Review

NLRP3 Inflammasome: A Starring Role in Amyloid-β- and Tau-Driven Pathological Events in Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) is the most prevalent neurodegenerative disease commonly diagnosed among the elderly population. AD is characterized by the loss of synaptic connections, neuronal death, and progressive cognitive impairment, attributed to the extracellular accumulation of senile plaques, composed by insoluble aggregates of amyloid-β (Aβ) peptides, and to the intraneuronal formation of neurofibrillary tangles shaped by hyperphosphorylated filaments of the microtubule-associated protein tau. However, evidence showed that chronic inflammatory responses, with long-lasting exacerbated release of proinflammatory cytokines by reactive glial cells, contribute to the pathophysiology of the disease. NLRP3 inflammasome (NLRP3), a cytosolic multiprotein complex sensor of a wide range of stimuli, was implicated in multiple neurological diseases, including AD. Herein, we review the most recent findings regarding the involvement of NLRP3 in the pathogenesis of AD. We address the mechanisms of NLRP3 priming and activation in glial cells by Aβ species and the potential role of neurofibrillary tangles and extracellular vesicles in disease progression. Neuronal death by NLRP3-mediated pyroptosis, driven by the interneuronal tau propagation, is also discussed. We present considerable evidence to claim that NLRP3 inhibition, is undoubtfully a potential therapeutic strategy for AD.

Keywords: Alzheimer's disease, amyloid-β, astrocytes, inflammasome, MCC950, neuroinflammation, NLRP3, microglia, pro-inflammatory cytokines, pyroptosis, tau

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease among the elderly population without approved therapies to halt or reverse its progression [1]. AD is associated with a specific pattern of pathological changes in the brain that result in neurodegeneration, loss of synaptic connections, and progressive memory deficits and cognitive impairment [2].

AD can be divided into different types, based on the age of onset and genetic predisposition, which are comparable in most clinical aspects [2]. Sporadic or late onset AD accounts for over 90% of AD cases and begins after the age of 65 years. Familial AD, a small subset of AD cases (<10%), is caused by

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genetic mutations and has an early-onset (fourth to sixth decade of life) [1].

PROTEIN AGGREGATES IN AD

One of the pathological hallmarks of AD is the extracellular accumulation of senile plaques in the brain. Senile plaques are composed of insoluble aggregates of amyloid-β (Aβ) peptides, firstly described by Alois Alzheimer in 1907 as a "(...) *deposition of a pathological metabolic substance in the neuron*" [3]. Alzheimer also described "(...) *the nucleus and the cell itself disintegrate and only a tangle of fibrils indicates the place where a neuron was previously located*." [3], referring to what is now recognized as the intracellular formation of neurofibrillary tangles (NFTs), consisting of hyperphosphorylated twisted filaments of the microtubule-associated protein tau [4]. It was also mentioned that "*Many neurons, especially the ones in the upper layer, have completely disappeared*." [3], which corroborates the observed progressive loss of synapses and neuronal death [2], leading to an atrophy of the brain confirmed in postmortem samples [3]. In AD, this phenomenon starts to affect tissue from the frontal and temporal lobes progressing to other areas of the neocortex [2].

Accumulation of abnormally folded proteins is a key histopathological feature of many neurodegenerative diseases. In Huntington's disease, the polyglutamine protein huntingtin occurs in intranuclear inclusions; in prion disease, prion particles bind to the membrane-bound protein and in Parkinson's disease (PD), aggregates of α-synuclein comprise the Lewy bodies present in the cytoplasm of neurons. In this sense, AD is rather unusual, since both intracellular and extracellular aggregates are observed—intraneuronal tangles of hyperphosphorylated tau and extracellular amyloid plaques consisting of aggregated Aβ peptides—which can potentially result in neuronal dysfunction and death. Indeed, the symptomatic phases of the disease have the duration of 8–10 years and are preceded by prodromal and asymptomatic stages that can last for two decades, where the formation of Aβ plaques and NFTs starts [2].

Formation and toxicity of Aβ species

AD pathology is dominantly explained by the "amyloid cascade hypothesis", which suggests Aβ as the initiator of all subsequent events that drive the progressive neuronal damage and cognitive dysfunction characteristic of the disease [5].

Aβ is a peptide produced in all neurons throughout life [2]. At physiological levels, Aβ is a normal, soluble product of neuronal metabolism, present in the brain of healthy people where it has important physiological functions, such as regulator of synaptic plasticity and enhancer of learning and memory, as well as antioxidant and metal chelator [6, 7]. Evidence indicates that synaptic activity directly evokes the release of Aβ at the synapse [6].

Aβ is produced through sequential proteolytic cleavage of amyloid-β protein precursor (AβPP), a type-1 transmembrane glycoprotein, by two enzymatic complexes: the β- and γ-secretases [7]. The function of AβPP remains unknown, although involvement in synaptic transmission and plasticity was hypothesized [8]. Recently, it was shown that full length AβPP has cell adhesion properties, mediating the stability of synaptic structures and maintaining adequate spine numbers [9]. Most of intracellular Aβ is distributed in the neuronal cytosol, colocalizing with different organelles dependent on where AβPP, β- and γ-secretases occur. It has been reported to be produced in the secretory pathway related organelles (endoplasmic reticulum, medial Golgi and trans-Golgi network), and to be associated with mitochondria [10].

AβPP can be processed through two enzymatic pathways: the amyloidogenic pathway and the non-amyloidogenic one. In the amyloidogenic pathway, the process of Aβ biogenesis, AβPP is first cleaved by the β-secretase, generating a soluble fragment from the N-terminal domain of AβPP and the β-cleaved C-terminal fragment, which is then processed by γ-secretase complex releasing the AβPP intracellular domain and Aβ [7]. Aβ is a heterogeneous mixture of peptides, composed of 37–43 amino acids, with different solubility, stability, biological and toxic properties [11]. The major isoforms of Aβ are peptides with 40 amino acids in length ($A\beta_{40}$), and 42 amino acid species ($A\beta_{42}$) [6]. $A\beta_{40}$ is continuously and abundantly produced in both healthy and AD-affected brain tissue, whereas other Aβ peptides are continuously produced at lower levels [11]. In physiological conditions, more than 90% of Aβ is in the form of $A\beta_{40}$, while less than 5% is generated as $A\beta_{42}$ [10]. The latter shows a higher propensity to aggregate into different states ranging from dimers to soluble oligomers to insoluble aggregates of fibrils that develop into plaques [6, 10]. However, one must not forget that aggregation and toxic properties were

mainly studied with solutions containing a single type of Aβ, and these properties are strongly influenced by the presence of other Aβ species [11]. Indeed, polymerization of Aβ is complex and occurs via a process called "nucleated conformational conversion", which involves several metastable intermediates. So, not only quantitative increases of individual Aβ peptides, but also qualitative changes in the spectrum of Aβ peptides, can determine the biophysical and pathological properties of Aβ [11].

In AD patients, $A\beta_{42}$ is present at a higher percentage and, in the classic view, its aggregation is considered the primary cause for the toxic Aβ effects, such as synaptic dysfunction, excitotoxicity, mitochondrial dysfunction, oxidative stress, and alteration of cell signaling pathways [7]. The most toxic forms are the high β-sheet structures, such as oligomers and fibrils [12, 13]. Thus, Aβ accumulation in the brain and its conformational changes are considered to play a pivotal role in the pathogenesis of AD [14]. At the diagnostic level, $A\beta_{42}/A\beta_{40}$ and $A\beta_{42}/A\beta_{38}$ ratios in the cerebrospinal fluid (CSF) are considered significantly better biomarkers for AD than $A\beta_{42}$ alone [15]. Indeed, decreased $A\beta_{42}/A\beta_{40}$ and $A\beta_{42}/A\beta_{38}$ ratios in CSF reflect AD pathology better, whereas decline in $A\beta_{42}$ alone is also associated with non-AD dementias [15].

The non-amyloidogenic pathway is the innate mechanism which is thought to antagonize Aβ generation, as AβPP is firstly proteolyzed by α-secretase within Aβ domain, generating big soluble AβPP fragments, further cleaved by γ-secretase, and producing non-toxic products [10].

It is well accepted that limiting the concentration of Aβ in the brain tissue, either by reducing Aβ production or enhancing Aβ clearance, is a putative way to inhibit the cascade of Aβ-induced pathological events [10]. Given the crucial role of β- and γ-secretases in Aβ production, manipulation of these secretases disturb the generation of Aβ [10]. Proteolytic degradation of Aβ into shorter soluble fragments, devoid of toxic effect, prevents Aβ aggregation or deposition into plaques. Lysosome-mediated endocytosis of Aβ also contributes to the clearance of toxic Aβ peptide and Aβ deposits [10].

