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The interplay between microglial states and major risk factors in Alzheimer's disease through the eyes of single-cell RNA-sequencing: beyond black and white

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von Maydell D, Jorfi M. The interplay between microglial states and major risk factors in Alzheimer's disease through the eyes of single-cell RNA-sequencing: beyond black and white. *J Neurophysiol* 122: 1291–1296, 2019. First published July 31, 2019; doi:10.1152/jn.00395.2019.—Microglia constitute ~10–20% of glial cells in the adult human brain. They are the resident phagocytic immune cells of the central nervous system and play an integral role as first responders during inflammation. Microglia are commonly classified as "HM" (homeostatic), "M1" (classically activated proinflammatory), or "M2" (alternatively activated). Multiple single-cell RNA-sequencing studies suggest that this discrete classification system does not accurately and fully capture the vast heterogeneity of microglial states in the brain. In fact, a recent single-cell RNA-sequencing study showed that microglia exist along a continuous spectrum of states. This spectrum spans heterogeneous populations of homeostatic and neuropathology-associated microglia in both healthy and Alzheimer's disease (AD) mouse brains. Major risk factors, such as sex, age, and genes, modulate microglial states, suggesting that shifts along the trajectory might play a causal role in AD pathogenesis. This study provides important insight into the cellular mechanisms of AD and underlines the potential of novel cell-based therapies for AD.

Alzheimer's disease; microglia; risk factor; RNA-seq; single cell

INTRODUCTION

The β-amyloid cascade hypothesis of Alzheimer's disease (AD) defines the current understanding of AD pathogenesis and has strong experimental support from multiple studies (Karran et al. 2011). The hypothesis postulates that toxic amyloid peptides accumulate in the brain during a decades-long preclinical phase and trigger the formation of intracellular neurofibrillary tangles. Amyloid oligomers and neurofibrillary tangles are implicated in neurodegeneration and synaptic deficits through astrogliosis, vascular alterations, global inflammation in the brain, and inhibition of neurotransmission and synapse formation (De Strooper and Karran 2016). However, quantity, quality, and temporal or spatial progression of amyloid deposition do not reliably predict the neurofibrillary tangles, neuronal death, and cognitive decline associated with AD (Jagust 2018; Karran et al. 2011). The disconnection between early amyloid deposition and explicit late clinical dementia suggests that a multifaceted cellular response governs AD pathogenesis (De Strooper and Karran 2016). Conceivably, multiple AD risk factors, including amyloid, age, sex, genetic variants, and neuropathology, determine clinical progression of the disease by modulating this cellular response. Specifically, microglial-mediated phagocytosis and neuroinflammation are likely effectors of AD risk genes implicated in inflammation and amyloid clearance. Compared with other glial cells and neurons, microglial transcriptomes are enriched for the expression of major AD risk genes, including apolipoprotein E (APOE) and triggering receptor expressed on myeloid cells 2 (TREM2) (Gosselin et al. 2017; Mathys et al. 2019). TREM2 deletion in a mouse model of familial AD (i.e., 5XFAD) interfered with microglial tropism toward amyloid plaques and anabolic microglial metabolism, while increasing microglial cell death (Ulland et al. 2017). This suggests that hypomorphic variants in AD risk genes might increase the disease risk, in part, by eliciting a dysregulated and pathogenic microglial response to amyloid plaques. Previously, inflammatory microglial activation states were discretely classified into "M1" and "M2" based on a limited number of cell-surface and secretion markers. However, clustering of single-cell transcriptomes has demonstrated that this discrete classification does not accurately capture the diverse multifunctional states of microglia (Ransohoff 2016). Discussing the "M1" and "M2" states is out of the scope of this article and readers are referred to a commentary by Richard Ranshoff (Ransohoff 2016).

