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Review

Regulated intramembrane proteolysis, innate immunity and therapeutic targets in Alzheimer's disease

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Abstract: The critical discovery of the presenilins and their association with familial Alzheimer's disease (AD) prompted an intensive research effort to understand the molecular mechanisms of that disease. The presenilins were subsequently found to be the catalytic component of the multi-protein enzyme complex, γ-secretase, the enzyme that is known to act on the amyloid precursor protein (APP) to generate amyloid beta (Aβ) peptides that comprise the neuritic plaques implicated in AD pathology. Here, we discuss the background of γ-secretase- mediated proteolysis of APP and its association with familial AD. We discuss the association of neuroinflammation with AD, focusing on the link between the innate immune response, the clearance of the Aβ peptides and disease progression. Currently, there are limited treatments for AD that strive to ameliorate the symptoms of the disease but do not address the molecular basis of the disease. The greater understanding of γ-secretase functions has provided new insights into potential therapeutics for AD, a number of which are in clinical trials.

Keywords: Presenilin; gamma-secretase (γ-secretase); regulated intramembrane proteolysis; Alzheimer's disease (AD); innate immune system; neuroinflammation

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; APH-1, anterior pharynx-defective 1; CNS, central nervous system; CTD, carboxyl terminal domain; CCR2, chemokine (C-C motif) receptor 2; CSF-1, colony stimulating factor-1; COX, cyclooxygenase; ErbB4, epidermal growth factor receptor; FDA, federal drug administration; FAD, familial Alzheimer's disease; GSK-3β, glycogen synthase kinase 3β; GWAS, genome-wide

association studies; I-CLiPs, intramembrane-cleaving proteases; IL-1R1, interleukin-1 receptor, type I; IL-1RII, interleukin-1 receptor, type II; interleukin; IFN, interferon; ICD, intracellular domain; LPS, lipopolysaccharide; MCP-1/CCL2, monocytes chemotactic protein-1; MCI, mild cognitively impaired; MAC, membrane attack complex; NFTs, neurofibrillary tangles; NMDA, N-methyl-D-aspartate; NSAIDs, non-steroidal anti-inflammatory drugs; PEN-2, presenilin enhancer 2; $p75^{NTR}$, p75 neurotrophin receptor; PS1, presenilin-1; PS2, presenilin-2; RIP, regulated intramembrane proteolysis; SLE, systemic lupus erythrematosis; SPP, signal peptide peptidase; TNFα, tumour necrosis factor-α; TNFR-I, tumour necrosis factor receptor type I; TLR, Toll-like receptor; TMD, transmembrane domain.

1. Introduction

Alzheimer's disease (AD) is an age-related dementia that is characterized by the presence of amyloid protein (Aβ) plaques formed from the cleavage of the amyloid precursor protein (APP), formation of neurofibrillary tangles (NFTs), neuroinflammation and neuronal loss. The cleavage of APP is a sequential two-step process, with the initial proteolysis by α- or β-secretase (BACE) releasing the APP ectodomain into the extracellular milieu (Figure 1). This is followed by a second cleavage event of the membrane-anchored carboxyl-terminal domains of APP by the γ-secretase proteases [1], generating an APP intracellular domain (AICD) and Aβ peptides of various sizes. Mutations in two related genes, *PSEN1* and *PSEN2*, are associated with familial AD [2], and these genes were subsequently found to encode the highly homologous presenilin (PS1 and PS2) proteins, respectively [3]. Mutations in PS1 are associated with amyloidogenesis in AD from the outset, in particular with production of the longer cleavage product of APP, Aβ42 [3].

According to the amyloid hypothesis, aberrant accumulation or defective clearance of the Aβ peptides leads to plaque formation and the onset of AD pathology, reviewed in [4]. While the Aβ hypothesis is not the only mechanism proposed to explain the pathology of AD, it is clear that there is a role for the Aβ peptides in the disease and this is likely due to detrimental effects on neuronal survival as a result of immune responsiveness or disturbances in calcium signalling, reviewed in [5,6]. In addition, there is evidence that the presence of Aβ peptides could be causally linked to the formation of NFTs via activation of apoptotic signalling pathways [7]. Thus, the presence of excess Aβ peptides could lead to the formation of the NFTs, providing a causal link between these two hallmarks of AD pathogenesis and supporting the Aβ hypothesis. Since the accumulation of aggregated Aβ, generated as a result of γ-secretase activities, is associated with the pathogenesis and progression of disease, both the γ-secretase proteases and Aβ peptides serve as points of entry in the development of AD therapeutics. In this review, we discuss the γ-secretase complexes and highlight the emerging importance of the immune system in the pathogenesis of AD. The challenges of designing therapeutics to γ-secretase due to the plethora of substrates other than APP are discussed, along with promising new immunotherapies to the Aβ peptide that are in clinical trials.

