HHS Public Access

Author manuscript

J Neurochem. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

J Neurochem. 2019 June ; 149(5): 562–581. doi:10.1111/jnc.14674.

Inflammatory Mechanisms in Neurodegeneration

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Abstract

This review discusses the profound connection between microglia, neuroinflammation, and Alzheimer's disease (AD)[1]. Theories have been postulated, tested, and modified over several decades. The findings have further bolstered the belief that microglia-mediated inflammation is both a product and contributor to AD pathology and progression. Distinct microglia phenotypes and their function, microglial recognition and response to protein aggregates in AD, and the overall role of microglia in AD are research areas that have received considerable research attention and yielded significant results. The following article provides a historical perspective of microglia, a detailed discussion of multiple microglia phenotypes including dark microglia, and a review of a number of areas where microglia intersect with AD and other pathological neurological processes. The overall breadth of important discoveries achieved in these areas significantly strengthens the hypothesis that neuroinflammation plays a key role in AD. Future

[1]Abbreviations used – ABCA7, ATP-binding cassette sub-family member 7; AD, Alzheimer's disease; Aβ, amyloid-β protein; ApoE, apolipoprotein E; ApoJ, apolipoprotein J; APP, amyloid precursor protein; ASC, apoptosis-associated speck-like protein containing a CARD; CARD, caspase recruitment domain; CLU, clusterin; CNS, central nervous system; CSF1R, colony stimulating factor receptor 1; COX-2, cyclooxygenase-2; CR1, complement component (3b/4b) receptor 1; CR3, complement receptor 3; DAM, disease-associated microglia; DAMP, danger-associated molecular pattern; EPHA1, ephrin type-A receptor 1; FPRL1, formyl-peptide-receptor-like-1; GFP, green fluorescent protein; GWAS, genome-wide association study; Iba1, ionized calcium binding adaptor molecule 1; IL-1β, interleukin-1β; INPP5D, inositol polyphosphate-5-phosphatase; LCA, leucocyte common antigen; LPS, lipopolysaccharide; MEF2C, myocyte enhancer factor 2C; MGnD, microglia neurodegenerative phenotype; MHCII, major histocompatibility complex class II; MS4A6A/MS4A4AE, membrane-spanning 4-domains subfamily 4; Mw, molecular weight; NFκB, nuclear factor kappa B; NLR, nod-like receptor; NSAID, nonsteroidal anti-inflammatory drug; RAGE, receptor for advanced glycation end products; PAMP, pathogen-associated molecular pattern; PET, positron emission tomography; PS1, presenilin-1; RA, rheumatoid arthritis; SR, scavenger receptor; SEC, size exclusion chromatography; EM, electron microscopy; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TREM2, triggering receptor expressed on myeloid cells 2; YS, embryonic yolk sac.

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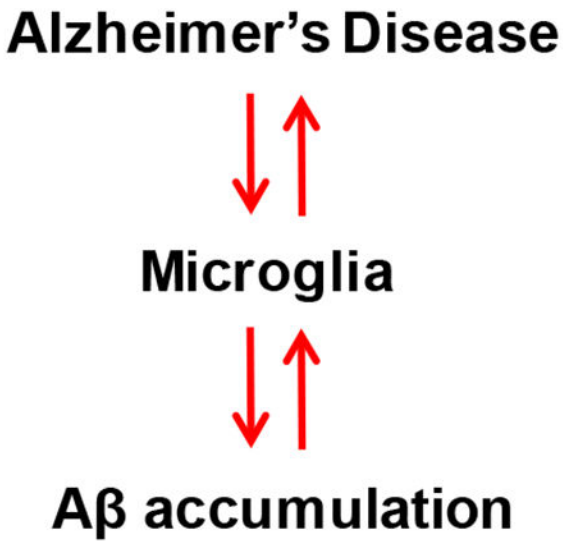
determination of the exact mechanisms by which microglia respond to, and attempt to mitigate, protein aggregation in AD may lead to new therapeutic strategies.

Graphical Abstract

This review will discuss the nexus between microglia, neuroinflammation, and Alzheimer's disease (AD). Areas of emphasis in the article are distinct microglia phenotypes and their function, microglial recognition and response to amyloid-β (Aβ) aggregates in AD, and the overall contributory role of microglia in AD. The cumulative data point to a dynamic interrelationship between microglial phenotype and function, as well as Aβ accumulation.



Introduction

Brief history of glia

In 1856 Rudolf Virchow coined the term "neuroglia" or "nerve-cement" to describe the brain connective tissue which consists of the non-neuronal or glial cells (Virchow. R 1856, 1858; Kettenmann and Verkhratsky 2008; Kettenmann et al. 2011; Ginhoux et al. 2013). The glial elements were first characterized by Robert Remak in 1838 and Heinrich Muller in 1851 (Muller 1851). Later in the 19th century, they were broadly introduced in various forms by Otto Deiters, Jacob Henle, Gustav Retzius, Camillo Golgi, and Michael von Lenhossek who introduced the term "astrocyte" or "astroglial cell" in 1893 (Deiters 1865; Henle 1869; Lenhossek 1893; Retzius 1894–1916; Golgi 1903). In 1899, Franz Nissl used the term "rod cells" (Stäbchenzellen) to describe the reactive glial elements with migratory, phagocytic, and proliferative functions and mesodermal origin (Nissl 1899). These phagocytic glial elements, which originated from the mesoderm and thus separate from neuronal and neuroglia origins, were termed "mesoglia" by W. Ford Robertson in 1900. He described that mesoglial cells, unlike astrocytes (macroglial cells), were not connected to blood vessels and acquired granular morphology and removed their processes in injured brains (Robertson 1900). Mesoglia, the third element of the central nervous system (CNS), were differentiated

from neurons (first element) and astrocytes (second element) by Santiago Ramon y Cajal in 1913. The “microglia” (the plural form of microgliocyte) or “Hortega cells” were thoroughly described by Pio del Rio-Hortega between 1919 and 1927 using a modified silver carbonate impregnation technique. He hypothesized that microglia were phagocytic cells with amoeboid features which migrated from mesoderm, pia mater, to entire brain regions through vessels and white matter tracts during early development. In the more mature brain they acquired a branched, ramified morphology (resting microglia) which transformed to an amoeboid morphological phenotype during pathological conditions (Del Rio-Hortega 1919a, 1919b, 1924, 1925, 1927, 1932). However, another component of mesoglia, neurectodermal oligodendroglia or oligodendrocytes, had no phagocytic activity (Ginhoux et al. 2013). There were controversies over the neuro-ectodermal (Fujita and Kitamura 1975; Kitamura et al. 1984; Hao et al. 1991; Fedoroff et al. 1997) or mesodermal (Murabe and Sano 1982; Hume et al. 1983; Murabe and Sano 1983; Perry et al. 1985) origins of microglia in several investigations. Light microscopy, electron microscopy (EM), and immunohistochemistry techniques supported the mesodermal origin of microglia hypothesis, distinguished the morphological similarities between microglia and macrophages, and supported the monocyte/macrophage-derived microglia hypothesis via phenotypic homologies (Murabe and Sano 1982; Hume et al. 1983; Murabe and Sano 1983; Perry et al. 1985). However, the emergence of immature macrophages within blood islands of the embryonic yolk sac (YS) at fetal day 9 was determined in mouse and rat by Takashi and Naito (Takahashi et al. 1989; Takahashi and Naito 1993). Following circulation formation around E8.5, the primitive macrophages, generated around E8.0, leave the YS blood islands, the only embryonic hematopoietic site at an early stage, and give rise to microglia after neuroepithelium colonization from E9.0/E9.5 (Takahashi and Naito 1993; Alliot et al. 1999; Ginhoux and Prinz 2015).

