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Microglial malfunction: the third rail in development of Alzheimer's disease

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Abstract

Studies of Alzheimer's disease (AD) have predominantly focused on two major pathologies: amyloid-ß (Aß) and hyperphosphorylated tau. These misfolded proteins can accumulate asymptomatically in distinct regions over decades. However, significant Aß accumulation can be seen in individuals who do not develop dementia, and tau pathology limited to the transentorhinal cortex, which can appear early in adulthood, is usually clinically silent. Thus, an interaction between these pathologies appears to be required to initiate and propel disease forward to widespread circuits. Recent multi-disciplinary findings strongly suggest that the third factor required for disease progression is an aberrant microglial immune response. This response may initially be beneficial; however, a maladaptive microglial response eventually develops, fueling a feed-forward spread of tau and Aß pathology.

Keywords

microglia; Alzheimer's disease; GWAS; epigenomics; transcriptomics; Aß peptide; tau phosphorylation; Braak stages

The pathogenesis of Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease, doubling every five years after the age of 65 and affecting one-third of those above the age of 85. Its prevalence is expected to triple by 2050 as a result of an expanding aging demographic

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(Hebert et al., 2003). Recent studies indicate that the pre-clinical development of AD begins years to decades before onset of memory deficits (Braak et al., 2011). Over this time frame, the two pathological hallmarks of amyloid ß accumulation and tau phosphorylation proceed asymptomatically, and are well underway by the time of initial diagnosis. Once predictive biomarkers for AD risk are identified, this preclinical phase will become a critical time window in which to intervene preventively to delay progression to AD. Later interventions at the stage of dementia are less likely to be disease modifying, as there is already extensive loss of neurons and circuitry at this point.

The "amyloid cascade hypothesis" of AD causation posits that accumulating amyloid-beta$_{42}$ (Aß$_{42}$) peptides in the brain lead to hyperphosphorylation of tau and neurofibrillary tangles (NFTs), neuronal loss, and cognitive decline (Hardy and Higgins, 1992). Since this initial hypothesis, neuropathological studies examining the temporal dynamics and anatomic distribution of amyloid and tau pathologies have expanded our understanding of the pathogenesis of AD (Figure 1). Interestingly, onset of tau pathology can precede Aß plaque accumulation by decades, and begins to appear in the transentorhinal cortex (TEC) as early as age 20 (Braak et al., 2011). By age 50, this Braak stage I–II pathology, also called the "transentorhinal" stage (Braak and Braak, 1995), is found in 50% of subjects (Braak et al., 2011), but remains clinically silent. Cognitive decline occurs with spread of tau pathology to allocortical regions, also known as the "limbic" stage (Braak Stages III–I), and later to neocortex, or the "isocortical" stage (Braak stages V and VI). At these advanced stages, tau pathology is always found in association with "neuritic" amyloid plaques that contain neurofibrillary tangles (NFTs) composed of aggregated hyperphosphorylated tau (Nelson et al., 2012).

In contrast, amyloid accumulates initially into "diffuse" plaques as early as age 40 in an anatomical pattern that does not overlap with the early entorhinal stage of tau pathology. Extracellular amyloid deposits initially appear in the neocortex, spreading subsequently to allocortical regions, including the entorhinal cortex and hippocampus, and caudally to subcortical structures (Thal et al., 2002). However, unlike tau pathology, extensive amyloid deposition can be seen in cognitively intact subjects (reviewed in (Sperling et al., 2014)). The progression of memory impairment, from episodic memory loss to loss of executive, visuospatial, and language abilities is tightly correlated with spread of tau pathology from the hippocampal formation to connected association neocortex, and is always accompanied with neuritic Aß plaque pathology. Thus, tau and Aß pathologies can develop asymptomatically and independently of one another in non-overlapping anatomical distributions for decades before onset of cognitive symptoms. An interaction between Aß pathology and nascent tau pathology appears critical for the subsequent spread of tau pathology beyond the TEC. Over the last few years, advances in genetics and modeling in AD mice now suggest that a defective microglial innate immune response may be the crucial factor that triggers a synergistic interaction between tau and Aß, tipping the balance to spread of tau pathology and progression to dementia.

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Maladaptive microglial responses: a dominant role in risk of AD

Recent human genome wide association studies (GWAS) and systems biology approaches have identified an unexpectedly dominant role of the microglial innate immune response in increasing the risk of developing AD. In this review, we will discuss immune genes identified in recent GWAS (Figure 2 and Table 1) as well as gene-regulatory network and epigenomic studies in human subjects (Box 1) that highlight a role for microglia in AD more consequential than previously appreciated. In parallel, studies in AD model mice have demonstrated that healthy microglial function is lost with advancing AD pathology, and have identified disease-modifying components of the innate immune response that could be targeted to slow disease progression. Taken together, these findings suggest that an unhealthy microglial response is triggered in response to Aß peptides and tau pathology, leading to a synergistic and toxic interaction between inflammation, Aß, and tau pathology that propels tau spread from TEC out to connected circuits. In that regard, this maladaptive microglial immune response may function as the third rail in energizing and initiating runaway tau and Aß pathologies. If microglia could ultimately be "reprogrammed" to a healthy phenotype, this could provide the critical disease-modifying effect needed to stem the expanding tide of late onset AD (LOAD).

GWAS of microglial gene variants associated with risk of AD

Apolipoprotein E4 (ApoE4)

The most frequent variant in LOAD is the E4 allele of ApoE (ApoE4). Of the three alleles, E2, E3, and E4, the E2 allele decreases risk for AD, whereas the E4 allele accelerates both age of onset and risk for AD. In brain, both astrocytes and microglia produce ApoE. In addition to its role in cholesterol transport and lipid metabolism, ApoE also modulates the innate immune response, eliciting variant- and gene dose-dependent differences in proinflammatory cytokine production. Targeted replacement of ApoE4/E4 worsens proinflammatory phenotype, and ApoE3/-mice generate a more vigorous inflammatory response compared to ApoE3/E3 mice (Vitek et al., 2009). ApoE also modulates microglial chemotaxis in a genotype-dependent manner (Cudaback et al., 2011), potentially through astrocytic secretion of the chemokine CCL3 (Cudaback et al., 2015). Microglial p38MAPK-dependent cytokine secretion and secondary neurotoxicity is highest in ApoE4/E4 cultures (Maezawa et al., 2006a, Maezawa et al., 2006b). Moreover, with activation of TLRs, ApoE4 potentiates the pro-inflammatory PGE_2 response but decreases expression of anti-inflammatory TREM2 in microglia in targeted E4/E4 mice (Li et al., 2015b). Finally, ApoE containing lipoprotein particles bind extracellular Aß peptides, and perform a crucial role in Aß transport and clearance.