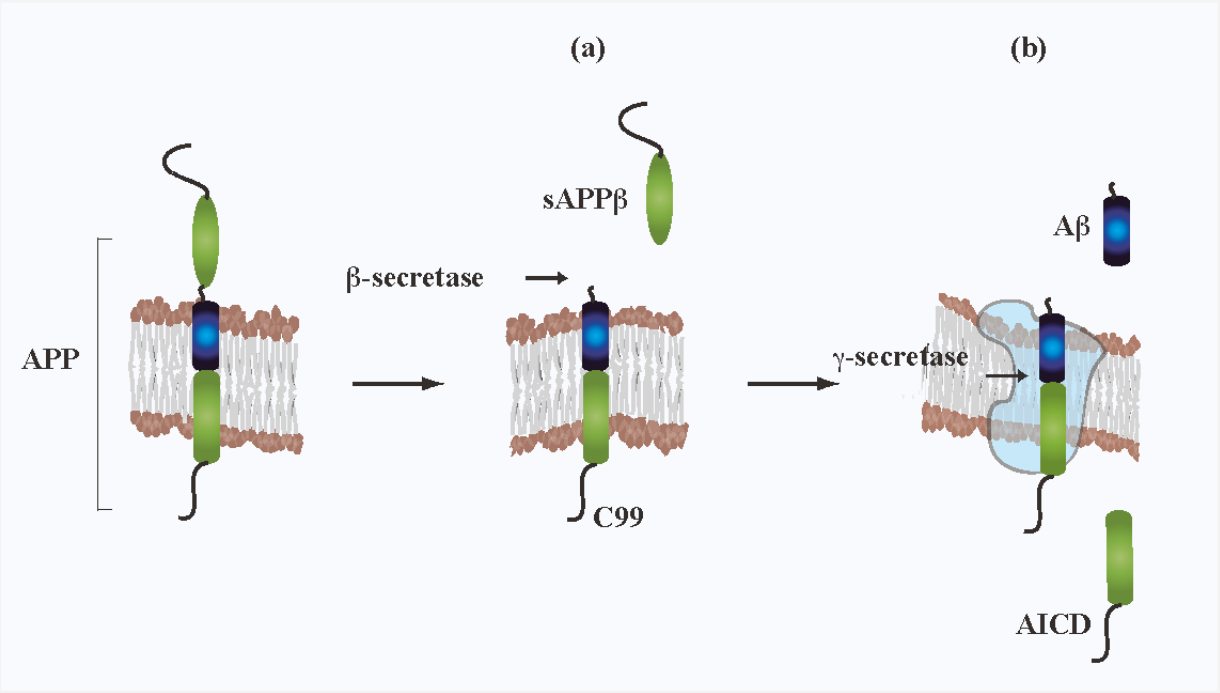

Figure 1. Regulated intramembrane proteolysis of APP by ectodomain shedding and γ-secretase cleavage. In this model, the progressive proteolytic cleavage of amyloid precursor protein (APP) is illustrated in which (a) the action of the sheddase, β-secretase, releases the soluble ectodomain (sAPPβ) and the membrane-anchored C99 carboxyl-terminal domain; and then (b) the γ-secretase complex is recruited to the APP C99 membrane stub and cleaves to liberate the APP intracellular domain (AICD) and Aβ peptide fragments.

2. Pathogenesis of Alzheimer's disease

The majority of AD cases are considered to be sporadic, with a non-Mendelian pattern of inheritance and a complex etiology [8,9]. However, genetic risk factors causing early- and late-onset FAD are well documented, where mutations in *APP* and both *PS1* and PS2 are strongly associated with early-onset FAD [2]. In addition to several risk factors, including aging and prior traumatic brain injury, inheritable polymorphisms are causally linked to AD pathogenesis, as reviewed in [8,9]. The search for susceptibility loci makes use of population studies, such as genome-wide association studies (GWAS) and more recent, exome and genome sequencing studies to identify common genetic variants associated with sporadic late-onset AD, reviewed elsewhere [8-10]. These studies have helped identify other loci that have a strong, weak or rare AD risk-association, and have also helped to delineate some biological processes that may be involved in the pathogenesis of AD, including the innate immune system and inflammation, cholesterol metabolism and endosomal vesicle recycling [10,11]. So far, such studies have identified the ε4-allele of the apolipoprotein E (*APOE)* gene, *CLU* (which encodes clusterin), the sorting protein-related receptor gene (*SORL1*), *PICALM* (also known as *CALM*) encodes phosphatidylinositol-binding clathrin assembly protein, which is involved in clathrin-mediated endocytosis) and *CR1* (which encodes the major receptor of C3b, a protein involved in complement activation) as a critical risk factor in sporadic late-onset AD [8,11]. It

is therefore possible that the production of and response to Aβ peptides is associated with both sporadic and familial occurrences of AD.