Spatial and temporal diversity of microglial phenotypes

Although early morphologic studies described microglia as either resting or activated, it has become clear that their behavior is characterized by a remarkable degree of basal plasticity. *In vivo* imaging of murine microglia has demonstrated that even in a healthy brain microglia are not at rest. Instead these immune cells are actively involved in homeostatic functions. Their highly motile processes continually interact with neuronal and glial cells, blood vessels (Nimmerjahn et al. 2005), as well as synapses (Wake et al. 2009; Tremblay et al. 2010). It is estimated that the entire brain parenchyma is sampled by these homeostatic microglia once every few hours (Nimmerjahn et al. 2005). Indeed, microglia appear to be involved in a spectrum of homeostatic functions including neurogenesis, neuronal migration, axon growth, and modulating synaptic connections (Sato 2015; Frost and Schafer 2016; Reemst et al. 2016; Li and Barres 2018). In addition to this range of normal behavior, studies show that microglia also differ in their phenotype across brain regions and lifespan in an extraordinarily complex fashion (Silvin and Ginhoux 2018). An early description of these differences by Lawson et al. examined microglial morphology and distribution in the adult mouse brain using the F4/80 antiserum. They reported greater microglial abundance in grey matter with highest concentrations in the hippocampus, basal ganglia, substantia nigra, and olfactory telencephalon with reduced immunoreactivity in the brainstem and cerebellum (Lawson et al. 1990). The authors estimated 3.5×10^6 total microglia per mouse brain

(Lawson et al. 1990). They also compared microglial morphology noting that this differed depending upon the brain region with round amoeboid cells near areas lacking a blood brain barrier, longitudinally branched cells in fiber tracts, and ramified cells throughout the neuropil (Lawson et al. 1990). A recent study by De Biase et al. increased our understanding of microglial phenotype regulation by characterizing adult mouse microglia via RNA sequencing, electrophysiology, and morphology to demonstrate region specific phenotypes within the basal ganglia (De Biase et al. 2017). Additionally, transient depletion of microglia by treating the animals with a colony stimulating factor receptor 1 (CSF1R) antagonist, PLEX5622, for 2 weeks followed by a 21 day recovery resulted in repopulation of basal ganglia microglia with diverse properties similar to pre-treatment supporting the view that local cues contribute to establishing microglial phenotype (De Biase et al. 2017; O'Neil et al. 2018). Similar findings were observed using 11-12 week old C57BL/6 mice to demonstrate regional diversity of microglial protein expression via flow cytometry (de Haas et al. 2008). Microglia isolated from the striatum, hippocampus, spinal cord, cerebellum, and cerebral cortex demonstrated clear expression of CD11b, CD40, CD45, CD80, CD86, F4/80, TREM-2b, CXCR3, and CCR9 with no evidence of MHCII or CCR7 immunoreactivity (de Haas et al. 2008). However, individual protein levels of these markers varied depending upon the brain region with no clear pattern of overall low or high levels for all proteins relevant to a region (de Haas et al. 2008). It is clear that these regional selective morphologies and phenotypes are not static, as microglia are able to transform from amoeboid to ramified and vice versa in response to varying conditions (Ling and Wong 1993; Ling et al. 2001). It has become clear that microglia also have an altered phenotype associated with normal aging. For instance, increased microglial Iba1/MHCII double immunoreactivity increases throughout the hippocampus in rats with age suggesting a phenotype change occurs (VanGuilder et al. 2011). However, this simple measure of change did not correlate with any behavioral dysfunction suggesting that microglial phenotypes unique to disease or injury states are superimposed upon age-associated changes (VanGuilder et al. 2011). A more recent study corroborates an aging phenotype of microglia using direct RNA sequencing from microglia isolated from 5 and 24-month-old mice (Hickman et al. 2013). The authors demonstrated that microglial are distinct from peripheral macrophages and down regulate gene pathways involved in neurotoxicity while upregulating those involved in neuroprotection with age (Hickman et al. 2013).

Changing role of microglia in Alzheimer's disease

The age-associated neurodegenerative disease, Alzheimer's disease (AD), is characterized by a robust increase in morphologically reactive microglia. Moreover, these microglia are often associated with extracellular plaque deposits of amyloid β (Aβ) peptide. *In vivo* imaging using [^{11}C] (R)-PK11195 positron emission tomography (PET) against the peripheral benzodiazepine receptor, abundantly expressed by mononuclear phagocyte lineage cells, has demonstrated microglial activation in patients with mild to moderate AD-type dementia compared to healthy individuals. Such observations from the living human brain suggests the possibility of microglia phenotypic alteration as an early AD pathological hallmark (Cagnin et al. 2001). A subsequent study of 64 AD patients and 32 controls using an 18-kDa translocator protein PET radiotracer coupled with amyloid imaging using Pittsburgh compound B PET reached a similar conclusion, i.e. that microglial activation

appears during prodromal and perhaps preclinical disease (Hamelin et al. 2016). To add to this complexity, the typical late-onset, sporadic form of disease suggests that any disease-associated changes in microglial phenotype are compounded by age-dependent differences. Based upon this plethora of regulatory influences, the study of microglial behavior in AD remains an area of intense study and will be the subject of the remainder of this review. As will be discussed in later sections, the specific nature of Aβ-microglia interactions together with their causes and consequences, remain a significant effort in the field. In addition, there is a remarkable array of microglial phenotypes in AD.

Early work focused on ultrastructural studies of extracellular amyloid-beta (Aβ) plaques and their associated microglia in the cortex of AD patients to reveal Aβ fibrils in altered cisterns of the microglial endoplasmic reticulum. Also, exogenous Aβ fibrils taken up by cultured microglia remained intact in phagosomes up to 20 days. Based on these observations, Wisniewski and Frackowiak hypothesized that Aβ fibrils are produced but not phagocytosed by microglia in AD (Wisniewski et al. 1989; Wisniewski et al. 1990; Frackowiak et al. 1992). In agreement with this, the *in vitro* production of amyloid-beta precursor protein (APP) by rodent microglia was further reported (Haass et al. 1991; Mönning et al. 1995; LeBlanc et al. 1996). However, lack of APP mRNA in microglia clustering in the vicinity of plaques in postmortem AD brains decreased support for microglial ability to synthesize Aβ as a contribution to plaques (Scott et al. 1993). Subsequently, HLA-DR, a major histocompatibility complex class II (MHC II) cell surface receptor expressed by antigen presenting cells, MHC I to a lesser extent, leucocyte common antigen (LCA), and the Fc gamma RI and Fc gamma RII receptors were all immunohistochemically identified on reactive microglia of postmortem AD brains (McGeer et al. 1987; Itagaki et al. 1988; McGeer et al. 1989; Tooyama et al. 1990). These receptors are indicative of immunoreactivity and phagocytic properties of microglia suggesting that microglia were not producing Aβ but instead responding to it. Subsequently, detection of Aβ in intracytoplasmic membranes, excluding secretory organelles, strengthened a phagocytosis-associated role of microglia in AD (el Hachimi and Foncin 1994). Perhaps most importantly, it has become clear that the type of Aβ and putative cell surface receptors that mediate microglia-Aβ interaction add significant difficulty to understanding how these cells behave in AD. As discussed in detail later in this review, it is now recognized that microglial phenotype is not simply phagocytic in AD. For example, interaction of microglia with Aβ deposits, via scavenger receptors (SRs) and the receptor for advanced glycation end products (RAGE), in an effort to phagocytose Aβ and clear up the extracellular milieu, triggered microglia immobilization, production of cytokines, reactive oxygen species, and nitrogen species, and results in neuronal death supporting a detrimental role for microglia in AD (Banati et al. 1993; El Khoury et al. 1996; Paresce et al. 1996; Lue et al. 2001a).

Heterogenous microglial population in AD

The diversity of the microglial population in AD has been the subject of several studies in recent years and numerous transgenic AD mouse models have contributed to these discoveries. The APP(/)PS1 transgenic strain carries the human transgenes for APP bearing the KM670/671NL Swedish mutation and presenilin-1 (PS1) containing an L166P mutation (Duff et al. 1996; Radde et al. 2006). These mice develop cerebral Aβ deposition within the

first few months of life. The 5XFAD strain carries the Swedish and two other APP mutations as well as two PS1 mutations (Oakley et al. 2006). Studies in these mouse models have led to the definition of multiple microglia phenotypes seen under various pathological conditions. One of these phenotypes has been termed disease-associated microglia (DAM), which are prevalent in the 5XFAD mice. DAM were recently characterized using single-cell RNA sequencing (Keren-Shaul et al. 2017). This phenotype is associated with (1) a downregulation of several homeostatic genes such as *P2ry12* (purinergic receptor P2RY12), *Tmem119* (transmembrane protein 119), and *Cx3cr1* (C-X3-C motif chemokine receptor 1); (2) *TREM2* (triggering receptor expressed on myeloid cells 2)-dependent upregulation of genes including *Axl* (receptor tyrosine kinase), *Clec7a* (C-type lectin domain containing 7A), *lpl* (lipoprotein lipase), *Spp1* (osteopontin) and *ITGAX* or *Cd11c*, (integrin subunit alpha X); and (3) *TREM2*-independent upregulation of *ApoE* (apolipoprotein E) and *Tyrobp* (TYRO protein tyrosine kinase binding protein) (Keren-Shaul et al. 2017). The microglia neurodegenerative phenotype (MGnD) is another recently-characterized population that similarly presents with reduced expression of homeostatic genes, including *P2ry12* and *Tmem119*, and increased expression of *ApoE, Axl*, and *Clec7a* genes (Krasemann et al. 2017b). These MGnD cells were only observed when neuronal apoptosis also occurred, for instance in the APP/PS1 mouse model, and found to be regulated by *TREM2* and *ApoE* expression (Krasemann et al. 2017a). Another microglial phenotype notably associated with AD and that will be covered in the last part of this review, is dark microglia (Bisht et al. 2016b). Although dark microglia share similar morphological features with typical microglia, pertaining to their size, morphology, association with the extracellular space and relationships with neurons and synapses, they possess distinct characteristics that are observed by EM. Typical microglia strongly express the homeostatic markers CX_3CR1, Iba1 and P2RY12. By contrast, dark microglia do not express P2RY12, and downregulate Iba1 and green fluorescent protein (GFP) in CX_3CR1-GFP reporter mice (Bisht et al. 2016b). Phenotypic differences between normal and disease-related microglia are summarized in Table 1.