Triggering Receptor Expressed on Myeloid cells 2 (TREM2)

Variants of TREM2 are strongly linked to AD. The most common variant rs75932628 (R47H) dampens TREM2 function, and correlates with increased severity of disease (Cruchaga et al., 2013, Guerreiro et al., 2013). TREM2 encodes a type I transmembrane protein expressed in myeloid cells that signals through DAP-12 and Syk-mediated tyrosine phosphorylation to promote phagocytosis. TREM2 signaling additionally suppresses

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cytokine production and promotes alternative activation of myeloid cells. Recent gene regulatory network analyses have highlighted DAP12 as a key network regulator in LOAD (Zhang et al., 2013). TREM2 function has been investigated in several different mutant APP models. In APP23 model mice, TREM2 expression is increased in myeloid cells around amyloid plaques, and in vitro promotes Aβ clearance (Melchior et al., 2010). TREM2 expression is also increased around plaques in 5xFAD and APP-PS1 mice (Jay et al., 2015, Wang et al., 2015b). TREM2-deficient 5xFAD mice show decreased microglial accumulation around amyloid plaques, potentially reflecting poor microglial viability from loss of trophic TREM2 signaling (Wang et al., 2015b). In another study, TREM2-deficient APP-PS1 mice similarly showed reduced numbers of myeloid cells around amyloid plaques, and TREM2 expression was found to be specifically associated with $CD11b^{+}/CD45^{hi}$ macrophages, suggesting that TREM2 functions in peripheral infiltrating macrophages (Jay et al., 2015). The varying outcomes found in these studies may reflect differences between the different mutant APP models as well as the time points examined for each model. TREM2 deficiency is also associated with impaired clearance of debris and prolonged demyelination after injury (Cantoni et al., 2015, Poliani et al., 2015). One proposed function of TREM2 is that it acts as a sensor of cellular damage by binding classes of anionic lipids released during cellular damage (Wang et al., 2015b). Indeed, studies using reporter cell lines indicate that the R47H variant reduces binding of anionic lipids to TREM2 (Wang et al., 2015b).

TREM2 can be cleaved at the membrane surface to release its soluble extracellular domain. This soluble protein is believed to act as a decoy for the endogenous ligand of TREM2. Linking the APP/Aß pathway to TREM2 is the finding that, like APP, a C-terminal fragment of TREM2 remains after release of the TREM2 extracellular domain, and this intramembranous portion is then cleaved by γ-secretase. Inhibition of this cleavage by γ-secretase inhibitors, currently being developed to reduce generation of Aß, may adversely affect TREM2 signaling, as failure to cleave the TREM2 CTF may lead to accumulation and sequestration of DAP12 at the cell surface (Wunderlich et al., 2013). In addition, evaluation of the TREM locus has uncovered an intronic variant of the family member TREM1, which like TREM2 is highly expressed on myeloid cells. TREM1 amplifies rather than suppresses the innate immune response (Schenk et al., 2007, Weber et al., 2014). This novel intronic TREM1 variant is associated with increased neuritic plaque burden and cognitive decline (Replogle et al., 2015).

Cluster of differentiation 33 (CD33)

CD33 encodes a type I transmembrane protein expressed by hematopoietic progenitors and myeloid cells. Several CD33 variants have been linked with LOAD (Jiang et al., 2014) that either increase or decrease AD risk. CD33 is a member of the sialic acid-binding immunoglobulin-like lectin family (Siglecs) and binds sialylated ligands through an extracellular V-type immunoglobulin domain. This binding leads to both suppression of NF-kB and JAK-STAT inflammatory gene expression as well reduction of Aß peptide uptake through the recruitment of SH2-domain containing tyrosine phosphatases, SHP-1 and SHP-2.

Amyloid plaques are typically associated with sialylated proteins and glycolipids. Binding of these Aß-associated proteins and lipids to CD33 suppresses the clearance of accumulating Aß peptides. Elevated levels of CD33, as seen in subjects with the C allele of rs3865444, located in the proximal promoter of CD33, are associated with a higher amyloid burden and decreased $Aß_{42}$ uptake (Bradshaw et al., 2013). Conversely, the protective T allele rs3865444 is associated with lower CD33 expression and lower $Aß_{42}$ levels (Griciuc et al., 2013); this allele is associated with a co-inherited polymorphism (rs12459419) that leads to a splicing defect of exon 2 which encodes the V-type immunoglobulin domain, resulting in more CD33 lacking the sialic acid binding site (Malik et al., 2013). In AD, levels of CD33 increase, suggesting a pathological upregulation of this protein in microglia. CD33 appears to suppress clearance of Aß peptides, as global deletion of CD33 in APP-PS1 mice significantly reduced Aß load (Griciuc et al., 2013).

Complement Receptor 1 (CR1)

CR1 is one of the four complement receptors and is a membrane-bound glycoprotein that binds the complement fragments C3b and C4b. CR1 is expressed in myeloid cells as well as erythrocytes, and attenuates complement activation by removing C3b-bound components and targeting them for degradation. In AD, accumulating Aß peptides activate the complement system in an antibody-independent manner (Bradt et al., 1998). Activation of C3 convertase cleaves C3 to C3a and C3b, and C3b molecules bind covalently to Aß peptide assemblies; these complexes then bind to CR1 and are phagocytosed by microglia or transported on erythrocyte CR1 receptors to the liver, where they are degraded by liver macrophages. In the periphery, clearance of Aβ42 from the serum has been shown to be dependent on C3b binding to CR1 (Rogers et al., 2006). In addition to Aß peptides, hyperphosphorylated tau derived from NFTs also potently activates the complement system to levels similar or greater than those elicited by Aß peptides (Shen et al., 2001). Given that the innate immune response will exacerbate both Aß peptide accumulation and tau hyperphosphorylation, complement activation by either of these pathologies may initiate a deleterious feed-forward cycle of Aß and tau pathology and neurotoxic inflammation.

Multiple variants of CR1 have been identified, some of which are located in the intronic region of the CR1 locus and may influence gene expression (Lambert et al., 2009). How these variants may influence clearance of $Aß_{42}$ or $Aß_{42}$-associated inflammation is not clear. The *CR1* variant rs6656401 (Lambert et al., 2009) is associated with increased AD neuropathology and rate of cognitive decline (Chibnik et al., 2011) and with increased severity of vascular amyloid deposition (Biffi et al., 2012). Additional CR1 variants are associated with increased AD risk (Luo et al., 2014) and increased $Aß_{42}$ levels in CSF (Brouwers et al., 2012), suggesting a role of CR1 in Aß peptide metabolism. Gene-brain structure association studies have determined that young *CR1* rs6656401 carriers show decreased gray matter volumes in the entorhinal cortex (Bralten et al., 2011), leading to the intriguing hypothesis that defective CR1-mediated Aß clearance at young ages may impact on subsequent risk of tau spread and LOAD, a question that will need to be tested in longitudinal studies.

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Clusterin or apolipoprotein J (CLU)

Clusterin or ApoJ is a chaperone glycoprotein that is secreted by both astrocytes and microglia. ApoE and clusterin are the two most abundantly expressed apolipoproteins in brain. Like ApoE, clusterin is released by microglia, functions in lipid transport, can bind Aß peptides (Xu et al., 2000), and functions in the clearance of Aß peptides via lipoprotein scavenger receptors (Cole and Ard, 2000), thus influencing the balance between Aß generation and removal. Recent studies indicate that clusterin may interfere with microglial and astrocytic uptake of Aß peptides in human primary microglia and astrocytes (Mulder et al., 2014). In AD, clusterin levels increase in brain and CSF (Lidstrom et al., 1998). Pathologically, clusterin is found in diffuse amyloid plaques, an early stage of amyloid deposition that precedes tau pathology. Plasma clusterin levels correlate with brain atrophy and rapid clinical progression in patients with AD (Schrijvers et al., 2011). Interestingly, deletion of clusterin in the PDAPP mouse model did not significantly alter total amyloid load but rather reduced fibrillar Aß load and neuritic dystrophy (DeMattos et al., 2002).