In sporadic AD, the balance between the amyloidogenic and the non-amyloidogenic pathways becomes altered. There is an excessive production of Aβ, via the amyloidogenic pathway, and a lower degradation due to a decreased activity of the degrading enzymes, such as neprilysin, insulin-degrading enzyme, and angiotensin converting enzyme I, thus causing impaired clearance mechanisms of Aβ from the brain [16]. The familial AD form is caused by autosomal dominant mutations in genes related to AβPP processing, leading to Aβ overproduction [2].

Tau aggregation in NFTs

Human tau protein can display six isoforms, of 37–46 kDa, which result from alternative splicing of a 6 Kb mRNA transcript originated from the transcription of the Microtubule Associated Protein Tau gene, located on chromosome 17 [17]. Regardless of the isoform, this protein can be divided in four main domains: a N-terminal acidic projection domain; a proline-rich domain; a microtubule binding domain and, finally, a C-terminal tail [18].

Tau is a soluble protein acting as a microtubule stabilizer in neuronal cells [19]. It participates in the stabilization of microtubules, by binding to their surface, and fosters microtubules' self-assembly [18]. Although this is the most prominent function of tau in healthy brain cells, it has also been reported that tau interacts with constituents of the neural plasma membrane, regulates signal transduction, and can interact with DNA and RNA [20]. The hydrophilic quality renders tau to be a "natively unfolded" protein with high mobility and flexibility. However, it displays intradomain interactions forming a "paperclip" like shape where the N-terminal, the microtubule binding domain and the C-terminal are brought close to each other [21].

Tau can occur in several forms, such as monomers (highly soluble proteins of 55–74 kDa), dimers (composed of two tau monomers in anti-parallel orientation linked by disulfide bonds), small soluble oligomers, granular tau oligomers (composed of an average of 40 densely packed tau monomers) and filamentous forms (such as the paired helical filaments and the straight filaments) [22]. Under pathological conditions tau becomes hyperphosphorylated and eventually forms intracellular aggregates. Tau aggregation is a hallmark of AD and other tauopathies, such as some forms of frontotemporal dementia and Pick's disease, among others [22].

Tau function is regulated by a broad range of posttranslational modifications, including phosphorylation, acetylation, glycation, isomerization, nitration, SUMOylation, and ubiquitination, and changes in these modifications can affect tau functions and lead to pathological events [20].

Tau phosphorylation is highly increased in AD and has gain a particular interest for disease pathology.

The effect of increased phosphorylation varies depending on the site of phosphorylation, but overall, it reduces tau's affinity for microtubules leading to cytoskeleton destabilization [20]. Moreover, the tau-tau interaction site is coincident with the tau-microtubule interaction site, so when tau is detached from microtubules it tends to aggregate [18]. Therefore, tau is shielded from aggregation when bound to microtubules, but loses this protection when it is detached from them due to phosphorylation [18]. Self-aggregation of tau and further recruitment of more tau monomers and dimers creates a nucleation center that harbors the process of oligomerization [23]. Tau oligomers then acquire a β-sheet structure, with subsequent aggregation into fibrils, culminating in the formation of NFTs [19, 20]. As observed for Aβ, intermediate species generated during the formation of tangles, are the most toxic ones affecting neuronal and synaptic function [20]. Hence, aberrant hyperphosphorylation of tau leads to the loss of tau's biological activity, its dissociation from microtubules, and promotion of tau aggregation [20].

Tau is normally phosphorylated by regulation of both kinases and phosphatases [20]. Important tau kinases comprise the glycogen-synthase kinase-3β, cyclin-dependent protein kinase 5, cAMP-dependent protein kinase and stress-activated protein kinases, while protein phosphatase 2 has been highlighted as the most important tau phosphatase [24]. Tau kinase 5 was found to be upregulated in AD brain, whereas protein phosphatase 2 was downregulated. Moreover, decreased addition of a single N-acetyl-glucosamine residue to specific serine/threonine residues, a protein posttranslational modification known as O-GlcNAcylation, is observed in AD brains, and is correlated with tau hyperphosphorylation. The decreased O-GlcNAcylation may be caused by the compromised glucose metabolism observed in the AD brain [24]. Tau can also undergo proteolytic cleavage by caspases, generating products that not only are toxic for the cell, but also disturb the paperclip-like conformation of tau, triggering its aggregation [18].

In light of the above, it is crucial to understand how toxic tau species mediate dysfunction and degeneration in AD and other tauopathies [22].

NEUROINFLAMMATION

In many acute and chronic diseases of the CNS, the neural-centric vision fails to elucidate the mechanisms underlying disease onset and progression. Non-neuronal cells, such as astrocytes and microglia, which monitor brain parenchyma and are in constant communication with neurons, actively contribute to maintain brain homeostasis.

Definition

Neuroinflammation is defined as the reactive response of the CNS against elements that interfere with its homeostasis and this response is involved in all neurological diseases, including developmental, traumatic, ischemic, metabolic, infectious, toxic, neoplastic, and neurodegenerative diseases. Emerging evidence suggests that neuroinflammation is not only a consequence of neurodegenerative diseases, but also a risk factor for many of them [25, 26]. Thus, understanding and controlling neuroinflammation might be key for the prevention or delay of late onset CNS diseases.

Neuroinflammation is mediated by the production of cytokines, chemokines, reactive oxygen species (ROS), and secondary messengers. These mediators are mostly produced by activated microglia and astrocytes, the resident CNS immune cells, and by peripherally derived immune cells, in the case of increased blood-brain barrier (BBB) permeability. However, although microglia and astrocytes have a crucial role as effectors of neuroinflammation, other cells, including neurons, express receptors for cytokines and other inflammatory mediators and can therefore participate in the coordinated inflammatory response within the CNS [25, 26].

Neuroinflammation is often a term with negative connotation. However, the degree of neuroinflammation depends on the context, duration, and course of the primary stimulus or insult [27]. There is evidence of active microglia and production of cytokines in early brain development, providing support, and ensuring synaptic pruning within the CNS. Furthermore, neuroinflammation is a protective response essential for neural tissue repair after injury, infection or trauma [28]. It helps to eliminate the cause of injury, and can fix and prevent further tissue damage [29, 30]. Such degree of neuroinflammation influences cellular biochemistry, physiology, and development, and is beneficial [27]. On the other hand, chronic neuroinflammation, characterized by excessive and sustained neuroinflammatory events, is detrimental, perpetuates tissue injury and neural dysfunction and, ultimately, impairs neuronal regeneration [31–34].

Glial cells: key players in neuroinflammation

Although there is irrefutable evidence for a neurotoxic role of Aβ in AD, neuroinflammatory cascades and long-lasting activation of microglia and astrocytes promote alterations in the crosstalk between glial cells and neurons which, together with the sustained exposure of neurons to pro-inflammatory mediators, can cause neuronal dysfunction and contribute to cell death. Indeed, glia-based neuroinflammatory events driven by Aβ are nowadays considered a major contributing factor for the pathogenesis of AD [35–37]. Furthermore, activation of the NLRP3 inflammasome (NLRP3), a cytosolic multiprotein complex mediator of inflammation, is believed to have a leading role in the process [38–42].

In physiological conditions, the Aβ peptide is produced continuously, undergoes degradation and is transferred to the CSF and local blood vessels [13]. However, in pathological conditions there is an abnormal accumulation and consequent aggregation of Aβ in the brain, which leads to the formation of aggregates capable of triggering a chronic activation of the CNS immune system [13, 43].

Microglia

Microglia are the first line of defense of the brain's immune system [44], and due to this immunological function, they are considered the macrophage-like cells of the brain [45]. These cells originate from primitive macrophages that exist in the yolk sac and colonize the CNS very early in development [46]. In the normal physiological state, microglia surveil the diverse brain regions being involved in multiple developmental events, such as the establishment of neuronal circuits, synaptic pruning and remodeling, keeping the homeostasis and assuring the proper development and neuroprotection of the CNS [47]. As an immune cell, microglia act as sentinels, detecting the first signs of invasion of pathogens or tissue damage.

When microglia survey the microenvironment—resting state—they have a ramified appearance with multiple branches and rapidly extend and retract their processes [48]. In the case of injury or infection, microglia become activated, adopt different morphologies and produce inflammatory mediators [49]. Based on gene expression profiles, activated microglia can be divided into several different populations, with the M1 and M2 subtypes representing the extremes of the spectrum. Activated microglia is essential for the resolution of CNS damage, but under

a long-lasting over-activation microglia excessively produces cytotoxic factors that perpetuate neuroinflammation [50]. Besides that, reactive microglia stop the surveillance of synapses leading to a disruption of neuronal circuits [50].

Astrocytes

Astrocytes derive from the neuroectoderm lineage and are the most abundant cells in the CNS parenchyma playing an essential role in the tripartite synapse [51]. In addition to participating in the fine tuning of synaptic transmission, structural support, glutamate uptake, metabolism of neurotransmitters, regulation of extracellular pH and K^+ levels, synaptic pruning, and clearance of debris and dead cells [52], astrocytes are also of major importance for the maintenance of the BBB [53–55].