Much more progress has been made in understanding the autosomal dominant inheritance of mutations in the presenilin genes that are associated with early-onset familial AD [2]. While this represents only a small proportion of AD patients, the research into FAD mutations has elucidated the molecular mechanisms of Aβ peptide production and Aβ peptides appear to be a common feature of both sporadic and familial AD. Although much is now known about the molecular mechanisms of Aβ production, the initial factor that leads to the release and subsequent accumulation of these peptides is not fully understood, although a genetic predisposition for aberrant APP cleavage is indicated. One hypothesis is that infections with viruses that replicate within cells of the central nervous system (CNS) could be a danger signal that triggers the onset of AD. Herpes viruses are able to remain latent in neurons for years until viral replication is reactivated by changes in the brain, such as those due to aging, reviewed in [12]. Reactivation of latent herpes simplex virus 1 (HSV1) has been found to cause local inflammation and neurotoxicity, as well as accumulation of Aβ [13], and HSV1 DNA is associated with Aβ plaques in AD patients [14]. HSV-1 infection has also been found to alter autophagosomal processing of Aβ and to inhibit the non-amyloidogenic pathway of APP cleavage [15]. Interestingly, incidences of sporadic AD were found to be associated with a cluster of genes in a GWAS [12] and this association was confirmed by meta-analyses of other GWASs [16]. The genes identified by these analyses were all known to be associated with viral infection and replication, particularly with that of herpes viruses, reviewed in [12,17]. There is thus some evidence for an association between reactivation of latent viruses and AD, underscoring the importance of the immune response in the pathogenesis of this disease.

3. The γ-secretase protease complex

The γ-secretase protease is a multi-protein complex consisting of PS1 or PS2, nicastrin, anterior pharynx defective-1 (APH-1) and presenilin enhancer-2 (PEN-2), reviewed in [18]. The unconditional requirement for each of these four integral membrane proteins for γ-secretase activity was verified following genetic ablation or RNAi knockdown of one or the other of the components, as well as by genetic reconstitution of γ-secretase activity in *Saccharomyces cerevisiae*, which lacks endogenous γ-secretase, [19]. All four proteins associate with each other and their co-expression resulted in increased γ-secretase activity in mammalian cells [20], *Drosophila* and *S. cerevisiae,* reviewed in [18,20,21]. The multi-protein identity of the γ-secretase proteases was subsequently corroborated by several studies reporting the purification of the active γ-secretase protease complexes [22-24]. Of these γ- secretase components, several variants exist of the presenilins (PS1 and PS2) and APH1 due to multiple genes and to alternative splicing thereof. In all species examined, there are two *PSEN* genes (*PSEN1* and *PSEN2*); while in humans there are also two *Aph-1* genes, *Aph-1a* and *Aph-1b*, which are alternatively spliced [25]. Further, in rodents, gene duplication of *Aph-1b* produces a third gene, Aph-1c [25]. Furthermore, it has now been demonstrated that multiple combinations of the four proteins can exist, depending upon which *PSEN* and *APH1* gene product is included therein, and that γ-secretase complexes have different functions based on their precise co-factor composition since the gene products of *PSEN* and *APH1* are not redundant [24-26].

While the presenilins were mainly examined in the context of APP cleavage and AD pathogenesis, they have been found to have many substrates other than APP, as well as biological functions independent of γ- secretase, reviewed elsewhere [27-29]. It is becoming apparent that the mechanism of cleavage of transmembrane proteins by membrane-embedded enzymes like γ-secretase is widely-employed in cell biology, with such enzymes classed as intramembrane-cleaving proteases or I-CLiPs, as reviewed in [30]. Further, this sequential proteolysis is now called regulated intramembrane proteolysis, and has emerged as a highly conserved signalling system that involves the cleavage of certain type I transmembrane proteins, reviewed in [31]. This mechanism allows the direct transfer of an extracellular signal into the cytoplasm or nucleus via the production of functional intracellular domains (ICDs), several of which have been found to regulate gene expression [32]. This novel mechanism is distinct from the classic membrane-to-nucleus signalling model via a cascade of post-translational modifications and intracellular messengers. To date, over 100 different γ-secretase substrates have been identified, including several cytokines and cytokine receptors TNFR1[33], IL-1R1 [34], IL-1R2 [35], IL-6R [36], CX3CL1 and CXCL16 [37], indicating a generic role in the regulation of receptor-mediated signalling pathways and highlighting the importance of γ- secretase in the regulation of growth factor and cytokine signalling, reviewed in [29].

