The microglial lysosomal system in Alzheimer's disease: guardian against proteinopathy

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Abstract

Microglia, the brain-resident immune cells, play an essential role in the upkeep of brain homeostasis. They actively adapt into specific activation states based on cues from the microenvironment. One of these encompasses the activated response microglia (ARMs) phenotype. It arises along a healthy aging process and in a range of neurodegenerative diseases, including Alzheimer's disease (AD). As the phenotype is characterized by an increased lipid metabolism, phagocytosis rate, lysosomal protease content and secretion of neuroprotective agents, it leaves to reason that the phenotype is adapted in an attempt to restore homeostasis. This is important to the conundrum of inflammatory processes. Inflammation per se may not be deleterious; it is only when microglial reactions become chronic or the microglial subtype is made dysfunctional by (multiple) risk proteins with single-nucleotide polymorphisms that microglial involvement becomes deleterious instead of beneficial. Interestingly, the ARMs up- and downregulate many late-onset AD-associated risk factor genes, the products of which are particularly active in the endolysosomal system. Hence, in this review, we focus on how the endolysosomal system is placed at the crossroad of inflammation and microglial capacity to keep pace with degradation.

Keywords

Late-onset Alzheimer's disease – endolysosomal homeostasis – microglia – membrane transport – phagocytosis.

1. Introduction

Improved living standards and a high-quality health care contribute to today's global life expectancy, which is higher than in any country a century ago. With longer lives comes, however, an increased incidence of age-related diseases, including dementias dominated by Alzheimer's disease (AD). For decades, the focus has been on understanding the neuronal pathology with an emphasis on amyloidogenic processing and production of toxic Aβ profiles as well as on how this interconnects with downstream tau pathology and disease spreading. Non-neuronal cell populations, including microglia, astroglia and oligodendrocytes, have been largely overlooked. Recent technological breakthroughs such as in single cell transcriptomics have more rapidly changed that picture (Olah et al., 2020; Sala Frigerio et al., 2019; Wingo et al., 2021). Many risk loci for late-onset AD (LOAD) prominently associate to (micro)glial populations and locate in genes whose products functionally converge onto the endocytic, phagocytic and lysosomal system (Van Acker et al., 2019a). This emphasizes that the contribution and interplay of different cell types should be amalgamated to fully grasp the underlying mechanisms that contribute to the manifestation of a clinical AD phenotype.

Microglia are the resident members of mononuclear phagocyte system in the human brain, in which they make up 10–15% of all cells. They monitor the nervous systems continuously and counter damage-associated signals through phagocytosis, inflammatory mediators and tissue remodelling. The homeostatic microglial state constitutively expresses low levels of CD45 and presents with a specific transcriptional profile (FCRLs, GPR34, HEXB, OLFML3, P2RY12, Siglech, SOCS3, and TMEM119) that distinguishes it from central nervous system-infiltrating macrophages and activated microglia (Frank et al., 2019). However, the annotation 'activated microglia' encompasses a complete range of heterogeneous subsets with diverse immunological and tissue-supportive features. Microglia and macrophages can display different activation phenotypes that were initially classified − for simplicity reasons − into two major principal states of activation: pro-inflammatory (M1) and anti-inflammatory (M2) microglia. This M1-M2 subdivision, however, does not reflect the high degree of region specialization and complexity of the brain that provides microglia with a multiplicity of signals, requiring different responses. Single-cell data analysis approaches have substantiated that the initial M1-M2 subdivision is only the tip of the iceberg. Depending on the micro-environment and external stimuli, different other activation states have been described (Fig. 1); e.g. activated response microglia (ARMs; (Sala Frigerio et al., 2019)), interleukin (IL)-33-responsive microglia (Lau et al., 2020), interferon response microglia (Sala Frigerio et al., 2019), lipid-droplet-accumulating microglia (Marschallinger et al., 2020), and proliferative region-associated microglia (Li et al., 2019). The most studied subtype entails the ARMs activation state that can be found in a range of neurodegenerative diseases, including AD, and has therefore alternatively been designated as disease-associated-microglia (DAM). However, given ARMs can also be found along a healthy aging process and are not necessarily associated with disease, the term 'disease-associated' may be misleading and one should opt to use the ARMs annotation instead (Sala Frigerio et al., 2019), which we will use hereafter. The exact mechanism that drives the ARMs phenotype is yet to be explored, but it is believed that general neurodegeneration-associated molecular patterns like debris from dead cells and myelin, lipid remnants, protein aggregates and other danger signals (ATP, NAD^+) drive conversion (García-Revilla et al., 2019). The phenotype is indeed characterized by an increased lipid metabolism, showing e.g. high lipoprotein lipase levels for lipid uptake as well as increased levels of the apolipoprotein E (ApoE), triggering receptor expressed on myeloid cells 2 (TREM2) and macrophage colony-stimulating factor 1, involved in lipoprotein clearance (Keren-Shaul et al., 2017). The promoted lipid metabolism and phagocytic

activity is a co-occurring event in ARMs' activation. The latter further entails an increased phagocytosis rate, lysosomal protease content and the secretion of neuroprotective agents, leaving to reason that the phenotype is adapted in an attempt to restore homeostasis [Fig. 1, (Keren-Shaul et al., 2017; Sala Frigerio et al., 2019)].

The primary beneficial aspect of microgliosis emerges clearly from microglia devoid of functional TREM2 (Parhizkar et al., 2019). TREM2 functions as an amyloid-β (Aβ) scavenger for its uptake and subsequent lysosomal degradation. In the absence of TREM2, microglia are locked in a homeostatic state and, hence, fail to acquire an ARMs phenotype; i.e. their potential to cluster around amyloid seeds and reduce the amyloidogenic cascade by phagocytic amyloid plaque clearance (Parhizkar et al., 2019). Interestingly, TREM2 is not only a signature marker for the ARMs phenotype, it is also a LOAD risk factor protein (Guerreiro et al., 2013). Actually, a broader range of LOAD risk factors now turns out to be up- or downregulated in the ARMs phenotype. Amyloid precursor-like protein 2 (Aplp2), the cathepsins (Cts) Cts-B and Cts-D, H-2 class II histocompatibility antigen I-E beta chain (H2-Eb1), phospholipase D3 (PLD3), TREM2 and TYRO protein tyrosine kinase-binding protein (TYROBP), are all upregulated in the ARMs cluster, while levels of myc box-dependent-interacting protein 1 (BIN1), cas scaffolding protein family member 4 (Cass4), phosphatidylinositol-binding clathrin assembly protein (PICALM) and sialic acid-binding Ig-like lectin H (Siglech) are downregulated (Sala Frigerio et al., 2019). One can clearly appreciate that many of these LOAD risk factor proteins are active in the endocytic, phagocytic and lysosomal system, underscoring a converging point of the degradative and inflammatory pathways. Of note, while alterations to risk factor proteins do not by themselves drive disease pathology, they may do so once multiple risk factors are present within the same pathway or mechanism. Add to this the fact that ageing and the AD pathology affect expression of quantitative trait loci (Yang et al., 2020), induce histone modifications (Nativio et al., 2020), and change other epigenetic (methylation) imprints (Blanco-Luquin et al., 2018); dysregulating transcription- and chromatin-gene feedback loops of which many can also be linked to the lysosomal-autophagy pathway (Yang et al., 2020), and LOAD risk SNPs in particular (Blanco-Luquin et al., 2018). Together, this makes it reasonable to hypothesize that the more risk factor single-nucleotide polymorphisms (SNPs) in one's genome (active in the same pathway), the more incapable microglia get in responding to the steady loss of normal functioning associated with ageing or a progressing neurodegeneration-linked proteinopathy (Table 1.).

These results are important to the conundrum of inflammatory processes in AD. Inflammation per se is not deleterious. It is only when microglial reactions become chronic or the microglial subtype is made dysfunctional by (multiple) risk proteins with SNPs that microglial involvement becomes deleterious instead of beneficial. As such, this review ties up (dysfunctional) cell mechanisms of the endolysosomal system and how to link AD risk factor proteins with microglial (in)capacity to keep pace with degradation. For this, we focus on the role of surface and endolysosomal receptors, the impact of lipid profiles on vesicular transport, the lysosomal degradative capacity as well as the exosomal escape route. However, we start with the impact of senescence on microglial functioning as this is the major risk factor for developing a neurodegenerative pathology (Hou et al., 2019).

2. Aging process of microglia

Microglia are generated from peripheral erythromyeloid progenitors (mesoderm) during embryonal development. They travel to the brain before the blood-brain barrier is closed, after which the

microglial population is sustained by self-renewal mechanisms (Matcovitch-Natan et al., 2016). Such homeostatic replenishment is achieved by specific regulatory programs involving colony-stimulating factor 1 receptor signalling and a unique transforming growth factor beta (TGF-β)-dependent signature (Butovsky et al., 2014). However, this mechanism is not without limits. Overall, about 2% of the microglial population is replicating at every point in life, making microglia to go through hundreds of renewal rounds over an average lifetime of 80 years (Askew et al., 2017). Hence, they may not be immune to age-related reductions in telomere length and may enter a senescent, dystrophic state at one point. This is important to keep in mind when studying age-related disorders. In addition, different neurodegenerative pathologies appear to accelerate the microglial turnover further. Microglia in brains of patients with frontotemporal lobar degeneration associated with progranulin (PGRN) mutations show more microglia with condensed nuclei (Sakae et al., 2019), as do microglia in other neurological diseases of different aetiology; subacute sclerosing panencephalitis, Wilson's disease and AD (Lewandowska et al., 2004). A study on mouse neocortices of amyloid precursor protein $(APP)^{KM670/671NL}$ and presenilin 1 $(PSEN1)^{L166P}$ transgenics showed that amyloidosis actually increases the replication rate up to three times the normal rate (Füger et al., 2017).

A large part of the aging phenotype revolves around post-mitotic cells failing to uphold their proteostatic lysosomal capacity. As they are not able to dilute debris through cell division anymore, this accumulation will initiate inflammaging-related disease mechanisms. In fact, while senescent cells lose their replication potential, they remain metabolically active, though less efficient. Aged cells show an inability to maintain the lysosomal acidic pH (Yambire et al., 2019), display age-related changes in activities and localizations of Cat-B, D, E and L (Stoka et al., 2016), as well as many other catalytic proteins functioning in lysosomes, including amidases, thioesterases, proteases and glycosyl hydrolases (Burns et al., 2020). Aside from the lysosomal degradative capacity, senescent cells exhibit an altered proteome with an increased representation of proteins involved in lysosome biogenesis [mechanistic target of rapamycin/transcription factor EB (mTOR/TFEB)], vesicle trafficking and organelle fusion (Burns et al., 2020). The levels of phagocytosis- and recycling-related receptors decline with age and along the AD process (Heckmann et al., 2019; Yanguas-Casás et al., 2020). Aforementioned dysfunctions are especially important in view of the accumulative waste theory of aging. This may over-demand an already sub-optimally working system. By way of example, a myelin overload upon age-related myelin degradation has been shown to generate lysosomal inclusions in microglia (Safaiyan et al., 2016). An accumulation of cellular remains and protein aggregates may even further aggravate the proteinopathy phenotype, given that aged microglia display a diminished migration potential, grounded in a decreased motility and inability to respond to different chemotactic cues (Yanguas-Casás et al., 2020).