In the 21st century, astrocytes are increasingly viewed as having a pivotal role in neuroinflammation and as critical contributors to CNS pathologies [56]. Indeed, astrocytes also become reactive in response to noxious stimuli and, together with microglia, are important players in neuroinflammatory processes influencing aspects of inflammation and immune reactivity highly relevant to neurological diseases [57].

Reactive astrogliosis is a graded and non-homogeneous response that fluctuates according to the type, severity, time, and duration of the insult [54, 56, 58, 59]. It is defined as the process whereby astrocytes undergo changes in transcriptional regulation, as well as biochemical, morphological, metabolic, and physiological remodeling, leading to gain of new function(s) or loss/upregulation of homeostatic ones [56].

Reactive astrocytes undergo this remodeling in response to diverse pathologies and have the capacity to adopt distinct state(s), characterized by a specific molecular profile, specific functions, and distinct impact on diseases [56]. The spectrum of reactive astrocytes was recently considered to be between two extreme states: the neurotoxic A1, induced by neuroinflammatory insults, and the neuroprotective A2, associated with ischemia. However, these two distinct reactive states most likely co-exist, constituting a part of a heterogeneous population that contain both [56, 60, 61]. Thus, reactive astrogliosis can have a pro-inflammatory or an anti-inflammatory outcome, depending on the signaling pathways that prevail [40, 56]. Reactive astrogliosis and scar formation are essential for confining CNS inflammation to the lesion site [58]. However, under pathological

conditions, reactive astrocytes are known to aggravate and perpetuate inflammatory responses, producing an exacerbated amount of pro-inflammatory mediators [40]. Furthermore, it was shown that microglia can induce the neurotoxic phenotype in astrocytes through the secretion of three cytokines (Interleukin (IL)-1α, Tumor Necrosis Factor (TNF)-α, and Complement component 1q) [61], which prompts the hypothesis that astrocytes are indeed crucial in neuroinflammatory processes. However, whether astrocytes can directly respond to an insult, or if they always require a priming by microglia is still debated.

Inflammasomes

The innate immune system comprises pattern recognition receptors (PRRs) that trigger signaling cascades and promote gene transcription through: 1) Nuclear factor kappa B (NF-κB) and activator protein 1, driving proinflammatory cytokine/chemokine production, and 2) members of the interferon regulatory factor, which mediate type I interferon-dependent antiviral responses [62, 63].

PRRs can be divided into two major classes based on their subcellular localization. Toll-like receptors (TLRs) and C-type lectin receptors are transmembrane proteins found in the plasma membrane and endosomes, where they can survey pathogen-associated molecular patterns (PAMPs) and damaged-associated molecular patterns (DAMPs) in the extracellular milieu [62]. A second class of PRRs resides in the intracellular compartment and includes the nucleic-acid sensing PRRs, such as the RNA-sensing Retinoic acid-inducible gene I-like receptors, and the DNA-sensing DNA receptor Absent in Melanoma 2-like receptors [62]. Another set of intracellular PRRs, distinct from those mentioned above, are the nucleotide-binding domain and leucine-rich repeat-containing (NLR) receptors, that recognize a wide range of PAMPs and DAMPs [62].

A subset of PRRs assemble into caspase-1-activating platforms called "inflammasomes". These cytosolic multiprotein complexes were first described by Martinon and co-workers [64] and generally have three main components: the cytosolic PRR (the sensor domain), the adaptor domain and the cysteine protease pro-caspase-1 as the effector domain. Through canonical activation inflammasomes convert pro-caspase-1 into the catalytically active enzyme, and mediate immune responses through caspase-1, upstream the production of the pro-inflammatory

cytokines IL-1β and IL-18 [62, 65, 66]. They can be activated by a wide range of exogenous stimuli, such as lipopolysaccharides (LPS) and viral RNA [67], and also by endogenous signals, such as ROS [65], urate crystals [68], cholesterol crystals [69], and aggregated forms of Aβ peptide [70, 71] and α-synuclein [72].

The NLR family of inflammasomes has been highly explored. It is characterized by the presence of a central nucleotide-binding and oligomerization domain, which is commonly flanked by a C-terminal leucine-rich repeats and a N-terminal caspase recruitment (CARD) or pyrin (PYD) domains [62].

NLRP3 inflammasome

The NLRP3 (nucleotide-binding oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing 3) inflammasome is the most studied inflammasome in the CNS. It belongs to the NLR family, the sensor protein is the NLRP3 domain, and the adaptor protein is the apoptosis-associated speck-like protein (ASC) containing a CARD [40]. The NLRP3 domain itself is composed of three distinct subdomains: 1) a C-terminal leucine-rich repeat domain that mainly recognizes the activating signal and mediates the resulting interactions [73, 74]; 2) a central nucleotide-binding and oligomerization domain that has ATPase activity being necessary for inflammasome assembly [75]; and 3) a N-terminal PYD that enables homotypic interactions between NLRP3 and the adaptor ASC [76]. In turn, ASC is composed of two domains: a N-terminal PYD that binds to the N-terminal PYD of NLRP3 through a homotypic interaction resulting in ASC dimer assembly [77], and a C-terminal CARD that interacts with the CARD domain of the pro-caspase-1 [78].

Activation and consequent assembly of NLRP3 leads to proximity-induced autocatalysis of pro-caspase-1 producing mature caspase-1 which, in turn, cleaves the inactive pro-inflammatory cytokines—pro-IL-1β and pro-IL-18—in their secreted forms, IL-1β and IL-18, respectively [62, 65]. Caspase-1 also cleaves another substrate, Gasdermin D (GSDMD), generating a pore-forming fragment that translocates to the plasma membrane leading to a lytic form of cell death called pyroptosis (further detailed in a forthcoming section) [79].

The basal levels of NLRP3 domain in resting cells are considered not sufficient to activate the inflammasome, and it is nowadays accepted that the canonical NLRP3 activation requires two-signals: 1) a priming signal, which is provided by a NF-κB-activating

stimuli to transcriptionally enhance the expression of NLRP3 domain and pro-IL-1β; and 2) an activating signal that promotes the assembly of the complex [62, 65, 67, 80].

The priming step enhances the levels of NLRP3 and pro-IL-1β, but not of ASC, pro-caspase-1 and pro-IL-18 [81], and besides promoting this de novo protein synthesis, controls NLRP3 domain post-translational modifications [82]. The NLRP3 domain is, normally, in an ubiquitinated state that prevents its oligomerization, and the priming event stimulates its deubiquitination allowing for NLRP3 to oligomerize [83]. Thus, there are two types of priming: the transcriptional priming and the post-transcriptional priming, also denominated the non-transcriptional priming [83]. Priming signals can be provided by TLRs' ligands, as LPS, or endogenous molecules as TNF-α [84].

The second step is characterized by the assembly (oligomerization) and activation of the inflammasome, resulting in a ring-like multiprotein complex. A hallmark of inflammasome activation is the ASC speck, a micrometer-sized structure formed by filamentous clusters of the adaptor protein ASC [85]. ASC specks can be released and taken up by neighboring cells promoting ASC assembly in the recipient cells, and consequently providing another form of inflammasome amplification [86]. The numerous NLRP3-activating stimuli are highly different at the chemical and structural level, making the possibility of a direct interaction with each activator unlikely [67]. It was therefore assumed that NLRP3 either senses a common secondary signal downstream of these stimuli or responds to cellular stress associated with physiological damage [86]. ROS production, potassium (K^+) efflux, calcium (Ca^{2+}) signaling disturbance, and lysosomal rupture were proposed as upstream signals required for NLRP3 activation [65, 67, 86]. Recently, ceramide was also found to trigger NLRP3 activation in microglia [87].

Non-canonical NLRP3 activation involves the engagement of caspases-4/5 (in human) and caspase-11 (in murine) either directly by LPS or downstream of TLR4. In relation to caspase-1, these caspases display a lower affinity for pro-IL-1β and pro-IL-18, but a comparable affinity for GSDMD [63]. Initially caspases-4/5/11 activity promotes membrane pores without cytokine release. However, membrane pores induce ionic flux and subsequent NLRP3 assembly with caspase-1 activation. Thus, signaling by the non-canonical pathway indirectly leads to IL-1β and IL-18 secretion via caspase-1 [63, 88].

NLRP3 inflammasome regulated cytokines: IL-1β and IL-18

Cytokines are low molecular weight proteins or glycoproteins secreted by several cell types. As signaling molecules, cytokines provide communication between cells and play a crucial role in the modulation of the innate and adaptive immune response. Cytokines are classified into lymphokines (regulators of the immune response secreted by T cells), pro-inflammatory cytokines (which amplify and perpetuate the inflammatory process), anti-inflammatory cytokines (that negatively modulate the inflammatory process), growth factors (which promote cell survival) and chemokines (that coordinate cell migration) [89].

Of these, this review will focus on NLRP3 regulated cytokines IL-1β and IL-18, which have emerged as critical signaling pathways in the initiation and perpetuation of the inflammatory reactions in the CNS.