Support for immune contribution to AD

The biochemical and histologic characterizations of microglial phenotypes in AD certainly support a role for these cells during disease, but genetic data provides even more compelling evidence of microgliosis contributing to disease. Genome wide association studies (GWAS) have revealed risk loci associated with immune response besides the well-known *APOe4* allele (late-onset AD) and pathogenic mutations of *APP*, *PSEN1*, and *PSEN2* (early-onset autosomal-dominant AD) which are carried by 50% and 2% of individuals with AD, respectively. For example, a large GWAS of 2,032 AD patients and 5,328 controls from France identified loci in *CLU* or *APOJ* (clusterin or apolipoprotein J) and *CR1* (the complement component (3b/4b) receptor 1), which are involved in Aβ clearance (Lambert et al. 2009). *ABCA7* (ATP-binding cassette, sub-family A, member 7), *MS4A6A/MS4A4E* (membrane-spanning 4-domains, subfamily A), *EPHA1* (Ephrin type-A receptor 1), *TREM2, HLA-DRB5-DRB1* (MHC II, DR beta 5-1), *INPP5D* (Inositol polyphosphate-5-phosphatase), *MEF2C* (myocyte enhancer factor 2C*)*, and *CD33*, also have polymorphisms associated with risk of late-onset AD (Lambert et al. 2009; Hollingworth et al. 2011a; Naj et al. 2011b; Guerreiro et al. 2013b; Jonsson et al. 2013; Lambert et al. 2013a; Karch et al.

2014). Collectively, there is continuing support for the idea that the immune system, and microglial behavior particularly, contributes to disease progression. Defining the phenotype of microglia during AD, as discussed below, as well as identifying tools for their manipulation, will offer novel intervention strategies.

Immunomodulatory trials for AD

Consistent with the idea of microglial inflammatory contribution to disease, epidemiological studies have revealed a remarkable decline of AD prevalence in individuals with rheumatoid arthritis (RA), most likely as a result of long-term anti-inflammatory therapy for this well-established chronic inflammatory disease (Jenkinson et al. 1989; McGeer et al. 1990; Li et al. 1992; Myllykangas-Luosujärvi and Isomäki 1994). Other epidemiological investigations focused on the contribution of long-term nonsteroidal anti-inflammatory drug (NSAIDs) usage to the risk of AD development and also confirmed an inverse association between NSAID use and AD (Andersen et al. 1995; Breitner et al. 1995; Stewart et al. 1997; Beard et al. 1998; Broe et al. 2000; in ‘t Veld et al. 2001; Lindsay et al. 2002; Zandi et al. 2002). In agreement with the robust biochemical and histologic evidence, an inflammatory hypothesis of AD arose suggesting anti-inflammatory interventions to decrease the incidence and progression of AD. Clinical trials for different NSAIDs with various durations of drug treatment have been performed to validate their potential protective role in mild to moderate AD. Although, 6-month administration of the NSAID indomethacin demonstrated cognitive deficit improvement of AD patients, the statistical data was inconclusive because of the small scale of the clinical trial (Rogers et al. 1993). Furthermore, treatment with a combination of the NSAID diclofenac and the synthetic prostaglandin misoprostol for 25 weeks, the NSAID rofecoxib (a cyclooxygenase-2 (COX-2)-selective inhibitor) or the NSAID naproxen (non-selective COX inhibitor) for 1 year, rofecoxib up to 4 years, the NSAID celecoxib (a COX-2-selective inhibitor) for 52 weeks, and celecoxib or naproxen for 3 years exerted no significant beneficial effect (Scharf et al. 1999; Aisen et al. 2003; Reines et al. 2004; Thal et al. 2005; Group et al. 2007; Soininen et al. 2007; Group et al. 2008). NSAID treatment initiation at the more advanced course of AD, the short durations of treatment, inappropriate dosage to ameliorate the inflammation compared to the epidemiological studies, and side effects, like gastrointestinal dysfunction, fatigue, dizziness, headache, hypertension, and stroke have all likely contributed to clinical trial failure. Similarly, a randomized, placebo-controlled multicenter trial of low-dose prednisone (a synthetic glucocorticoid) treatment reported no significant amelioration effect on cognition of AD patients in primary and secondary analysis (Aisen et al. 2000). Additional study of microglial behavior during AD is required to determine whether immunomodulatory therapies are feasible. The remainder of this review is focused on summarizing current understanding of microglial contributions to AD.

Conformational aspects of Aβ relevant for neuroinflammation

Aβ conformational species

The conformation or aggregation state of Aβ has a substantive impact on the cellular inflammatory response to the peptide. Numerous studies have shown that monomeric Aβ is unstructured and fairly innocuous. Yet, when monomers begin to non-covalently assemble

and aggregate, a β-sheet structure develops and predominates. This conversion renders Aβ a more toxic and inflammatory molecule. Aβ aggregation typically does not progress in discrete defined steps and can yield a polydisperse solution of species that differ in size, conformation, and solubility. Several classes of Aβ aggregates have emerged that include oligomers, protofibrils, and fibrils (Figure 1). The most classical and well-characterized Aβ aggregates are fibrils, which make up the core of AD neuritic plaques (Terry 1985). Fibrils are high molecular weight (M_w) rod-like structures greater than 1 μm in length and contain multiple layers of β-sheet linked monomers (Colvin et al. 2016; Walti et al. 2016). Protofibrils also have a defined β-sheet structure, but are smaller in both diameter and length, and are soluble precursors to fibrils (Harper et al. 1999; Walsh et al. 1999; Nichols et al. 2015). Oligomers are also soluble and tend to have less β-sheet structural characteristics than the other more developed Aβ aggregates. Oligomers generally fall into two groups, smaller (dimers through hexamers) (Lambert et al. 1998; Bitan et al. 2003; Jin et al. 2011) and larger M_w species (Hepler et al. 2006).

Technical considerations for the preparation of Aβ for cellular studies

It is challenging to directly compare studies of Aβ biological activity when there is frequently a lack of rigorous definition of the Aβ solution species. In many cases, these solutions contain multiple Aβ species, which complicates interpretation. Numerous studies over the years have identified solution conditions that promote formation of one Aβ species over another. The combination of these conditions and additional separation steps can be successfully employed to isolate a particular species. Reconstitution of dry Aβ peptide will most assuredly yield a mixed solution. Thus, additional steps are usually required to obtain monomeric Aβ. This is especially the case for Aβ42 due to its significantly higher aggregation propensity than Aβ40 (Jarrett et al. 1993). Pretreatment of dry Aβ peptide with hexafluoroisopropanol and/or in alkaline conditions decreases the amount of preformed aggregates (O'Nuallain et al. 2006; Teplow 2006), but the best method for Aβ monomer purification is size exclusion chromatography (SEC) (Walsh et al. 1997). A well-established method for preparing oligomers entails resuspension of Aβ in dimethyl sulfoxide, dilution into cold Ham's F-12 cell culture medium without phenol red, and incubation at 4 °C (Stine et al. 2003). Conditions for preparing, isolating, and characterizing soluble protofibrils were initially reported 20 years ago (Walsh et al. 1997; Harper et al. 1999; Walsh et al. 1999) and thoroughly chronicled in subsequent years (Lashuel and Grillo-Bosch 2005). Insoluble Aβ fibrils have commonly been prepared by 37 °C incubation at neutral (Harper et al. 1997) or acidic pH (Stine et al. 2003). Higher ionic strength encourages Aβ fibril formation (Harper et al. 1999; Nichols et al. 2002; Nichols et al. 2015) and the fibril insolubility allows isolation in the pellet fraction after centrifugation of the sample at 17,000*g* (Walsh et al. 1997). Thoughtful preparation and rigorous characterization for a given Aβ preparation is important to fully interpret the interactions between Aβ and biological targets.