4. γ-secretase cleavage in immune regulation

The link between γ-secretase and the immune system has been demonstrated by the phenotypic characterization of *in vivo* models of presenilin deletion or mutation. Since deletion of *PSEN1* is embryonic lethal [38], transgenic mouse models have been developed to circumvent this effect, including *PS1* null mice that have been rescued with a *PS1* transgene [39] and brain region-specific gene knockout techniques based on the Cre/LoxP system [40-42]. In addition, the selective knockout of one or more alleles of the presenilins, with the retention of at least one functional copy, has been used since the early days of presenilin research to define its function with the $Psen1^{+/-}Psen2^{-/-}$ mouse having the most severe reduction in presenilin alleles that permits post-natal survival [43]. The phenotype of $Psen1^{+/-}Psen2^{-/-}$ "partial deficient" mice is normal up to approximately six months, when the majority of the mice develop skin and autoimmune defects similar to those observed in systemic lupus erythematosus (SLE) [44]. Thus, PS1 deficiency is associated with an abnormal immune response and the development of an autoimmune phenotype. The requirement for the presenilins in adaptive immune system function has since been demonstrated in lymphocytes [45-47]. Deficiency in presenilins antagonized T-cell homeostasis and signalling [45,46], while presenilin-deficient B-cells were defective in responsiveness to LPS and B-cell receptor-induced proliferation and signal transduction [47]. From these studies, it is clear that the presenilins have essential functions in the immune system in general, outside of the CNS. Within the brain, studies using conditional knockouts of the presenilins identified a role for it in negatively regulating the inflammatory response mediated by microglia [48,49]. Further, it was found that PS2, rather than PS1, was critical in inhibiting microglia-mediated inflammation, with IFNγ selectively up-regulating PS2 expression and with PS2 deficiency resulting in enhanced cytokine expression [49]. Interestingly, it has recently been found that TLR activation in macrophages regulates the γ-secretase-mediated cleavage of the colony-stimulating factor 1 (CSF-1) receptor, which is required for monocyte/macrophage

differentiation [50]. Although this work was done in macrophages, a similar system could be in operation in microglia and the activation of TLRs in response to Aβ peptides could lead to microglial activation via promotion of CSF-1 receptor proteolysis by TACE and γ- secretase. From the data, a model is emerging whereby the functions of TLRs and γ-secretase in inflammation and microglial activation combine to regulate the inflammatory response in the brain in general and to Aβ peptides in particular. Dysfunction of this system could be fundamental to the pathophysiology of AD.

Further to this, γ-secretase-mediated cleavage of innate immune receptors has recently been shown to be important in cytokine signalling. The pro-inflammatory cytokine IL-1 and its receptors, IL-1RI and IL-1RII, have long been associated with AD [51]. While IL-1RI is essential for signal transduction to NF-κB activation following IL-1 binding, IL-1RII is a decoy receptor that binds to excess ligand and does not transmit a signal, as reviewed in [52]. Both of these receptors can be found in a membrane-bound and soluble form, both forms of which are known to have biological functions [52]. In an early study, elevated levels of the soluble form of IL-1RII were found in the cerebrospinal fluid of AD patients [51]. Thus, the finding that IL-1RII was subject to cleavage by α- and β- secretase followed by intramembrane proteolysis by γ-secretase [35] fits with the observed elevation of soluble Il-1RII in AD [51]. Work in our laboratory has shown that the type I IL-1R was also subject to cleavage by metalloproteases and γ-secretase [34]. We demonstrated that the IL-1RI ectodomain could be cleaved constitutively and that this cleavage was enhanced by activation of metalloproteases as well as exogenous addition of IL-1 [34]. Ectodomain shedding released a soluble IL-1RI into the extracellular milieu, while the remaining membrane-bound CTD was a substrate for further cleavage by γ-secretase to liberate an IL-1RI ICD into the cytoplasm. Importantly, we demonstrated that inhibition of γ-secretase activity antagonized IL-1-induced cytokine secretion, suggesting that γ-secretase regulated cytokine responsiveness [34].

The effect of the presenilins on IL-1 signalling could be mediated by its interaction with downstream signalling molecules. The over-expression of TNF receptor-associated factor 6 (TRAF6) or interleukin-1 receptor-associated kinase 2 (IRAK2) enhanced the cleavage of IL-1RI, suggesting that these adaptors up-regulated γ-secretase activity [34,53]. This observation is consistent with the finding that TRAF6 and IRAK2 are also novel interaction partners for the presenilins [34,54]. In addition to the effects on signalling adaptors and cascades, the γ-secretase cleavage of IL-1RI could directly affect gene expression. As with other γ-secretase substrates, it is feasible that the ICD of IL-1RI could act as a transcription factor. There are nuclear localization signals within the ICD and IL-1RI has previously been reported to localize to the nucleus [55]. It thus remains to be determined if the induction of cytokine gene expression following IL-1 treatment is due, in part, to ICD transactivation as well as to the usual signal transduction pathway. IL-1 signalling is complex, with numerous regulatory mechanisms, and it is possible that proteolysis of the receptors following ligand binding simply adds another level of regulation to this system. In addition to IL-1R, other cytokine receptors could be regulated by γ- secretase. There is evidence that TNFR1 and TNFR2 are subject to ectodomain shedding [56] and we recently demonstrated that TNFR1 is also cleaved by γ-secretase [33]. The physiological role of the presenilins in signalling pathways downstream of IL-1R and TNFR1 is not yet fully known but it is feasible that it acts as a scaffold or chaperone for adaptor proteins facilitating the spatial segregation of divergent signalling events arising from each receptor. Several lines of evidence

suggest a role for the presenilins in trafficking within the cell and the presenilins are known to localize to the endosome [57,58]. Thus, it is possible that the presenilins help to recruit adaptors to such organelles and this could be critical for signal transduction given the endosomal localization of certain innate immune receptors, such as the TLRs in the phagosomes of microglia.

