced a marked upregulation of the senescence marker p16, which was significantly reduced by indomethacin treatment (LPS vs. LPS_INDO, $P = 0.014$). For another senescence-associated marker, CD11c, treatment with indomethacin demonstrated a trend toward reduction; however, the decrease did not reach statistical significance (LPS vs. LPS_INDO, $P = 0.073$) (Figure 11).

These results suggest that COX-2 inhibition via indomethacin effectively mitigates LPS-induced senescence-like changes in microglial cells, with significant reductions in key markers of cellular senescence. This highlights the potential role of prostaglandin signaling in driving immunosenescence in the context of AD.

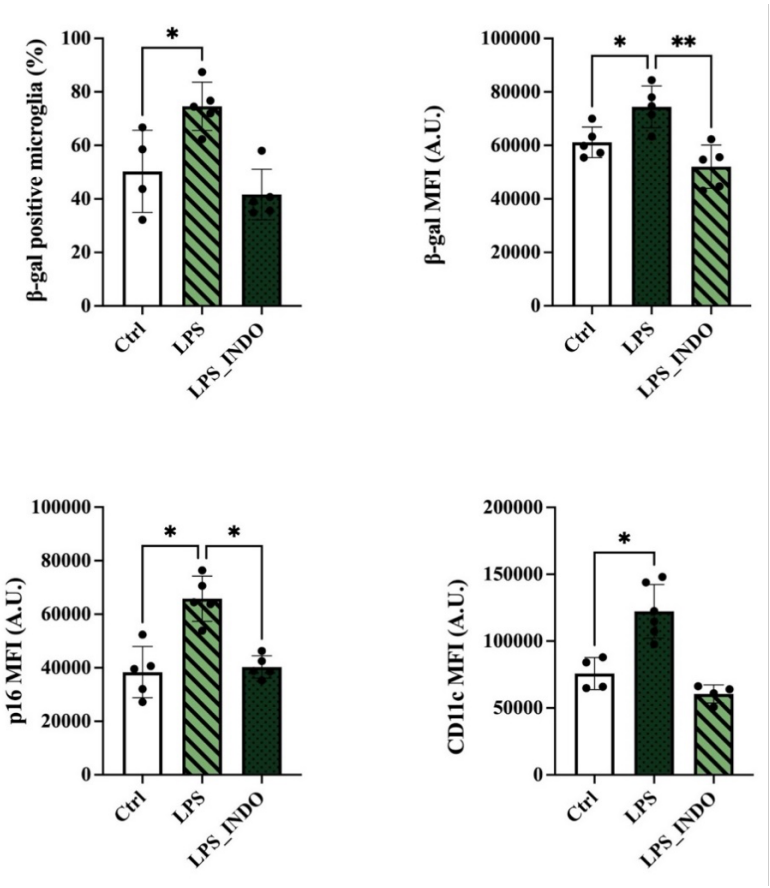

Figure 11: Analysis of Senescence Markers in LPS-treated Tg2576 Microglia after Pharmacological Inhibition of Diacylglycerol Lipase (DAGL) and Cyclooxygenase (COX). Microglia were chronically treated with lipopolysaccharides (LPS, 50 ng/mL) alone or in combination with indomethacin (INDO, 5 μM) three times on alternate days over a week. Histograms represent senescence markers, including β-galactosidase (percentage of positive cells and mean fluorescence intensity [MFI]), p16 MFI, and CD11c MFI, measured by flow cytometry. Data are presented as means ± SD from 4–5 independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test (*P < 0.05, **P < 0.01).

5. DISCUSSION

This study provides critical insights into the biochemical interplay between microglial senescence, endocannabinoid signaling, and AD. By exploiting an original ex vivo approach, we demonstrated that chronic inflammatory stimulus, when coupled with embryonic Aβ exposure, induces a senescent phenotype in primary microglia, a hallmark of immune dysfunction in AD. Through modulation of key ECS enzymes—FAAH and DAGL—we effectively reversed markers of senescence, underscoring the therapeutic potential of ECS targeting in alleviating inflammation-driven microglial dysfunction.