Clusterin variants are associated AD (Lambert et al., 2009). The risk allele rs11136000(C) is associated with a change in hippocampal network activation and a subtle reduction in hippocampal volume (Lancaster et al., 2014) and reduced CSF Aβ in AD patients (Elias-Sonnenschein et al., 2013). Intriguingly, cognitively intact older individuals with intracranial Aß deposition and increased CSF clusterin levels demonstrate volume loss in entorhinal cortex by MRI (Desikan et al., 2014), suggesting that clusterin may contribute to preclinical neurodegenerative changes in the EC.

ATP-binding cassette, subfamily A, member 7 (ABCA7)

ABCA7 encodes a member of the ATP-binding cassette transporter family that functions in lipid transport across membranes. Increased levels of ABCA7 are reported in AD brains and positively correlate with disease severity (Karch et al., 2012, Zhao et al., 2014). Variants in the *ABCA7* locus (Hollingworth et al., 2011, Naj et al., 2011) include the rs3764650 variant that has been linked to increased neuritic plaque burden and LOAD risk (Shulman et al., 2013).

ABCA7 is highly enriched in microglia (Kim et al., 2006) and to a lesser extent hippocampal neurons (Kim et al., 2005). ABCA7 promotes phospholipid and cholesterol transport to lipid acceptors, notably apolipoproteins E and I. ABCA7 is a mammalian ortholog of *CED-7*, a *C. elegans* gene involved in phagocytosis of apoptotic cells. In mammalian systems, in the setting of an innate immune response, ABCA7 will associate with the scavenger receptor LRP1 (low-density lipoprotein receptor related protein 1) on the cell membrane; LRP1 mediates phagocytosis by binding C1q coated complement activators, triggering an ERK-dependent phagocytic signaling cascade (Jehle et al., 2006). Absent or defective ABCA7 disrupts this interaction and impairs phagocytosis. Consistent with this, bone marrow-derived macrophages from ABCA7$^{-/-}$ mice show a marked reduction in Aβ phagocytic activity (Kim et al., 2013) and deletion of ABCA7 in the J20 APP mouse model increases amyloid accumulation (Kim et al., 2013). Thus, at pre-clinical AD stages, microglial ABCA7 may function not only in clearing Aß peptides but also in removing

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apoptotic cellular debris from early neurodegenerative injury, both of which generate a vigorous innate immune response leading to additional secondary neurotoxicity.

Membrane-spanning 4-domains subfamily A (MS4A cluster)

The *MS4A* cluster encodes a family of four-transmembrane domain proteins, with both N- and C-termini residing intracellularly. Variants at this locus are associated with either increased or decreased AD risk (Hollingworth et al., 2011, Proitsi et al., 2014). Although these proteins are expressed in myeloid cells, their function in the pathogenesis of AD is currently unknown. Src homology 2 (SH2) and SH3 domain-binding sites at the N- and C-termini suggest that these proteins might act as docking centers for other signaling proteins. Alternatively, the 4-transmembrane domain structure of MS4A proteins is reminiscent of ion channels, suggesting that MS4A in multimeric form may function to flux ions (reviewed in (Eon Kuek et al., 2015)), potentially calcium ions (Koslowski et al., 2008).

HLA-DRB1 and HLA-DRB5 (Human leucocyte antigen Major histocompatibility complex, class II, DR β 1 and DR β 5)

HLA-DRB1 and HLA-DRB5 are members of the major histocompatibility complex II. MHCII proteins are found on antigen-presenting cells; peptide epitopes from phagocytosed and digested antigens are carried out to the surface membrane by MHCII for antigen presentation. HLA-DR positive microglia are observed in multiple neurodegenerative conditions, including AD (McGeer et al., 1988). Recently, methylation in the *HLA-DRB5* locus was found to be associated with increased Aß load and NFT density (Yu et al., 2015). The mechanism underlying the HLA-DR susceptibility is not known, however may involve aberrant microglial activation and secondary neurotoxicity. In a mouse model of Parkinson's disease, overexpression of α-synuclein led to MHCII expression in microglia and secondary degeneration of dopaminergic neurons (Harms et al., 2013).

Myocyte enhancer factor 2C (MEF2C)

MEF2C encodes a transcription factor that functions in brain and muscle development. MEF2C is also expressed in monocytes where LPS significantly increases its transactivation activity (Han et al., 1997). Additional evidence suggesting that MEF2C regulates inflammatory gene expression has emerged from chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq) analyses examining the pattern of genomic binding of MEF2C in a lymphoblastoid cell line. This study demonstrated that MEF2C binding sites were enriched near inflammation genes found to be associated with GWAS of bone mineral density (Johnson et al., 2014), a process regulated in part by bone specific macrophages or osteoclasts. Taken together, these findings suggest that in microglia, where MEF2C is highly expressed (Zhang et al., 2014), this transcription factor may be playing an important role in regulating inflammatory gene expression in response to Aß peptides, tau pathology, and cellular contents released with neurodegeneration (DAMPs).

Inositol polyphosphate-5-phosphatase (INPP5D)

INPP5D encodes an inositol phosphatase that is a negative regulator of the PI3K/Akt pathway. In myeloid lineage cells, studies examining the mouse homologue *SHIP1* have

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shown that this phosphatase functions broadly as a negative regulator of immune signaling (reviewed in (Zhang et al., 2000)). Interestingly, INPP5D/SHIP1 deficient mice show increased osteoclast formation, osteoporosis, and generation of the pro-inflammatory cytokine IL6 (Takeshita et al., 2002). INPP5D/SHIP1 is implicated in multiple human disease states characterized by a dysregulated immune response, including cancer, diabetes, and atherosclerosis (Viernes et al., 2014), and most recently LOAD (Lambert et al., 2013). Intriguingly, two SHIP1 binding partners are functionally linked to other GWAS variants associated with LOAD: DAP12 (Peng et al., 2010) and CD2AP (Bao et al., 2012). DAP12 is an adaptor molecule for TREM1/2 signaling (see above) and CD2AP (CD2-associated adaptor protein) is implicated as potential risk factor for LOAD (Lambert et al., 2013).

Additional GWAS genes potentially linked to aberrant microglial inflammation

The Erythropoietin-producing hepatocellular (Eph) receptor (*EPHA1*) is associated with LOAD risk (Carrasquillo et al., 2015, Naj et al., 2011, Wang et al., 2015a). The Eph receptors are receptor tyrosine kinases and their ligands, ephrins, are involved in mediating cell adhesion and migration, and may function in immune cell migration. EphA1 is expressed by both lymphocytes and monocytes (Sakamoto et al., 2011), suggesting a potential role in the microglial immune response in AD. Additional genes associated with LOAD risk expressed in microglia (Zhang et al., 2014) include *BIN1*, *PICALM*, *PTK2B*, and *CASS4*, however their functions in neurodegeneration have not been examined.