Aβ conformation and inflammatory receptor interaction

Due to the different conformation states and variable pathogenicity of Aβ, several studies have focused on how different species interact with neuroinflammation-associated receptors (Figure 1). These receptors have been found in pre-synaptic and post-synaptic neurons as well as intracellularly and on the cell surface of astrocytes and microglia (Jarosz-Griffiths et

al. 2016). Microglial receptors continue to be of particular interest due to the cells' key role in the spread of inflammation (Doens and Fernandez 2014). The inclusion of Aβ as a pathogen-associated molecular pattern (PAMP) and danger-associated molecular pattern (DAMP) further expands the number of cell-surface Aβ receptors and adds intracellular receptors such as nod-like receptors (NLRs) to the growing list (Heneka 2017). Although there are numerous Aβ receptors, this section will highlight a few that interact with different forms of Aβ and mediate inflammatory processes. Early studies provided evidence for a receptor complex comprised of CD36, $\alpha_6\beta_1$ integrins, and CD47 that recognizes fibrillar Aβ and initiates a pro-inflammatory cascade involving tyrosine kinases. Individual components were determined by systematic testing of selective antagonists for each component (Bamberger et al. 2003). In addition to the integrin receptor complex, an impressive number of reports have implicated Toll-like receptors (TLRs) as major mediators of inflammation induced by fibrillar Aβ (Walter et al. 2007; Jana et al. 2008; Udan et al. 2008; Reed-Geaghan et al. 2009; Stewart et al. 2010). TLRs 2, 4, and 6 along with CD36 and the TLR accessory protein CD14 have all been shown to contribute substantially to the overall proinflammatory response. As interest grew in soluble aggregated forms of Aβ, receptors were examined for their ability to recognize these species. Terrill-Usery et al demonstrated that TLRs (via the adaptor protein MyD88) were the major mediators of tumor necrosis α (TNFα) and interleukin-1β (IL-1β) production induced by protofibrillar Aβ42 in primary wild-type and $MyD88^{-/-}$ mouse microglia (Terrill-Usery et al. 2014). RAGE is another microglia receptor involved in neuroinflammation. RAGE was implicated as a mediator of Aβ-induced synaptic depression in entorhinal cortex slices after bath application of the slices with a solution of Aβ42 monomers and small oligomers (dimers and trimers) (Origlia et al. 2010). NLRs are potential intracellular receptors for Aβ, and although their direct interaction has not been demonstrated, it has been known for some time that Aβ can enter microglia, trigger NLRP3 inflammasome activation, and produce mature IL-1β (Halle et al. 2008). Furthermore, CD36/TLR4/6-mediated uptake of soluble Aβ triggered inflammasome activation and conversion of the Aβ into a fibrillar form (Sheedy et al. 2013). This finding provided evidence that the NLRP3 inflammasome contributes to Aβ nucleation and aggregation inside the cell. An intriguing receptor for Aβ is APP itself. A recent report showed that oligomeric Aβ42 oligomers and fibrils stimulated tyrosine kinase pathways and TNFα production at similar levels in wild-type microglia, yet only fibrils induced an inflammatory response in $APP^{-/-}$ mouse microglia (Manocha et al. 2016). The finding suggested that APP may serve as a receptor for oligomeric Aβ. Additional Aβ receptors that may be sensitive to Aβ conformation include formyl-peptide-receptor-like-1 (FPRL1), additional SRs, complement receptors, and TREM2 (Doens and Fernandez 2014; Zhong et al. 2018).

Aβ conformation and cytokine production

The relationship between Aβ aggregation structure and cellular stimulation of pro-inflammatory cytokine release (Figure 1) has been explored by numerous groups. An early study demonstrated that fibrillar forms of Aβ40, and the fragment Aβ25-35, stimulated IL-1β release from lipopolysaccharide (LPS)-treated THP-1 human monocytes (Lorton et al. 1996) While non-aggregated Aβ was not stimulatory. This paradigm of LPS-primed immune cells continues to be used as a model for infection in neurodegeneration or to study two-

signal activation of the inflammasome. Despite the utility of LPS priming, it was subsequently shown that fibrillar Aβ40 alone stimulated tyrosine kinase-mediated pathways leading to increased TNFα and IL-1β gene expression in THP-1 cells (Combs et al. 2001). Stimulation of cytokines (TNFα and IL-1β) by fibrillar Aβ40 was also corroborated in primary murine microglia (Yates et al. 2000). More recent work has focused on Aβ42 and other types of aggregated Aβ species. Golenbock and colleagues demonstrated production of IL-1β in LPS-treated primary murine microglia by fibrillar, but not nonfibrillar reverse Aβ peptide (Halle et al. 2008). To further probe the impact of Aβ conformation, White el al. directly compared the stimulatory effect of oligomeric and fibrillar Aβ42 in rat astrocytes and found that the two species stimulate different types of inflammatory responses. They observed that Aβ42 oligomers were more potent than fibrils and induced a profound, early inflammatory response. Fibrils caused a delayed, more chronic response (White et al. 2005). Similar findings were obtained in a later study where both low and high M_w Aβ42 oligomers (L-Aβol and H-Aβol respectively) were compared to Aβ42 fibrils (Heurtaux et al. 2010). Both oligomeric species induced similar levels of TNFα in MMGT12 murine microglial cells, and these levels were significantly higher than those induced by fibrils. This robust proinflammatory activity by soluble Aβ42 aggregates has also been observed in another report that directly compared TNFα secretion from primary murine microglia in response to carefully isolated Aβ42 monomers, protofibrils, and fibrils (Paranjape et al. 2012). Monomers and fibrils triggered very low levels of TNFα, while protofibrils provoked ~20-fold higher TNFα levels than the other species. This low level of TNFα production by fibrils was despite the finding that fibrils displayed significantly higher thioflavin T fluorescence, a marker of β-sheet structure. Interestingly, Aβ40 protofibrils were much less effective than Aβ42 protofibrils. The normal aging process may play a role in the responsiveness of microglia to different conformations of Aβ. In a study comparing TNFα secretion in postnatal and adult murine microglia, both oligomeric and fibrillar Aβ42 stimulated TNFα in young microglia. However, only Aβ42 oligomers induced TNFα in adult microglia (Floden and Combs 2006). Alternate Aβ conformations may impact levels of particular inflammatory molecules differently. Combs and colleagues demonstrated that oligomers were better than fibrils at stimulating IL-6 in murine microglia, yet the reverse was true for stimulating keratinocyte chemoattractant chemokine (Sondag et al. 2009). The cumulative data emphasizes the importance of Aβ conformation in multiple mechanisms of microglia interaction and phenotypic transformation. This is particularly true for Aβ42, with its increased aggregation propensity, ability to form multiple aggregation species, and high relevancy in neuroinflammatory processes linked to AD pathology.

Aβ conformation and microglial internalization

Microglia are the primary phagocytic cell in the brain. The interaction between microglia and extracellular brain Aβ is a significant area of AD research. Much of this focus is on the ability of microglia to internalize Aβ as this would provide an efficient way to clear Aβ and reduce or prevent accumulation. However, even this interaction is sensitive to Aβ conformation or aggregation state. There are many reports of microglial internalization of different forms of Aβ (monomer, oligomer, protofibril, fibril), but fewer have directly compared these species within a single published report. Maxfield and colleagues utilized radio- and fluorescent-labeling to study primary murine microglial internalization of soluble

(presumably monomeric) and fibrillar Aβ at low concentrations (0.2-1.0 μM) (Chung et al. 1999). For Aβ fibrils, they observed saturable uptake, minimal degradation, and slow release. Soluble Aβ was internalized by a non-saturable process and was released rapidly from microglia also with minimal degradation. Two major mechanistic classes of cellular internalization include phagocytosis for particles greater than 0.5 μm, and macropinocytosis for extracellular fluid containing smaller, soluble particles (Gordon 2003). The two processes have commonalities in receptors and signaling pathways, particularly the formation of cups in the plasma membrane (Swanson 2008). The mechanistic aspects of Aβ internalization by microglia were further probed by Landreth and colleagues who again delineated between fibrillar and soluble Aβ. In their study using both BV-2 and primary murine microglia, it was determined that fibrillar Aβ was internalized by phagocytosis and soluble Aβ through fluid-phase macropinocytosis (Mandrekar et al. 2009). The concept of "soluble" Aβ was investigated more closely by inclusion of soluble aggregated species such as oligomers and protofibrils. Grutzendler and colleagues demonstrated in an *in vivo* study that microglia were highly effective at protofibrillar Aβ uptake but were incapable of phagocytosis of fibrillar congophilic Aβ (Liu et al. 2010). Further differences between Aβ species were subsequently reported in another study using primary murine microglia (Taneo et al. 2015). Aβ42 oligomers and fibrils were internalized more readily than monomers, but only the oligomers induced endo/phagolysosome rupture releasing cathepsin B into the cytoplasm. In another study conducted by Nichols and colleagues, a rigorous separation of "soluble" Aβ was implemented. Size-exclusion chromatography was used to isolate fluorescently-labeled monomeric and protofibrillar Aβ42. This methodology produced a more nuanced picture of Aβ internalization and showed that primary mouse microglia internalize protofibrils rapidly and robustly compared to monomers (Gouwens et al. 2016). Additional experimentation by the group concluded that very little degradation of the protofibrils occurred. The overall data indicate that microglia-mediated internalization is greatly dependent on Aβ conformation or aggregation state. The molecular details of the interactions driving and directing the internalization processes remain to be elucidated (Figure 1). One fate of internalized Aβ may be packaging into microvesicles and release from the cells back into the extracellular space. Microvesicles are spherical structures ranging in size from 100-1000 nm that bleb from the cell surface carrying a variety of biomolecules (Budnik et al. 2016). Several studies have reported the release of previously internalized Aβ in association with microvesicles (Joshi et al. 2014; Sollvander et al. 2016; Gouwens et al. 2018). This process appears to be sensitive to Aβ conformation and has implications for spreading of Aβ and proliferation of neuroinflammation.