In our approach, we used primary microglial cells isolated from the brains of Tg2576 mice and exposed them to chronic inflammatory stimuli such as LPS. This setup replicated the aging and pathological microenvironment of AD. Trough Flow cytometry and functional analyses we revealed key senescence markers, including β-galactosidase activity, p16, ferritin, and lipofuscin, while also highlighting the regulatory role of endocannabinoid signaling.

In our senescent model we revealed a consistent upregulation of β-galactosidase activity and other markers like ferritin and lipofuscin, reinforcing their role as reliable indicators of cellular aging. Notably, p16 expression was elevated, whereas p21 did not show significant upregulation. This discrepancy is likely due to the transient nature of p21, which limits its reliability as a marker of sustained senescence. In contrast, p16 plays a pivotal role in maintaining long-term senescence by stabilizing cell cycle arrest through the p16/RB pathway (Kudlova et al., 2022).

As demonstrated by Matsudaira and colleagues, CD11c expression is predominantly observed in microglia with elevated p16 levels, establishing a robust connection between cellular senescence and the DAM (Disease-Associated Microglia) phenotype (Matsudaira et al., 2023). Its

upregulation, associated with immune activation and the pro-inflammatory SASP, suggests that senescent microglia not only lose protective functions but also actively contribute to a neurotoxic environment. Consistent with these findings, we also analyzed CD11c expression in our model and observed a concurrent increase alongside elevated p16 levels. This highlights the link between DAM characteristics, senescence markers, and the progressive inflammatory dysfunction observed in AD models.

Consistent with prior studies demonstrating that FAAH inhibition reduces neuroinflammation (Oddi et al., 2024, *accepted for publication, FEBS Journal*), our findings reveal an additional and significant potential of FAAH inhibition in reversing microglial senescence. This is evidenced by the reduction of key senescence markers such as p16, SA-β-gal, ferritin, CD11c, and lipofuscin following treatment with URB597. The underlying mechanism appears to involve enhanced AEA signaling, which critically suppresses NF-κB activation, a master regulator of the senescence-associated secretory phenotype (Sancho et al., 2003).

Correa et al., have demonstrated that AEA modulates NF-κB activity by impairing its nuclear translocation in microglial cells, thereby attenuating its pro-inflammatory effects and promoting anti-inflammatory responses, predominantly via CB2 receptor pathways (Correa et al., 2010). Our findings corroborate these mechanisms, as we observed a significant reduction in NF-κB activation after FAAH inhibition with URB597. This suggests that elevated AEA levels can disrupt NF-κB-driven inflammatory cascades, restoring microglial homeostasis and mitigating cellular senescence.

We found that Tg2576 microglia exhibit a constitutive imbalance in the regulation of 2-AG metabolism, leading to elevated levels of this endocannabinoid. In the context of the senescent phenotype, pharmacological inhibition of DAGL using KT172 significantly reduced LPS-induced

senescence markers, including β-galactosidase activity, p16 expression, and CD11c upregulation. These findings underscore the therapeutic potential of targeting DAGL to restore microglial homeostasis and alleviate dysfunction associated with cellular senescence.

AEA and 2-AG exhibit distinct roles in endocannabinoid signaling, highlighting a complex balance, especially in pathological contexts like neuroinflammation and senescence. AEA primarily exerts anti-inflammatory and homeostatic effects through CB_1 and CB_2 receptors (Correa et al., 2010). In contrast, the role of 2-AG is more intricate due to its higher endogenous levels and metabolic versatility. While 2-AG has anti-inflammatory properties via CB2 receptor activation, it also generates pro-inflammatory metabolites through its degradation into arachidonic acid and subsequent oxidative metabolism, leading to prostaglandin-like substances (e.g., PGE2-G). This duality underscores its potential to both mitigate and exacerbate inflammation, depending on the context (Leuti et al., 2020; Valdeolivas et al., 2013).