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BOX 1

Systems biology approaches: gene regulatory networks and epigenomics

Advances in sequencing technologies now allow for sophisticated transcriptome and epigenome profiling. In conjunction with GWAS, these techniques have yielded recent insight into the role of inflammatory genes and networks in the onset and progression of AD. Microarray and RNA-seq technologies measure changes in gene expression across the whole genome during different disease stages. RNA-seq allows for detection of novel transcripts and variants since it does not rely on arrays of probes, as in microarray analysis. A variety of sequencing and alignment methods exist for RNA-seq, which result in lower noise during analysis and higher sensitivity in detecting transcripts (Maze et al., 2014). Complementary to transcriptome studies, ChIP-seq measures changes in histone methylation and transcription factor binding to assess changes in gene regulatory networks. Recent studies employing these techniques have strongly implicated the innate immune response in the early stages of AD.

Comparing microarray or RNA-seq data across conditions or samples reveals differential gene expression. Hundreds, even thousands, of genes can show significant increases or decreases in expression that correlate with markers of disease progression. Clustering these differences into co-expression patterns helps identify key genes that account for a significant proportion of changes in gene expression. Weighted gene co-expression network analysis (WGCNA), one of the most common approaches, clusters genes into modules and pathways based upon the strength of their expression correlation. WGCNA was used to identify 12 modules in AD CA1 samples that related to synaptic, metabolic, immune, and white matter processes (Miller et al., 2008). Interestingly, CA1 showed greater enrichment for signal transduction, immune response, and cell motility genes in comparison to CA3 (Miller et al., 2013). The CA1 region is a very early target of tau pathology, with NFTs appearing in the EC first, and then in the CA1 apical dendrites of the stratum lacunosummoleculare (SLM) that receive afferent input from CA3 and the EC. These results suggest the involvement of immune processes at the earliest stages of tau spread from the EC.

Alternative gene network analyses have employed different correlation methods or have taken into account prior information about biological functions of genes (Aubry et al., 2015, Villaverde and Banga, 2014) and provide support for the role of immune responses in AD development. Tau overexpression in mice resulted in higher expression of inflammatory genes (Wes et al., 2014). Clustering genes from LOAD patients and mouse models of AD showed a shared over-representation of inflammatory pathways (Augustin et al., 2011). Zhang et al used Bayesian inference to identify regulatory genes upstream of networks significantly changed in AD (Zhang et al., 2013). In this analysis, the immune module was overrepresented in LOAD patients and TYROBP (DAP12) was one of the highest ranked regulators. Reanalysis of this data set with driver-gene inference by genetical-genomics and information theory (DIGGIT), which also explores upstream regulatory elements, validated TYROBP as a significant regulator of AD (Chen et al., 2014).

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CHIP-Seq allows the characterization of histone modifications or transcription factor binding. Gjoneska et al. profiled histone methylation and identified a pattern of down-regulation of synaptic plasticity genes and up-regulation of immune response genes in AD patients (Gjoneska et al., 2015). The CK-p25 mouse model of AD exhibited the same changes in gene expression and the up-regulation of immune functions was found to result from an increase in both immune cell types and expression of inflammatory genes (Gjoneska et al., 2015). In other studies, changes in BIN1, CLU, MS4A6A, ABCA7, CD2AP, and APOE explained 16.8% of variability of plaque burden (Chibnik et al., 2015). In epigenome-wide association studies (EWAS) examining patterns of DNA methylation, Ank1, a cytoskeletal protein known to be involved in inflammation, was found to be hypermethylated in AD patients (De Jager et al., 2014, Lunnon et al., 2014). These epigenomic studies have provided insight into genes linked to AD that did not reach genome wide significance (Gjoneska et al., 2015).

Reprogramming microglia to healthier phenotypes in AD

In addition to the GWAS and systems biology approaches detailed previously, mouse modeling has provided significant support for the importance of the innate immune response in demonstrating interactions between Aß peptides, tau, and microglial responses. In addition, as age is the primary risk factor for AD, increased levels of low grade chronic inflammation, also known as “inflammaging” (reviewed in (Frasca and Blomberg, 2015)), will occur with advancing age and influence microglial function. The evidence establishes two narratives: both a detrimental loss of healthy microglial activity and a gain of synaptotoxic function.

In the unique environment of the central nervous system, microglia maintain tissue homeostasis and are continually sampling their microenvironment. In the developing brain, microglia play a major role in sculpting neuronal circuits and eliminating apoptotic neurons (Schafer et al., 2012, Wake et al., 2013). In the adult brain, microglial brain-derived neurotrophic factor (BDNF) functions in excitatory neurotransmission and hippocampal and motor learning (Parkhurst et al., 2013), suggesting that microglia continue to participate in synaptic plasticity into adulthood. This is underscored by the broad range of neuropeptide and neurotransmitter receptors expressed by microglia (Pocock and Kettenmann). However, microglia are very sensitive to their microenvironment and can lose their normal beneficial functions. After seven days in culture, microglia lose genome-specific programming (Gosselin et al., 2014). Transplantation of myeloid cells to new tissues results in reprogramming of their enhancer and promoter landscape (Lavin et al., 2014), indicating that these cells are quite plastic and readily adapt to their environment. It is therefore likely that nascent accumulation of immunogenic Aß peptides, phosphorylated tau, and/or cellular debris from early synaptic degeneration will alter microglial transcriptional and epigenomic states. This may begin in the hippocampal formation, and specifically in the TEC-EC-CA1 circuit, which is considered “ground zero” for subsequent acceleration of tau pathology.

Tau pathology triggers a robust microglial inflammatory response (Wes et al., 2014, Zilka et al., 2009). This in turn likely sets off a vicious cycle where maladaptive

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inflammation further aggravates tau pathology, leading to an intensifying neurotoxic inflammatory response. In the P301S tauopathy model, this tau-induced inflammatory response is prevented by administration of the immunosuppressant FK506. Moreover, in the P301S model, microglial inflammation precedes tangle formation (Yoshiyama et al., 2007). This is in keeping with human neuropathological studies by Streit et al. (Streit et al., 2009) demonstrating that microglia become dystrophic and fragmented prior to the appearance of abnormal tau in the TEC and EC at early Braak stages I–III. This suggests that aberrant microglial immune responses could trigger tau pathology, particularly in the context of aging, which is not only the primary risk factor for AD but also is characterized by progressive increase in synaptotoxic inflammation. In support of this, the rTg4510 tauopathy mouse model demonstrated that CD45 positive microglia increased with age in parallel with tau pathology (Lee et al., 2010a). Additional studies support a tight and reciprocal link between microglial immune responses and aggravation of tau pathology. In 3xFAD mice, which harbor mutant forms of APP, PS1, and tau, LPS administration accelerated microglial reactivity (Kitazawa et al., 2005) and tau hyperphosphorylation (Bhaskar et al., 2010). In hTau and rTg4510 tau mice, LPS similarly exacerbated tau pathology (Lee et al., 2010a). In vitro, co-culture of neurons with microglia pre-stimulated with LPS or pro-inflammatory cytokines induced the accumulation of aggregation-prone forms of tau in neurons (Gorlovoy et al., 2009). Of relevance to the synergistic interaction between Aß, tau, and microglial immune responses, co-cultures of neurons with microglia pre-activated with Aß peptides led to increased neuronal tau phosphorylation and synaptophysin loss in a p38-MAPK dependent manner (Li et al., 2003).