Furthermore, microglia isolated from aged animals display a bioenergetic shift from glucose to fatty acid utilization, clearly having its repercussion on mitochondrial function and oxidative phosphorylation as well (Flowers et al., 2017). Once the autophagic and lysosomal capacity gradually subsides to a point in which the recycling of obsolete, misfolded and aggregated proteins gets compromised, this will further impact on organelles such as mitochondria. Hence, not only do lysosomes of old cells show a heightened propensity to leak (Ni et al., 2019). Dysfunctional mitochondria that do not get cleared also release mtDNA into the cytosol as well as in the extracellular milieu, propagating injury from microglia to astrocytes and to neurons (Joshi et al., 2019; Nakahira et al., 2011). Together with damage-associated molecular patterns (DAMPs) as reactive oxygen species

(ROS) and metabolites such as nicotinamide adenine dinucleotide (NAD^+) or adenosine triphosphate (ATP), mtDNA will initiate a chronic subclinical inflammatory cascade, including toll-like receptor (TLR)-signaling. As such, senescent cells will constitutively secrete higher levels of i.a. IL-6 and TNF-α in comparison to their younger counterparts (Njie et al., 2012). Microglia from aged mice further not only express higher levels of inflammatory cytokines, they show a predominant induction of ROS upon activation instead of an increase in nitric oxide levels as their younger counterparts (Von Bernhardi et al., 2015). The iron-driven Fenton reaction is one source of increased ROS levels, generating ·OH radicals. Being essential players in upholding brain iron homeostasis and having naturally aged brains as well as those with neurodegenerative pathologies accumulating iron (Van Acker et al., 2019b), it may not be surprising that many dystrophic microglia that show an increased iron content and high levels of the iron scavenger protein L-ferritin can be found within these brains (Angelova and Brown, 2018; Swanson et al., 2020). Overloading microglia with iron is actually one of the model systems that are being used to recreate a senescent-like phenotype with increased endoplasmic reticulum (ER) stress (Angelova and Brown, 2018). Alternatively, microglia can be isolated from aged animals. However, this approach has the disadvantage of being costly, to result in lower yields and to be more difficult regarding sustaining undamaged aged cells in culture than when using neonates/young animals (Angelova and Brown, 2018; Swanson et al., 2020). In conclusion, while microglial functioning gets affected by aging, this aging effect has been hard to recapitulate in a model system, leading to a generally low usage of aged microglia model systems. Hence, one should be aware of the expected additional impact of microglial senescence in studies on neurodegenerative diseases.

3. Microglia maintain key Aβ scavengers

AD is characterized by an increased production of toxic Aβ species from APP through a promoted amyloidogenic pathway in neurons. Particularly in familial AD, wherein mutations are found in the *APP* and *PSEN* genes, Aβ overproduction and aggregation may be an early pathological instigator. Brain-resident phagocytes, on the other hand, are pivotal for the clearance of soluble Aβ and the Aβ fibrils. Microglia and macrophages can take up extracellular Aβ by four different routes: (I) exosomal transfer, (II) phagocytosis, (III) pinocytosis and (IV) receptor-mediated endocytosis. Different factors can affect these processes, including alterations to the membrane composition (Köberlin et al., 2016), cytoskeleton organization [e.g. APOE4 influences actin structures, (Muth et al., 2019)] and signaling mechanisms. For one, Aβ and Tau induce the removal of negatively-charged sialic acids from cell surface proteins of primary microglia. Such desialylation promotes, in turn, phagocytosis of neurons through the microglial complement receptor (CR)-3 (Allendorf et al., 2020). What is more, complement expression and activation are increased in AD patient's brains (Wu et al., 2019), promoting phagocytosis further. The importance of complement involvement in the microglial-AD pathology is emphasized by CR1 being one of the phagocytic receptors identified as a LOAD risk factor. Together with TREM2, sialic acid binding Ig-like lectin 3 (Siglec3, or CD33) and Sortilin Related Receptor 1 (Sorl1), CR1 has been recently reviewed in-depth (Podleśny-Drabiniok et al., 2020). Here we will focus on endocytic and degradative routes linked to low-density lipoprotein receptor (LDLR) and TLR cascades on which several LOAD risk factors impinge.

3.1. LOAD risk proteins affect LRP1-mediated uptake of Aβ

The LDLR-related protein 1 (LRP1; also known as CD91) is ubiquitously expressed in the central nervous system, including on microglia. Its microglial function is linked to the phagocytic clearance of apoptotic cells and the endocytosis of a variety of AD-relevant ligands such as ApoE, α2-macroglobulin, Aβ

peptides and the soluble ectodomain of APP as well as SorLA/LR11 (Van Gool et al., 2019). Although LRP1 mediates microglial clearance of Aβ peptides, this effect is also ascribed to the binding of LRP1 with ApoE. The ε4 allele of the *ApoE* gene confers the highest genetic risk for sporadic AD after age (Schmechel et al., 1993). The three-fold increased risk with one copy of the ApoE4 variant is unrelated to Aβ production, but rather affects its microglial uptake (Strickland and Holtzman, 2019). In addition, LRP1 endocytosis can be affected by LOAD risk factor proteins as the complement component 1q (C1q) (Duus et al., 2010), PICALM (Storck et al., 2018), and the ATP-binding cassette sub-family A member 7 (ABCA7, (Jehle et al., 2006)). ABCA7 not only promotes the cell surface localization of LRP1 to support phagocytosis of Aβ and the removal of (amyloid-loaded) apoptotic cells (Jehle et al., 2006), it also affects pro-inflammatory signaling by regulating surface levels of the TLR co-receptor CD14 (Aikawa et al., 2019). The impact of this dual Aβ endocytosis-inflammatory regulation is reflected in heterozygous variations in ABCA7 conferring a predisposition to LOAD (e.g. rs3764650 SNP; odds ratio: 1.23) and possibly even to early onset AD as well (De Roeck et al., 2017).

3.2. LOAD risk factor proteins affect TLR-uptake of Aβ

Microglia sense potentially harmful structures with their pattern recognition receptors (PRRs). Among them are the TLRs (1 to 9) that reside in the plasma membrane and in endocytic organelles of phagocytes (Fig. 2). TLRs contain a leucine rich repeat (LRR) motif in the ectodomain for substrate recognition and share further structural and signaling cascade resemblance with the type 1 IL-1 receptor. Where oligodendrocytes and astrocytes only express a limited set of TLRs, microglia express TLRs 1 to 9 (Bsibsi et al., 2002). Expression of TLR10 and TLR11 has not yet been detected. TLR10 is selectively expressed in B cells and weakly in dendritic cells; TLR11 is only present as a pseudogene in humans (Hornung et al., 2002; Roach et al., 2005). Through TLR1-9, microglia recognize pathogen-associated molecular patterns (PAMPs) or DAMPs. The latter are not associated with infectious micro-organisms but are rather endogenous danger signals from stressed or dying cells, including phospholipids, proteins and nucleic acids (Fig. 2). TLR responses to PAMPs have been widely studied, but their responses towards DAMPs is less characterized. However, these are highly relevant in a neurodegenerative context as apoptotic cells and pathogenic debris are highly likely triggers for TLR signaling.

Aβ has been found to bind different TLRs at the plasma membrane, including TLR2 (Rubio-Araiz et al., 2018), TLR4 (Balducci et al., 2017), and a heterodimer of TLR4:TLR6 (Stewart et al., 2010). In addition, LOAD risk factor proteins are found to impact the process. The clathrin-binding protein PICALM regulates TLR4's intracellular vesicle transport (Mertins et al., 2017), as do CD2-associated protein (CD2AP) and BIN1 (Fujikura et al., 2019). ABCA7 does not only affect TLR4 signaling by regulating surface levels of the TLR co-receptor CD14 (Aikawa et al., 2019), it also regulates CD14-TLR4 endocytosis (Fig. 2). As such, ABCA7 haplo-deficiency diminishes pro-inflammatory microglial responses under acute inflammation (Aikawa et al., 2019). Unexpectedly, microglia of ABCA7$^{+/-}$ mice also show higher levels of intracellular Aβ (Aikawa et al., 2019). Besides an enhanced endocytosis, this abnormal endosomal build-up could indicate a reduced degradation as well as a reduced recycling, as for instance ABCA7 has been shown to also promote plasma membrane localization of LRP1 and its associated signalling (Jehle et al., 2006). While a role for ABCA7 in recycling remains to be investigated, it has been shown that TLR4 can recycle via the LC3-Associated eNDOcytosis (LANDO) pathway, which shares proteins like ATG5 and Rubicon with LC3-associated phagocytosis, or LAP (Heckmann et al., 2019). The pathway runs, however, independently from canonical autophagy and especially the WD

(tryptophan-aspartate) domain of Atg16L was found essential in the process (Heckmann et al., 2020). Knockout of the domain by itself is enough to generate AD pathology of endogenous murine Aβ and Tau, without affecting autophagy markers P62 (Sequestosome-1) and LC3-II (Heckmann et al., 2020). Of note, as some $LC3^{+}$ Aβ-loaded vesicles are also positive for markers as clathrin and Ras-related protein Rab5, it remains to be shown to which extent microglia specifically and predominantly use the LANDO pathway to recycle Aβ-receptors such as TREM2, CD36 and TLR4 back to the cell surface. Given that levels of proteins involved in LANDO (e.g. ATG5 and Beclin1) decline with both age and AD, this makes a progressive LANDO failure to likely contribute to the amyloid etiopathology (Heckmann et al., 2019). Moreover, deactivation of LANDO proteins (e.g. Rubicon and myeloid ATG5) increases Aβ-induced reactive microglial activation (Heckmann et al., 2019).

4. Endosomal TLR signalling indirectly impacts Aβ pathology

An increasing number of data involves TLRs in the AD pathogenesis, in particular, those localised in the endolysosomal system (e.g. TLR9; Fig. 2). Most recently, a family-based genetic study in a multigenerational Belgian family identified a TLR9 p.E317D mutation segregating across generations. The mutation is situated in the TLR9 substrate sensor pocket and reduces its activity by half (Cacace et al., 2020). While no causative mutation had been identified before, the sensing role of TLR9 for unmethylated CpG oligodeoxynucleotides (CpG-ODN) had already been negatively correlated with cerebral amyloid angiopathy levels and cortical amyloid burden (Scholtzova et al., 2017, 2009). In 3xTg-AD mice, TLR9 agonists reduce tau pathology burden (Scholtzova et al., 2014), and patients with mild cognitive impairment and high TLR8/9 levels are less likely to progress to AD than those with lower levels (La Rosa et al., 2017). The exact underlying mechanisms remain largely elusive. While a link with autophagosome formation has been described, no uniformity has been found yet. Initially, it was reported that stimulation of the substrate of TLR9 in microglia increases uptake of $A\beta_{1\text{-}42}$ through upregulation of the G protein-coupled formyl peptide receptor (Tahara et al., 2006). On the other hand, TLR9-myeloid differentiation primary response 88 (MyD88) signaling, mediated through mTORC1 activation, was shown to rather inhibit autophagy induction ((Kader et al., 2017); Fig. 2). In addition, no difference in classical downstream inflammatory mediator levels (e.g. nitric oxide and TNF-α) has been recorded when microglia were co-treated with $A\beta_{1\text{-}40}$ and CpG versus CpG alone (Lotz et al., 2005).