Pharmacological interventions targeting 2-AG levels, such as DAGL or MAGL inhibition, illustrate this complexity. DAGL inhibition reduces 2-AG levels and alleviates neuroinflammation, whereas MAGL inhibition increases 2-AG but may exacerbate inflammation by boosting AA-derived pro-inflammatory prostaglandins. This dynamic was apparent in rodent models of AD, where COX-2 inhibition ameliorated 2-AG-related neuroinflammation (R. Chen et al., 2012; Leuti et al., 2020; Piro et al., 2012). These findings emphasize the need for caution in modulating 2-AG pathways to harness its anti-inflammatory benefits while minimizing risks associated with its pro-inflammatory metabolites

Our study highlights the complex role of 2-AG in modulating microglial responses and its involvement in LPS-induced senescence. Interestingly, the effects of 2-AG on murine macrophages and microglia appear contradictory. On one hand, 2-AG has been shown to promote an anti-

inflammatory phenotype in innate immune cells (Gallily et al., 2000). On the other hand, it stimulates NO production in LPS-activated macrophages (Chang et al., 2001) and microglia, as corroborated by our findings (Scipioni et al., 2023).

Notably, we demonstrated that chronic administration of 2-AG alongside LPS does not induce a senescent phenotype. This suggests that the discrepancies may arise from its conversion into arachidonic acid and subsequent bioactive COX-2 metabolites. Indeed, inhibiting COX-2 with indomethacin significantly mitigated senescence markers, including β-galactosidase activity, p16 expression, and lysosomal dysfunction, highlighting the role of prostaglandin signaling in driving microglial senescence, thus suggesting the role of prostaglandin signaling in driving microglial senescence may be due to the reduction of 2-AG-derived AA and pro-inflammatory prostaglandins (Alhouayek & Muccioli, 2014).

6. CONCLUSIONS

In conclusion, our study demonstrates that chronic exposure to beta-amyloid alone is insufficient to induce microglial senescence, requiring persistent inflammatory stimulation to trigger this phenotype. Enhancing anandamide levels through FAAH inhibition effectively counteracts senescence by modulating the NF-κB signaling pathway and reducing key cellular aging markers.

A possible explanation for the role of cyclooxygenase-2 in microglial senescence involves enzyme competition in inflammatory environments. Elevated levels of substrates like 2-arachidonoylglycerol may lead to competition between enzymes such as cyclooxygenase-2 and monoacylglycerol lipase for substrate metabolism. This competition can influence the efficiency and pathway of metabolism, depending on the enzyme's affinity for the substrate and the available substrate concentration. The resulting shift in metabolic products, such as bioactive prostaglandins, contributes to the immune dysfunction and cellular aging observed in senescent microglia (C. Chen, 2023).

These findings underscore the therapeutic potential of targeting the ECS and metabolic pathways like cyclooxygenase-2 to alleviate microglial senescence and restore immune functionality.

7. MATERIALS AND METHODS

7.1 Reagents

Chemicals were of the purest analytical grade. DMEM, foetal bovine serum (FBS), and other cell culture reagents were purchased from Corning (Corning, NY, USA). LPS from Escherichia coli O111:B4, URB597, KT172 and Indometacin was purchased Sigma Aldrich (St. Louis, MO, USA)

7.2 Mice model

Mice (Tg2576), expressing high levels of human mutant (K670N/M671L) APP transgene (hAPPK670N/M671L) under transcriptional control of the hamster prion protein promoter and develop amyloid plaques at 11–13 months of age predominantly in neocortex and hippocampus. In particular, transgenic hAPP expression itself, reported to be neuron-specific (Castaneda et al., 2013), were purchased from Taconic Biosciences (strain: B6;SJL-Tg(APPSWE)2576Kha; cod. 1349-M). A colony of Tg2576 was obtained by crossing heterozygous transgenic male mice with C57BL/6J x SJL hybrid female mice. All the experiments were performed according to ethical regulations on the use and welfare of experimental animals of the European Union (EU Directive 2010/63/EU) and the Italian Ministry of Health (D.lgs 26/2014), and the procedures were approved by the bioethical committee of Fondazione Santa Lucia of Rome (n. 421/2019-PR).