A recent study by Sala Frigerio et al. (2019) provides further insight into microglial transcriptomic heterogeneity and its association with multiple AD risk factors. The study revealed that microglia exists along a continuous transcriptomic trajectory in AD mouse models and non-AD controls. Moreover, major AD risk factors, including sex, age, and genes, altered the distribution of microglial states along this spectrum. The study raises multiple questions: *1*) Are these microglial states

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consistent with previous single-cell studies? *2*) What underlying mechanisms mediate the association between risk factors and shifts along the trajectory? *3*) What role do these shifts play in AD pathogenesis? *4*) How can one leverage the microglial transcriptomic spectrum to find a novel AD intervention? Here, we discuss the current understanding of microglial transcriptomic heterogeneity and explore answers to the aforementioned questions.

TRANSCRIPTOMIC HETEROGENEITY IN MICROGLIA

Compared with selected cell-surface markers, whole single-cell transcriptomes capture the functional state of a cell with increased granularity. Single-cell transcriptomes are influenced by cell-intrinsic and -extrinsic factors that define the functional state of a cell, including differential epigenetic priming, temporal, or spatial location in the brain and proximity to pathology. Single-cell RNA-sequencing of microglia derived from mice and human brains, followed by clustering, has revealed systematic and heterogeneous transcriptomic changes that correlate with development and neurodegenerative pathology. Broadly, prior single-cell studies have classified microglia into two states: *1*) homeostatic microglia (HM), conserved across multiple healthy adult brain regions, and *2*) neuropathology-associated microglia (NAM), enriched with progressive neurodegenerative disease pathology (Keren-Shaul et al. 2017; Li et al. 2019; Masuda et al. 2019; Mathys et al. 2017, 2019). HM are characterized by extensive outward processes, decreased phagocytic and lysosomal activity, and expression of various homeostatic marker genes, including microglial checkpoint genes. NAM show rounded enlarged bodies, increased phagocytic activity, expression of AD risk genes (e.g., APOE and TREM2) and downregulation of homeostatic checkpoint genes. HM and NAM delineate broad trends across multiple single-cell studies. However, substantial heterogeneity exists among the HM and NAM populations identified to date.

A number of studies have already shown that NAM cells are enriched in embryonic development and a variety of progressive pathologies, including AD, amyotrophic lateral sclerosis, multiple sclerosis, and aging (Keren-Shaul et al. 2017; Masuda et al. 2019; Mathys et al. 2017, 2019). For example, Keren-Shaul et al. (2017) found that NAM cells were increased in AD mouse cortex (5XFAD) as pathology progressed, while the HM population declined. The authors termed this particular NAM population "disease-associated microglia." These microglia cells were characterized by decreased expression of HM markers, such as CX3C chemokine receptor 1 (CX3CR1) and purinergic receptor P2Y12 (P2RY12), and increased APOE and TREM2 expression over time. In a separate study, Mathys et al. (2017) characterized two groups of NAM cells using a CK-p25 mouse model of severe neurodegeneration: *1*) early response microglia, an intermediate NAM population preceding severe amyloidosis, neuronal loss, and cognitive decline, and *2*) late-response microglia, whose emergence correlated with neuronal and synaptic loss and cognitive decline. In line with disease-associated microglia, late-response microglial transcriptomes were enriched for APOE and inflammatory genes. Interestingly, within the late-response population, subsets of cells showed differential enrichment for major histocompatibility complex (MHC) II- and interferon-associated genes, suggesting that late-stage NAM populations are functionally heterogeneous. Collectively, these studies show a trend in the transition of microglia from HM to heterogeneous NAM populations in AD mouse models. This transition correlates with progressive increases in AD pathology and is characterized by increased APOE expression by NAM cells. Importantly, an analogous NAM population was identified in a single-cell study of human AD cortices, suggesting that the pathology-associated transition toward NAM is conserved in humans (Mathys et al. 2019). The human NAM population shared a core signature of 27 markers with disease-associated and late-response microglia, including APOE, tyrosine kinase binding protein (TYROBP), cluster of differentiation 74 (CD74), ferritin light chain (FTL), and hypoxia inducible factor 1 subunit alpha (HIF1A) (Mathys et al. 2019, Supplementary Table 7). The shared genes make up 11, 5, and 35% of the disease-associated microglial, late-response microglial, and human NAM signatures, respectively, emphasizing that the transcriptomic overlap between the three NAM populations is incomplete. Variable neuropathologies, distinct genetic backgrounds, and technical differences might explain the transcriptomic differences among these NAM populations.