IL-1β and IL-18 bind to their respective receptors—IL-1R1 and IL-18R—on microglial cells, astrocytes, neurons, and endothelial cells, triggering NF-κB-dependent transcriptional events, which result in the generation of multiple inflammation-associated genes [90]. IL-1β is also associated with the integrity of the blood barriers modulating the infiltration of peripheral immune cells into the CNS and IL-18 regulates the function of interferon-γ in T cells and natural killer cells [90].

There is also evidence that increased IL-1β levels are implicated in the response to Aβ deposition [91] and high levels of IL-1β and IL-18 were found in the CSF, brain tissue and plasma of patients with CNS infections, brain injury and neurodegenerative diseases, such as AD [92]. These pro-inflammatory cytokines are also powerful regulators of synaptic function and have been shown to inhibit long-term potentiation, a form of neuronal plasticity believed to reproduce learning and memory [93].

It is worthwhile to emphasize that IL-1β and IL-18 maturation by caspase-1 is sufficient for slow, caspase-1/GSDMD-independent secretion of the active cytokines [94]. However, one cannot forget that caspase-1, besides processing the pro-IL-1β and pro-IL-18, also cleaves GSDMD generating membrane pores which boosts cytokine release, even in the absence of pyroptosis. Indeed, data suggest that cytokine secretion via GSDMD pores starts before cell rupture during pyroptosis [88]. When the membrane-repair mechanisms do not annihilate the GSDMD pore burden and the cell undergoes pyrop-

tosis, the whole intracellular content is released to the surroundings, including IL-1β and IL-18.

In the non-canonical inflammasome activation, caspase-4/5/11 cleaves GSDMD and the occurrence of membrane pores causes ionic flux that triggers NLRP3 assembly and caspase-1 activation, leading to caspase-1–dependent IL-1β/IL-18 processing with enhanced secretion through GSDMD pores [63, 88].

The role of the blood-brain barrier

The brain is an immunoprivileged site, due to the existence of the BBB, a structure localized at the interface between the blood and the cerebral tissue, that regulates the exchange of substances and blood cells in and out of the brain [55, 95]. The BBB is mainly composed of endothelial cells, pericytes and astrocytes, that create the neurovascular unit with the adjacent neurons, protecting the brain and maintaining its homeostasis [96]. BBB disruption involves phenotypical changes in endothelial cells and astrocytes and allow for blood cells to enter the brain parenchyma and engage in a crosstalk with brain cells, contributing to the modulation of the immune responses in the nervous tissue [91]. IL-1β signaling plays a major role in the initiation and continuation of the inflammatory reactions in the CNS in response to various adverse stimuli. High levels of IL-1β, and other inflammatory mediators, are deleterious to BBB integrity, which prompts the infiltration of peripheral immune cells into the brain upon a chronic neuroinflammatory context. These cells can enter the brain through the defective BBB, proliferate at the site of inflammation and further enhance neuroinflammation, either directly or through glial and neuronal cells [34].

Moreover, the inflammasomes, and more specifically the NLRP3 inflammasome, is a crucial component of the peripheral immune response. It was already reported that Aβ oligomers can directly interact with the NLRP3 domain and ASC, present in the infiltrated macrophages and cause inflammasome activation, further contributing to the neuroinflammatory milieu [97].

NLRP3 INFLAMMASOME IN AD

The association between pro-inflammatory cytokines, NLRP3 and AD was mentioned in several reports. In 1995, high concentrations of IL-1β and IL-6 were observed in the CSF of patients with AD [98]. Years later, a study pointed to the induction of

tau-hyperphosphorylation by high levels of IL-1β, aggravating AD pathogenesis [99]. In microglia cell cultures, NLRP3 was identified as a sensor for fibrillar Aβ in a process involving Aβ phagocytosis, lysosomal damage, and Cathepsin B release [70]. More recently, the *in vivo* relevance of the NLRP3 in AD was confirmed by Heneka and colleagues [38].

NLRP3 priming and activation by Aβ species

Several hypotheses on how Aβ species guides NLRP3 priming and activation in microglia are represented in Fig. 1.

One of the hypotheses relates to its role as a priming stimulus to NLRP3 (middle panel in Fig. 1). This happens by the postulated connection Aβ-CD36 [100], promoting the formation of a TLR4-TLR6 heterodimer, forming then a receptor complex composed by the previous three: CD36/TLR4/TLR6. This leads to the translocation of NF-κB to the nucleus, promoting the transcription of the NLRP3 domain of the inflammasome and pro-IL-1β [101]. Then, a second signal is needed to promote the oligomerization of the NLRP3 domain and assembly with ASC which recruits caspase-1 through its CARD, leading to the assembly of the inflammasome [78].

Regarding the Aβ-mediated NLRP3 activation, one mechanism that has gained increasing interest, is the phagocytosis of the peptide (right panel in Fig. 1). The peptide is incorporated in lysosomes promoting its destabilization, activation and content release, namely Cathepsin B, a lysosomal proteolytic enzyme [70]. The latter promotes the assembly of NLRP3, by a still unknown mechanism, and consequently, maturation and release of IL-1β and IL-18 [70]. Heneka's work [26] mentioned that NLRP3-ASC oligomerization pathway together with CD36/TLR4/TLR6 complex formation and lysosomal damage were necessary for aberrant host proteins to promote the assembly of NLRP3. Another possibility of Aβ-mediated activation of the NLRP3 complex involves the release of ATP by dying neurons which, in turn, activates the purinergic receptor P2X7 [102, 103], predominantly expressed by microglia and in lesser amounts by astrocytes [104], causing K^+ efflux and inducing the recruitment of the membrane pore pannexin-1 [105]. This mechanism postulates that extracellular molecules/components can enter the cell through the hemichannel pannexin-1 causing homeostasis alterations in the cytosol and activating NLRP3 [105]. To note that the expression
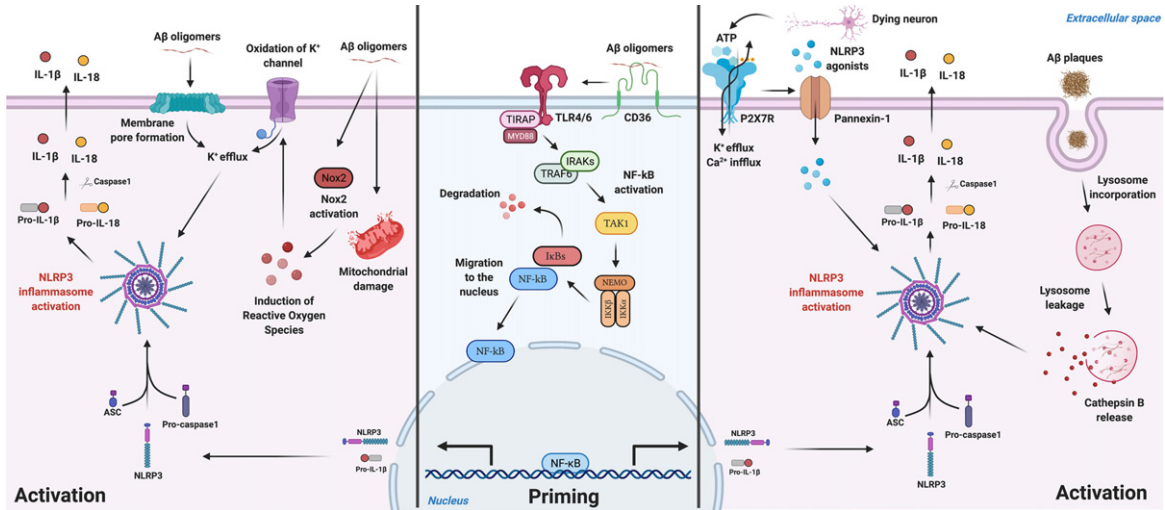
Fig. 1. Schematic illustration for Aβ-mediated NLRP3 inflammasome priming and activation mechanisms described in microglia. Aβ species can work either as a priming stimulus (middle panel) or as an activating stimulus (left and right panels). As a priming signal, Aβ oligomers bind to the CD36 surface receptor, triggering the formation of a TLR4-TLR6 heterodimer. This heterodimer activates a cascade of signaling molecules resulting in the activation and nucleus translocation of the transcription factor NF-kB, that promotes the transcription of NLRP3 domain and of pro-IL-1β. Aβ plaques can act as an activating signal by two main mechanisms (right panel). Aβ is known to cause synaptic dysfunction and neuronal damage. Considering this, P2X7R is activated by ATP released from dying neurons and recruits the Pannexin-1 channel that allows the entrance of NLRP3 agonists to the cell. Also, ATP binding to the purinergic receptor induces K^+ efflux and Ca^{2+} influx, known to promote NLRP3 activation. On the other hand, Aβ plaques can also be phagocytized and incorporated into lysosomes, boosting lysosomal destabilization and consequent content release. Cathepsin B, a lysosomal proteolytic enzyme, promotes the assembly of the inflammasome by a still unknown mechanism. Aβ oligomers are also able to activate NLRP3 through a mechanism that does not involve phagocytosis (left panel). Soluble oligomeric Aβ species can induce pore formation in the cell membrane and ROS production, which then promotes oxidation of K^+ channels. These events might culminate in K^+ efflux promoting the activation of the inflammasome. The assembly and activation of NLRP3, independently of the mechanism involved, ultimately results in the production and release of the inflammatory cytokines IL-1β and IL-18. Created with BioRender.com.

of P2X7 receptor is increased in human brains with AD [106].