Another major microglia-mediated immune pathway that is also sensitive to Aβ conformation or aggregation state is the classical complement pathway. A long-established finding showed that the assembly of Aβ aggregates was critical for binding of C1q and activation of complement (Jiang et al. 1994). Furthermore, Aβ, complement-dependent pathways, and microglia cause elevated synaptic pruning and synaptic loss in AD (Hong et al. 2016). There are numerous aspects to the relationship between microglial activation and neurodegeneration. While many of these mechanisms have been elucidated *in vitro*, studies of AD patients have shown that the degree of microglial activation was positively correlated

with clinical dementia rating, Braak staging, and neuritic plaque development (reviewed in (Mrak 2012). Further discussion of this relationship is provided in the next section.

The relationship of microglia and Aβ plaque deposition

Aβ-induced alterations of the microglial phenotype

Microglia readily react to disturbances of their environment; accordingly, the deposition of Aβ as insoluble plaques in the brain parenchyma of AD patients changes microglial function. Early studies already described pronounced microglial accumulation around amyloid plaques (microgliosis) in AD brain tissue (Dickson et al. 1988; Haga et al. 1989; Itagaki et al. 1989), combined with dramatic morphological changes of plaque-associated microglia (Akiyama et al. 2000). These features are recapitulated in the aforementioned mouse models of AD pathology, which reproduce amyloid plaque deposition. These models are useful for studying the relationship between amyloid plaque deposition and microglial phenotypes, allowing also for experimental manipulation (Ashe and Zahs 2010; Jucker 2010).

In line with the clear increase in microglial numbers in the direct vicinity of amyloid plaques, recent studies confirmed increased microglial proliferation rates in living mice in response to cerebral β-amyloidosis (Fuger et al. 2017). Additional *in vivo* mouse studies demonstrate that CSF1R signaling appears to play a critical role in regulating this proliferation (Kamphuis et al. 2012; Gomez-Nicola et al. 2013; Olmos-Alonso et al. 2016). In addition, long-term two-photon imaging of microglia in APP/PS1 mice revealed that plaque-distant microglia proliferate and subsequently migrate to amyloid plaques (Fuger et al. 2017), providing a mechanism for the commonly observed microgliosis in AD models and indicating that soluble factors (rather than aggregated amyloid itself) may be the trigger for microglial proliferation.

Alterations of microglia in the vicinity of Aβ plaques are not restricted to changes in morphology and cell number. For example, various studies demonstrated impairments in microglial phagocytosis in AD models (Floden and Combs 2011; Krabbe et al. 2013; Wildsmith et al. 2013). Such changes in microglial clearance of Aβ are of immense interest to the field of AD research, as microglial phagocytic activity would in principle be able to degrade Aβ aggregates and may therefore alleviate amyloid-induced pathology (D'Andrea et al. 2004; Heneka and O'Banion 2007; Heneka et al. 2015). As such, improving microglial phagocytosis using Aβ-directed antibodies is still being intensely studied as a therapeutic strategy in clinical trials for AD patients (Fu et al. 2010; Graham et al. 2017). For example, the anti-Aβ antibody aducanumab was shown to strongly enhance recruitment of microglia to amyloid plaques and simultaneously reduce the amount of plaques in AD patients, suggesting that improving microglial clearance of Aβ might indeed represent a promising therapeutic avenue (Sevigny et al. 2016).

In addition to reduced phagocytosis, extensive studies suggest upregulated pro-inflammatory microglial activity (Rogers et al. 1992; Lue et al. 2001b; Butovsky et al. 2005; Lindberg et al. 2005; Floden and Combs 2006). Aβ induces complex changes in the microglial phenotype since it acts on a diverse array of microglial signaling receptors, including TLRs

and SRs (El Khoury et al. 1996; Paresce et al. 1996; Landreth and Reed-Geaghan 2009), and activates complex signaling cascades such as the NLRP3 inflammasome (Halle et al. 2008). The considerable heterogeneity in gene expression between individual microglia and particularly between microglia associated with plaques and in plaque-free areas was highlighted by recent single-cell transcriptional profiling studies (Keren-Shaul et al. 2017; Korin et al. 2017; Krasemann et al. 2017b; Mathys et al. 2017). In these studies, microglial subtypes associated with neurodegenerative disease, including DAM and MGnD (as described in Table 1), implicated TREM2 and ApoE as key mediators of these phenotypes (Keren-Shaul et al. 2017; Krasemann et al. 2017b). Furthermore, it was shown that a switch in the microglial activation state is necessary for their response to amyloid deposits. For example, microglia in a homeostatic state (e.g. shown by their expression of P2RY12) do not cluster around amyloid plaques. Moreover, altering the balance between microglial disease-associated and homeostatic phenotypes significantly affects the microglial reaction towards amyloid pathology by altering microglial clustering, phagocytosis, and cytokine secretion (Jay et al. 2015a). For example, a signaling role for DAM markers ApoE and TREM2 in the induction of microglial activation in response to Aβ deposition has now been demonstrated through analysis of microglial gene expression profiles in knockout mouse models (Keren-Shaul et al. 2017; Krasemann et al. 2017b; Ulrich et al. 2018). Interestingly, genetic variants of both proteins have been linked to AD susceptibility, with variants in *APoE* and *TREM2* being the two strongest known risk factors for late-onset AD (Corder et al. 1993; Guerreiro et al. 2013a; Jonsson et al. 2013). In the future, single-cell studies will aid in identifying heterogeneous subpopulations of microglia and define disease-specific subgroups. Correlations with functional phenotypes could shed new light on therapeutic targets to specifically manipulate microglia in areas of pathology.

While these studies have provided considerable evidence that Aβ plaques alter microglial activity and function, changes in microglial function are not a mere bystander effect of Aβ deposition. Instead, many studies demonstrate that the microglial functional state itself modulates amyloid pathology, demonstrating a bi-directional relationship between microglial function and amyloid plaque deposition (Figure 2).

Modulation of amyloid pathology by microglia

Strong evidence for the idea that microglia may directly impact Aβ aggregation was provided by genome-wide association studies that linked variants in microglial genes to an increased risk for the development of late-onset AD (Hollingworth et al. 2011b; Naj et al. 2011a; Lambert et al. 2013b; Zhang et al. 2013; Gagliano et al. 2016; Efthymiou and Goate 2017; Huang et al. 2017). These studies have intensified the interest in studying the microglial contribution to AD pathogenesis.

Due to the identification of TREM2 as a strong genetic risk factor for AD, its role in AD is being intensely studied. TREM2 binds a variety of membrane lipids and is involved in microglial phagocytosis of damaged neurons and Aβ (Wang et al. 2015b; Wang et al. 2015a; Zhao et al. 2018). In addition, TREM2 has recently been shown to directly bind oligomeric Aβ (Zhao et al. 2018). As mentioned above, recent studies also demonstrated that TREM2 is controlling the balance between homeostatic and pathology-associated microglial states

(Keren-Shaul et al. 2017; Krasemann et al. 2017b; Mazaheri et al. 2017). However, while knockout of *TREM2* robustly impairs microglial clustering around amyloid plaques in mice, it has produced inconsistent results on amyloid plaque load (Jay et al. 2015b; Wang et al. 2015b; Wang et al. 2015a), which may be due to different roles of TREM2 in different stages of disease (Jay et al. 2017). However, a recent study found that overexpressing human *TREM2* in mouse microglia leads to a robust reduction in cerebral amyloidosis, clearly indicating that activation of this receptor may be therapeutically useful (Lee et al. 2018).