Extensive data in mouse AD models demonstrates that, similar to tau, oligomeric and fibrillar Aß peptides also elicit a vigorous microglial innate immune response (reviewed in (Heneka et al., 2014)). Whether Aß peptide assemblies are generated locally in the hippocampal formation or enter the TEC and EC from the temporal neocortex remains to be determined; the expansion of Aß plaque pathology is less predictable than that of abnormal tau, and injury from oligomeric Aß peptides has been postulated based on animal data. If there is an interaction between nascent tau pathology and Aß peptide assemblies in the hippocampal formation, both would be associated with a vigorous microglial response. In this context, homeostasis would be achieved only with a beneficial microglial response consisting of healthy chemotaxis, phagocytosis, and lysosomal degradation of misfolded proteins (including Aß and tau) and DAMPs, and an effective termination of the inflammatory response. However, exposure to accumulating Aß peptides leads to loss of these critical microglial functions, resulting in a persistent pro-inflammatory state and failure to clear misfolded proteins (Hickman et al., 2008, Krabbe et al., 2013).

A loss of healthy microglial function, however, might be prevented by targeting selected microglial receptor pathways. Non-steroidal anti-inflammatory drugs (or NSAIDs), which block cyclooxygenase (COX-1/COX-2) activity and PGE_2 production, have shown preventive effects against development of AD in cognitively normal aging populations (in t' Veld et al., 2001, Stewart, 1997, Vlad et al., 2008), however are not a viable strategy, as NSAIDs inhibit toxic as well as beneficial prostaglandin pathways (Funk and

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FitzGerald, 2007, Liang, 2011). Modeling the mechanism of action of NSAIDs in AD mice using cell-specific knockout strategies of PGE_2 receptors has accordingly revealed both toxic and beneficial PGE_2 microglial signaling pathways. Ablation of microglial EP2 receptor restored microglial chemotaxis, Aß clearance, and Igf1 generation, and led to termination of the pro-inflammatory response and prevention of memory dysfunction in AD model mice (Johansson, 2013, Johansson et al., 2015). Conversely, the anti-inflammatory and neurotrophic PGE_2 EP4 receptor enhanced microglial Aß clearance, beneficial inflammatory responses, and synaptic viability in the APP-PS1 mouse (Liang, 2011, Shi et al., 2010, Woodling et al., 2014). The microglial fractalkine receptor CX3CR1 is another beneficial signaling pathway that is specifically involved in neuron-to-microglia signaling. Ablation of CX3CR1 aggravates tau pathology in mouse models of tauopathy and amyloid accumulation (Bhaskar et al., 2010, Cho et al., 2011) and increases pro-inflammatory cytokine signaling (Cho et al., 2011) and LPS-induced tau hyperphosphorylation (Bhaskar et al., 2010). While these studies suggest a beneficial role for fractalkine signaling, the role of the CX3CR1 in AD may be time and/or context-dependent. For instance, in contrast to the synaptotoxic effects of receptor deletion for tauopathy, CX3CR1 deficiency reduces Aβ deposition in mutant APP mouse models (Lee et al., 2010b). In the 3xFAD model, receptor deletion had no effect on Aβ deposition, but prevented neuronal injury (Fuhrmann et al., 2010). Other microglial membrane proteins discovered by GWAS and detailed above, including TREM2 and CD33 also represent attractive targets to reprogram microglia to a healthier pro-clearance state.

Perhaps most compelling would be the possibility that microglial dysfunction underlies the interaction between tau and Aβ at early pre-clinical AD stages (Figure 3). In the human hippocampal formation, the initiating event might be the microglial response to early accumulation of synaptotoxic Aß oligomers or to nascent tau pathology, which are both immunogenic. Moreover, both Aß oligomers and tau pathology cause synaptic and neuronal injury, so cellular debris released from injured synapses will function as a third potent stimulator of microglial innate immune responses. A three-way synergistic interaction between Aß oligomers, nascent tau pathology, and microglial inflammation may occur at early Braak stages and initiate the spread of tau pathology. In non-susceptible individuals, the microglial response may be more effective at removing immunogenic Aß, tau, and/or cellular debris, and homeostasis is readily reestablished. In susceptible individuals however, recent GWAS and systems biology approaches suggest that aberrant microglial responses will accelerate tau phosphorylation and Aß peptide accumulation, propelling tau pathology.

Future directions

The concept that microglial function is central to the development of AD is now gaining traction, after decades of focus on Aß peptide and tau biology. Characterization of the microglial response longitudinally, as has been done for tau and Aß in comprehensive neuropathological studies, has yet to be done for AD-related neuroinflammation. A significant barrier to achieving this has been the lack of a specific molecular signature for dysfunctional human microglia. The microglial/myeloid response is now understood to be much more complex than the originally proposed M1 and M2 polarization states (Xue

et al., 2014). Moreover, microglial states are likely to shift dynamically over time and space in synch with expanding Aß and tau pathologies. One promising approach to better understand the temporal and spatial dynamics of microglial responses will be to characterize the neuroinflammatory response longitudinally using PET imaging (Box 2). As both Aß deposition and tau can be imaged by PET, it will be important to understand the timing and localization of their interactions with the inflammatory response. With the development of more sensitive and specific radioligands to detect microglial immune responses, it may be possible to identify when and where the earliest microglial changes occur in AD, setting the stage for development of disease-modifying preventive therapies.

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BOX 2

Positron emission tomography (PET) is becoming an increasingly important imaging modality for improving the way we diagnose and stage AD (Ossenkoppele et al., 2013). Additionally, PET is being used to monitor the effects of novel AD therapeutics in clinical trials, and is providing a non-invasive picture of the molecular underpinnings of this complex neurodegenerative disease in a longitudinal manner (Berti et al., 2011). Over the last few decades, numerous PET radioligands have been specifically developed for imaging AD – many of which target Aβ and/or tau pathology. The in vivo binding profile of Aβ-PET radioligands is believed to provide an estimate of Aβ neuritic plaque density in AD patients, as evidenced by uptake in parietal and frontal cortices, posterior cingulate, and precuneus regions. In terms of specific tau-PET tracers, several studies have shown that these tracers bind specifically to areas of the brain exhibiting extensive NFTs in keeping with Braak staging, and that they do not bind Aβ in human AD postmortem tissue.

There are also specific radioligands for imaging neuroinflammation, including translocator protein 18 kDa (TSPO), cyclooxygenase-2 (COX-2), cannabinoid receptors, P2X7, and monoamine oxidase-B enzyme (MAO-B) for imaging reactive astrocytes (Jacobs and Tavitian, 2012). Of these, the most widely investigated is TSPO. TSPO is a highly hydrophobic protein that forms part of a complex with voltage-dependent anion channel and adenine nucleotide carrier in the outer mitochondrial membrane. Key roles of TSPO include transporting cholesterol to the inner compartments of the mitochondria (i.e., the rate-limiting step of steroidogenesis) and maintaining mitochondrial homeostasis (Batarseh and Papadopoulos, 2010). While TSPO is moderately expressed in the healthy brain, it is upregulated under inflammatory conditions and has been used as a marker of “activated” microglia in imaging studies (Chen and Guilarte, 2008, Ching et al., 2012).