Recent work by Sala Frigerio et al. (2019) found two distinct NAM populations in an APP-knock-in mouse model of AD. These populations share a limited number of markers, including APOE and CD74, with the aforementioned NAM cells. The authors revealed a continuous spectrum of microglial states from HM to two distinct NAM end points: interferon-response microglia, enriched for interferon response genes, and activated-response microglia, enriched for MHC II genes. This finding is consistent with the transcriptomic heterogeneity observed in the aforementioned late-response microglial population. In line with transcriptomic heterogeneity observed in previous single-cell studies, Sala Frigerio et al. (2019) found that multiple transitioning microglial states, including cycling proliferative microglia and transition-response microglia, existed on the HM-NAM trajectory. Consistent with the study by Keren-Shaul et al. (2017), which showed a small disease-associated microglial population emerge in aged nontransgenic mice, Sala Frigerio et al. (2019) found the full transcriptomic trajectory of transitioning microglia from HM to NAM in both transgenic AD mice and nontransgenic wild-type mice (Keren-Shaul et al. 2017; Sala Frigerio et al. 2019). Moreover, key AD risk factors, including age, sex, amyloid, and genes, altered the microglial distribution along the HM-NAM trajectory (Sala Frigerio et al. 2019). Although the study by Sala Frigerio et al. provides important insights into the spectrum of microglial states associated with major AD risk factors, the mechanisms by which risk factors modulate the shift along this spectrum and the implications of such a shift for AD pathology remain unclear.

MECHANISMS DRIVING TRANSCRIPTOMIC TRAJECTORY SHIFTS

APOE genotype is one of the strongest known genetic risk factors for AD (Karran et al. 2011). In the brain, APOE is secreted primarily by astrocytes and microglia, where the lipoprotein mediates cholesterol transport and uptake by signaling through receptors. Importantly, APOE is implicated in activating the innate anti-amyloid immune response and mediating amyloid clearance from the brain (Karran et al. 2011).

Studies have found that the transition from HM to heterogeneous NAM states depends on APOE expression (Krasemann et al. 2017) and APOE expression positively correlates with NAM induction (Keren-Shaul et al. 2017; Mathys et al. 2017), suggesting that APOE might drive amyloid clearance through NAM phagocytosis. More recently, Sala Frigerio et al. (2019) found that the induction of only one subtype of NAM cells (activated response microglia) depended on APOE expression, while lack of APOE expression induced the alternative NAM subtype (interferon response microglia). Moreover, they showed that microglial clustering around amyloid plaques was also APOE dependent. This may suggest that activated response microglia preferentially cluster around amyloid plaques compared with interferon response microglia. However, the mechanisms by which APOE gates microglial transitions along the trajectory remain unclear. In a recent review, Deczkowska et al. (2018) proposed that microglia express specific receptors, through which they sense neurodegeneration-associated signals and subsequently transition into various NAM subtypes. Recent findings support this hypothesis by showing that microglial phagocytosis of apoptotic cells induced the transition toward a NAM-like transcriptomic state (Krasemann et al. 2017). This transition depended on microglial expression of APOE and TREM2. These findings strongly suggest that AD risk genes, such as APOE, might mediate sensing of neurodegeneration-associated signals (Deczkowska et al. 2018). Alternatively, APOE might gate NAM transitions by altering the intrinsic quality of these signals. For example, Sala Frigerio et al. (2019) showed that APOE deletion decreased the dense core amyloid burden in a mouse model of AD without affecting total amyloid load. This could suggest that APOE-dependent formation of dense core amyloid induces the transition from HM to activated response microglia.