It was also shown [107] that soluble oligomeric Aβ species were able to activate NLRP3 through a mechanism that does not involve phagocytosis of the peptide (left panel in Fig. 1). Soluble oligomeric Aβ species were described to induce pore formation in the cell membrane [108], and were shown to induce ROS production in microglia [109], which then promotes oxidation of K^+ channels [110]. These events might culminate in K^+ efflux promoting the activation of the inflammasome [107].

The sequence of events that culminate in the activation of the inflammasome are tightly regulated so that the inflammatory response proceeds its correct path. Some known regulators of the inflammasome are Double-stranded RNA-dependent protein kinase, Guanylate-Binding Protein 5 [111, 112], NLRP10 [113]. and Nek7 [114]. The last one is the best characterized member of the Never in Mitosis Gene A (NIMA)-related kinases (NEK proteins) and the way Nek7 regulates NLRP3 has been well studied and characterized, being recognized that K^+ efflux is

involved by promoting conformational changes in the inflammasome allowing the connection of Nek7 to the catalytic domain of the leucine rich repeat domain, thus regulating the activation and oligomerization of NLRP3 [114].

NLRP10 is a negative regulator of NLRP3 inflammasome, once it interacts with ASC, decreasing its availability and preventing ASC oligomerization with the NLRP3 domain [113, 115]. The work of Murphy and colleagues [115] showed that in LPS-primed mixed glial cultures treated with aggregated Aβ$_{42}$ and Aβ$_{40}$, the NLRP10 inflammasome is degraded allowing ASC to assembly with the NLRP3 domain. This degradation was proposed to be via phagocytosis and consequent release of Cathepsins. In this manner, ASC is free to associate with the NLRP3 domain and form a functional inflammasome.

The role of microglia

Neuroinflammation in AD is primarily driven by the resident microglia population. Microglia express the NLRP3 components, namely NLRP3 domain,

ASC and caspase-1 [116] and NLRP3 assembly, with the subsequent caspase-1 activation and IL-1β and IL-18 secretion, has been well explored in microglia. Several works point to a relevant role of these cells for AD progression through neuroinflammatory responses mediated by NLRP3 [38, 42, 70, 116, 117].

Under healthy conditions, microglia activation leads to engulfment and clearance of Aβ. However, prolonged exposure to Aβ species leads to a persistent priming and activation of NLRP3 in microglia [38] causing inflammasome components, such as the adaptor protein ASC, to be released to the extracellular space. There, Aβ clusters around exogenous ASC fibrils and forms ASC-Aβ composites which can boost NLRP3 activation in the surrounding microglia, reducing the capacity of microglia for Aβ clearance and leading to pyroptotic cell death. During pyroptosis, vast quantities of ASC are set free, starting the vicious cycle of ASC-Aβ composites all over [118, 119]. Upon Aβ accumulation, microglial cells become progressively impaired in Aβ clearance [120].

It is reported that microglia are recruited to plaques [121] and can phagocytose Aβ [117], starting an immunological response towards it [122]. When phagocytosed by microglial cells, Aβ induce lysosomal swelling and loss of lysosomal integrity, triggering the release of the lysosomal contents, as Cathepsin B, into the cytosol, that activates NLRP3 and initiates the IL-1β pathway in microglial cells. In fact, an increased expression of this cytokine was detected in microglia surrounding the Aβ plaques in patients with AD [117]. Released IL-1β is then capable of stimulating the production of other pro-inflammatory molecules [70], and the excessive production of these molecules may lead to neuronal damage, by activating harmful signaling cascades [123]. It was also reported that microglial cells are incapable of efficiently degrading fibrillar Aβ for a period of weeks after its internalization [124], which can be explained by the structural damage of Aβ-containing lysosomes mentioned above.

Heneka and co-workers [38] deeply assessed the involvement of NLRP3 in the Aβ-mediated inflammatory responses *in vivo*. These authors showed a significant increase in cleaved caspase-1 in the brain lysates from hippocampus and frontal cortex derived from AD patients. This increase, as well as an increase in IL-1β in the tissue, was replicated in the APP/PS1 mice model of AD, that carry mutations in AβPP and presenilin-1, together with the chronic deposition of Aβ, neuroinflammation, and cognitive impairment. Furthermore, NLRP3 or caspase-1 deficiency protected APP/PS1 mice from memory deficits, long-term potentiation suppression, and reduction in spine density in the pyramidal neurons, supporting a fundamental role of the NLRP3/caspase-1 axis in behavioral and cognitive dysfunction in AD. Additionally, APP/PS1 mice deficient for NLRP3 or caspase-1 revealed a considerable reduction in brain concentrations of aggregated forms of Aβ, further suggesting that NLRP3 acts through caspase-1 to exert the observed effects. It was also reported that NLRP3 activation negatively impacts the microglial Aβ clearance function in AD. Prolonged exposure to Aβ leads to persistent activation of microglial cells and it was observed that the M1 phenotype was reduced in NLRP3- or caspase-1-deficient APP/PS1 mice, while markers of M2-like microglia were increased. This led to the hypothesis that impairments in NLRP3/caspase-1 cascade promotes a switch of activated microglial cells toward an M2-like state, characterized by increased Aβ clearance and enhanced tissue remodeling.

The contribution of astrocytes

NF-kB pathway is associated with neuroinflammation, controlling cytokine production, and reactivity in glial cells, and it is strongly activated during neurodegeneration [125–127]. In animal models of AD, studies have demonstrated activation of this pathway in astrocytes, proving that these cells play a role in neuroinflammation in this context [122]. Furthermore, astrocytes express several proteases and receptors involved in the biogenesis of Aβ, which includes neprilysin, insulin-degrading enzyme and endothelin-converting enzyme 1/2, being involved in the degradation and clearance of the peptide [128, 129]. These cells are also the major producers, in the brain, of Apolipoprotein E, one of the major risk factors of late-onset AD [130]. Moreover, the neurotoxic phenotype of astrocytes is characterized by the upregulation of NF-kB and, consequently, of the complement component 3 (C3) [60, 131], that was shown to be significantly increased in the brain synapses and in the CSF of AD patients [132]. These cells secreted C3 in response to Aβ, which resulted in the shrinkage of some synapses and increased activity of others, process that was associated with NF-κB signaling [133]. Besides, C3 was shown to bind to C3a receptor in microglia, inhibiting microglia's ability to phagocytose Aβ [134].

Recently, several astrocytic receptors and transporters were proposed to be involved in the pathogenesis of AD and its targeting was considered a viable approach for treating this pathology [135].

It is not well established the neuroinflammatory response of astrocytes towards Aβ [130], but evidence suggests an Aβ-induced pro-inflammatory profile in astrocytes [136]. It was reported that, in the presence of Aβ, astrocytes adopt a phagocytic phenotype mediated by CD36, CD47 and RAGE [137]. In fact, the RAGE/NF-kB pathway seems to be an important contributor to the inflammation observed in AD [130]. NF-kB, as mentioned earlier, is involved in NLRP3 priming and, in rats treated with $Aβ_{1-42}$ oligomers, IL-1β was shown to be expressed in reactive astrocytes that surrounded the Aβ injection site [138]. Given the relevance of NLRP3 signaling in IL-1β maturation and release, it can be hypothesized that astrocytes' contribution to the inflammatory milieu observed in AD is NLRP3-dependent. However, this is not entirely clear, with studies stating opposite results regarding the expression of NLRP3 components and its function in these cells.

Primary cultures of astrocytes primed with LPS and other classical NLRP3 stimuli did not result in significant expression of NLRP3 domain, ASC and IL-1β, pointing to a lack of a functional inflammasome in astrocytes [116]. The authors postulated that this could be due to a weak expression of NLRP3 domain and to the lack of ASC in astrocytes. This observation was also corroborated in neurosphere-derived astrocytes [139]. This study tested the potential of $Aβ_{25-35}$ of inducing the expression of NLRP3 in astrocytes, obtaining similar results to the previous ones, and suggesting that astrocytes do not react to $Aβ_{25-35}$, at least not in *in vitro*. Also, IL-1β mRNA was not translated into protein by human astrocytes [139]. These authors have excluded the possibility of a rapid protein degradation rate and postulated the occurrence of a highly unusual repression of the translational mechanism.