Recently, a previously unappreciated role for TREM2 in the formation of a protective microglial barrier around amyloid plaques has been suggested. A number of studies showed that microglia, which cluster around Aβ plaques, use their processes to form a tight barrier to shield the surrounding environment from toxic Aβ species. Interestingly, TREM2 appeared to be specifically expressed by microglial processes contacting amyloid plaques and TREM2 knockout abolished microglial clustering. As a result, *TREM2* knockout mice and also human TREM2 R47H cases of late-onset AD had increased amounts of diffuse forms of Aβ surrounding amyloid plaques (Wang et al. 2016; Yuan et al. 2016). Importantly, this change in Aβ plaque structure was associated with increased neuronal damage, indicating that, in addition to the total amount of amyloid, the particular structure of amyloid deposits could also impact disease progression and can be influenced by microglial function. Similarly, other factors, such as deletion of CX3CR1 or anti-Aβ immunization, also modulate amyloid plaque structure and neuronal damage (Condello et al. 2015), implicating microglial barrier function as a novel mechanism in amyloid-plaque compaction and the restriction of amyloid-associated toxicity. Deciphering cause or consequence remains difficult in this context. In the brains of AD patients, a variety of plaque structures occur, ranging from dense core to diffuse plaques (D'Andrea et al. 2004; Yuan et al. 2016; Rasmussen et al. 2017). The amount of microglial accumulation differs markedly between dense core and diffuse plaques; however, it is unclear whether the change in plaque structure is caused by differences in microglial barrier function or whether different plaque conformations trigger differential migration/turnover of microglia. Adding to the complexity, it was previously demonstrated that even the dense core fibrillar plaques may possess Aβ conformational diversity by serving as reservoirs of oligomeric Aβ (Koffie et al. 2009).

In addition to the recent interest in TREM2, a multitude of studies have analyzed the effect of overexpression or knockout of certain cytokines on amyloid pathology. However, these studies have again shown conflicting results (Chakrabarty et al. 2010a; Chakrabarty et al. 2010b; Vom Berg et al. 2012). For example, overexpression of IL-1β was found to exert beneficial effects on Aβ deposition (Shaftel et al. 2007), while other studies demonstrated ameliorated disease progression upon blockage of IL-1 signaling or knockout of the IL-1β-producing NLRP3 inflammasome (Kitazawa et al. 2011; Heneka et al. 2013). Recently, the inflammasome adaptor protein ASC, which forms aggregates (so called specks) upon inflammasome activation and can subsequently be released from microglia, was implicated in cross-seeding Aβ pathology (Venegas et al. 2017), suggesting another microglial mechanism in the modulation of cerebral β-amyloidosis. Similarly, a two-photon *in vivo* study indicated that microglial uptake of aggregated Aβ, followed by microglial death and release of residual Aβ, could lead to the spreading of amyloid deposition (Baik et al. 2016).

Another factor that needs to be considered when studying microglial function in AD is the extraordinarily long lifetime of individual microglia. While an early study already indicated that microglia have very low turnover rates (Lawson et al. 1992), recent studies have readdressed this question (Askew et al. 2017; Fuger et al. 2017; Tay et al. 2017). Two studies employing genetic labeling of individual microglia reported very long lifetimes of more than 15 months for cortical microglia (Fuger et al. 2017; Tay et al. 2017). Of note, the turnover of microglia varies between brain regions, proving once more the heterogeneity among individual microglia (Askew et al. 2017; Tay et al. 2017). This extremely long lifetime of microglia has important implications for microglial function. In aged mice, microglial are skewed towards adopting a pro-inflammatory phenotype with impaired homeostatic functions (Sierra et al. 2007; Norden and Godbout 2013; Hefendehl et al. 2014; Grabert et al. 2016; Galatro et al. 2017). Furthermore, some studies suggest that microglial senescence occurs with age and might contribute to the development of neurodegenerative diseases (Streit et al. 2004; Streit et al. 2009).

Due to their extraordinarily long lifespan, alterations of the microglial phenotype in response to disturbances in the environment may change the microglial reaction to much later occurring stimuli, a concept known as innate immune memory (Wendeln et al. 2018). Accordingly, it was recently shown that microglia are capable of immune memory, proving that external stimuli can induce long-lasting alterations of the microglial epigenome and thereby alter the microglial reaction to secondary stimuli, including Aβ deposition (Wendeln et al. 2018). Importantly, the concept of innate immune memory might also explain the many confounding studies on the role of microglia in AD, since a combination of stimuli over a long period of time (possibly across the whole microglial lifetime) might impact the microglial phenotype in disease, but this is a field that is only beginning to be explored.

Effects of microglia depletion

The studies described above convincingly demonstrate that alterations of microglial activity impact Aβ deposition. An alternative approach to study the contribution of microglia to amyloid pathology is the use of microglia-depletion models. For example, using expression of a ganciclovir-activated suicide gene in microglia, a 90% reduction in the number of microglia was achieved, but this reduction in microglial numbers did not alter amyloid plaque load in APP/PS1 mice over a four week incubation period (Grathwohl et al. 2009). Moreover, upon discontinuation of ganciclovir treatment, peripheral myeloid cells rapidly repopulate the brain in this model (Varvel et al. 2012; Varvel et al. 2015). However, the resulting replacement of microglia with peripherally derived myeloid cells did not alter amyloid plaque load (Prokop et al. 2015; Varvel et al. 2015), suggesting that the impairment of microglial clearance function in AD cannot be rescued by the infiltration of peripheral cells, as these are exposed to the same altered brain environment. Interestingly, a recent study using diphtheria toxin based ablation of microglia in APP/PS1 mice reported no changes to plaque number over 2 weeks, but a 13% increase in plaque size, indicating once more that the microglial barrier contributes to amyloid plaque compaction (Zhao et al. 2017).

A different approach to achieve microglial depletion is based on the inhibition of microglial CSF1R signaling. With certain doses of CSF1R inhibitors, a nearly complete ablation of microglia can be achieved (Elmore et al. 2014). Depletion of microglia using CSF1R inhibitor in a mouse models of AD did not alter Aβ load, but improved cognition (Dagher et al. 2015). In line with this finding, using a CSF1R inhibitor at a dose that blocks microglial proliferation without ablating microglia also improved cognitive measures without altering amyloid load in an AD mouse model (Olmos-Alonso et al. 2016), indicating that targeting of microglial turnover with CSF1R modulators might exert beneficial effects independent of amyloid load. In the latter study, CSF1R inhibition shifted microglia to adopt an anti-inflammatory phenotype, indicating once more the importance of the microglial activity state in modulating disease progression. Strikingly, a recent study indicated that early long-term depletion of microglia in the 5XFAD mouse model of Alzheimer's pathology led to a dramatic decrease in plaque load (Sosna et al. 2018), implying that in addition to amyloid plaque compaction, microglia may also play a significant role in inducing plaque formation.

Dark microglia: a new phenotype predominantly associated with pathological states

Similar to the DAM and MgND, dark microglia are a disease-associated phenotype that is abundant in AD pathology. These cells were described in EM and their ultrastructural features will be discussed below. They are rare in healthy young adult mice, but drastically increase in number in the APP/PS1 mouse model of AD, and under conditions considered risk factors for AD, i.e. aging and chronic stress (Bisht et al. 2016b). Previous studies have indicated that chronic stress is both a cause and consequence of sporadic AD, which represents approximately 95% of all cases (for more on this topic, see the extended review by (Machado et al. 2014). Using paradigms of chronic unpredictable stress, an increased density of dark microglia was observed after two weeks of stress in wild-type mice compared to controls housed without any disturbance (Bisht et al. 2016b). Upon stress, dark microglia accounted for about one-fourth of the typical microglial population (Bisht et al. 2016b). Additionally, at 14 months of age, which corresponds to middle age in mice, dark microglia became highly prevalent, and even more in APP/PS1 mice of the same age. In this context of AD pathology, dark microglia accounted for two-third of the typical microglial population (Bisht et al. 2016b). In these APP/PS1 mice, where Aβ plaques are seen as early as 4 months of age (Malm et al. 2011), TREM2-positive dark microglia were detected nearby the amyloid plaques and in regions of neuronal dystrophy, showing engulfed amyloid deposits, as well as juxtaposing synaptic elements, which contained an accumulation of autophagosomes (Bisht et al. 2016b). As discussed above, TREM2 has been associated with the modulation of amyloid pathology (Wang et al. 2015a), while variants of this gene were also identified as an important risk factor for sporadic AD (Guerreiro et al. 2013a; Jonsson et al. 2013). The importance of TREM2 in dark microglia in AD is still undetermined. However, during early postnatal development, where dark microglia are also observed, these cells required the expression of TREM2, being strongly reduced in numbers in the hippocampus of TREM2 knockout mice (*versus* wild-type controls) (Bisht et al. 2017).