In addition to TNFR1, IL-1RI and IL1-RII, several additional γ-secretase substrates are receptors with important biological functions in the immune system. The type I interferons (IFNs) bind surface receptors (IFNARs) and induce a signalling cascade via the Janus kinase (JAK)-signal-transducer and activator of transcription [59] pathway to induce IFN-stimulated gene (ISG) expression, reviewed in [60]. At least one IFN receptor, IFNAR2, was found to be proteolytically cleaved by γ-secretase and the ICD was shown to repress gene transcription [61]. In addition to receptors, regulated intramembrane proteolysis of other cell surface transmembrane proteins has been described. There are a number of chemokines that are expressed in a transmembrane form, including CX3CL1 (fractalkine) and CXCL16, which are cleaved by α-secretase and γ-secretase [37]. Ectodomain shedding has been found to be required for chemokine recruitment of immune cells and in microglial activation, as reviewed in [62]. The mucin-type sialoglycoprotein, leukosialin or CD43, was cleaved during neutrophil activation in a γ-secretase-dependent manner and generation of the ICDs was found to be required for neutrophil adhesion [63]. Unlike in previous instances of regulated intramembrane proteolysis, CD43 is first cleaved by cathepsin G, not a metalloprotease [63]. The γ-secretase-generated CD44 ICD has been shown to localize to the nucleus of macrophages in which it promotes the activation of NF-κB and macrophage fusion [64]. Major histocompatibility class I (MHC I) proteins are found on most nucleated cells and are essential in many immune processes, including antigen presentation to T cells [65]. An MHC class I protein, human leukocyte antigen-A2 (HLA-A2) was found to be cleaved by α-secretase and then by γ-secretase [65]. Unlike other ICDs generated by γ-secretase cleavage, the HLA-A2 ICD was very unstable and rapidly degraded [65], supporting the hypothesis that regulated intramembrane proteolysis of some transmembrane proteins could serve to clear them from the membrane rather than in signal transduction. There is evidence that both ectodomain shedding and γ-secretase cleavage are critical in normal immune system function.

5. Innate immune signalling in Alzheimer's disease

5.1. Complement activation in AD

Although amyloid plaques in the brains of AD patients are primarily composed of Aβ, other molecules including activated complement fragments have been identified in close association with the plaques. In the CNS, components of the complement system are synthesized by microglia, astrocytes and neurons and contribute to the local inflammatory response, reviewed in [66,67]. There are conflicting reports of beneficial and detrimental effects of activation of complement in AD. A lot of this confusion arises from different AD mouse models used to study complement by either knocking out complement genes or using inhibitors of complement components, reviewed in [68]. For example, the complement component C1q has long been associated with AD since it was found to co- localize with amyloid deposits in the brains of AD patients [69]. In mouse models of AD (Tg2576 and APP/PS1), deletion of the C1q gene reduced the severity of AD

neuropathology and this suggested that C1q expression was detrimental in AD [70]. However, the binding of Aβ peptides by C1q also enhanced phagocytosis of these peptides by microglia, which express the receptor for C1q and can thus bind to C1q-bound Aβ peptides [69]. Subsequently, C1q was found to help selectively remove apoptotic neurons and to suppress pro-inflammatory cytokine production in an *in vitro* model using primary rat neurons and microglia [71]. This suggests that C1q could be involved in a mechanism to limit the inflammatory response within the brain.

In order to resolve the conflicting roles of complement in AD, the model system must be chosen carefully. Importantly, the differences between mice and humans could limit the usefulness of mouse models in this area. For example, it has been found that Aβ42 peptides are also bound by the complement component C3b, which then binds to the complement receptor type I (CR1) on erythrocytes and is subsequently cleared from the circulation by macrophages in the liver [72]. Importantly, mice do not have complement receptors on their erythrocytes and thus would not utilize this mode of Aβ clearance, reviewed in [73]. Moreover, while mice have complement receptors on other cells, such as macrophages and neutrophils, murine complement is poorly activated by human Aβ peptides [74]. Thus, differences in murine and human complement biology must temper results of AD mouse models geared towards looking at complement activation. Interestingly, polymorphisms in the above-mentioned complement receptor type I (*CR1*) gene locus have also been identified as a risk factor for sporadic AD, reviewed in [8]. Further, herpes viruses are known to modulate complement function and to target complement- associated proteins (CR1 and APOJ) that are also linked to sporadic AD, reviewed in [12]. Complement is thus likely to be important in the clearance of Aβ peptides by promoting uptake by liver macrophages or by enhancing phagocytosis by microglia.