Two LOAD risk factor proteins have been identified that work exactly on this crossroad of inflammation and endolysosomal degradation, namely PLD3 and PGRN (Cruchaga et al., 2014; Minami et al., 2014). PLD3 is a 5'-3' exonuclease that regulates inflammatory responses by degrading single stranded DNA (ssDNA); i.e. the substrate of TLR9 (Cappel et al., 2021). Mice deficient for both PLD3 and its family member PLD4 go into an inflammatory state that is already lethal early in life (Gavin et al., 2018). Reduced PLD3 levels also lead to an accumulation of extracellular Aβ levels (Cruchaga et al., 2014). This phenotype corresponds to what is observed in another neurological disorder, cystic leukoencephalopathy. Herein, RNase T2 dysfunction in patients causes rRNA to aggregate in the lysosomes that, in turn, get enlarged and increase in numbers, being indicative of lysosomal dysfunction (Haud et al., 2011). RNAse T2-depleted microglia further show lysosomes that are engorged with Aβ and other protein substrates as well as with undigested apoptotic material and they adopt an inflammatory signature (Hamilton et al., 2020). In addition, PLD3 directly interacts with PGRN and PGRN has been identified as a co-receptor for TLR9 through binding CpG-ODNs and transporting them to endolysosomes for interaction (Park et al., 2011). In this way, PGRN enables macrophages to

respond to very low amounts of CpG-ODNs (15 nM) that would otherwise not elicit TLR9 activation ((Park et al., 2011), Fig. 2). In agreement, depletion of PGRN in phagocytes lowers production of TNFα and IL-6 upon CpG-ODNs addition (Park et al., 2011). PGRN deficiency significantly upregulates the global ratio of $CD68^+$ microglia in the brain of APPswe/PSEN1ΔE9 mice, with a specific increase near Aβ plaques, and promotes the transcription of the TYROBP network of inflammation-related genes (C1qA, CD22, CD68, TREM2, TYROBP/DAP12). On the other hand, expression of typical pro-inflammatory (iNOS, IL1β, TNFα, and IL6) and anti-inflammatory genes (Arg-1, TGFβ, Fizz1, and Ym1) remains unaffected by PGRN depletion (Takahashi et al., 2017). These results are in line with those of ablating PGRN expression in myeloid cells of APP mice, primary microglia and the spontaneously transformed microglia cell line C8-B4. PGRN deficiency in these microglial cells compromises autophagy, leading to three-fold increased plaque loads, a fast-tracked deposition and phosphorylation in human tau-expressing mice as well as worsened spatial memory deficits (Minami et al., 2014; Suárez-Calvet et al., 2018). Conversely, increasing PGRN levels in primary wild-type microglia and C4B8 cells promoted endocytosis of $A\beta_{1-42}$ *in vitro* (Pickford et al., 2011). Hence, these data would point to a defective signaling being the primary culprit and the lysosomal build-up being a downstream consequence.

Much less is known about endolysosomal TLRs other than TLR9. Nonetheless, TLR3 transcript and protein levels were shown elevated in AD compared to non-demented cases and were linked to a lower plaque or tangle burden in human brain middle temporal gyrus samples (Walker et al., 2018). As for TLR7, its stimulation was shown to induce autophagy through MyD88 activation (Delgado et al., 2008). These studies further strengthen a potential general and central role for the TLR-MyD88-mTORC1 signaling in AD pathogenesis (Fig. 2).

5. Impact of lipid profiles on the vesicular degradation pathway

Lipid homeostasis is affected in many neurodegenerative diseases (Meng et al., 2020), being vulnerable to excess levels of glutamate and lowered oxygen availability. Given above-mentioned processes are all linked to membrane structures, either at the cell membrane or linked to intracellular vesicles, it may be of no surprise that alterations in the cellular lipid content or metabolism will impact on intracellular transport and degradative systems. Adaptations to the microglial activation state may represent an answer to these deviations from homeostasis. The microglial ARMs signature exhibits an enhanced lipid metabolism and phagocytic pathway, showing upregulated levels of for instance lipoprotein lipase (Lpl), cathepsin L-inhibiting cystatin-F (Cst7), and the exosome marker CD9 in a TREM2-dependent fashion (Keren-Shaul et al., 2017). This reactive microglial subtype represents a high-energy demanding state that resembles a developing microglia signature. The study of Hammond *et al*. detected a clear overlap between microglial expression of i.a. ApoE and Lpl between microglia of postnatal life day 5 (P5) and those responding to injury (Hammond et al., 2019). Similar work on P7 microglia identified a proliferative region-associated microglia (PAM) subset, primarily detected in developing white matter, with an ARM-overlapping gene signature regarding, among others, Lpl, Cd9, and ApoE (Li et al., 2019). In addition, site-specific phosphorylation of PSEN1 at serine 367 has also been linked with microglial development protein hubs (Ledo et al., 2020b). These studies pinpoint the importance of immuno-metabolism, with a particular notion for lipid and lipoprotein changes during neurodegeneration (Loving and Bruce, 2020).

Lysosomal lipid storage problems are, in turn, accompanied by aberrant cytokine releases, substantiated in TLR-related processes that show a functional crosstalk with membrane lipids (Köberlin et al., 2016). Active TLR2, TLR4 and TLR9 are drawn to membrane microdomains, containing combinations of high cholesterol and sphingolipid levels. Hence, when lipid compositions and membrane fluidity of phagocytes are affected, as during AD progression, TLR signaling gets dysregulated as well (Köberlin et al., 2016). The inverse effect is also seen. Stimulation of TLR4 promotes lipid droplet formation in microglia, concomitantly with increased levels of storage or neutral lipids (monoacylglycerols and triacylglycerols, (Marschallinger et al., 2020)). These TLR4-induced lipid-laden microglia show a transcriptional signature that resembles those of aged microglia that accumulate lipids; so-called LDAMs (Marschallinger et al., 2020). LDAMs not only release excessive levels of pro-inflammatory chemokines and cytokines as CCL3, CXCL10, and IL-6, they also exhibit an upregulation in genes linked to nitric oxide and ROS generation (e.g. *CAT, KL, PPP1CB, JAK,* and *RAP1B*), and are defective in phagocytosis (Fig. 1, (Marschallinger et al., 2020)). These results are in accordance with *in vivo* data, showing cellular debris and proteins to accumulate once lipid-rich microglia arise (Jaitin et al., 2019). In support, chaperone-assisted lipophagy enables neutral lipid transfer from lipid deposits in microglial somata to the lysosome-phagosome system where these fatty acids and sterols are linked to microglial phagosome genesis and maturation (Chali et al., 2019). Microglia also accept lipids from stressed neurons through fatty acid transporters or by ApoE. The latter is the forefront lipid transporter associated with sterol movement between cells and gets significantly upregulated in activated microglia (Chali et al., 2019). ApoE-deficient, iPSCs-derived microglia show an impaired cholesterol transport and high levels of cholesteryl esters, similarly as to microglia lacking functional TREM2 (Nugent et al., 2020). Interestingly, a broad array of anionic and zwitterionic lipids robustly triggers microglial TREM2 signalling to increase the expression of lipid metabolism genes, degrade myelin cholesterol and, as such, keep the cholesterol ester levels in check (Nugent et al., 2020). In this regard, myelin damage has been found to induce TREM2-linked transcriptional changes in microglia, including of ApoE and Apoc1 (for general cholesterol transport), of Niemann-Pick type C Intracellular Cholesterol Transporter 2 (NPC2; for egress of cholesterol from lysosomes) as well as of the lysosomal acid lipase (Lipa; for the deacylation of triacylglyceryl and cholesteryl ester core lipids (Nugent et al., 2020)). Interestingly, phospholipase C γ2 (PLCγ2), which generates the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), was recently identified as a LOAD risk factor (Andreone et al., 2020). PLCγ2 signals downstream of TREM2 to regulate the cellular cholesterol metabolism, process neuronal debris and mediate inflammatory responses (Andreone et al., 2020), and also turned out to be one of the highly affected proteins in the proteome of aged microglia (Flowers et al., 2017). The accompanying effects on cholesterol efflux and lipid clearance could, however, aggravate or induce lysosomal defects. Median cholesterol levels in lipid bilayers support the formation of $A\beta_{1-42}$ ion channels that cause an unregulated ionic leakage, while high cholesterol levels promote inter-leaflet uncoupling, affecting the membrane's mechanical property (Gao et al., 2020).

6. Lysosomal degradative capacity

6.1. Metabolome-adjustable lysosomal biogenesis

The concept of metabolome-adjustable lysosomal biogenesis that was first described by Karageorgos and colleagues in the late nineties, has regained much interest in light of the increasing awareness of profound proteopathic inflammatory responses in neurodegenerative diseases (Karageorgos et al., 1997; Rudnik and Damme, 2020), including in AD (Heckmann et al., 2019), but also in ageing (Wingo et al., 2019). The concept entails that the cell can control the size and number of lysosomal organelles

with genetic programs, depending on its catabolic needs. Such lysosomal adaptability is highlighted in a recent large-scale proteomic analysis of AD brains and cerebrospinal fluid. The study identified the "microglial metabolism" module to be one of the most affected by AD pathology (Johnson et al., 2020). In addition, proteomes of aged microglia, independent from pathology, also showed a bioenergetic shift from glucose to fatty acid metabolism (upregulating *HADHA*, *HEXA*, *HEXB*, and *PLCG*) as well as an alteration in phagosome maturation and FCγ receptor-mediated phagocytosis (Flowers et al., 2017). Important upstream regulators were mTOR and the transcription factor c-MYC; not surprisingly both key drivers in the biogenesis of lysosomes (Flowers et al., 2017). c-MYC senses levels of extracellular nutrients through the ERK1/2 kinases, while the lysosomal content is monitored by the v-ATPase/mTORC1 sensing module. These signals are passed on by phosphorylation events to the transcription factor EB (TFEB; Fig. 3). TFEB regulates the transcription of the CLEAR (Coordinated Lysosomal Expression and Regulation) gene network. Being its master regulator, TFEB sits in the driver's seat to control lysosomal biogenesis, autophagy, cellular metabolism, the immune response, and exo-/endocytosis (Palmieri et al., 2011).