7.3 Neonatal microglia cell cultures and treatment

Neonatal microglia cells were obtained from mixed glia cultures derived from the cerebral cortices of post-natal day 0–2 (p0–p2) C57BL/SJL wild-type and Tg2576 mice as described (Lian et al.,

2016) After 5-7 days, cells were collected after vigorously taping the flask. These procedures gave almost pure (no more than 2% contamination) microglial cell population.

Cells were then seeded at a density of 10,000 cells onto plastic 6-well dishes coated with poly-L-lysine (100 µg/ml) in plating medium composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.5% glucose, 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. At this stage, treatments were delivered three times on alternate days over the course of a week with LPS (50 ng/mL) in the presence and absence of the drugs URB597 (5 µM), KT172 (0.1 µM), and Indomethacin (5 µM) and 2-AG (20 nM).

7.4 Measurements of endogenous levels of the endocannabinoids by liquid chromatography-mass spectrometry

The analytical standards utilized in this study included AEA, N-arachidonoylethanolamine-d8 (AEA-d8), 2-AG, and 2-arachidonoylglycerol-d8 (2-AG-d8). Lipids were extracted from primary microglia using a chloroform–methanol–water mixture (2:1:1 v/v) with internal standards (ISs) added (1 ng/mL of AEA-d8 and 100 ng/mL of 2-AG-d8). The organic layer was evaporated under a gentle stream of nitrogen and subjected to micro-solid phase extraction (µSPE) for rapid purification using OMIX C18 tips from Agilent Technologies (Santa Clara, CA, USA). Analyses were conducted using a Nexera XR LC 20 AD UHPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Kinetex XB-C18 column (1.7 µm, 100 × 2.1 mm) from Phenomenex (Torrance, CA, USA), and coupled to a 4500 Qtrap mass spectrometer from Sciex (Toronto, ON, Canada) featuring a Turbo V electrospray ionization (ESI) source. AEA and 2-AG levels were expressed as pmoles per cell count.

7.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted with a ReliaPrep RNA Miniprep System kit (Promega). SuperScript IV VILO Reverse Transcriptase (Invitrogen) was used for cDNA synthesis. Transcripts were quantified by qRT-PCR using a StepOne Real-Time PCR System sequence detector (Applied Biosystems). The predesigned TaqMan Gene Expression Assays probes used were all obtained from the Applied Biosystems and are specified in the Table 1. The relative expression of different amplicons was calculated by the Delta–Delta Ct (ΔΔCT) method and converted to $2^{-\Delta\Delta Ct}$ for statistical analysis (Livak & Schmittgen, 2001).

Table 1. List of genes analyzed in the study along with their Taqman probes (Assay ID)

No.	Gene	Assay ID
1	*βactin*	Mm04394036_g1
2	*daglα*	Mm00813830_m1
3	*daglβ*	Mm00523381_m1
4	*mgll*	Mm00449274_m1

7.6 Western blotting

Western blotting was carried out following established protocols. In summary, cell pellets were resuspended in an appropriate volume of ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a cocktail of protease and phosphatase inhibitors. The samples were then sonicated on ice with continuous pulses for 1 minute. Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (30 µg per lane) were denatured at 95 °C for 10 minutes, separated using SDS-PAGE

(10%), and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies that are specified in the Table 2. Following incubation with the appropriate peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA), signals were developed using the Lightwave Plus enhanced chemiluminescence detection system (GVS, Bologna, Italy). Chemiluminescence signals were captured using a C-DiGit blot scanner (LI-COR, Lincoln, NE, USA) and analyzed with Image Studio Software 4.0.21 (LI-COR). The densities of protein bands were quantified, and the mean protein-to-actin ratios were calculated and reported.