Distinct properties of dark microglia

Dark microglia are recognized by their electron-dense cytoplasm and nucleoplasm, making them appear as “dark” as mitochondria by EM (Bisht et al. 2016b). Bisht et al. hypothesized that this distinctive feature of the dark microglia could reflect cellular shrinkage, associated with oxidative stress in other cell types (Bisht et al. 2016a). Indeed, dark microglia display various features of oxidative stress including the dilation of their endoplasmic reticulum and Golgi apparatus, modifications related to their nucleus (condensation of the nucleoplasm, partial to complete lack of heterochromatin pattern), and alterations to mitochondria’s integrity, all of which might be attributed to oxidative stress (Bisht et al. 2016b). Under homeostatic conditions, enzymes and other antioxidant molecules are produced to neutralize reactive species (Rahal et al. 2014). However, this equilibrium is lost in non-homeostatic conditions, due to elevated reactive species levels or an altered production of antioxidants. The toxic reactive species can compromise cellular functions by affecting lipids, proteins, and nucleic acids, thus resulting in organelle impairments as well as morphological alterations (Rahal et al. 2014).

Another feature of dark microglia is their highly ramified and extremely thin processes that encircle various types of synaptic elements, i.e. synaptic clefts, pre-synaptic axon terminals, as well as post-synaptic dendritic branches and spines (Bisht et al. 2016b). By comparison, typical microglia make focal contacts with synaptic elements (Tremblay et al. 2010). The classical complement pathway might underlie dark microglia’s interactions with synapses considering that they strongly express CD11b, a component of the complement receptor 3 (CR3), in their distal processes encircling synaptic elements (Bisht et al. 2016b). As discussed above, previous studies have demonstrated a crucial involvement of this complement pathway, involving binding of CR3 to different complement components, in synaptic loss under various conditions (Stephan et al. 2013; Hong et al. 2016; Vasek et al. 2016). In AD pathology, 4 month-old APP/PS1 mice lacking the component 3 (C3) showed reduced synaptic loss in the hippocampus when compared to age-matched APP/PS1 mice (Hong et al. 2016). Likewise, 16 month-old APP/PS1 mice deficient in C3 had less extensive synaptic loss in the CA3 region of the hippocampus compared to age-matched APP/PS1 mice (Shi et al. 2015). Additionally, dark microglia associate with enlarged pockets of extracellular space (Bisht et al. 2016b), even more than typical microglia (Tremblay et al. 2010). This finding suggests an increased remodeling of the extracellular matrix by dark microglia, which could modulate the concentration of various glio- and neuroactive substances within the perisynaptic environment (Tremblay et al. 2011).

Beyond these ultrastructural differences, dark microglia’s distribution seems to be quite distinct from typical microglia as well. Typical microglia are localized both in the gray and white matter, being most present in the former, in young adult female BALB/c mice (Lawson et al. 1990), and they are found throughout the brain (Lawson et al. 1990). Dark microglia, however, appear so far to be restricted to specific brain regions, e.g. *stratum lacunosum moleculare* of the hippocampus CA1, the subgranular layers of prefrontal cortex, the basolateral nucleus of the amygdala, and the median eminence of the hypothalamus (Bisht et al. 2016b). Interestingly, the *stratum lacunosum moleculare* of the hippocampus CA1 display a pronounced loss of dendritic spines in the APP/PS1 mouse model (Siskova et

al. 2014). While the brain regions where dark microglia are prevalent exert distinct functions, structurally, they have one characteristic in common: the presence of large blood vessels. Interestingly enough, dark microglia were found to tightly juxtapose the vasculature (Bisht et al. 2016b), with more than half of these cells directly touching one blood vessel in ultrathin section (Bisht et al. 2016b). Whether these cells could help to maintain blood brain barrier integrity, which is compromised in AD (Zenaro et al. 2017) still remains undetermined. Furthermore, dark microglia were mostly found within clusters, a microglial feature usually seen increasingly with aging (Hefendehl et al. 2014). An abnormal distribution of microglial parenchymal surveillance could lead to areas that are more prone to damage and pathology. In addition, clusters may also be the result of fast repopulating microglial cells (Bruttger et al. 2015). Bruttger et al. found after microglial ablation, through the administration of diphtheria toxin into 8-week old mice in which these cells selectively express the diphtheria toxin receptor, that microglia formed clusters during repopulation at the peak of proliferation (i.e. day 7 post-injection) then spread out to their specific territory (at day 14 post-injection). Dividing astrocytes were also shown using EM to associate with the vasculature and proliferate in response to acute injury (Bardehle et al. 2013). These overall observations raise the intriguing possibility that dark microglia may be newly formed microglial cells that have yet to spread out to their designated area.

Even though dark microglia’s distinct ultrastructural features and interactions with synapses as well as the vasculature were described and their regional distribution within the brain is being characterized, some of their essential properties including their nature are still shrouded in mystery. However, some pieces of puzzle have been put together to unravel the riddle behind their origin. In particular, the first clue that dark microglia originate from the embryonic yolk sac comes from the fact that these cells are found in the hippocampus of adult CCR2 knockout mice (Bisht et al. 2016b). In this model, most of the monocytes remain in the bone marrow, due to the lack of CCR2, a chemokine receptor that is required for their egress and recruitment to inflammatory sites (Tsou et al. 2007). CCR2 is also considered necessary for the infiltration of monocytes into the brain (Mildner et al. 2009). Nevertheless, as mentioned by Bisht et al. (2016b), the presence of dark microglia in the CCR2 knockout mice does not preclude the possibility that these cells could be monocytes that infiltrate the brain via CCR2-independent pathways. Dark microglia do, however, express the microglia-specific marker 4D4 (Krasemann et al. 2017b), which provides support for a microglial origin (Bisht et al. 2016b). In addition, dark microglia do not express 4C12, which is considered a marker of inflammatory monocytes (Bisht et al. 2016b). Although the physiological significance of dark microglia has yet to be elucidated, the findings presented in this section together suggest that dark microglia could represent a subset of microglial cells that become stressed as a result of their hyperactive involvement with the maintenance of brain physiology.

Summary/Conclusion

Microglia survey the central nervous system and are responsible for a multitude of immune, inflammatory, and clearance functions. Their role in neurodegenerative disease has been clearly established and this role may be a potential avenue of therapeutic intervention. The presence of different phenotypes of microglia and how they change or respond to pathologic

changes during disease processes is of significant importance in the understanding of complex etiologies. Much attention and research has focused on Aβ, as the primary component of neuritic plaques in AD, and a potent stimulator of microglial activation. The extent, manner, and mechanism by which microglia respond to the presence and accumulation of protein aggregates such as Aβ ultimately may determine their impact and the overall outcome. A multitude of research demonstrates a bidirectional relationship between amyloid plaque deposition and microglial phenotypes, proving that changes in immune-associated molecules alone are sufficient to modulate Aβ pathology. These effects on Aβ pathology are mediated by alterations of microglial phenotypes and functions, e.g. by impacting microglial phagocytosis, barrier function, or cytokine secretion. Recent studies also suggest that some of the molecules implicated in microglial modulation of Aβ pathology might act together in previously unappreciated ways and converge on common microglial signaling pathways, e.g. pathways controlling metabolism (Chan et al. 2015; Ip et al. 2017; Krasemann et al. 2017b; Ulland et al. 2017). Future studies will reveal whether a common theme of clearly definable master regulators underlies the complex interplay between different microglial functions and thereby impacts neurological disease progression.

Acknowledgements / Conflict of Interest Disclosure

This review was, in part, aided by work conducted under the support of National Institutes of Health Grants R15AG033913 (MRN). Marie-Ève Tremblay is recipient of a Canada Research Chair (Tier 2) of *Neuroimmune Plasticity in Health and Therapy*. Her work for this project was supported by a Foundation Grant from the Canadian Institutes of Health Research (CIHR). Marie-Kim St-Pierre is a recipient of CIHR and Fonds de recherche du Québec-Santé (FRQS) master's scholarships. We respectfully include a conflict of interest statement disclosing that Colin K. Combs is an author on this publication and also serves as an editor for the Journal of Neurochemistry.

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--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

⇒ if yes, please ensure that the info “Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee.” is included in the Methods.

ARRIVE guidelines have been followed:

No

⇒ if it is a Review or Editorial, skip complete sentence ⇒ if No, include a statement: “ARRIVE guidelines were not followed for the following reason:” (edit phrasing to form a complete sentence as necessary).