However, in the transgenic Tg2576 mice that develops Aβ plaques in the aged brain, a strong expression of IL-1β in the reactive astrocytes that surrounded the Aβ deposits was shown [140]. Corroborating this study, others [141] demonstrated that astrocytes express all NLRP3 components (NLRP3, ASC, and caspase-1) and can produce IL-1β in several CNS lesions, like active demyelinating lesions of multiple sclerosis, active necrotic lesions of neuromyelitis optica, and acute necrotic lesions of cerebral infarction. The expression of all NLRP3

components was also confirmed in cortical astrocytes in a mouse model of traumatic brain injury [142]. Recently, Johann and colleagues [143] examined human samples of sporadic amyotrophic lateral sclerosis and found that human astrocytes were the major cell type expressing NLRP3 and ASC domains, inferring that astrocytes were involved in motoneuron degeneration via NLRP3 activation.

Altogether, the studies suggest that, as happen in other CNS pathologies (multiple sclerosis, traumatic brain injury, amyotrophic lateral sclerosis), the expression and activation of NLRP3 in astrocytes and the subsequent release of IL-1β and IL-18, might play a role in the neuroinflammatory milieu observed in AD, aggravating, and perpetuating the damage made by the primary line of defense in the CNS: the microglia. This controversial NLRP3 activation in astrocytes certainly deserves to be elucidated in the context of AD, both in animal models and human samples of the disease.

CELL DEATH BY NLRP3 INFLAMMASOME-MEDIATED PYROPTOSIS

Many physiological processes require cell death, as happen during embryonic development, and this mechanism occurs efficiently under normal conditions [144]. However, cell death is also the final solution for a cell when numerous stresses are accumulated up to a level beyond its recovery capacity, a situation depicted in neurodegenerative diseases [145].

For a long time, cell death was discussed dichotomously as either necrosis or apoptosis, and these were believed to be the two major death pathways for neurons in brain pathologies [145]. However, the knowledge of cell death has changed a lot throughout decades, and several other types of cell death were described, and associated to neurodegenerative diseases [145, 146]. Nowadays, the best-studied forms of programmed cell death are apoptosis, necroptosis, and pyroptosis [144].

Necrosis is defined as a passive, accidental cell death arising from environmental perturbations with uncontrolled release of cellular contents and thus highly inflammatory, while apoptosis is an active, programmed process of autonomous cellular disassembling that does not elicit inflammation, thus immunologically silent [144, 147].

Necroptosis is an alternative mode of regulated cell death that mimics features of apoptosis and necrosis [148]. Although TNF-α has long been considered an inducer of apoptosis, necroptosis also occurs following the activation of the TNF receptor-1 by TNF-α and requires protein receptor-interacting protein kinase 3 (RIPK3) [144, 148]. Multiple studies have focused on TNF-α, RIPK3, and caspase-8 to understand the molecular mechanism of necroptosis and it was shown that inhibition of caspase-8 shifts apoptosis to necroptosis due to activation of RIPK3 and its ligand [148]. Once activated, necroptosis triggers a process of cellular auto destruction, resulting in passive release of cytokines and other DAMPs to the extracellular space leading to robust inflammation [144, 148].

Pyroptosis is the newest form of programmed cell death inherently associated with inflammation. It was long regarded as caspase-1-mediated cell death but was redefined as a GSDMD-dependent and programmed necrosis [149]. Recent observations confirmed that the pyroptosis executioner, GSDMD, is also a substrate of inflammatory caspases-4/5/11 [150]. Gasdermins, a protein family bearing membrane pore-forming activity, were associated with various genetic diseases, but their cellular function and mechanism of activation, except for GSDMD, are still under study [150].

GSDMD is constitutionally auto-inhibited by the binding of the C-terminal repressor domain (GSDMD-CT) to the N-terminal pore-forming domain (GSDMD-NT). Full-length GSDMD is inactive, but inflammatory caspases relieve this inhibition by catalyzing the proteolytic cleavage of the interdomain loop and promoting the release of the pyroptosis inducer GSDMD-NT [149, 150]. GSDMD-NT can translocate to the inner leaflet of the plasma membrane (or the bacterial plasma membrane), where it binds with high specificity to selected phosphoinositides (or cardiolipin) and oligomerizes, generating membrane pores. The membrane repair mechanisms, through the endosomal sorting complexes required for transport machinery, first attempts to contain the damage inflicted by GSDMD pores. However, if the membrane repair mechanisms are not able to overcome the GSDMD pore burden in the plasma membrane, the cell will die by pyroptosis [88, 144]. The outer membrane of endosomes and phagosomes contain the same phospholipids, suggesting that GSDMD-NT may also bind to these organelles. To note, that because of its lipid-binding preferences, GSDMD-NT kills from within the cell, and does not

harm neighboring cells when it is released during pyroptosis [79, 150].

Summarizing, pyroptosis is a novel form of regulated cell death recently implicated in the pathogenesis of multiple neurological diseases [151, 152]. It is characterized by the formation of GSDMD-mediated membrane pores, downstream of canonical and non-canonical inflammasome activation [152]. The pore formation in the plasma membrane of pyroptotic cells is dependent on GSDMD cleavage by inflammatory caspases, resulting in the permeability of plasma membrane, water influx, cell swelling and osmotic lysis, prompting the release of pro-inflammatory cytokines and cytosolic content to the extracellular space [153, 154]. As happens in apoptosis, cells undergoing pyroptosis depict chromatin condensation and DNA fragmentation [153]. On the other hand, apoptosis is initiated by perturbations of the microenvironment, does not comprise osmotic lysis, involves packaging of cellular contents in apoptotic bodies, and caspase-3 is the main downstream executioner caspase that cleaves several other procaspases (as caspase-2, -6, -8 and -10) into their active forms, creating an apoptosis-amplifying cascade [144, 147, 152]. Furthermore, necrosis, necroptosis and pyroptosis result in the release of alarmins and other proinflammatory signals into the cellular surroundings, while apoptosis is considered "silent" and does not elicit subsequent immune responses [144]. Although cell death pathways have long been considered to act in parallel with little or no overlap, it is now clear that apoptosis, necroptosis and pyroptosis are closely related and can regulate each other [144].

NLRP3-mediated pyroptosis, mainly associated to microglia, was found to be critical in the pathogenesis of AD [42]. However, the expression of the inflammasome domains has also been reported in other cell types [41], including astrocytes, neurons, oligodendrocytes, and endothelial cells, implying that these cells can also undergo a NLRP3-mediated cell death by pyroptosis. Indeed, NLRP3 activation within dopaminergic neurons was recently proposed to contribute to neurodegeneration in a context of PD [155].

The impact of neuronal death by pyroptosis

AD is characterized by a progressive neuronal dysfunction, which, regardless of the contributing mechanisms, leads to neuronal death [145].

Neuronal death contributes to the neuroinflammatory milieu through the release of elements (ATP, pro-inflammatory cytokines, and other cytosolic components) that can recruit a NLRP3-mediated inflammatory response in neighboring glial cells and boost cytokine release. However, the mechanism under which NLRP3 activation may occur within neurons, in a context of AD, and the link between inflammasome activation in neurons and neuronal death by pyroptosis was not explored.

The next paragraphs argue that NLRP3-mediated pyroptosis in neurons may constitute a cell death mechanism that contributes to neurodegeneration in AD. The proposed intercellular communication among microglia, astrocytes, and neurons in the presence of Aβ species is illustrated in Fig. 2.

Tau–mediated neuronal pyroptosis

Extracellular accumulation of Aβ in senile plaques, together with the intraneuronal formation of NFTs, are considered hallmarks of AD, with plaque formation preceding NFTs. Although Aβ still is the major target for AD therapy, there is a growing focus on strategies aiming at other pathogenetic players in AD, as the tau protein [156].