Based on the aforementioned studies, which have provided substantial insight into the transition from HM to NAM, it is interesting to consider the mechanisms that might cause the simultaneous induction of two heterogeneous NAM populations, such as activated and interferon response microglia. We hypothesize the following nonexclusive mechanisms: *1*) Proximity-derived transition. For cells in direct proximity to amyloid plaques, APOE-mediated plaque signaling may induce a transition from HM to activated response microglia. In contrast, distal microglia may transition to interferon response microglia in response to signaling by ubiquitous cytokines and soluble amyloid oligomers. *2*) Heterogeneous HM populations. HM might be epigenetically primed to transition into interferon or activated response states. Activated response precursor cells may express a unique set of receptors that mediate APOE-dependent transition into the activated state in response to a variety of factors, including amyloid plaques and cytokines. Exploring these ideas further to understand the mechanisms by which AD risk genes mediate microglial transitions along a transcriptomic trajectory may be integral to elucidating the cellular mechanisms of risk, the protective or pathogenic function of different microglial subgroups, and potential therapeutic approaches to modulate these trajectories.

Age is the strongest risk factor for AD. Aging is associated with increased neuroinflammation and with accumulation of neuropathologies such as amyloid plaques, neuronal death, and mitochondrial dysfunction. Multiple studies have seen a correlation between aging and NAM enrichment. Mathys et al. (2019) found a significant overlap of aged human microglial markers with the human NAM signature, including CD74, multiple human leukocyte antigen genes, and complement protein, but not APOE. In another study, Holtman et al. (2015) found shared coexpressed gene groups, including lysosomal and phagocytic modules, in aging microglia and NAM. Likewise, upregulation of c-type lectin domain containing 7A (CLEC7A) and CD74 (activated response- and interferon response microglial markers) was found in aged microglia (Hickman et al. 2013). Importantly, Keren-Shaul et al. (2017) found disease-associated microglial populations emerge in super-aged nontransgenic wild-type mouse brains, showing that NAM appear with age. Along the same lines, Sala Frigerio et al. (2019) found that aging accelerates the transition from HM to interferon response and activated response microglial subpopulations of NAM in nontransgenic wild-type and transgenic AD mouse models. Together, these studies suggest a functional overlap between the microglial states induced in aging and those states induced in neurodegeneration. Multiple mechanisms may explain the age-associated transition to NAM. *1*) Microglial expression of proteins for sensing endogenous signals and microbes changes with age: Aged microglia upregulate the expression of transcripts involved in the host defense response and sensing microbes (Hickman et al. 2013). This raises an interesting question: Do these transcriptomic changes cause a microglial shift to NAM by increasing microglial sensitivity toward triggering pathologies? These transcriptomic changes could occur in response to intrinsic age-associated processes, such as telomere shortening, oxidative stress, and protein aggregation. In support of this hypothesis, Böttcher et al. (2019) found a positive correlation between aging and expression of TREM2, a cell-surface receptor necessary for phagocytic disease-associated microglial induction (Böttcher et al. 2019; Keren-Shaul et al. 2017). *2*) The burden of neurodegenerative-associated signals increases with age: NAM signatures have been identified in neurodegenerative disease models other than AD, including models of amyotrophic lateral sclerosis and multiple sclerosis (Keren-Shaul et al. 2017; Krasemann et al. 2017; Masuda et al. 2019), showing that NAM emerge as a nonspecific response to multiple pathologies that are also enriched with age, such as neuronal apoptosis, proteinopathies, and global inflammation. Altogether, age-associated processes could facilitate microglial transition toward NAM by increasing neurodegeneration-associated signal burden, modifying microglial sensitivity to these signals, and upregulating genes that are necessary for NAM induction.