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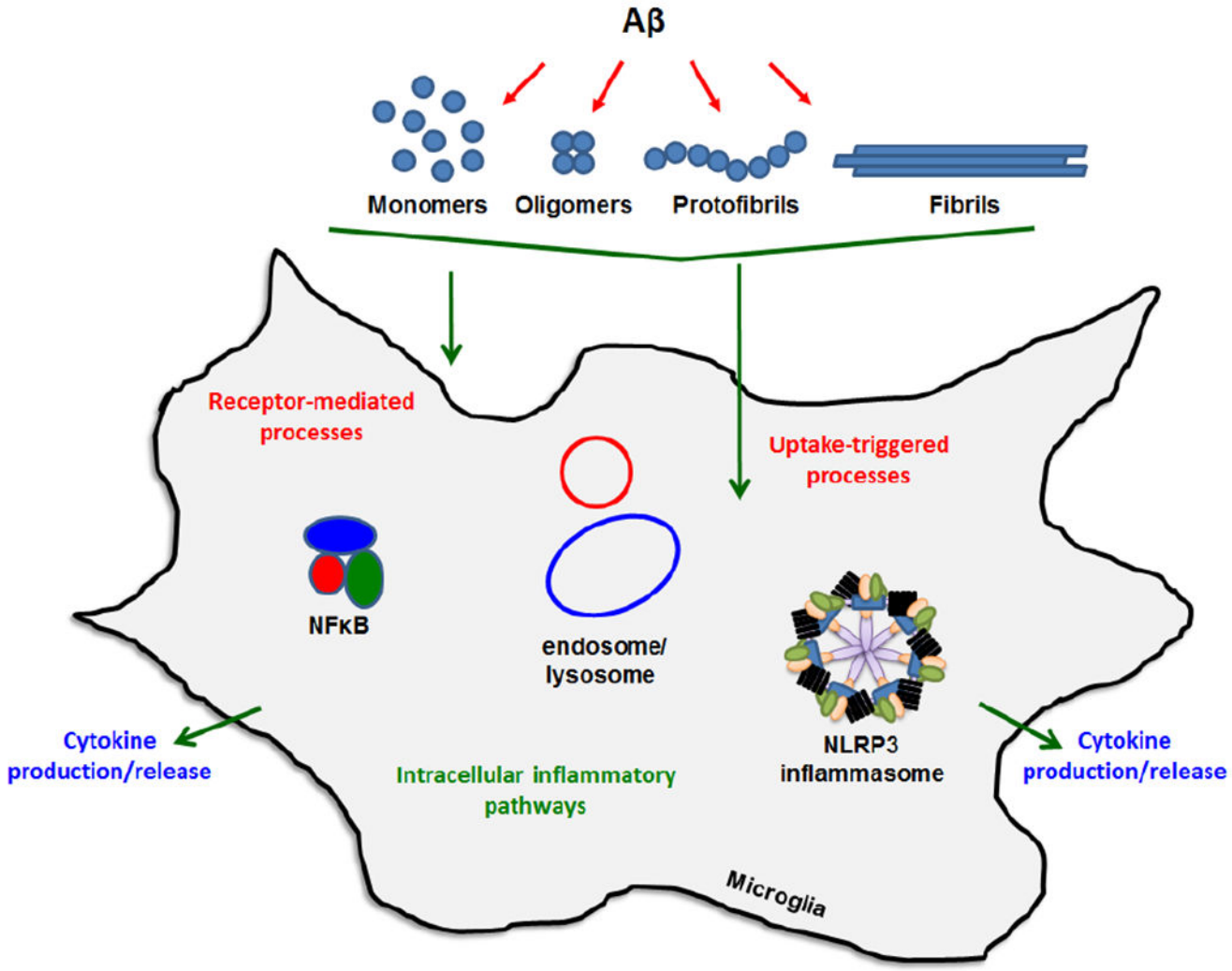

Figure 1.

Mechanisms of Aβ-mediated neuroinflammation. Interactions between microglia and various conformational forms of Aβ involve stimulation of cell-surface receptors and internalization by non-receptor mechanisms. Either interaction can provoke pro-inflammatory cytokine release through multiple intracellular pathways. Two key processes involve NFκB-mediated transcription/translation of cytokines such as TNFα and pro-IL-1β followed by NLRP3 inflammasome-mediated cleavage of pro-IL-1β to mature IL-1β. Internalized Aβ can trigger inflammasome activity and this may occur via endosomal or lysosomal factors.

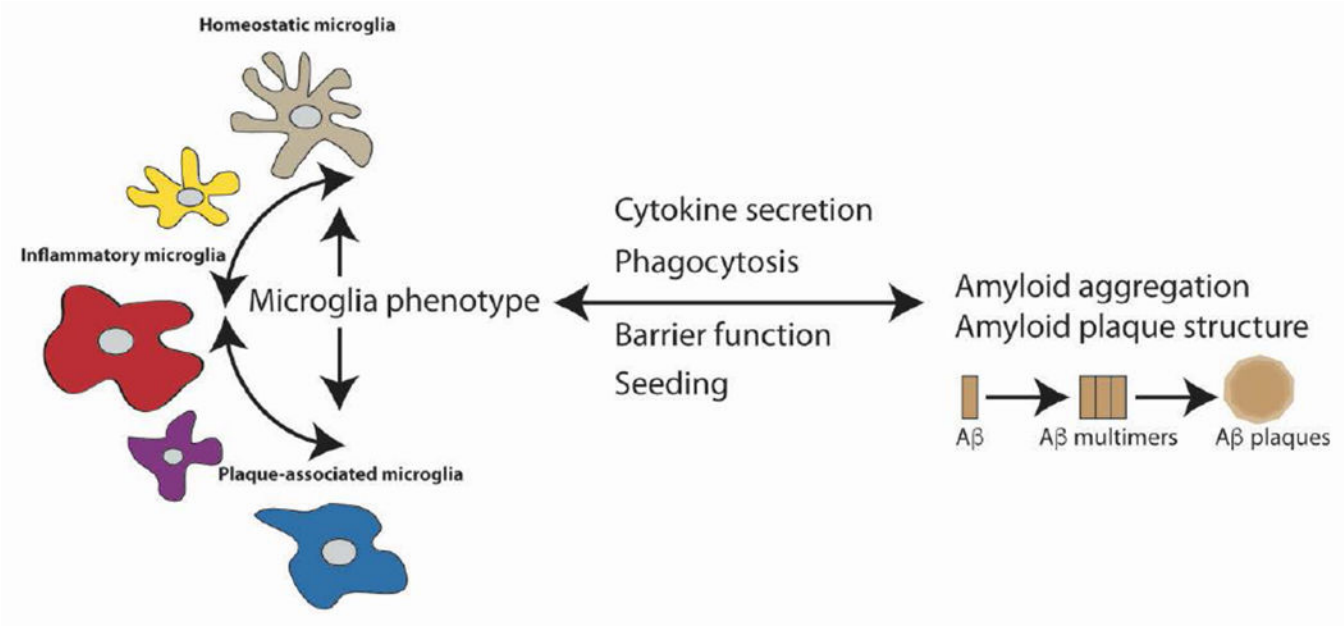

Figure 2.

Bi-directional relationship between microglia and plaque deposition. Significant evidence indicates that Aβ aggregation and accumulation in plaque deposits leads to changes in microglia phenotype and microglia-mediated neuroinflammation. However, many studies also demonstrate that the microglial functional state can, in turn, modulate Aβ aggregation, plaque deposition, and pathology. Mechanisms by which microglia may negatively modulate Aβ aggregation or spread may include phagocytosis or the formation of a tight barrier around plaques, while positive modulation may involve secretion of cytokines or seeding of Aβ by microglial cellular factors.

Table 1.

Phenotypic properties of microglia in AD pathology. Current observations and characterizations of normal and distinct phenotypes of microglia. Corresponding references are [1] (Bisht et al. 2016b) [2] (Peters et al. 1991) [3] (Savage et al. 2018) [4] (Tremblay et al. 2010) [5] (Keren-Shaul et al. 2017) [6] (Krasemann et al. 2017b) [7] (Bisht et al. 2016a) [8] (Hui et al. 2018).

Sample	Typical microglia [1-4]	Dark microglia [1, 7, 8]	DAM and MGnD [5, 6]
AD observation	Clustered and activated around Aβ plaques	APP-PS1 mice	DAM: post mortem AD human brains, 5XFAD mice
			MGnD: APP-PS1, APP-PS1 *Trem2*$^{-/-}$ mice
Morphology	Ramified processes when surveying the parenchyma	Hyper ramified by TEM	Not detemined
	Short and thick processes when reactive		
Ultrastructural Identification	Distinct chromatin pattern	Loss of chromatin pattern	Not detemined
	Small oval cell body	Dark appearance possibly due to condensed nucleo- and cytoplasm [7]	
	Cytoplasmic phagocytic inclusions, lipofuscin granules and/or lipid bodies	Oxidative stress features (ER and Golgi apparatus dilation)	
	Long/narrow ER cisternae		
Immunoreactivity	High immunoreactivity for homeostatic markers (GFP in CX3CR1-GFP mice, P2RY12, IBA1, etc.)	Low immunoreactivity for homeostatic markers (GFP in CX3CR1-GFP mice, IBA1), no expression of P2RY12	DAM: immunoreactive for IBA1 MGnD: low or no immunoreactivity for P2RY12
Regional Distribution	Throughout the white and grey matter	Median eminence of the hypothalamus	Cerebral cortex
		Dentate gyrus [8]	Hippocampus (near Aβ plaques)
		Strata radiatum and lacunosum moleculare of the hippocampus CA1	DAM are not found in the cerebellum
		Basolateral amygdala	