This is in particular evident when an overload of proteins or aggregates is targeted for degradation as in the case of neurodegenerative diseases in general and AD in particular. The weight that the TFEB homeostatic pathway has on the Aβ load, which is at least equal to the impact of the overproduction of (toxic) Aβ species, has been elegantly shown by Ledo *et al.,* using a microglia model that is phospho-deficient for the familial AD-linked PSEN1 protein ($PSEN1^{S367A/S367A}$, (Ledo et al., 2020a)). $PSEN1^{S367A/S367A}$ microglia show a dysfunctional autophagy and failure to degrade Aβ, owed to decreased TFEB mRNA levels independent of γ-secretase activity (Ledo et al., 2020a). In this line, an altered expression of PSEN1/2 through PSEN protein depletion or AD-associated mutations in fibroblasts and iPSCs also results in an attenuated CLEAR gene network (Reddy et al., 2016). The PSENs are multifaceted proteins that are not only involved in catalyzing γ-secretase activity, but promote several other cellular processes, including calcium signaling (Escamilla-Ayala et al., 2020; Pizzo et al., 2020). Hence, when PSEN-deficient, cells struggle to upkeep normal calcium stores, leading to dysfunctional mTOR dynamics and an inability to re-localize TFEB into the nucleus ((Reddy et al., 2016), Fig. 3). In addition, TFEB efflux from the nucleus may be Aβ-triggered, independent of calcium dysregulations. Translocation of TFEB to the nucleus has been found to be a transient event after oligomeric Aβ stimulation, with it being sequestered in the cytoplasm after prolonged exposure (Yao et al., 2019). However, the microglial degradative capacity can be re-instated by contact with colony-stimulating factor (Daria et al., 2017), IL-33 (Lau et al., 2020), or TLR targets (Rubio-Araiz et al., 2018). Similarly, microglia of old mice will start to proliferate again and clear amyloid plaques when subjected to conditioned media of young microglia (Daria et al., 2017).

Of interest, TFEB is not only linked to AD as part of the cell's reaction to the lysosomes being incapable of coping with the degradative load. At least ten genome-wide association study (GWAS) loci for AD (*BIN1*, *CLDN11*, *POLN*, *STK32B*, *EDIL3*, *AKAP12*, *HECW1*, *WDR5*, *LEMD2*, and *DLC1*, (Grubman et al., 2019)), are also downstream genes regulated by TFEB. Many of these have a clear inflammatory link. By way of example, the rs6733839 SNP of BIN1 resides in a microglia-specific enhancer region (Nott et al., 2019). This affects the normal human microglial expression levels that present with high levels of the ubiquitously transcribed isoform 9 and the microglia-specific isoforms 6 and BIN1-13 (Crotti et al., 2019). Other disease-risk variants identified by GWAS and localized in microglial super-enhancer

regions include MEF2C, STAT6, CD14, ITGAM, AIF1, APOE, CASS4, INPP5D and PICALM (Nott et al., 2019).

6.2. The proteolytic milieu of acidic lysosomes

An important number of the proteolytic enzymes degrading Aβ can be found in the lysosomes (Table 2). These include the tripeptidyl peptidase (TPP) and the Cts family of cysteine proteases: i.e. Cts-B, -D, -E and -L. Others modulate or promote amyloidogenic processing, such as the membrane type 1-matrix metalloproteinase (MT1-MMP). It goes without saying that these enzymes perform a central role in the cell to cope with amyloid proteinopathy. As such, microglia and brain macrophages that are found at sites of amyloid plaques in brains of both patients with AD and familial AD animal models do show upregulated MT1-MMP levels (Langenfurth et al., 2014). Given MT1-MMP cuts upstream of the β-cleavage (BACE1) site, it also promotes BACE1 cleavage and, hence, the amyloidogenic pathway (Liao and Van Nostrand, 2010). Being not the primary Aβ-producing cells, the importance of such a side effect has not been investigated in microglia yet. It has, however, been shown that the accretion of oligomeric Aβ could render lysosomes of aged microglia leaky, allowing Cat B to leak into the cytosol (Ni et al., 2019). Not only is Cat B unable to perform its lysosomal functions, it also starts degrading mitochondrial transcription factor A. This leads to a compromised mtDNA biosynthesis and, hence, higher mitochondria-derived ROS levels as well as an increased production of pro-inflammatory mediators (Ni et al., 2019). A decreased degradation of Aβ in microglia could be further explained by a spontaneous isomerization and epimerization of long-lived peptides like Aβ, which further impedes their degradation by the lysosomal Cts (Lambeth et al., 2019).

Most importantly, lysosomal acidification is a prerequisite for the lysosomal enzymes' digestive role as well as to drive efflux of digested materials. Microglia in the resting state have a sub-optimal lysosomal pH, which hinders them to degrade fibrillary Aβ well despite the whole set of proteases being present (Majumdar et al., 2007). This changes when microglia get activated and their lysosomes acquire a pH~5. While lysosomes can still acidify to some extent without a Cl^- transporter (ClC-7), it is especially the effect of this transporter that drives lysosomal acidification in activated microglia (Fig. 4). Only low levels of ClC-7 can be found in resting microglia as ClC-7 gets transported to the ER for breakdown in the ER–associated degradation (ERAD) pathway (Majumdar et al., 2011). The mislocalization has been attributed to low osteoclastogenesis Associated Transmembrane Protein 1 (Ostm1) levels in quiescent microglia (Fig. 4). ClC-7 and Ostm1 form a molecular complex, which is essential for the correct structure, stability, and transport of ClC-7 (Majumdar et al., 2011). It was further shown that the Ostm1 transport is disrupted in activated microglia surrounding amyloid plaques. These microglia show a diffuse ClC-7 staining pattern and, hence, lysosomal alkylation even though they are activated. This may contribute to the inability of plaque-associated microglia to efficiently degrade Aβ (Majumdar et al., 2011). In addition, lower luminal Cl^- levels in lysosomes have been linked to impaired Ca^{2+} effluxes, a distorted functioning of Cts-C and a dysfunctional arylsulfatase B activity (Chakraborty et al., 2017). Alternatively, $Aβ_{1-42}$ itself (as opposed to $Aβ_{1–40}$ oligomers, fibers, and monomers) may create non-selective ion channels, that might completely abolish the ion gradients (Bode et al., 2017).

7. The digestive route as a source of disease spreading

7.1. Exosomal release spreads Aβ pathology

Dysregulations of aforementioned processes do not only lead to congestion in the intracellular endolysosomal system (Escamilla-Ayala et al., 2020; Sannerud et al., 2016), but may also contribute to

another pathological hallmark of AD, namely extracellular Aβ aggregates. It has been postulated that such aggregates may originate in part from multivesicular bodies (MVB) that release their content in the brain parenchyma (Willén et al., 2017). MVBs are endosomal organelles that arise from inward budding towards intraluminal vesicles that can either be transported to the lysosomes for degradation or be secreted as exosomes. Originating from endosomal maturation, exosomes contain a wide range of cargos, including nucleic acids (e.g. mRNA, miRNA, non-coding RNAs, and DNA), proteins (e.g. tetraspanins, heat shock proteins, 14-3-3 protein family members, chemokines and cytokines), and bioactive lipids (e.g. prostaglandins, leukotrienes, and fatty acids). While being an efficient signaling and clearance mechanism between cells under homeostasis, the beneficial effect might turn detrimental during AD progression (van Niel et al., 2018). AD-affected neurons will not only produce exosomes with higher levels of glycosphingolipids that assemble Aβ40 and Aβ42 species (Paolicelli et al., 2019), they have also been found to promote this secretory route as an alternative for degradation and waste removal. The five lysines in the APP cytosolic domain are key to the process as they enable the transport of APP from endosomal intraluminal vesicles to the endosomal limiting membrane (Williamson et al., 2017). In addition, neuronal cells with the APP^{swe} mutation exhibit distended MVBs that fuse more often with the plasma membrane for exosomal release, attributed to a promoted CD63-dependent exosome formation (Willén et al., 2017). However, neurons are not the only cells that will use exosomal release as a loophole to get rid of undigestible material.

Aside from a high endocytic capacity, microglia also possess a clear exocytic capacity as part of their antigen presentation and cytokine release mechanism (Joshi et al., 2014). Hence, while they are efficient in clearing exosomes or protein aggregates, they can also aid in propagating the disease via increased exosomal release rates. Joshi and colleagues demonstrated that when intracellular pathways of Aβ degradation are saturated in microglia, extracellular insoluble $Aβ_{1–42}$ aggregates are not completely degraded but converted in soluble neurotoxic Aβ species that are secreted through exosomes. The exosomes collected from AD patients are particularly neurotoxic, thereby acting as effective promotors of neuronal injury (Joshi et al., 2014). Moreover, exosomes of microglia have also been tightly linked to their immune functions and responses. Microglia treated with IFN-γ produce extracellular vesicles with increased major histocompatibility complex class II (MHCII) levels and different (inflammatory) cytokines, including IL-2, IL-4, IL-12p70, IL-17, IL-21, IL-22, IL-33, IFN-γ, ITAC, TGF-β, and TNF-α (Fitzgerald et al., 2018). Hence, exosomes of microglial origin can be regarded as important transport vehicles for the regulation of inflammatory responses. Microglial exosomes could further affect neighboring pathways by transferring signaling molecules. By way of example, inflammatory microglia produce increased amounts of miR-146a-5p-containing exosomes for microglia-to-neuron transfer. Prolonged exposure to miR-146a-5p causes neurons to downregulate *Syt1* and *Nlg1* expression, decreasing dendritic spine densities and causing a loss of excitatory synapses (Prada et al., 2018). Comprehensive and up-to-date lists can be consulted at ExoCarta, the exosome database (http://exocarta.org/).

7.2. Exosomal release spreads tau pathology and inflammasome specks

Tau is a major microtubule-associated protein within axons of differentiated neurons and functions to some extent in the somatodendritic compartment in a phosphorylated form as well (Kanaan and Grabinski, 2021). Its abnormal hyperphosphorylation and deposition in neurofibrillary tangles is one of the cardinal pathological features of the AD pathology. With little to no expression of tau being found in astrocytes or microglia (Kanaan and Grabinski, 2021), its effect on the glial system appears to be

rather indirect, though of impact. A prompt microglial activation alleviates Aβ-induced lipid bilayer damage (and repair), of which tau hyperphosphorylation and tangle formation has been described as a down-the-line consequence (Lee et al., 2021). When homeostatic rescue mechanisms become insufficient to avert the formation of Aβ fibril-induced dystrophic neurites, microglia also start phagocytosing tau-containing synapses. Once taken up, microglia exhibit high ubiquitinated levels of tau, being representative of a failed proteasomal or lysosomal degradation (Asai et al., 2015). The latter is an incentive for tau inclusion within multivesicular bodies destined for exosomal release, which promotes tau spreading to adjacent brain regions (Asai et al., 2015; Xu et al., 2020). Such microglia-promoted tau spreading could account for the pattern of pathologic tau staging (i.e. Braak staging) that disseminates across non-synaptically connected areas; e.g. going from the trans-entorhinal cortex to the occipito-temporal gyrus (Asai et al., 2015). The process of mutant tau exocytosis involves the lysosomal Ca^{2+} channel - transient receptor potential cation channel, mucolipin subfamily (TRPML1/Mcoln1) - and is inversely correlated with intracellular pathology and accelerated spreading (Xu et al., 2020). Interestingly, TRPML1-mediated Ca^{2+} release could also affect microglial motility, as seen in dendritic cells. When Ca^{2+} is released from the lysosomal store through TRPML1/Mcoln1, myosin-2 gets activated to provide in a directed cell motility (Bretou et al., 2017); a feature important for microglia recruitment. In addition, this process was found to be self-enforcing: lysosomal Ca^{2+} promotes TFEB activation, which in turn promotes further expression of TRPML1/Mcoln1 (Bretou et al., 2017). PSEN1/γ-secretase activity is further linked to the process, phosphorylating myosin-2 to enable EphA3-dependent axon protrusion (Louvi et al., 2004). In accordance, loss of PSEN1 function had already been described to both affect the radial and tangential migration of neuronal cells through the cortex (Javier-Torrent et al., 2019); increasing the likelihood of a similar impact of the AD pathology and/or causative mutations on microglial functioning.