Epidemiological evidence suggests that females have an increased risk of developing dementia (Tower 2017). However, the genetic, molecular, and cellular basis driving this modified risk remains largely unexplored. Multiple fundamental sex-differences exist that may play a role in modifying the progression of and/or response to neuropathology by sex, including *1*) sex-chromosome dosage and *2*) maternal inheritance of mitochondria (Tower 2017). Readers are referred to a review by John Tower for further details on fundamental sex differences that may modify risk of dementia and other diseases (Tower 2017). A number of studies have observed differential enrichment of microglial states between the sexes, which suggests that sex-specific distributions along microglial trajectories might play a role in modifying risk of dementia. For example, Mathys et al. (2019) found that the human NAM

population is preferentially enriched in females. Similarly, Sala Frigerio et al. (2019) found an increased progression of HM to activated response microglia in female AD mice compared with their male counterparts. Here, we suggest potential mechanisms that explain how sex might modify the shift from HM to NAM: *1*) Developmental priming: Neonatal females show increased microglial phagocytosis and expression of NAM genes, such as TREM2 (Nelson et al. 2017). This finding suggests that differential developmental trajectories cause NAM enrichment early in development, which might predispose female microglia to a shift toward NAM in response to upstream pathology. *2*) Sex-epigenetic axis: Differential Lysine Demethylase 5 (KDM5D/C) expression was found in human female versus male microglia (Gosselin et al. 2017), which suggests sex-specific epigenetic priming of microglia. Sex-specific differential priming of HM may regulate transitions to NAM, as epigenetic priming of NAM loci has been observed in precursor HM (Keren-Shaul et al. 2017), *3*) Cell-extrinsic factors: Sex converges on multiple cell-extrinsic factors, including hormones and immune response (Tower 2017), which may modulate transitions along the transcriptomic trajectory at a sex-specific rate. Together, these studies show that multiple sex-associated factors may facilitate the transition from HM to activated response microglia in females, likely in response to aging- and neuropathology- associated triggers.

LEVERAGING TRANSCRIPTOMIC SHIFTS IN CELLULAR THERAPIES FOR AD

Multiple microglial transition states, epigenetic HM priming, and reverse transitions from NAM to HM upon disease resolution (Keren-Shaul et al. 2017; Krasemann et al. 2017), suggest that microglial states are plastic and might be responsive to therapeutic modulation, analogous to immunotherapies in cancer. Immunotherapies have shown strong efficacy in activating a productive anti-tumor immune response in a wide range of cancers by facilitating immune cell recognition of transformed cancer cells (Chen and Mellman 2013). Immunotherapy-like approaches may translate to AD as a therapeutic intervention. For example, activation or inhibition of specific NAM-gating genes, such as APOE or TREM2, may constitute a therapeutic strategy to induce protective microglial responses to triggering pathology and other risk factors. Interestingly, anti-programmed death-1 (anti-PD1) immunotherapy reduced amyloid burden and cognitive decline in mouse models of AD (Baruch et al. 2016). The therapy-induced influx of monocytes observed in the study likely contributed to increased amyloid clearance (Baruch et al. 2016). However, it is interesting to speculate whether acute microglial transition to phagocytic NAM in response to anti-PD1 treatment might also have played a role in decreasing amyloid burden. The interferon response microglial-marker interferon regulatory factor 7 (IRF7) has been shown to induce programmed death ligand-1 (PDL1) expression in cancer cells (Lai et al. 2018). This raises the question whether PDL1 expression is also enriched in interferon response microglia and whether blocking the PD1-PDL1 axis may induce a shift from interferon response microglia to phagocytic activated response microglia to reinforce amyloid clearance. As the study by Baruch et al. (2016) did not specifically address the involvement of microglia in anti-PD1-induced amyloid clearance, this question needs to be investigated. Importantly, since it is still unclear to what extent and under what circumstances NAM play a pathogenic or protective role in AD, further research must address whether acute induction of phagocytic NAM is beneficial in AD.

Understanding the checkpoints that gate HM, interferon response microglia, activated response microglia, and transition states connecting these extremes would be crucial to developing cellular therapies that modulate these trajectories. However, inducing transitions between HM and NAM may not be the only way to expand selected microglial populations. Microglia shift from low levels of random proliferation to selected clonal expansion upon acute neurodegeneration (Tay et al. 2017). This suggests that age- and neurodegeneration-associated damage to the brain may result in progressive recruitment and clonal expansion of NAM cells that already reside in the healthy brain. Thus, therapies that modulate the expansion of resident microglial subtypes may also be effective in altering the distribution of microglial states in AD. Further studies should investigate to what extent microglial transitions are mediated by clonal expansion versus sequential transition between states.