5.2. Microglia and clearance of Aβ plaques: TLRs, pro-inflammatory cytokines and defective phagocytosis

Microglia and astrocytes are the immune effector cells within the CNS and they are considered to be "activated" when they exhibit altered morphology and express specific marker proteins, such as IBA-1in the case of microglia [75]. The recruitment of microglia and astrocytes to the Aβ plaques results in localized inflammation around the plaques indicating that the glial cells are producing pro-inflammatory mediators following activation, reviewed in [76]. This is consistent with the finding that activation of microglia and, to a lesser extent, astrocytes is mediated by Toll-like receptors (TLRs) [77]. TLRs are normally activated in response to microbial ligands and TLR signalling results in the induction of cytokine and chemokine production, however TLRs have also been found to respond to endogenous ligands produced following tissue damage, which are referred to as danger-associated molecular patterns or DAMPs, reviewed in [78,79]. It is thus feasible that either the Aβ peptides, or other molecules produced as a result of the cytotoxic effect of Aβ on surrounding cells, are sensed as DAMPs by microglia and astrocytes. Microglia and astrocytes express TLRs, particularly TLRs 1-4 and TLR9, and TLR signalling is activated in response to uptake of Aβ peptides [77,79]. In particular, TLR2, TLR4 and TLR9 have been implicated in Aβ responsiveness since ligands for these TLRs enhanced phagocytic uptake of Aβ peptides [80-82]. TLRs are thus critical for activating glial cells and enhancing microglial phagocytosis, as further supported by research in AD mouse

models. In one AD mouse model that was also homozygous for a destructive mutation of TLR4, increased diffuse and fibrillar Aβ deposits were observed that were not seen in TLR4 wild-type AD mice [82]. Also, the administration of an acute injection of the TLR4 ligand, lipopolysaccharide (LPS), in transgenic mice reduced Aβ plaque burden, suggesting that TLR4 activation facilitated Aβ uptake [83,84]. TLR2 is the most highly expressed TLR in microglia and a number of studies have found it to be critical for the response to Aβ peptides, in particular since microglia from *TLR2*$^{-/-}$ mice were unable to respond to Aβ42 [85-87]. Deficiency of TLR2 in knockout mice also accelerated cognitive decline [85]. Further, it has been found that Aβ peptides activate typical TLR signalling pathways via MyD88 and NF-κB [86]. In an AD mouse model, knock-down of MyD88 expression reduced inflammation while also accelerating cognitive deficits, indicating that TLR signalling via MyD88 had a protective function in AD [88]. It has also been found that an NF-κB-regulated microRNA (miRNA-146a) is upregulated in response to Aβ [89]. It is thus feasible that TLRs are involved in the sensing of Aβ by microglia and that TLR-induced signalling could be implicated in AD.

One outcome of TLR signalling is the induction of pro-inflammatory cytokine production and elevated levels of these cytokines are observed in response to Aβ peptides. In general, pro-inflammatory cytokines are produced in response to a stimulus but their production is curtailed by anti-inflammatory signals. In AD, it is hypothesized that inflammation, while initially beneficial, if sustained can result in a self-propagating cycle that contributes to neurodegeneration, reviewed in [90]. In transgenic mice expressing wild-type or mutant APP, Aβ plaques were associated with activated microglia and astrocytes, as well as increased levels of the cytokines TNFα, IL-1β and IL-6 [75]. Studies using human brain tissue and *in vitro*, cell-based systems have shown that Aβ-activated glial cells over- produce pro-inflammatory cytokines and that this can lead to the degeneration of neurons [91,92]. Further, the deletion of either of the cytokine receptors TNFR-I or interferon (IFN)-γ receptor type I in transgenic mouse models resulted in decreased Aβ plaque formation, inflammation and prevented learning and memory deficits [93]. Several studies have identified potentially pathological consequences of IL-1β signalling in AD, including increased expression and proteolysis of APP [94], tau phosphorylation [95] and secretion of phospholipase A2-IIA (sPLA2-IIA) from astrocytes [96]. While these studies indicate that pro-inflammatory cytokine production in response to Aβ peptides could exacerbate the symptoms of AD, other work has shown a protective effect of inflammation. Cytokines can stimulate uptake of Aβ by microglia that are otherwise unable to phagocytose the peptides in the large quantities seen in AD [97]. Additional to this, sustained overexpression of IL-1β in the hippocampus of APPswe/PS1ΔE9 mouse model of AD resulted in activated microglia and astrocytes, increased levels of TNFα and IL-6 and decreased amyloid plaque load by enhancing Aβ phagocytosis [98]. It is thus possible that the induction of cytokines does enhance Aβ uptake and clearance. However, the continued presence of Aβ peptides in the CNS, which could be sensed as DAMPs by microglial TLRs, could lead to a perpetual activation of the TLRs and continuous pro-inflammatory cytokine release. The failure to down-regulate cytokine production appears to be able to induce some of the effects associated with AD but there is also evidence for improved Aβ clearance following IL-1β stimulation.