Tau, the major component of NFTs, is a micro-tubule-associated protein mainly expressed in neurons. Under physiological conditions, tau is involved in polymerizing microtubules and maintain their stability. As aforementioned, tau function is regulated by a broad range of posttranslational modifications and changes in these modifications can affect tau functions and lead to pathological events [157].
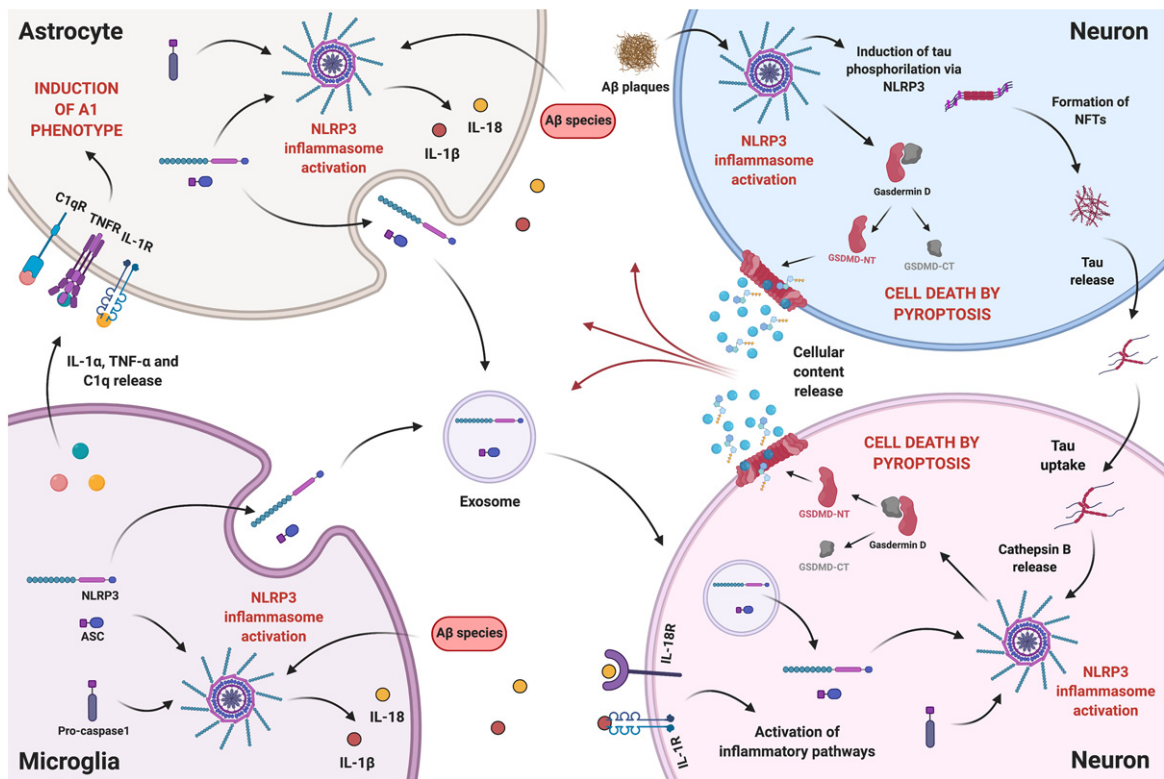

Fig. 2. The intercellular communication between neurons and glial cells in the presence of Aβ species is a complex and reciprocal process. Aβ-induced reactive microglia release IL-1α, TNF-α, and C1q, which can be received by resting astrocytes promoting the reactive A1 state. NLRP3 activation in reactive glial cells, boosts the release of pro-inflammatory cytokines IL-1β and IL-18, which can bind to their respective receptors in neurons activating inflammatory pathways. Reactive glial cells might also release NLRP3 components, namely NLRP3 domain and ASC, through exosomes that can be internalized by neurons. On the other hand, Aβ plaques can, in neighboring neurons, mediate the occurrence of pyroptosis and promote the development of NFTs, via NLRP3 activation. More importantly, tau can be released and taken up by other neurons, activating NLRP3 in the recipient cell, hypothetically through lysosomal destabilization and Cathepsin B release, and inducing pyroptosis. Therefore, proximity to Aβ plaques will not determine neuronal death by NLRP3-mediated pyroptosis, since, due to tau interneuronal propagation, pyroptosis can occur in neurons far from plaques. Ultimately, release of cellular content caused by pyroptosis will feed a self-propagating loop of neuroinflammatory mediators. Created with BioRender.com.

A cross-sectional neuropathological study showed that the tau pathology of AD typically spreads from one area to another along a neural network [157]. Masters and co-workers [2] suggested that NFTs extended into the dendrites, where tau could be released and taken up by other neurons. The mechanism underlying the stereotypical progression of NFTs in AD has not been clarified, but recent studies indicate that tau spreads by interneuronal transfer of the pathological form of tau, named "tau propagation". This process can be divided into three basic steps: 1) release of the intracellular pathological form of tau into the extracellular space by the donor cell, 2) uptake of tau by recipient cells, and 3) formation of new intracellular aggregates of tau in the recipient cells [157]. Passive leakage from degenerated cells and tau dissociation from tangles are likely to contribute to tau release into the extracellular space, although studies have suggested that active tau release could occur without neurodegeneration [157, 158]. Extracellular tau uptake can be mediated by endocytosis occurring on the cell surface, and studies have also suggested the involvement of exosomes in the mechanism of interneuronal tau transfer [157, 159, 160].

The association between Aβ and NLRP3 in microglia has been thoroughly explored over the years (this paper included) and is nowadays being extended to astrocytes. But the relationship between NLRP3 and tau was less examined. It was recently demonstrated that synthetic tau seeds, pre-aggregated tau fragments comprising the microtubule-binding domain of tau, induced IL-1β secretion in primed primary microglia, that was abolished by a specific NLRP3 inhibitor, indicating an inflammasome dependence [161]. These results were confirmed in TauP301S transgenic mice, fully proving the occurrence of tau-mediated NLRP3 activation. These authors further showed that tau seeds were incorporated by microglia and activated NLRP3 through lysosomal destabilization and Cathepsin B release. It was also described [2] that Aβ plaques induce tau aggregation in nearby neurons, mediating the development of NFTs by an unknown mechanism. Only recently, a study by Ising and co-workers [162] showed that NLRP3 acted as a link between Aβ plaques and NFTs formation. These authors demonstrated that the injection of Aβ-containing APP/PS1 brain homogenates induced tau hyperphosphorylation in Tau22 mice, but not in Tau22 mice deficient for NLRP3 or ASC, indicating that NLRP3 was an important mediator of Aβ-induced tau pathology.

Altogether, this work placed Aβ upstream NFTs formation.

We herein propose that Aβ plaques mediate, in nearby neurons, the occurrence of pyroptosis and promote the development of NFTs, via NLRP3 activation (Fig. 2, top right blue neuron). On the other hand, tau species can be released and taken up by other neurons, activating NLRP3 in the recipient cell (Fig. 2, bottom right pink neuron), through lysosomal destabilization and Cathepsin B release, as described for tau seeds incorporated by microglia [161], and thus promote pyroptosis. Therefore, it is reasonable to anticipate that, through tau interneuronal propagation, NLRP3-mediated pyroptosis can contribute to neurodegeneration in neurons far from Aβ plaques.

The contribution of extracellular vesicles for neuronal pyroptosis

Extracellular vesicles (EVs) are lipid bound vesicles secreted by most cells into the extracellular space. The three main subtypes of EVs are microvesicles, exosomes, and apoptotic bodies, with a diameter size typically ranging from 0.1–1 μm, 30–150 nm, and 50 nm–5 μm, respectively [163]. EVs provide a new type of intercellular communication, which transfer a box of information from a cell (either proteins, lipids, or nucleic acids) to another [164]. Their production changes dynamically in number and content in response to specific environmental signals, and their content, or cargo, is released into the cytoplasm of receiving cells, where it mediates several functional effects [163, 164].

In the nervous system, EVs participate in neuron-glial crosstalk, a bidirectional communication important to preserve brain homeostasis and, when dysfunctional, involved in several CNS diseases. The impact of this type of intercellular communication and its relevance at the clinical level is being strongly recognized by the scientific community [165].

Growing evidence correlates inflammasome activity with enhanced secretion of exosomes and modulation of their protein cargo [166]. This inflammasome-mediated secretion of proteins represents a novel paradigm in inflammatory responses [166], and has recently received some attention.

In the CNS, microglia, astrocytes, oligodendrocytes, and neurons have been reported to secrete exosomes into the extracellular space, and it was suggested that exosomes can carry bioactive cytokines, such as IL-1β, as well as inflammasome components [167]. Indeed, it was shown that NLRP1 inflammasome domains were present in exosomes derived

from CSF of spinal cord injury and traumatic brain-injured patients following trauma [168]. Also, Sarkar and co-workers [169] have shown that exposure to manganese, a transition metal linked to neurodegenerative disorders, such as AD and PD, activated NLRP3 in primed microglial cells *in vitro* and *in vivo*, with formation of ASC specks and enhanced exosomal release of ASC.

Given recent data [170] proving the occurrence of glia-to-neuron transfer of extracellular vesicles' cargo, it is herein proposed that NLRP3 activation in glial cells by Aβ species can induce an exacerbated release of exosomes, carrying inflammasome domains—NLRP3 and ASC—, which can be transferred to neurons contributing to the levels of NLRP3 components in the receiving cell (Fig. 2). Presuming the tau interneuronal propagation hypothesis, this will strengthen the impact of tau-induced NLRP3 activation in neurons, as explained above.

NLRP3 INHIBITION AS A POTENTIAL THERAPEUTIC TARGET IN AD

NLRP3 is, by far, the most studied and the best characterized inflammasome. Aberrant activation of this complex and IL-1β/IL-18 secretion has been detected in various diseases [40], including inflammatory diseases, diabetes, atherosclerosis, and neurodegenerative diseases, evoking a substantial clinical interest in exploring the effectiveness of its inhibitors.