Furthermore, lysosomal tau seeds have been shown to activate the microglial NLRP3-ASC inflammasome [NACHT, LRR and PYD domains-containing protein 3 (NLRP3)-Apoptosis-associated speck-like protein containing a CARD (ASC); (Stancu et al., 2019)]. While such inflammasome activation may eventually lead to pyroptotic cell death, it initially upregulates the microglial cytokine and chemokine production (Stancu et al., 2019). In addition, ASC specks display a similar 'seeding' behaviour as do tau-loaded exosomes (Franklin et al., 2014). ASC inflammasome specks are protease-resistant, get secreted by the activated microglial cell and are taken up by nearby recipient microglia in which they cause lysosomal swelling, damage and leakage, hence, activating the inflammatory IL-1 cascade (Franklin et al., 2014). The sequence of events outlined here may come full circle, with microglia-derived ASC specks cross-seeding Aβ (Venegas et al., 2017). Of note, while the initial cascade may be Aβ-driven, this is not a prerequisite. Tau pathology by itself is capable of inducing microglial changes and, hence, has been detected in other age-related neurodegenerative pathologies as well: e.g. frontotemporal dementia, progressive supranuclear palsy and corticobasal degeneration (Stancu et al., 2019).

8. Unanswered questions and challenges

8.1. Combining polygenic subcellular information with intercellular communication

Although most (LO)AD patients present with a polygenic risk profile (Harrison et al., 2020), studies have mainly been focussing on individual risk genes at the molecular and mechanistic level thus far. This is likely inevitable as for most proteins encoded by risk genes, too little information is available to comprehend their full functional contributions to, for instance, endolysosomal homeostasis. However,

as our knowledge on the individual risk gene level is slowly increasing, we should start initiating parallel studies in patient-derived cells, selected for a diverse and higher endocytic polygenic burden. Herein, however, the installation of proper (isogenic) control cells might provide an important hurdle. In addition, most information is still coming from GWAS and transcriptome-wide association studies. Only the more recent single cell-RNA studies have highlighted cell type specific up- or downregulations of clusters of risk genes and their involvement in pathways wherein their functions overlap: including lipid metabolism, phagocytosis and inflammation. Looking for such LOAD-affected pathways that coincide, de group of De Jager investigated the quantitative trait loci that regulate co-expression modules of genes with an unprecedented study design (Yang et al., 2020). They identified the TMEM106B locus as a master regulator of over 1000 genes, implicated in both AD and limbic-predominant age-related TDP-43 encephalopathy (LATE). The module contains many lysosomal biology and myelination related genes and may help to explain how TMEM106B and ApoE-Aβ dysfunctions converge in similar expression alterations and TDP-43 pathology (Yang et al., 2020). This necessity to functionally interconnect different LOAD risk genes needs to be extended to different cell contexts as well. For decades, neurons have been put at the center stage by nature of AD being a neurodegenerative disease and neurons being the major source of Aβ production and tau hyperphosphorylation. Although this now shifted with the emergence of a glial signature in the disease process, we should not remake the same mistake and only focus on glial cell types from now on. The interplay between the different brain cell types is important in the light of several features in the pathoetiology, including Aβ clearing, synapse loss, synapse pruning, and inflammation up to disease spreading. In this regard, Cserép *et al*. recently identified a direct microglia–neuron communication route surpassing exosomal and interstitial communication through specialized junctions between neuronal cell bodies and microglial processes (Cserép et al., 2020). Over 91% of neurons shows contacts with microglial processes in all main areas of the brain. The microglial molecular fingerprint of these sites implicates a functional role for P2Y purinoceptor 12 receptors (purinergic signaling) and a strong mitochondrial dependency, with levels of mitochondrial membrane protein TOM20 being 420% increased at these sites. Inhibiting contacts negatively influences injury–induced microglial neuroprotection, including the regulation of neuronal Ca^{2+} content (Cserép et al., 2020). Other non-neuronal populations are emerging in recent single cell studies of AD-affected brains as well (Wingo et al., 2021), of which the relevance to the pathoetiology is uncharted territory. In this regard, while only a small population in absolute numbers, it was found that addressing the accelerated dystrophy and decay of oligodendrocyte precursor cells in an AD context alleviates plaque-linked pro-inflammatory signatures and microglial activation status (Zhang et al., 2019). A spatial transcriptomic analysis study of cells in the immediate neighbourhood of amyloid plaques identified not only an intercellular crosstalk between astrocytes and microglia, the paper also revealed early alterations in the oligodendrocyte gene response (Chen et al., 2020). To quote another non-neuronal example, a normal processing of APP was found essential to uphold vasoprotective properties of endothelial cells; i.e. the activation of actin cytoskeleton-interacting proteins as well as of plasma membrane proteins, including VEGFR2 (Ristori et al., 2020). Moreover, an increased blood-brain barrier permeability in aged subjects and those with AD enables peripheral immune cells, including monocytes, neutrophils, and lymphocytic cells to interact with resident microglia. For a comprehensive overview of this topic, the reader is directed to a recent review of (Wyatt-Johnson and Brutkiewicz, 2020).

Secondly, the same LOAD SNPs are expressed in both neuronal and non-neuronal cells, underscoring that subsequent or parallel functional alterations in the different cell types could affect, re-enforce or

accelerate the pathology in surrounding cells (Szepesi et al., 2018). Whereas many LOAD risk factors are clearly upregulated in the ARMs phenotype during disease progression, several also do function in neuronal APP proteolysis. For instance, BIN1 and CD2AP have a majorly polarized distribution and regulate the endosomal sorting of APP in axons and BACE1 in dendrites, respectively, thereby affecting Aβ production differently (Ubelmann et al., 2017). In addition to the primary neuronal link and presynaptic localization of the BIN1:H isoform, BIN1:L co-localizes preferentially with oligodendrocyte markers (De Rossi et al., 2016). PICALM regulates PSEN1/γ-secretase internalization in neurons but is also involved in transcytosis of Aβ through endothelial cells (Kanatsu et al., 2016; Van Acker et al., 2019a). PLD3 is practically solely expressed by neurons under brain homeostasis, then again gets about halved during the course of AD (Cruchaga et al., 2014; Satoh et al., 2014). The only cells bucking this trend are the ARMs. This microglial subtype shows a 1,76- to 4-fold upregulation of PLD3 in 5XFAD and APP^{NL-G-F} mice (Keren-Shaul et al., 2017; Sala Frigerio et al., 2019). In this case, PLD3 SNP variants may have likely a primary neuronal impact, before any microglial pathology is emerging.

In conclusion, a recent concept of the AD pathology included a cellular phase in between the biochemical and clinical phases (De Strooper and Karran, 2016). Herein, the proteopathic stress of the biochemical phase, originating from the accumulation of Aβ and tau, gives rise to the cellular phase. When its homeostatic function cannot be sustained anymore, the clinical phase of AD is instigated (De Strooper and Karran, 2016). In the rare familial AD cases, the biochemical phase is most likely the trigger for this sequence of events. However, this may not necessarily apply to LOAD. In cases with a high polygenic endocytic burden, alterations in endolysosomal transport regulation in both neuronal and non-neuronal cells may slowly derail the normal APP proteolysis and clearance, respectively. This results in feed-forward loops that rupture endolysosomal compartments. Thus, in LOAD, still constituting over 95% of all AD cases, the cellular phase may as well precede and, next, run in parallel to the biochemical phase.

8.2. Therapeutic avenues for the ageing microglial system struck with AD pathology

Chronic (non-resolving) inflammation is likely aggravated by pathophysiological conditions that ageing populations in developed countries tend to acquire, including metaflammation from chronic overnutrition or obesity, metabolic syndrome and type-2 diabetes mellitus (Herradon et al., 2019). By way of example, the relative risk of developing AD increases 153% with type-2 diabetes as a comorbidity (Biessels et al., 2020). Hence, the idea of addressing inflammation and (immune-) metabolism in the treatment of age-related neurodegenerative disorders, particularly AD, has been gaining momentum. Current ongoing trials listed in the ClinicalTrials.gov trial registry and addressing one or more mechanisms linked to microglial phagocytic/lysosomal or inflammatory capacity, have been outlined in table 3. While some therapies still target the effect of the inflammatory pathway (e.g. neutralization of TNFα by XPro1595 or IL-1β by Canakinumab), many ongoing trials are currently targeting one or more mechanisms at the crossroad of inflammation and degradation that have been outlined in this review. One of these entails activation of the TREM2 pathway that pokes microglia towards an autophagic and neuroprotective (ARMs) activation state. Whilst the furthest ahead, the AL002 antibody (NCT04592874) is not the only TREM2 activator in the pipeline. Researchers at the German Center for Neurodegenerative Diseases generated a monoclonal antibody that aside from stabilizing TREM2 on the cell surface also activates phagocytic SYK signaling (Schlepckow et al., 2020). Preclinical analyses show the antibody to promote microglial conversion towards an ARMs phenotype and to stimulate phagocytosis of myelin debris and Aβ (Schlepckow et al., 2020). As TREM2 acts

downstream of inhibitory CD33 and given the generation of neutralizing antibodies to be generally less challenging as activating ones, Alector and Abbvie are currently also testing AL003 (NCT03822208); a monoclonal antibody against CD33 (Siglec3). At a preclinical level, small CD33-inhibitory compounds are being generated with the same aim, increasing microglial toxic Aβ42 peptide uptake and degradation (Miles et al., 2019). A recent tissue-specific expression study of the Cruchaga lab on protein quantitative trait loci substantiates this premise. Not only does CD33's genetic architecture associate with an AD risk but so do protein level affecting alterations, e.g. DNA methylation or histone acetylation variations (Yang et al., 2021). Of note, while being locked in the homeostatic state is detrimental to the microglial response, as e.g. by TREM2 dysfunction or depletion (Parhizkar et al., 2019), so is the reverse. Aberrant PGRN availability polarizes microglia towards a chronic hyperactivated state that results in a similar wide-spread brain dysfunction as does TREM2 depletion (Götzl et al., 2019). AZP2006 is aimed at stabilizing the interaction between PGRN and another regulator of lysosomal function, prosaposin, avoiding PGRN to be cleaved and promoting its secretion (NCT04008355). While no preclinical data has been published on AZP2006, it is envisioned that stabilization of the interaction restores microglial hyperactivation, reducing pro-inflammatory cytokine secretion and rather promoting the clearance of Aβ and tau (Nicholson et al., 2016).