The first TSPO-PET tracer evaluated in AD patients was [^{11}C]PK11195. Some studies have reported increased regional binding of [^{11}C]PK11195 in AD patients compared to healthy controls in a manner correlating with disease severity and in areas known to be affected by Aβ pathology (i.e., entorhinal, temporoparietal, and cingulate cortex) (Cagnin et al., 2001, Edison et al., 2008) while other studies reported no significant differences between AD, MCI, and controls (Schuitemaker et al., 2013, Wiley et al., 2009). While [^{11}C]PK11195 has provided unprecedented insights concerning the neuroinflammatory component of AD in living subjects, its suboptimal signal-to-background ratio and brain permeability, extensive plasma protein binding, and a poor signal-to-noise ratio (Chauveau et al., 2008) – all of which have limited widespread clinical utility. Second generation TSPO tracers have been developed with improved affinity and signal-to-background ratios compared to [^{11}C]PK11195. These second generation agents have also been evaluated in MCI and AD patients – with some encouraging yet mixed results. Yasuno and colleagues observed increased binding of [^{11}C]DAA1106 in AD patients compared to controls, although no correlation was found in terms of disease severity (Yasuno et al., 2008), and later identified higher radioligand binding in MCI subjects (Yasuno et al., 2012), suggesting that microglial immune responses occur early with onset of cognitive change. However, a recent study with [^{18}F]DPA-714 failed to detect a

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significant difference between AD patients and controls, although in this study TSPO genotyping had not been performed, potentially confounding the results (Golla et al., 2015). A separate study using [^{18}F]FEPPA, where corrections were made for different TSPO genotypes and partial volume effects, reported significantly increased tracer uptake in frontal, temporal, and parietal cortices, along with the posterior limb of the interior capsule, which was associated with cognitive deficits (Suridjan et al., 2015). Similarly, Kreisl and coworkers performed a TSPO-PET study using partial volume corrections and TSPO genotype adjustments and demonstrated increased binding of [^{11}C]PBR28 in AD but not in MCI patients (Kreisl et al., 2013). This finding is likely due to the limitation of TSPO as a target and/or the limited sensitivity of [^{11}C]PBR28 for detecting alterations in TSPO levels at the MCI stage. We know from histological studies that microglial activation is abundant in MCI patients. Different binding sites and/or oligomeric states of 18kDa TSPO protein may be responsible for variations in "sensitivity" and binding of different TSPO ligands. Recent x-ray crystallography studies of TSPO have provided valuable insights on the structure and function of TSPO, and could ultimately lead to improved design of TSPO tracers (Guo et al., 2015, Li et al., 2015a).

Thus there are limitations associated with TSPO as a marker of microglial function, including the lack of clarity around what a positive TSPO-PET signal actually represents. First, TSPO is not only expressed on microglia, but also to a certain extent on blood cells, vascular endothelium, and astrocytes. Second, TSPO levels may not distinguish between healthy versus dysfunctional microglial phenotypes. Additionally, a proportion of the population (~30% Caucasians and African Americans) has a polymorphism in the TSPO gene that weakens binding of second and third generation TSPO ligands. Lastly, the extent to which TSPO levels are altered with disease progression might be too subtle, especially in early stages of AD, to provide a sensitive read-out of neuroinflammation. Although TSPO-PET tracers have provided a unique opportunity to visualize microglial activation in living subjects with AD, there is a need for more specific and sensitive PET biomarkers of the various aspects of neuroinflammation. Longitudinal imaging studies with neuroinflammation PET agents in combination with Aβ- and tau-PET radioligands will undoubtedly shed light on the time-course and spread of these aspects of AD, and ultimately improve the way we diagnose and treat this disease.

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Outstanding Questions

- How do the polymorphisms in genes identified in GWAS alter protein function and increase risk of developing AD?
- Are there protective variants in healthy individuals carrying known AD risk factors, and will they also encode microglial immune genes?
- What are the molecular signatures of maladaptive microglia during the development of AD? Will they vary with changes in Braak stage, CERAD score, or anatomical location?
- Since the role of inflammation is increasingly implicated in AD progression, what novel markers might be used to study longitudinal changes in microglial activity?
- Which membrane proteins or transcription factors will prove to be attractive therapeutic targets to prevent maladaptive neuroinflammation?
- Can we target microglia and maintain their healthy function with aging, and will this result in a significant delay and reduction of AD risk?

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Trends Box

- The pre-clinical course of AD spans years to decades before the onset of cognitive decline. Early appearance of $Aß_{42}$ and tau pathologies are initially clinically silent and do not overlap anatomically. With progression to AD, these pathologies converge and spread from hippocampus to connected circuits.
- Microglia play critical roles in maintaining homeostasis in brain by clearing misfolded proteins and controlling inflammation.
- GWAS and systems biology approaches have identified the microglial immune response as a dominant factor in AD risk.
- Studies in AD model mice demonstrate that healthy microglial functions are lost with progression of amyloid pathology.
- A maladaptive microglial immune response may be the precipitating factor underlying the escalation of pathology to AD.
- Reprogramming microglia to more healthy states is a potential therapeutic direction.

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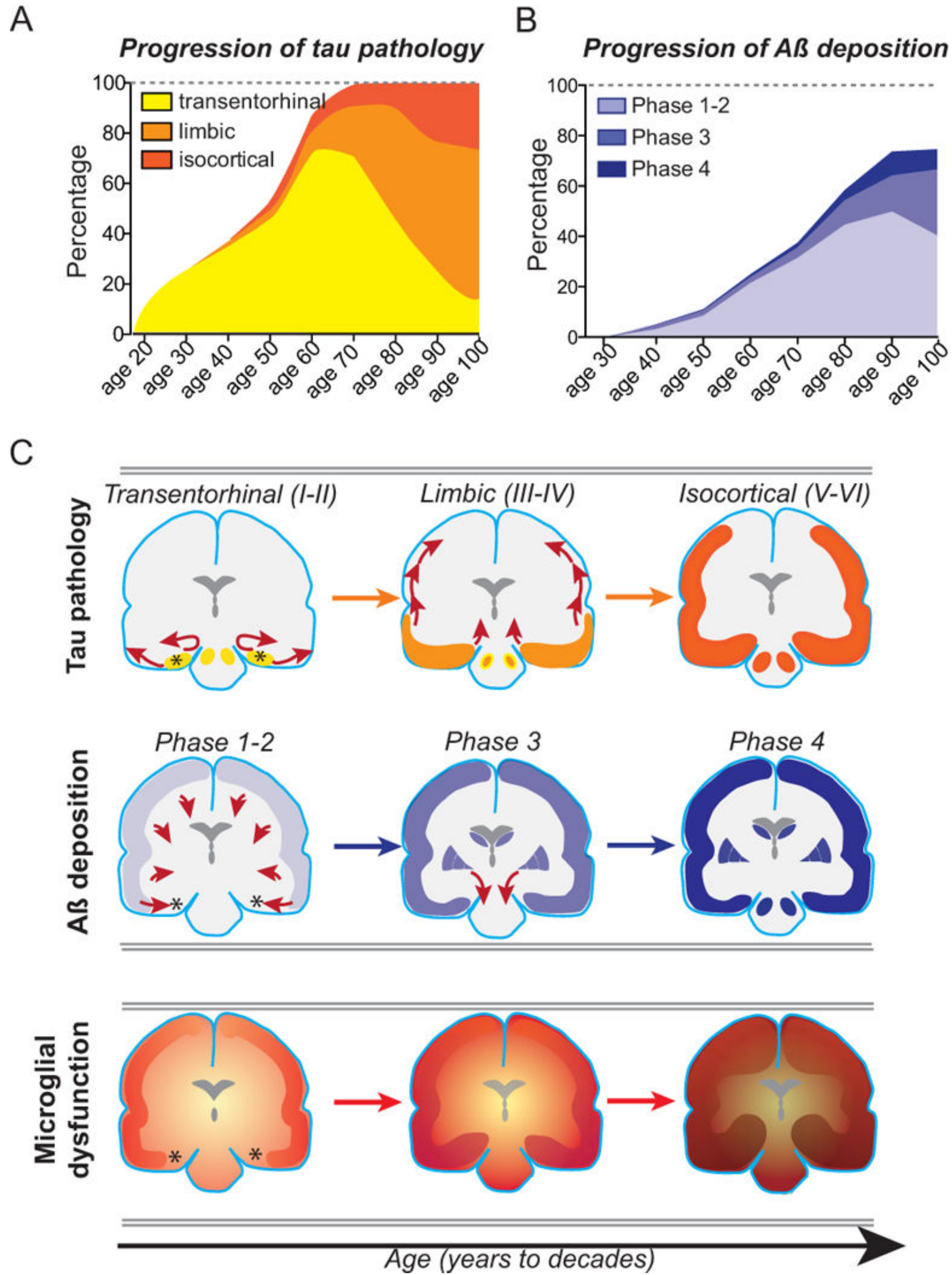