OUTLOOK

Multiple AD risk factors modulate the transcriptomic state of microglia, a cell type integral to brain homeostasis. These findings strongly suggest that microglial shifts along the trajectory play a causal role in AD pathogenesis. We hypothesize that differential distributions along the transcriptomic trajectory accelerate the progression of dementia in familial AD over sporadic AD and pathologic aging, by modulating the cellular response to upstream neuropathological triggers (Fig. 1). Clinically, familial AD, sporadic AD, and pathologic aging are characterized, in part, by extensive amyloid deposition yet are distinguished by variable cognitive decline. By altering microglial distributions, a combination of risk factors may initiate a protective or pathogenic cellular response, which extends or accelerates time-to-dementia, respectively. Therefore, modulating these distributions may constitute a novel therapeutic approach to extend time-to-dementia. Importantly, cellular therapies may be effective at later stages of the disease by circumventing the difficult task of blocking the accumulation of triggering pathology over decades. However, the functional implications of different microglial states for AD pathology and associated therapeutic approaches still remain unclear: *1*) Do NAM initially play a protective role in response to amyloid pathology and aging? *2*) Does induction of NAM drive dementia over time via chronic neuroinflammation and phagocytosis? *3*) What role do NAM subpopulations (e.g., interferon response or activated response microglia) play in protecting against or exacerbating the pathogenic cascades of AD? *4*) Are certain NAM subpopulations driving contributors to dementia, while others are protective? *5*) Should cellular therapies induce a balance of heterogeneous NAM populations or selectively induce one state over another? Scientists will need to address these and other questions of causality in an attempt to develop effective cellular therapies for AD.

Although the understanding of microglial involvement in AD is still in its infancy, recent single-cell RNA-sequencing studies show that select cellular markers insufficiently capture diverse microglial states and their multifaceted involvement in AD. How-