Studies have uncovered various inhibitors of the NLRP3 cascade, which were validated through *in vitro* studies and *in vivo* experiments in animal models of diseases. Pharmacological approaches to inhibit the NLRP3 inflammasome, either directly or indirectly, were exhaustedly reviewed by Zahid and coworkers [171], such as blocking the expression of NLRP3, ASC, caspase-1, and neutralizing the expression of inflammatory cytokines (JC124), inhibiting NLRP3 ATPase activity (CY-09, MNS and OLT1177), abolishing NLRP3-Nek7 interaction (Oridonin), disturbing ASC oligomerization (Glyburide and BHB), and inhibiting caspase-1 activation (VX-740 and VX-765), among others.

Nowadays, NLRP3 is considered a highly promising therapeutic target for many diseases and NLRP3 inhibitors have caught the attention of many pharmaceutical companies [172]. Academic chemists are still struggling with NLRP3 biology, as it is a multiprotein complex that requires priming and assembly

before triggering downstream signaling, but even so a portfolio of NLRP3 inhibitors are already in preclinical and clinical trials [172].

The diarylsulfonylurea-containing small molecule, termed as MCC950, was found to block canonical and non-canonical NLRP3 activation with no effect on other inflammasomes [173]. Later, studies [174] indicated that MCC950 binds non-covalently to the Walker B motif within the NLRP3 domain, thereby blocking the ability of NLRP3 to hydrolyze ATP and inhibiting NLRP3 oligomerization and activation. Nowadays, MCC950 is considered a potent and selective NLRP3 inhibitor and chemists from many NLRP3-devoted companies used it as a starting point to design other molecules [172].

However, few studies have explored the effect of MCC950 within the context of AD. Dempsey and coworkers [175] showed that LPS-primed microglia were able to induce an increase in IL-1β release in the presence of ATP or Aβ, and that MCC950 attenuated the effect of both stimuli. Also, these authors demonstrated that inhibition of the inflammasome by MCC950 improved Aβ phagocytosis by cultured microglia. These results were corroborated in the APP/PS1 mouse model of AD, where Aβ-containing plaques were significantly reduced after MCC950 treatment, indicating an increased Aβ phagocytosis as happened *in vitro*. Overall, it was shown that MCC950 was able to reduce Aβ-guided pathologic events and enhance cognitive function. Also, chronic intracerebral administration of MCC950 in TauP301S transgenic mice was shown to inhibit exogenously seeded tau pathology [161].

Thus, MCC950 and MCC950-based compounds are a promising therapeutic approach in AD and should be thoroughly examined in various *in vitro* and *in vivo* models to fully understand their value in this disease.

SUMMARY

The so-called amyloid cascade hypothesis [176], which postulates that the accumulation and aggregation of Aβ, the main component of senile plaques, in the brain parenchyma is the initiator of the subsequent events that lead to AD, has dictated and guided much of the academic and pharmaceutical research over the past two decades. Fundamental knowledge on the role of Aβ in this disease arise from studies that focused on how Aβ forms senile plaques and how these abnormal structures induce neurodegeneration
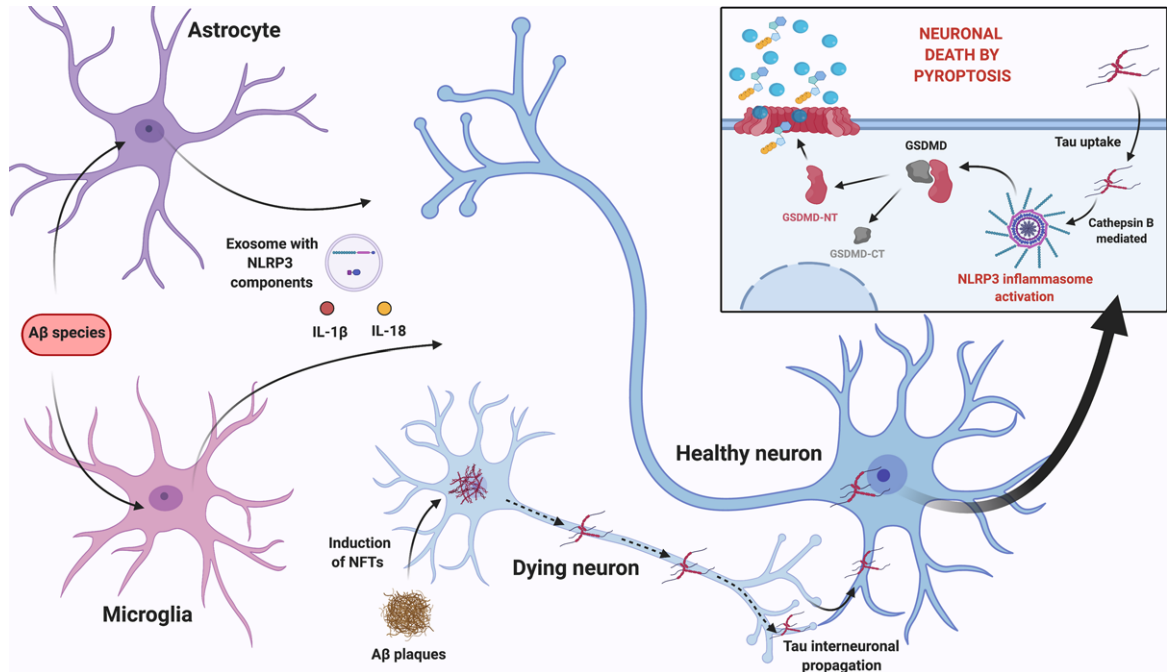
Fig. 3. Proposed multicellular feedback loop that feeds on NLRP3 inflammasome activation in glial cells and neurons. Aβ-induced NLRP3 activation in glial cells, prompts the release of pro-inflammatory cytokines, as well as exosomal release of NLRP3 domain and ASC. This neuroinflammatory milieu sums up to the neurotoxic effect of Aβ plaques in nearby neurons, which induce tau destabilization and NFTs formation, via NLRP3 activation. According to tau interneuronal propagation hypothesis, tau is then released and taken up by neighboring healthy neurons activating NLRP3 in these cells, most likely through lysosomal release of Cathepsin B, causing neuronal death by pyroptosis with cellular content release and perpetuating glial cells' activation. Created with BioRender.com.

and neuronal loss [177, 178]. However, pharmaceutical drugs against Aβ failed in various phases of clinical trials, and the idea that tau, and not only Aβ, can be a relevant factor underlying the development and progression of AD, has emerged [156, 179].

Furthermore, neuroinflammation and inflammatory mediators are known to be involved in CNS diseases, and research on the interaction between inflammatory pathways, triggered in glial cells via NLRP3 activation, and disease progression, has recently boosted in AD.

Microglia is the "maestro" of the CNS immune response and was always at the core of Aβ-induced NLRP3 activation. Indeed, the mechanisms of NLRP3-mediated immune response triggered by Aβ species were deeply highlighted in microglia and only recently these studies were extended to astrocytes.

After reviewing the most recent data on this subject, we propose that the neuroinflammatory milieu arising from Aβ-induced NLRP3 activation in glial cells, prompts the release of pro-inflammatory cytokines and the exosomal release of NLRP3 domain and ASC. We suggest that these events can sum up to the neurotoxic effect of insoluble Aβ

plaques that mediate the occurrence of pyroptosis and promote the development of NFTs, via NLRP3 activation. More importantly, considering the occurrence of tau interneuronal propagation, released tau can be taken up by neighboring neurons and activate NLRP3 in recipient cells causing neuronal death by pyroptosis. This multicellular feedback loop, summarized in Fig. 3, feeds on inflammasome activation in glial cells and neurons.

CONCLUSION

NLRP3 targeting was recently proposed as a therapeutic approach for AD [180]. Based on all the topics reviewed in this paper, we hereby claim that NLRP3 inhibition, due to its starring role in Aβ- and tau-driven pathological events, is undoubtfully a promising strategy for the development of therapies for this disease.

ABBREVIATIONS

Aβ, amyloid-β; AβPP, amyloid-β precursor protein; AD, Alzheimer's disease; ASC, apoptosis-

associated speck-like protein; ATP, adenosine triphosphate; BBB, blood brain barrier; CARD, caspase activating and recruitment domain; CNS, central nervous system; CSF, cerebrospinal fluid; C3, complement component 3; EVs, extracellular vesicles; GSDMD, gasdermin D; GSDMD-CT, C-terminal fragment of GSDMD; GSDMD-NT, N-terminal fragment of GSDMD; IL, Interleukin; LPS, lipopolysaccharides; NF-κB, Nuclear factor kappa B; NFTs, neurofibrillary tangles; NLRP3, NLRP3 inflammasome; PD, Parkinson's disease; PRR, pattern recognition receptor; PYD, pyrin domain; RIPK3, receptor-interacting protein kinase 3; ROS, reactive oxygen species; TLR, Toll-Like Receptor; TNF, Tumour Necrosis Factor

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Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/21-0268r1).

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