Table 2. List of the primary and secondary antibodies used in the study.

Antibody	Host	Company, #Catalog	Dilution
β-actin	Rabbit	Cell Signaling #4970	1:10000
DAGLα	Mouse	Santacruz #sc-390409	1:500
DAGLα	Rabbit	Invitrogen #PA5-26331	1:500
MAGL	Rabbit	Abcam #ab24701	1:500
CB_2	Mouse	R&D system	1:500
Anti-rabbit IgG-HRP	Mouse	Santa Cruz #sc-2357	1:5000
Anti-mouse IgG-HRP	Goat	Santa Cruz #sc-2005	1:5000

7.7 Immunofluorescence and confocal analysis

Cell surface CB2 receptors were examined through live confocal imaging using (Roche), a potent fluorescent CB2 agonist probe, as described in previous studies (Gazzi et al., 2022). In brief, primary microglia from WT and Tg2576 mice were seeded at a density of 2×10^4 cells/mL on LabTek chambers (Ibidi) and cultured for 24 hours. Cells were then incubated with 0.2 μM 8-SiR, and time-lapse imaging was conducted using a ZEISS LSM 800 confocal laser scanning microscope equipped with an Airyscan detection unit (Zeiss, Oberkochen, Germany). Live imaging was carried out at a temperature of 22 ± 2 °C, recording one frame every 15 seconds for 20 minutes.

After the recording session, cells were visualized in Airyscan mode. Images were captured at the equatorial plane of the cells using ZEN Blue 2.3 software (Zeiss, Oberkochen, Germany). Super-resolution processing was performed via the Airyscan toolbox within the ZEN software. The processed data were exported as TIFF files and further analyzed using Fiji software (National Institutes of Health) A Gaussian kernel filter with a standard deviation of 0.8 pixels was applied to the images. All intensity profiles were normalized and background-subtracted relative to the final frame of the recording. For display purposes, images were enhanced in Artstudio Pro version 2.0.13 (Lucky Clan, Lodz, Poland; http://www.luckyclan.com) by adjusting brightness and contrast.

7.8 Senescence β-galactosidase

Microglial cells were harvested after 7 days, resuspended in 1X PBS to a concentration of 0.5 × 10^6 cells per 100 µL, and incubated in 1% BSA in PBS with surface antibodies at +4°C for 30 minutes. Following incubation, cells were fixed with 2% paraformaldehyde for 15 minutes, washed, and resuspended in a working solution containing enzymes for the senescence assay. The CellEvent Senescence Green Flow Cytometry Assay Kit (Invitrogen, Waltham, MA, USA) was used to detect cellular senescence, targeting β-galactosidase activity, a biomarker for senescence. This kit includes a fluorescein-based reagent that emits a fluorogenic signal at 490/514 nm under acidic conditions. After a 1-hour incubation at 37°C in the dark, cells were washed and analyzed on a flow cytometer with a 488-nm laser and 530-nm/30 filter.

Senescence-associated β-galactosidase (SA-β-Gal) activity was also evaluated using a β-galactosidase staining kit (Cell Signaling Technology, #9860). In brief, microglia that had been subjected to chronic treatment with 50 ng/mL LPS were fixed and stained 24 h at 37°C with a solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The resulting SA-

β-gal activity led to the production of insoluble blue granules, which were examined using standard light microscopy (Olympus BX53).