Of note, both male and female patients are eligible for enrolment/participation in all listed studies, with the exception of clinical trial 'NCT04120233' that only includes females. Given the target of the studied compound (MW151) is undisclosed, the underlying reasoning is unknown but could include a female-specific pathway. In this regard, a study sequencing the microglial transcriptome of male and female adult mice revealed microglia to exhibit gender-specific expressions of a number of NF-κB-regulated genes of the inflammatory response (Villa et al., 2018). The same study detected female microglia to be better apt than male microglia to exert neuroprotective actions after ischemic damage (Villa et al., 2018). In line with these results, male microglia do become more rapidly developmentally mature and aged in response to an immune challenge as do female microglia, rendering the latter less vulnerable to such events (Hanamsagar et al., 2017). The lysosomal system may be gender-affected as well. Autophagic removal of dopamine receptors by microglia during adolescent nucleus accumbens re-shapement only occurs in males (Kopec et al., 2018). However, given the amount of studies on the sexually dimorphic nature of (adult) microglia to be still limited (reviewed in (Bordeleau et al., 2019)), well-promulgated research into gender-specific microglial subcellular mechanisms and responses to pharmacotherapies are further awaited. Overall, scrutinizing cell-specific and intercellular connection-related functions of AD proteins will attribute to a better definition of the neuronal and microglial deleterious or protective properties. As such, elucidation of these mechanisms will provide a better therapeutic understanding to modify responses towards neuroprotection and Aβ degradation without eliciting an inflammatory reaction.

Sample CRediT author statement

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Conflicts of Interest / Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Figures

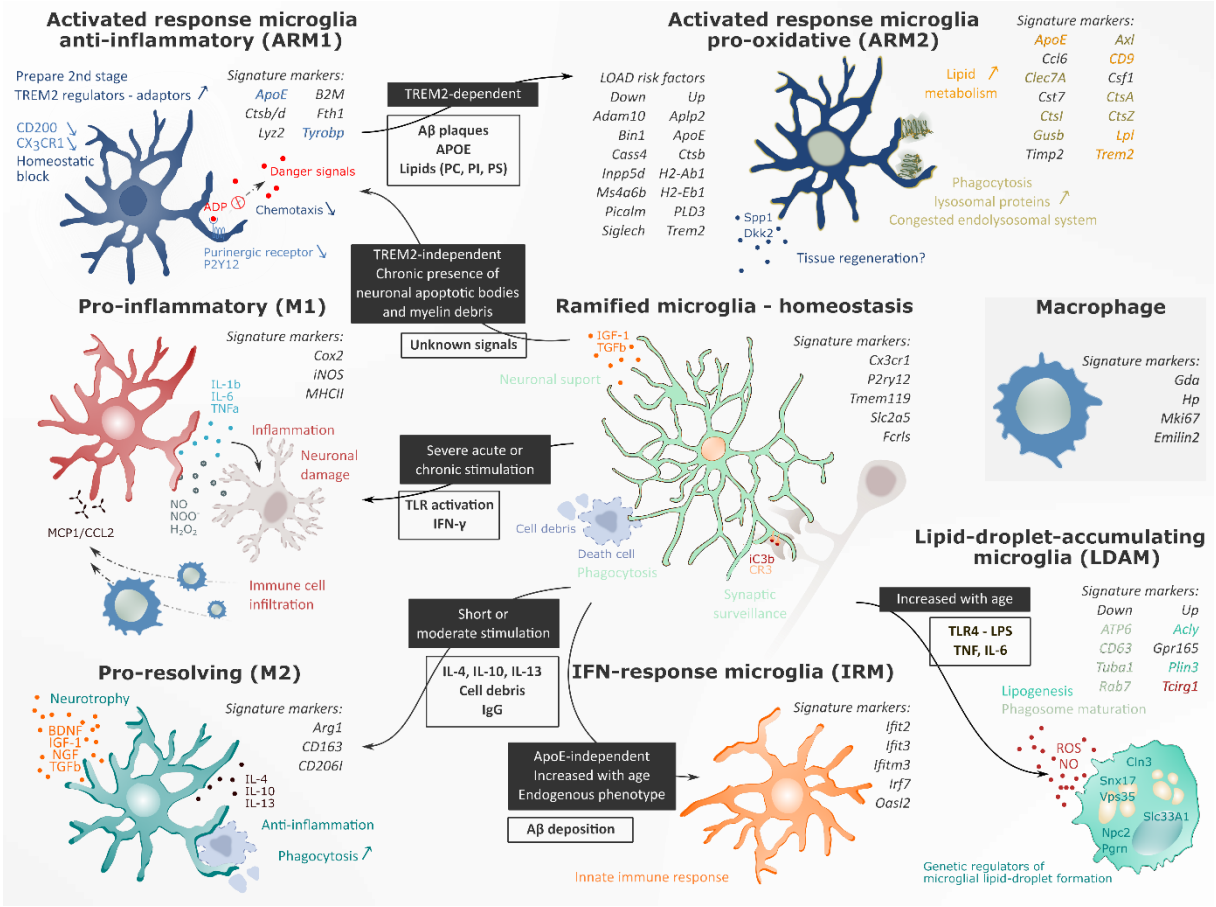

Fig. 1. Microglia activation states. Surveilling, homeostatic microglia can be identified in the healthy brain by their ramified morphology. While previously denoted as “resting” state, this subtype is all but static. Homeostatic microglia constantly extend and retract their ramified processes to sense synapses, release signal molecules and scan the environment for damage-associated molecular pattern molecules such as myelin debris or calcium release. Depending on the environment’s stimuli, microglia adapt into a wide range of phenotypic and functional states, which are plastic and may further cross-interact and interconvert in one another. Neuroinflammatory microglia promote a neurotoxic reactive astrocyte subtype that damages neurons and oligodendrocytes, mediates synapse loss, and promotes immune cell infiltration in the brain. Activated response microglia that are found at sites of neurodegeneration, are highly phagocytic and display intracellular particles filled with Aβ. The accumulation of protein fragments like Aβ could be facilitated by lipids accumulating in microglia along the ageing process. The partially overlapping lipid droplet-accumulating microglia state also shows a dysfunctional and pro-inflammatory state. Color codes indicate involvements in indicated mechanisms.

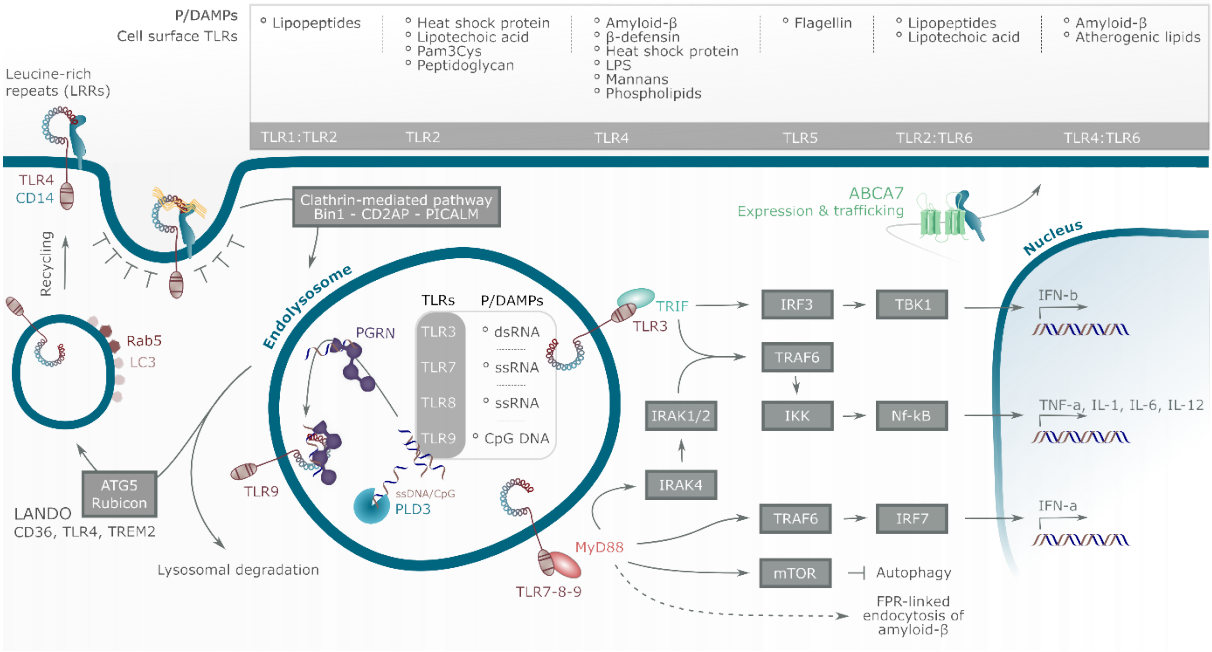

Fig. 2. Toll-like receptor activation. TLRs are membrane signaling receptors with a leucine-rich horseshoe structure that recognizes the P/DAMPs and a Toll/IL-1 receptor (TIR) domain to which adaptor proteins such as MyD88 and TRIF can bind. One subset of TLRs is localized at the plasma membrane to recognize extracellular components, where the second group of TLRs (including TLR3/7-9) can be found in endolysosomes to sense nucleic acid. TLR4 is unique in that it can signal both at the cell membrane and within endosomes, reacting to viral envelope glycoproteins. Different LOAD risk proteins are also connected to the pathway. ABCA7 is involved in the expression and cellular trafficking of the CD14 co-receptor. Bin1, CD2AP and PICALM are adaptor proteins functioning in clathrin-mediated endocytosis of TLRs and TREM2 gets trafficked back through the LANDO pathway that also transports TLR4. PGRN, PLD3 and TLR9 risk proteins can all be linked to the cellular response towards ssDNA. Where PLD3 functions as an exonuclease, PGRN works as a soluble cofactor for TLR9 and delivers/recruits its substrate. TLR9 itself has recently been ascribed with a risk factor profile in multigenerational Belgian family as well (Cacace et al., 2020).