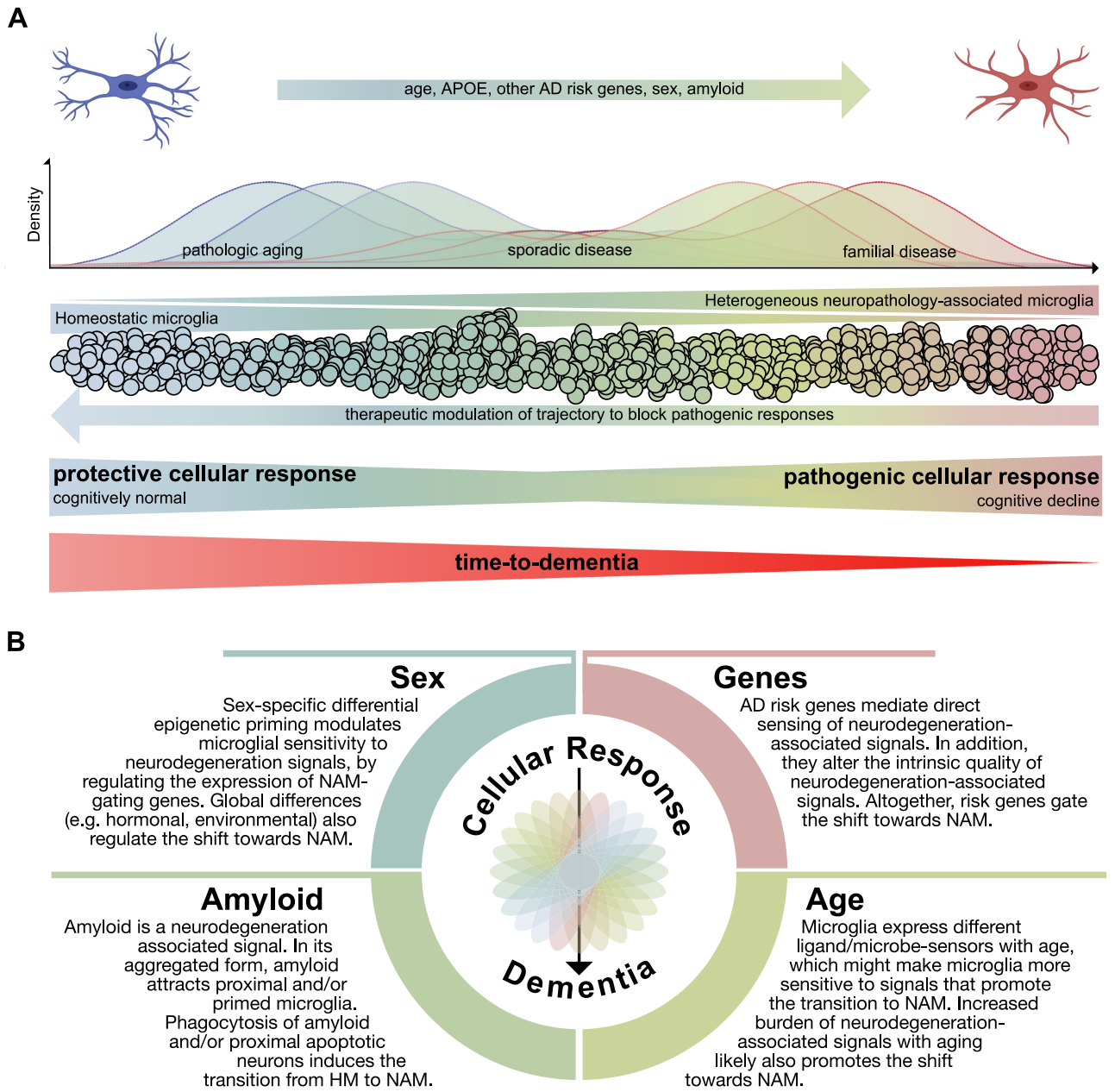

Fig. 1. A hypothetical model of microglial distributions along the homeostatic microglial (HM)-neuropathology-associated microglial (NAM) transcriptomic trajectory and risk of dementia. *A*: differential distributions of microglia along the transcriptomic trajectory may accelerate the progression of dementia in familial over sporadic Alzheimer's disease (AD) and pathologic aging. While acute induction of NAM (represented by the distribution centered on the far left of the spectrum) may be beneficial or protective against upstream triggers, such as amyloid, overwhelming and sustained presence of NAM (represented by the distribution centered on the far right of the spectrum) may promote AD pathogenesis. However, it is also conceivable that the distribution is reversed. Induction of NAM may support a protective cellular response, while failure to sufficiently induce NAM, for example due to hypomorphic variants in AD risk genes, may have pathogenic effects. *B*: age, AD risk genes, sex, and amyloid may confer increased risk of dementia by shifting the microglial distribution toward an overwhelmingly pathogenic response, which accelerates time-to-dementia. However, the induced shift toward NAM by age, sex, and amyloid may equally represent a protective response to these risk factors.

ever, single-cell transcriptomics still poses multiple challenges: *1*) It remains extremely difficult to identify and consolidate consensus populations across studies based on the limited number of biased markers that are reported to characterize individual states. Ideally, single-cell data from multiple studies would be pooled in a separate multivariate analysis to identify consensus states among studies. *2*) Although numerous studies have found overlap between microglial states in human brains and mouse models (Galatro et al. 2017; Mathys et al. 2019), microglial sensitivity to genes, aging, and pathology predicts substantial differences between transcriptomic trajectories identified from human and mouse microglia. These challenges must be addressed to translate the findings of multiple single-cell studies into viable cellular therapies for AD.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.v.M. and M.J. conceived and designed research; D.v.M. and M.J. prepared figures; D.v.M. and M.J. drafted manuscript; D.v.M. and M.J. edited and revised manuscript; D.v.M. and M.J. approved final version of manuscript.

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