Figure 1. Development of tau and amyloid pathologies and the neuroinflammatory response, adapted from (Braak and Braak, 1991, Braak and Braak, 1995, Braak et al., 2011, Streit et al., 2009, Thal et al., 2002)

(A) Early tau pathology begins in young adulthood in the transentorhinal cortex (TEC; Braak stages I–II, or transentorhinal stage which is clinically silent). With aging, tau pathology spreads beyond the TEC and entorhinal cortex into hippocampus and allocortex (Braak stages III–IV, or limbic stage where memory dysfunction begins). Subsequently, tau pathology spreads to connected neocortical association areas (Braak stages V–VI, or isocortical stage where cognitive deficits are very advanced). Note that 100% of subjects

have some degree of tau pathology after age 70. **(B)** Amyloid deposition begins later in life and may be present in cognitively normal subjects. **(C)** Tau pathology spreads in a caudal-rostral direction beginning in the locus ceruleus and TEC (asterisk), then progresses to limbic and isocortical stages. Amyloid pathology begins in neocortex, and expands caudally into allocortical and subcortical areas at later phases. With progression of both tau and Aß pathologies, neuropathological studies (Streit et al., 2009) together with results from PET imaging of microglial activation using TSPO radioligands (Cagnin et al., 2001, Edison et al., 2008, Kreisl et al., 2013, Suridjan et al., 2015, Yasuno et al., 2012, Yasuno et al., 2008) (see Box 2), indicate that microglial responses likely track with both Aß deposition and spread of tau pathology. Although TSPO-PET radioligands have some important limitations, they have provided unique insights concerning the neuroinflammatory component of AD in living subjects, and have highlighted the importance for such non-invasive imaging techniques to enhance our understanding of the in vivo time-course of neuroinflammation in the development and progression of AD.

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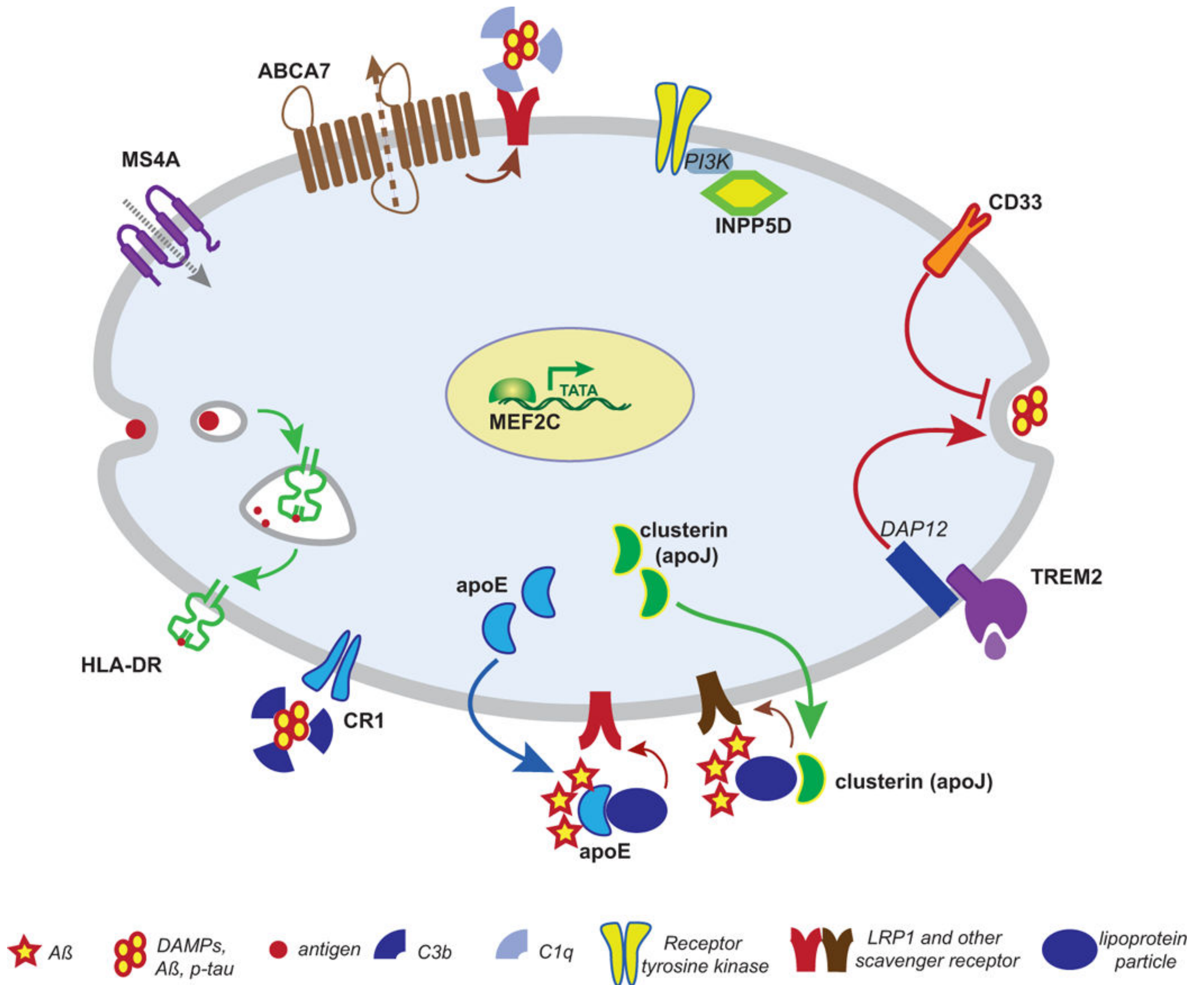

Figure 2. Immune-related GWAS variants in AD

Multiple genes with GWAS variants are present in myeloid cells, including microglia (diagrammed here), confirming the importance of microglial responses in the development of AD. Many GWAS genes encode transmembrane proteins that regulate complex signaling cascades for inflammatory gene expression, endocytosis and phagocytosis, and clearance of Aß peptides, phospho-tau, and damage-associated molecular pattern proteins (DAMPs). The apolipoproteins ApoE and clusterin (CLU or ApoJ) are produced by microglia as well as by astrocytes and are components of lipid/cholesterol lipoprotein particles that transport Aß peptides that are taken up by scavenger receptors.