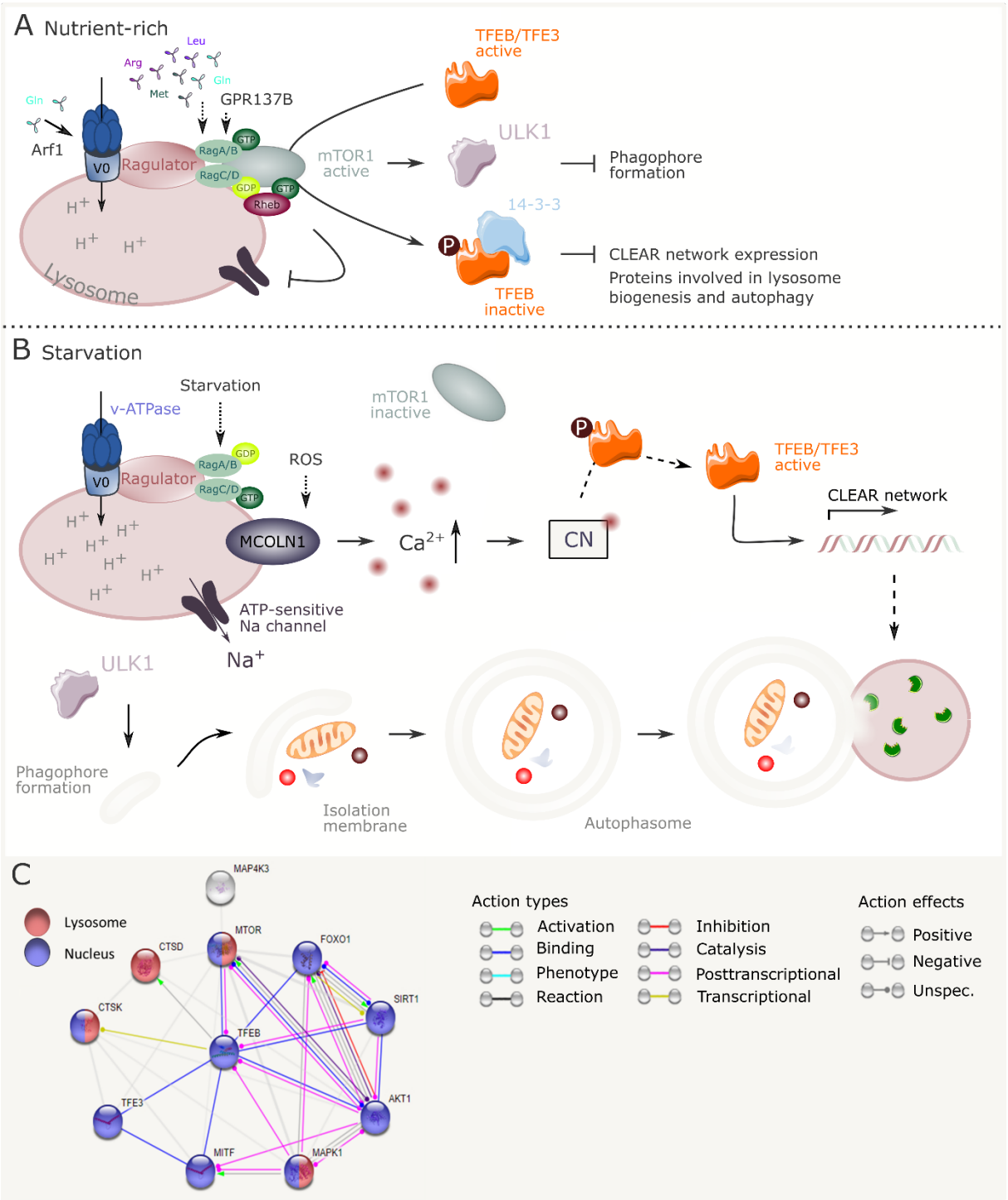

Fig. 3. TFEB regulation of autophagy – lysosomes. (A) Under nutrient-rich conditions, amino acid sensors (CASTOR1, SESN2 and SAMTOR) promote the active state of the Rag heterodimer ($Rag^{A/B}$*GTP- $Rag^{C/D}$*GDP), which attracts mTORC1 to the lysosomal surface. Rheb, another GTPase, subsequently interacts with mTORC1 to activate it. Active mTOR phosphorylates TFEB at different serine positions, enabling the interaction with the phospho-binding protein 14-3-3 that inhibits nuclear translocation of TFEB. (B) Following starvation or cellular stress, the inactive Rag heterodimer ($Rag^{A/B}$*GDP- $Rag^{C/D}$*GTP) does not recruit mTOR. Two-pore channels release Na^+ from the lysosomal lumen to depolarize the membrane and promote H^+ pumping by the V-ATPase. In addition, low nutrient availability switches ATP synthesis from amino acid and carbohydrate metabolism to partial oxidation of fatty acids. Reactive oxygen species (ROS) are formed as a byproduct that trigger the nonselective cation channel/ROS sensor TRPML1, alternatively known as mucolipin-1 (Mcoln-1). TRPML1 increases the cytoplasmic Ca^{2+} levels, activating calcineurin. Calcineurin in turn dephosphorylates TFEB, causing the 14-3-3 complex to dissociate and enabling fast translocation of TFEB to the nucleus for expression of autophagy/lysosomal genes. Inhibition of mTOR also promotes the serine/threonine protein kinase activity of ULK1, allowing ULK1 to activate the vacuolar protein

sorting 34 (VPS34) by phosphorylation. It also phosphorylates ATG9, allowing its recruitment to autophagy initiation sites and driving phagophore generation. Aside from this general baseline-starvation switching mechanism, microglia contain a newly identified lysosome-localized G protein-coupled receptor (GPCR)-like protein, GPR137B, that can activate mTORC1 even under amino acid-depleted conditions (Gan et al., 2019). GPR137B interacts with Rag GTPases, promoting the GTP-loaded, active state of lysosomal RagA and, hence, mTOR recruitment. Knockout microglia show a high expression level of the CLEAR network and expanded lysosomes. (C) TFEB protein interaction network. Representation of protein-protein associations of TFEB. The interaction network was obtained using the STRING database (http://string-db.org/). Edges represent protein-protein associations. Associations are meant to be specific and meaningful, i.e. proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other.

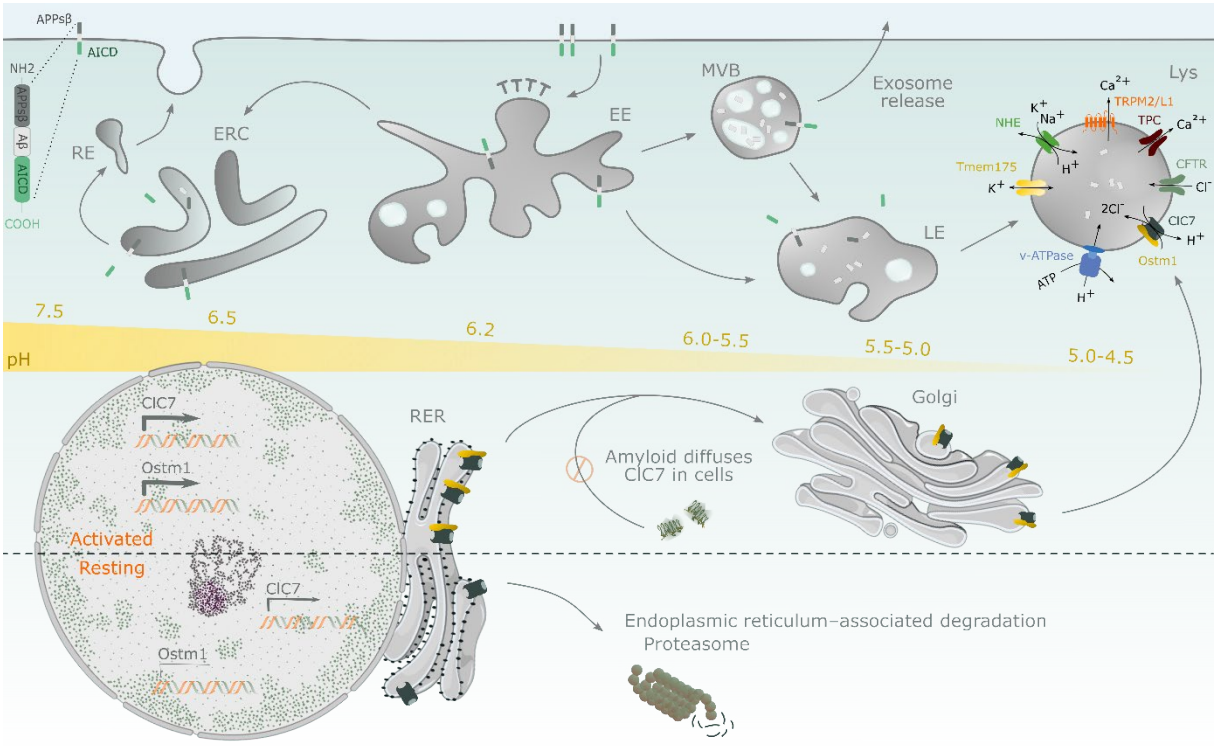

Fig. 4. pH-dependency across the endo-lysosomal pathway. The concentration of protons, or pH, within endocytic vesicles steadily decreases going from the plasma membrane to lysosomes. The optimal acidic environment for the lysosomal hydrolysis is ultimately reached by a coordinated functioning of the V-ATPase proton pump and an anion influx/a cation efflux. The pump functions as an electrogenic proton translocator, implying that the V-ATPase pump does not counterbalance the positive charge of the transferred H^+-ion. The counter-ion transport is established by either: (i) an efflux of positively charged ions including K^+ and Na^+, and (ii) an influx of negatively charged ions, e.g. Cl^- that gets imported by the CLC chloride/proton exchangers. Especially ClC-7 drives microglial lysosome acidification, which is only induced upon microglial activation. Both Ostm1 and ClC-7 are upregulated, making the latter to induce a correct transport of ClC-7 to the lysosomes where ClC-7 can efficiently lower the pH to a suitable level for degradative functions. In the amyloidogenic pathway, APP gets cleaved by BACE1 (β-secretase) and subsequently γ-secretase in (early) endosomes and recycling/degradative endosomes, respectively. Both enzymes have a pH optimum that does not correspond to their primary cellular localization. BACE1 shows an optimum pH value 4.0-4.5 and γ-secretase of 7. Given Aβ40-cleavage to be more pH-sensitive, the Aβ42/Aβ40 ratio rises with increasing deviation from the optimal pH. Generated Aβ can be sorted for either exosome release or lysosomal degradation. CFTR; cystic fibrosis transmembrane conductance regulator, ClC; chloride channel protein, NHE; sodium/hydrogen exchanger, Tmem175; endosomal/lysosomal potassium channel (transmembrane protein 175), TPC; two-pore channel, TRPM; transient receptor potential cation channel subfamily M member, v-ATPase; vacuolar-type H^+-ATPase.

Tables

Table 1. Load risk factors and familial AD genes in microglia from brains.