7.9 Flow cytometry

For flow cytometry analysis, 5 x 10^5 cells were processed in a V-bottom untreated 96-well plate, which included additional wells for isotype control, unstained, and single staining with Live/Dead dye. Each well, except for the unstained control, was stained with LIVE/DEAD™ Near-IR (Invitrogen, Waltham, MA, USA, #L10119) following the manufacturer's instructions. This was followed by the addition of 100 µL of FACS buffer to dilute the dye, and the plate was then centrifuged at 300xg for 5 minutes at room temperature (RT). Subsequently, the cells were washed by resuspending the pellets in 200 µL of FACS buffer and centrifuging again under the same conditions. Cell surface staining involved incubating each well with 30 µL of the antibody mix (refer to Table 3) for 25 minutes at 4°C, followed by the addition of 150 µL FACS buffer and centrifugation of the plate at 300xg for 3 minutes at RT. The wells were then washed again with 200 µL FACS buffer. After surface staining, the cells were fixed with 2% paraformaldehyde at RT for 15 minutes and washed twice. For nuclear antibody staining, the cells were resuspended in 50 µL FACS buffer, transferred to FACS tubes, and stored at 4°C until further analysis. The staining protocol continued with the addition of 200 µL of 1X Permeabilization Buffer to each well, followed by centrifugation at 400-600 x g for 5 minutes at RT, and discarding of the supernatant. This step was repeated, and the pellet was resuspended in the residual volume and adjusted to about 100 µL with 1X Permeabilization Buffer. Directly conjugated antibodies for intracellular antigens (refer to Table 3) were added without washing and incubated for at least 30 minutes at RT, protected from light. Afterward, 200 µL of 1X Permeabilization Buffer was added, and cells were centrifuged and washed as before. Following this step, the secondary fluorescent antibody (refer to

Table 3) was added. Finally, the cells were resuspended in Flow Cytometry Staining Buffer and analyzed by flow cytometer. The expression of the aforementioned proteins wasassayed by means of polychromatic flow cytometry in a Cytoflex analyzer (Beckman Coulter, Chaska, MN, USA) and the samples were analyzed using Flowjo analysis program (Treestar, Ashland, OR, USA). For each experiment, a minimum number of 10,000 events were analyzed.

Table 3. List of the primary and secondary antibodies used in the study.

Antibody	Host	Fluorochrome	Company	Dilution
CD11b	Mouse	Viogreen	Miltenyi #130-113-811	1:500
CD11b	Mouse	Alexa fluor 488	Invitrogen# 53-0112-82	1:500
CD45	Mouse	PerCp	Miltenyi #130-123-879	1:500
CD45	Mouse	Brilliant Violet 650	Invitrogen# 416-0451-82	1:500
TREM2	Rabbit	PE	Invitrogen# ANR-018	1:500
CD11c	Mouse	PerCp-Vio 700	Miltenyi # 130-114-110	1:500
p16	Mouse		Abcam #54210	1:50
p21	Rabbit		Abcam #188224	1:50
Ferritin	Rabbit		Abcam #109373	1:50
NF-kB	Mouse		Invitrogen #2A12A7	1:50
Anti-rabbit	Dokey	PE	Invitrogen #12-4739-81	1:200
Anti-mouse	Donkey	Alexa Fluor 647	Invitrogen #A-31571	1:200

UltraComp eBeads™ Compensation Beads (Invitrogen, Waltham, MA, USA) and MACS Comp KIT anti-REA (Miltenyi Biotec, Bergisch Gladbach, Germany) were incubated with each fluorophore, 1 μL of each antibody in one drop of the beads at RT for 15 min, in the dark. The beads were then washed with 1 mL of FACS buffer and centrifuged at 300xg for 5 min. The supernatant was discarded and the beads resuspended in 200 μL of FACS buffer.

7.10 Lipofuscin detection

Lipofuscin granules were detected using the Sudan Black B (SBB) staining method (Evangelou et al., 2017). Briefly, cells cultured on glass slides were fixed with 3% paraformaldehyde (PFA) and dehydrated in 70% ethanol for 2 minutes. Slides were then incubated on a plate shaker with freshly filtered SBB solution (0.7% w/v) in 70% ethanol. After staining, the slides were rinsed twice with 50% ethanol. Nuclei were counterstained with Nuclear Fast Red solution (0.1% w/v) for 5 minutes, followed by two washes in distilled water. Finally, slides were mounted with an aqueous mounting medium and observed under an Olympus microscope BX53 (Evangelou & Gorgoulis, 2017).

8. LIST OF PUBLICATION

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