The persistence of Aβ peptides is likely due to a defect in the uptake of these peptides by microglia, such as that observed in the brains of AD patients and transgenic AD mouse models [76,97]. It is feasible that the normal function of microglia is altered in AD by an

unknown mechanism, particularly since bone-marrow-derived phagocytes recruited into the CNS following irradiation in mouse models are able to clear Aβ plaques [97]. It has been suggested that the plaques in human AD patients are more insoluble than those in AD mouse models, making it more difficult for the microglia to clear the plaques, reviewed in [99]. In a model referred to as "frustrated phagocytosis", microglia respond to the presence of difficult-to-phagocytose material by enhanced secretion of inflammatory molecules, including potentially cytotoxic cytokines [99]. Further, a study found that inflammation and enhanced phagocytosis actually increased Aβ production [74]. Interestingly, IFNγ can stimulate γ-secretase activity within the phagosomes of macrophages and induce the cleavage of other phagosome proteins, such as CD44 [100]. This suggests that one effect of pro-inflammatory cytokine secretion is to enhance γ-secretase activity, ostensibly to activate phagocytosis but this could also have a detrimental effect in further enhancing APP cleavage. Thus, in AD, defective γ-secretase function could lead to abnormal phagocytosis and ineffectual clearance of Aβ peptides, as well as aberrant proteolysis of APP.

In addition to cytokines, activated microglia and astrocytes also secrete chemokines, proteins that recruit more immune cells to the site of inflammation; and up-regulation of chemokines and their receptors is associated with AD [97]. The deletion or inhibition of monocyte chemotactic protein-1 (MCP-1/CCL2) in an AD mouse model was associated with lower Aβ levels, as well as reduced microglia and astrocyte recruitment and cognitive impairment [101]. Consistent with this, when CCL2 was over-expressed in transgenic mice, it was found to have a deleterious effect, including increased cognitive impairment and Aβ production [101]. In contrast, deletion of the receptor (CC-chemokine receptor 2 or CCR2) for MCP-1/CCL2 in an AD mouse model had deleterious effects and promoted the rapid onset of disease symptoms and death [102]. The absence of the CCR2 impaired microglial activation and responsiveness to Aβ, which is hypothesized to account for the early onset of disease [102]. These findings appear to contradict one another but it has been suggested that they reflect distinct mechanisms of action. The CCR2 deficiency is associated with earlier onset of AD symptoms and this is hypothesized to be due to the role of CCR2 in recruiting macrophages from the circulation into the brain to clear Aβ plaques [101]. Thus, CCR2 plays a protective role in the early stages of AD by promoting the clearance of Aβ peptides, while the over-expression of MCP-1/CCL2 during the course of the disease could exacerbate AD symptoms. These results support the hypothesis of inflammation induced by microglia and astrocytes in response to Aβ accumulation.

6. Therapeutic targeting of γ-Secretase complexes

While there is currently no cure for AD, several pharmacological and lifestyle-based therapies are available to manage the most common symptoms. Research into the underlying biochemical and genetic causes of AD have however allowed the development of therapeutics aimed at the molecular basis of the disease. During the last decade, the pharmaceutical industry has focused on reducing Aβ formation and preventing the deposition and accumulation of Aβ in amyloid plaques. Given the importance of γ-secretase in the production of Aβ40 and Aβ42, this enzyme has been the focus of intensive research in order to identify compounds that could inhibit or modulate its activity, reviewed in [103]. These potential therapeutics target active and allosteric sites of γ-secretase [104] or modify the substrate so as to reduce Aβ42 formation or aggregation, reviewed in [105]. Several small molecules can inhibit γ- secretase activity *in vitro* [106-109], however some studies have

raised concerns that off-target effects associated with inhibition of γ-secretase cleavage of other substrates, including Notch, could interfere with critical cell signalling events in cellular homeostasis, haematopoiesis and cellular adhesion [110]. Nonetheless, some of these compounds have been put forward for clinical trials, the current state of which has been reviewed elsewhere [111-113]. One of the most promising AD therapeutics was the γ-secretase inhibitor semagacestat (LY450139), since results from initial clinical trials showed that it could cross the blood-brain barrier and reduce Aβ levels in the brain [114]. However, phase III clinical trials were halted in 2010 as a result of the drug worsening cognitive symptoms in patients, as well as being associated with a higher risk of developing skin cancer [114]. MK-0752 was also abandoned since Aβ levels rebounded and even exceeded baseline levels. Another prominent γ-secretase inhibitor, avagacestat (BMS-708163) [115], was discontinued during a second phase II trial when Bristol-Myers Squibb announced that it would halt all clinical development of avagacestat for AD. The recent failure of these and other compounds highlight the desperation of scientists to produce an AD therapeutic, but also highlights our limited understanding of the γ-secretase proteases as a therapeutic target in AD treatment [103,116]. The presence of several γ-secretase protease complexes, and multiple substrates means that off-target side effects or even the induction of new pathologies, such as skin cancers observed in the semagacestat trial, is likely [114,117]. This development is not surprising in light of the pleiotropic functions of γ-secretase, in particular the reported involvement of the presenilins and γ-secretase proteases in several cancers and with reduced PS1 levels associated with hyper-proliferation of the epidermis in mice [118]. One of the chief concerns with γ-secretase inhibitors is with respect to the concomitant inhibition of Notch cleavage, since this is essential in the differentiation of cell lineages in adults, such as the maturation of B- and T- lymphocytes, reviewed in [119]. Thus, there are fears that inhibition of γ-secretase proteases could impair vital biological processes even while inhibiting the cleavage of APP and Aβ formation [114].