Gene	Function	Healthy mouse	AD mouse	Healthy human	AD human
APP	Cell surface receptor	0.38	-0.34	0.36	-0.29
ABCA7	Phospholipid-transporting ATPase	n.d.	n.d.	0.00	n.d.
ACE2	Carboxypeptidase	n.d.	n.d.	0.00	n.d.
ADAM10	Sheddase	0.66	-0.41	4.55	0.26
ADAMTS1	Metalloproteinase	0.02	1.55	0.00	0.04
APOE	Lipoprotein-mediated lipid transport	2.92	3.43	0.00	0.25
BIN1	Control of plasma membrane curvature	1.49	-1.17	5.27	0.26
CASS4	Docking protein for tyrosine kinase signaling	0.06	-3.02	0.16	0.06
CD2AP	Scaffold adaptor protein	0.36	-0.57	0.00	0.17
CELF1	Binds RNA for post-transcriptional regulation	0.27	-1.36	2.41	0.11
CLU	Chaperone	0.01	1.10	0.00	-0.15
CR1	Immune adherence receptor	0.21	-0.38	0.00	n.d.
DSG2	Component of intercellular desmosome junctions	n.d.	n.d.	0.00	n.d.
EPHA1	Ephrin receptor tyrosine kinase	n.d.	n.d.	0.00	n.d.
FERMT2	Scaffolding protein that enhances integrin activation	n.d.	n.d.	0.00	0.04
PGRN	Growth factor, key regulator of lysosomal function	3.17	1.03	0.00	-0.02
INPP5D	PtdIns phosphatase	1.03	-0.83	9.49	0.61
IQCK	Binds EF-hand proteins	n.d.	n.d.	0.14	0.11
MAPT	Promotes microtubule stability and assembly	n.d.	n.d.	0.01	-0.03
MEF2C	MADS box transcription enhancer family member	1.34	-0.45	11.67	-0.14
NME8	Microtubule binding	n.d.	n.d.	0.00	n.d.
PICALM	Clathrin assembly protein	1.37	-1.01	9.27	0.50
PLD3	Lysosomal exonuclease	0.29	1.76	0.00	0.14
PSEN1	Catalytic subunit of the gamma-secretase complex	0.20	-0.24	0.26	0.01
PSEN2	Catalytic subunit of the gamma-secretase complex	n.d.	n.d.	0.00	n.d.
PTK2B	Actin cytoskeleton regulating tyrosine kinase	0.46	0.46	0.00	-0.06
RIN3	Ras effector protein	n.d.	n.d.	5.31	0.31
TREM2	Member of Ig receptor superfamily	2.48	1.23	0.00	0.07
ZCWPW1	Histone modification reader	0.03	1.40	0.00	n.d.

Healthy mouse values were obtained from (Keren-Shaul et al., 2017). Values represent the mean absolute number of observed transcripts per cell. AD values contain fold changes to the ARMs' activation state, relative to the baseline from the first column. Healthy human values were obtained from (Hawrylycz et al., 2012), displaying the log2(CPM+1) with CPM being the counts per million reads mapped. As for the mice, AD human values represent the fold changes occurring in AD-affected microglia (Del-Aguila et al., 2019).

Table 2. Proteases involved in Aβ degradation

Protease	pH optimum	Soluble and fibrillar Aβ cleavage sites		Cellular localization	Reference
Angiotensin-converting enzyme (ACE)	7.0-8.5	Asp7/Ser8		Plasma membrane	(Hu et al., 2001)
Cathepsin B	4.5-5.5	Gly33/Leu34 Val40/Ile41	Gly38/Val39 Aβ fibrils	Endosomes - lysosomes Phagolysosomes	(Mueller-Steiner et al., 2006)
Cathepsin D	3.5–5.0	Leu17/Val18 Phe20/Ala21	Phe19/Phe20 Leu34/Met35	Late endosomes - lysosomes	(Rogeberg et al., 2014)
Cathepsin E	3.5-4.5	Phe19/Phe20	Phe20/ala21	Endosomes - lysosomes	(Mackay et al., 1997)
Cathepsin K	5.5-6.5	Unspecified broad specificity		Phagolysosomes	(Bohne et al., 2004)
Cathepsin L	3.0-6.5	Unspecified broad specificity		Phagolysosomes	(Bohne et al., 2004)
Dipeptidyl peptidase (DPP)-IV	7.4-8.7	Ala2/Glu3		Plasma membrane	(Antonyan et al., 2018)
Endothelin-converting enzyme 1 (ECE-1)	~6.8	Lys16/Leu17 Phe19/Phe20	Leu17/Val18 Leu34/Met35	Plasma membrane	(Rogeberg et al., 2014)
Insulin degrading enzyme (IDE)	6.0-8.5	His13/His14 Lys16/Leu17 Phe19/Phe20 Lys28/Gly29 Met35/Val36	His14/Gln15 Val18/Phe19 Phe20/Ala21 Leu34/Met35	Cytosol Plasma membrane	(Rogeberg et al., 2014)
Matrix metalloproteinase (MMP)-2	~8.5	Lys16/Leu17 Met35/Val36	Leu34/Met35	Extracellular - secreted	(Roher et al., 1994)
MMP-9	5.4–6.5	Aβ fibrils Ala30/Ile31	Phe20/Ala21	Extracellular - secreted	(Yan et al., 2006)
membrane type 1 (MT1) MMP	~7.5	Val12/His13 Gln15/Ls16 Val18/Phe19	His14/Gln15 Leu17/Val18 Ala30/Ile31	Plasma membrane Endosomes - lysosomes	(Liao and Van Nostrand, 2010)
Neprilysin (NEP)	~7.0	Gly9/Tyr10 Gly29/Ala30 Gly33/Leu34	Phe19/Phe20 Ala30/Ile31 Leu34/Met35	Plasma membrane	(Rogeberg et al., 2014)
Plasmin	~7.5	Arg5/His6 Lys28/Gly29	Lys16/Leu17	Extracellular - secreted	(Tucker et al., 2000)
Tripeptidyl peptidase (TPP)	2.5-5.0	Aβ fibrils		Lysosome	(Solé-Domènech et al., 2018)

Aβ42 amino acid sequence – ASP (1), ALA (2), GLU (3), PHE (4), ARG (5), HIS (6), ASP (7), SER (8), GLY (9), TYR (10), GLU (11), VAL (12), HIS (13), HIS (14), GLN (15), LYS (16), LEU (17), VAL (18), PHE (19), PHE (20), ALA (21), GLU (22), ASP (23), VAL (24), GLY (25), SER (26), ASN (27), LYS (28), GLY (29), ALA (30), ILE (31), ILE (32), GLY (33), LEU (34), MET (35), VAL (36), GLY (37), GLY (38), VAL (39), VAL (40), ILE (41), ALA (42).

Table 3. Therapeutic avenues addressing microglia (inflammatory) responses

Compound	Target	Target group	(Envisioned) effect	Trial N°
Phase 3				
Cromolyn + ibuprofen (ALZT-OP1)	Uses a mast cell stabilizer & an anti-inflammatory drug	Early stage	Promotes microglial recruitment to and uptake of Aβ	NCT02547818
Donanemab	IgG2a anti-pyroglutamate Aβ, as found in plaques	MMSE 20-28	Facilitates targeting of microglia to the Aβ plaque, promotes phagocytosis	NCT04437511
Phase 2				
Allopregnanolone	GABA-A receptor modulator	MMSE 20-26, APOE4+	Inhibits inflammatory signals induced by activated MyD88-dependent TLRs, promotes neurogenesis	NCT04838301
AL002	Activating antibody against microglial TREM2	MMSE ≥ 22	Promotes recruitment of microglia around Aβ plaques that get reduced. Increases expression of microglial repair genes	NCT04592874
AZP2006	Stabilizing the prosaposin-progranulin complex	PSP and AD	Activates autophagy in microglia, clearing Aβ and tau aggregates	NCT04008355
Crenezumab	IgG4 anti-Aβ antibody	PSEN1 E280A carriers	Decreases Fcγ receptor-induced microglial overactivation, e.g. TNF-α secretion, and promotes microglial Aβ engulfment	NCT01998841
Canakinumab	Monoclonal antibody against IL-1β	MMSE 20-30	Neutralizes pro-inflammatory IL-1β signaling	NCT04795466
Dapagliflozin	Na-glucose co-transporter 2 inhibitor	MMSE 15-26	Increases osmotic diuresis and haematocrit. Decreases body weight and improves mitochondrial fitness	NCT03801642
GRF6019	Plasma fraction	MMSE 0-10	Replenish plasma components that decline with age	NCT03765762
Lenalidomide	Immunomodulator, binding cereblon	MMSE 22-28	Exerts proerythropoietic and anti-angiogenic properties, inhibits proinflammatory cytokine (e.g. TNF-α & IL-6) release	NCT04032626
Montelukast	Leukotriene receptor antagonist	MoCA < 26	Restores microglial phagosomal/lysosomal dysfunctions and reduces age-associated neuroinflammation	NCT03991988
Neflamapimod (VX-745)	Inhibitor of p38α	MMSE > 20	Promotes microglia switch from pro-inflammatory to a phagocytic state	NCT03435861
Simufilam (PTI-125)	Reshapes filamin A structures	MMSE 16-26	Halts filamin A and Aβ42 to TLR4, reducing glial inflammation, reduces IL-6 and soluble TREM2 levels	NCT04388254
Phase 1				
AL003	Inhibiting antibody against microglial CD33	Mild-moderate AD	Activates microglia by blocking CD33's inhibitory effect	NCT03822208
Edicotinib	Microglial colony stimulating factor-1 receptor antagonist	Mild cognitive impairment	Blocks microglial proliferation and secretion of IL-1β and TNFα.	NCT04121208
GlyNAC	Glutathione boost	MoCA 10-20	Reduced glutathione-SH neutralizes free radicals, electrophilic reagents and toxic peroxides	NCT04740580
Inzomelid	NLRP3 inflammasome inhibitor	AD	Abrogates microglial Aβ phagocytosis and ensuing lysosomal damage as well as IL1-β and IL-18 secretion	NCT04015076
LX1001	AAVrh.10-APOE2	APOE4+	Increase APOE4 levels to decrease Aβ levels and amyloid deposition	NCT03634007
MW151	Undisclosed target	First-in-human	Attenuates dysregulated glial proinflammatory cytokine production, does not impact plaque load	NCT04120233
Pepinemab	Inhibiting antibody against semaphorin 4D	MMSE ≥22	Impacts immune cell migration	NCT04381468
RO7126209	Fab fragment to TfR coupled to an IgG1 anti-Aβ antibody	MMSE 18-28	Promotes plaque clearance by binding Aβ and engaging microglia to phagocytose	NCT04639050
XPro1595	Selective TNFα inhibitor, neutralizing the soluble form	Mild-moderate AD	Reduces microglial activation	NCT03943264

Aβ, amyloid beta; AD, Alzheimer's disease; APOE4, apolipoprotein E4; CD, cluster of differentiation; GABA, gamma aminobutyric acid; IgG, immunoglobulin G; IL, interleukin; MMSE, mini-mental state examination; MoCA, Montreal cognitive assessment; NLRP3, NLR family pyrin domain containing 3; PSEN1, presenilin 1; PSP, progressive supranuclear palsy; TfR, transferrin receptor; TLR, toll-like receptor; TNF-α, tumour necrosis factor alpha.