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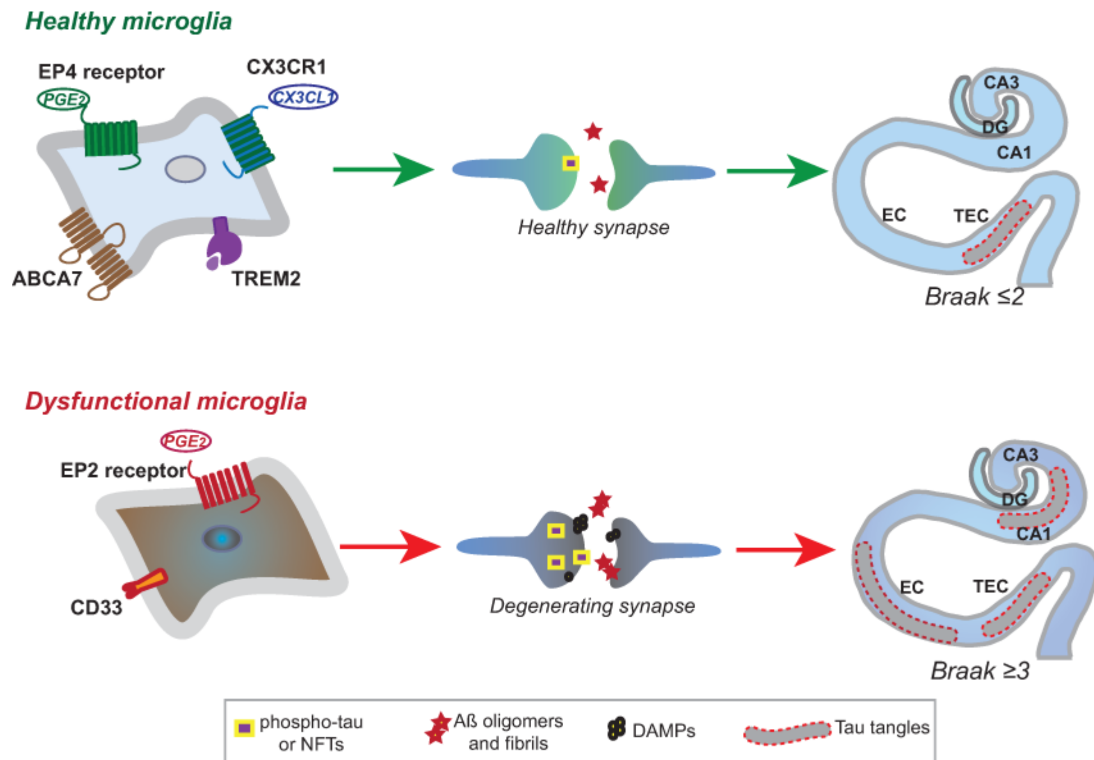

Figure 3. Model of candidate microglial receptors that function in healthy versus maladaptive immune responses in AD

Microglia generate an innate immune response to accumulating Aß peptide oligomers and fibrils, phosphorylated and misfolded tau proteins, and cellular contents (DAMPs) that are released as a result of synaptic and neuronal injury. Synaptic injury occurs both from direct $Aß_{42}$-oligomer toxicity and secondary neurotoxicity from microglial inflammatory responses. Secondary neurotoxicity involves generation of reactive oxygen species from oxidative enzymes like iNOS or COX-2 and release of neurotoxic cytokines and proteases. How microglia respond to and interact with immunogenic Aß, tau, and cellular proteins is likely to influence the initiation and progression of hippocampal pathology early in AD development, as modeled here. Microglial receptors examined in mouse AD models are shown that may represent attractive targets for reprogramming microglia to healthy states. TREM2, CD33, and ABCA7 have GWAS variants associated with AD risk and microglial PGE_2 EP2 and EP4 and CX3CR1 receptors modulate microglial function in AD model mice. (**Top**) The beneficial effects of CX3CR1 (Bhaskar et al., 2010, Cho et al., 2011, Fuhrmann et al., 2010), TREM2 (Jay et al., 2015, Melchior et al., 2010, Wang et al., 2015b), ABCA7 (Kim et al., 2013), and PGE_2 EP4 (Liang, 2011, Shi et al., 2010, Woodling et al., 2014) signaling may help maintain healthy synapses and limit early spread of pathology in hippocampus (Braak stages 2). (**Bottom**) Conversely, elevated expression of CD33 (Griciuc et al., 2013) and the PGE_2 EP2 receptor (Johansson, 2013, Johansson et al., 2015) may enhance synaptic injury and facilitate the spread of tau pathology beyond the TEC (Braak stages 3).

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Table 1

Genetic risk variants of AD identified *via* GWAS.

GWAS gene	Chromosomal location	Function	Cellular localization	Brain localization		
				Microglia	Astrocyte	Neuron
APOE4	19q13.2	Lipid processing pathway	Cytoplasm, Endosome	++	+++	+
TREM1	6p21.1	Immune response/Proinflammatory	Plasma membrane	++	+	+
TREM2	6p21.1	Immune response/Anti-inflammatory	Plasma membrane	+++	–	–
CD33	19q13.3	Immune response/Endocytosis	Plasma membrane	+++	–	–
CR1	1q32	Immune response/Complement system	Plasma membrane	++	–	++
CLU	8p21-p12	Lipid processing pathway/Immune response/Complement system	Cytoplasm, Extracellular space	+	+++	+
MS4A4E/6A	11q12.1 – q12.2	Immune response	Plasma membrane	++	–	–
ABCA7	19p13.3	Lipid processing pathway/Immune response/Complement system	Plasma membrane	+++	++	+++
MEF2C	5q14.3	Immune response/Endocytosis	Nuclear/Cytoplasm	++	+	+++
INPP5D	2q37.1	Immune response	Cytoplasm	+++	+	–
HLA-DR5/DR1	6q21.3	Immune response	Plasma membrane, Extracelluar space	+++	+	–
EPHA1	7q34	Immune response/Endocytosis	Plasma membrane	+	+	+
PICALM	11q14	Endocytosis*	Golgi apparatus, Vesicle, Nuclear	+++	+	+
BIN1	2q14	Endocytosis*	Nuclear, Cytoplasm	+++	+	++
PTK2B	8p21.1	Endocytosis*	Plasma membrane, nuclear, cytoplasm	+++	–	+
CASS4	20q13.31	Cytoskeleton/Axonal Transport*	Cytoskeleton	+++	–	+

*microglial function yet to be established. "+++" high expression; "++" moderate expression; "+" low expression, yet present; "–" absent/unknown expression.