7. Aβ immunotherapy

Given the difficulties associated with inhibition of γ-secretase, other avenues for the treatment of AD are being pursued. One of the most promising is antibody-based immunotherapies, reviewed in [114,120,121]. Both active and passive immunotherapies have been investigated for their efficacy in clearing Aβ peptides from the blood and thereby reducing plaque sizes [122]. Active immunization involves the administration of Aβ peptides to the circulation in order to trigger an immune response, including the production of anti-Aβ antibodies. In contrast, passive immunization involves the delivery of anti-Aβ antibodies. The merit of Aβ immunotherapies is thought to lie in the removal of the Aβ peptides by either opsonization, which enhances phagocytosis, or the creation of a peripheral Aβ sink to withdraw peptides from aggregates within the brain [112]. An alternative mechanism is that the anti-Aβ monoclonal m266 sequestered monomeric Aβ peptides in the brain, preventing plaque formation, though it is not known what happens to the sequestered monomers [123].

Immunization with Aβ peptides was found to attenuate the pathology of AD in mouse models [124,125]. Aβ immunization has been tested in humans and some benefits have been observed in terms of reduced cerebral Aβ levels and stabilized or improved cognition [126]. While there is evidence of clinical benefit of these therapies, there have been complications with

at least one trial reporting some cases of meningoencephalitis and without having any protective effect against the neurotoxic peptides [122]. Administration of the Aβ peptides generates an immune response that includes activation of T cells and can lead to inflammation [112]. The use of passive immunotherapies is thought to be safer and less likely to induce a T cell-mediated immune response [122]. Modifications of anti-Aβ antibodies for passive immunization have been reported to improve the safety of immunotherapy, including the use of antibody fragments [127,128] and humanized monoclonal antibodies [129]. Another alternative method of active immunization, that of Aβ-encoding DNA that is conjugated to gold particles and delivered by gene gun injection, has been found to elicit an antibody response without also inducing T cell proliferation or IFNγ and IL-17 secretion, thereby reducing the risk of inflammation in the brain [130]. A number of passive and active Aβ immunotherapeutic strategies are currently being tested in phase II and III clinical trials [122], including the humanized monoclonal antibody Bapineuzumab that produced disappointing results in two-phase III trials [85,131,132]. Developments in the area of Aβ therapeutics have been addressed in a number of recent reviews [112,120,121,133]. Overall antibody-based immunotherapies have failed to show a significant clinical benefit in patients with mild to moderate AD, leading to the proposal that therapeutic intervention is occurring too late in these patient groups and treatment should commence earlier when patients are at the asymptomatic stages of the disease. Likewise, these studies highlight the poorly understood relationship between the immune system and AD and encourage research aimed at increasing our understanding of the role of immune responses in AD and their impact on immunotherapy approaches in AD. Finally, evaluation of the efficacy of AD therapeutics is hindered by the lack of stage-specific biomarkers that are easy to measure. While Aβ peptides, plaque formation and hyperphosphorylated tau are reliable biomarkers, they are present in the CNS and so are monitored by either cerebrospinal fluid withdrawal or the use of brain imaging techniques [134]. These methods are invasive, costly and might not be useful predictive markers for the development of AD since they are only elevated following disease onset and not in all cases of AD [134]. Current research in AD biomarkers has identified differences in the immune system protein profiles of normal and AD patients and these proteins could be used to develop a test for the early stages of AD [134-136].

8. Conclusions and future perspectives

There is clearly a role for the immune response in AD, with markers of inflammation and complement activation being closely associated with the Aβ plaques. It is interesting to find that the critical enzyme implicated in AD is also important in immune system function and regulation. This suggests a close association between aberrant APP cleavage and immune system dysfunction in the AD brain since presenilin mutations could be the cause of both. While only a few cytokine receptors are yet known to be γ-secretase substrates, it is possible that more remain to be discovered. Further, a function is assumed but has not been demonstrated for the ICDs produced following γ-secretase cleavage. Given the gene transactivation observed with the ICDs of Notch and APP, it is feasible that the ICD of IL-1RI is also functional. This would be consistent with nuclear localization of this ICD. If this were a general mechanism employed by other innate immune receptors, it would greatly alter the current understanding of signalling via adaptor recruitment and cascades of post-translational modifications. The importance of

γ-secretase in the immune response underscores the pleiotropic functions of this enzyme complex that render it difficult to treat AD by γ-secretase inhibitors. The best therapeutic prospects at the moment lie with modulation of γ-secretase activity by certain NSAIDs, as well as with the use of monoclonal antibodies to bind to excess Aβ peptides to remove them from the system.

Conflict of interest

The authors declare no conflict of interest.

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