# White matter aging drives microglial diversity

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## SUMMARY

**Aging results in both grey and white matter degeneration, but the specific microglial responses are unknown. Using single-cell RNA sequencing from white and grey matter separately, we identified white matter associated microglia (WAM), which share parts of the disease-associated microglia (DAM) gene signature and are characterized by the activation of genes implicated in phagocytic activity and lipid metabolism. WAM depend on triggering receptor expressed on myeloid cells 2 (TREM2) signaling and are aging dependent. In the aged brain, WAM form independently of apolipoprotein E (APOE), which is in contrast to mouse models of Alzheimer's disease, in which microglia with WAM gene signature are generated prematurely and in an APOE-dependent pathway similar to DAM. Within the white matter, microglia frequently cluster in nodules, where they are engaged in clearing degenerated myelin. Thus, WAM may represent a potentially protective response required to clear degenerated myelin accumulating during white matter aging and disease.**

## INTRODUCTION

White matter is composed of mostly myelinated axons that connect neurons from different brain regions into functional circuits. The light appearance of the white matter results from the high lipid content of myelin, a multilamellar membrane structure that coats axons and constitutes almost half of the dry weight of the white matter (Stadelmann et al., 2019). Long thought to be passive part of the brain, it is now clear that dynamic, experience-dependent generation of white matter myelin content by oligodendrocytes affects learning and brain functions (McKenzie et al., 2014; Mount and Monje, 2017; Pan et al., 2020; Wang et al., 2020; Steadman et al., 2020). In humans, white matter volume reaches its peak at around 40-50 years, after which it

continuously declines (Sowell et al., 2003). White matter aging is not only associated with tissue shrinkage, but frequently also with focal lesions seen on magnetic resonance imaging (MRI) as hyperintensities, that are related with cognitive impairment, and increased risk of stroke and dementia (Prins and Scheltens, 2015). Electron microscopical studies performed in non-human primates have revealed that major pathological alterations that occur during aging are found in the white and not the grey matter (Peters, 2002), consisting of myelin outfolding, splitting and accumulation of multilamellar fragments (Peters, 2002; Safaiyan et al., 2016). A further understanding of such age-related white matter pathology and its associated cellular responses is essential, as aging is a major risk factor for the most prevalent neurodegenerative diseases. One cell population that respond to aging are microglia, which are long-lived, self-renewing cells with phagocytic scavenging and immune surveillance functions (Grabert et al., 2016; Fuger et al., 2017; Salter and Stevens, 2017; Prinz et al., 2019). Gene expression studies have shown that aged microglia develop a more inflammatory phenotype, particularly in the white matter (Poliani et al., 2015; Safaiyan et al., 2016). Age-dependent increase of inflammatory status of microglia is often referred to as primed or sensitized, but the nature of this response is only partially understood (Perry and Holmes, 2014). Several groups have applied single-cell genomics approaches to characterize the shift in microglial states after various pathological insults (Keren-Shaul et al., 2017; Mathys et al., 2017; Friedman et al., 2018; Hammond et al., 2019). This work has established that microglia undergo a relatively stereotypical conversion into disease-associated microglia (DAM) with a microglia-neurodegenerative (MGnD) or activated response microglia phenotype (ARM) (Keren-Shaul et al., 2017; Krasemann et al., 2017; Sala Frigerio et al., 2019). This conversion is dependent on TREM2 (triggering receptor expressed on myeloid cells 2) activity, which increases phagocytosis and lipid metabolism. Such DAM have the potential to restrict pathology by enhancing the clearance of misfolded and aggregated proteins that commonly accumulate in neurodegenerative diseases, but to what extent DAM/MGnD/ARM are also generated during

normal aging is under debate (Keren-Shaul et al., 2017; Friedman et al., 2018; Hammond et al., 2019; Sala Frigerio et al., 2019). As aging-induced damage to the brain involves the degeneration of myelinated nerve fibers, not characterized by proteins aggregates, but by the release of lipid-rich, tightly compacted, and therefore difficult to digest myelin debris, we hypothesized that microglial responses should differ between the aged grey and white matter. To characterize this microglial response, we combined genetic perturbation, single-cell RNA sequencing (scRNA-seq), immunohistochemistry and functional assays to comprehensively characterize microglial responses that occur in the aged white matter.

## RESULTS

### TREM2- and age-dependent formation of white-matter associated microglia

To characterize this microglial response, we took advantage of single-cell RNA sequencing (scRNA-seq), which is a powerful and sensitive technique to reveal transcriptomic cell-to-cell variation of microglia in the normal and diseased brain (Hammond et al., 2019; Keren-Shaul et al., 2017; Krasemann et al., 2017; Masuda et al., 2019; Mathys et al., 2019; Van Hove et al., 2019; Chen et al., 2020). However, transcriptional responses to dissociation have also been shown to alter microglial scRNA-seq analysis (Ayata et al., 2018; Haimon et al., 2018; Hammond et al., 2019; Li et al., 2019). To avoid isolation artifacts, we established an automated dissociation protocol for microglia that inhibits *ex-vivo* transcription by the addition of actinomycin D (ActD) (Wu et al., 2017). Using scRNA-seq, we tested the effect of the ActD addition, which was able to prevent significant inductions of four genes (*Jun, Lars2*, *Gm23935*, *CT010467.1*) and of the average expression of immediate early genes, known to respond to brain dissociation (Wu et al., 2017) (Figure S1A-C; STAR Methods). Using this optimized protocol, we dissociated grey matter from the frontal cortex and white matter tracts from the corpus callosum as well as the optical tracts and the medial lemniscus, from wild type aged mice (18-20 months old; Figure 1A). To compare aging and neurodegeneration effects on microglia, we also isolated microglia from a transgenic Alzheimer's disease (AD) mouse model that expresses five human familial AD gene mutations (5xFAD) (Oakley et al., 2006). Since the activation of DAM gene expression profile depends on TREM2 signaling (Keren-Shaul et al., 2017), we included aged *Trem2* knockout mice (*Trem2*$^{-/-}$) into the analysis. We performed Smart-seq2 (SS2), which has a high transcript capture rate that produces biologically meaningful clusters even for a small number of cells (Picelli et al., 2014; Gokce et al., 2016; Li et al., 2019). After eliminating low-quality SS2 libraries, we included 1038 microglia from 16 mice. Analysis of sorting data showed that aged wild-type white matter immune cells had significant increased levels of $CD45^+$ and $CD11b^+$ labeling compared to grey matter, which was

not observed in aged *Trem2*$^{-/-}$ animals (Figure S1D-F). Based on known immune cell markers, we identified 847 high-quality SS2 scRNA-seq microglia from aged mice and also cells expressing markers for granulocytes, perivascular macrophages and oligodendrocytes, which were excluded from downstream analyses (Figure S1I-J). Next, we analyzed the transcriptomes of single-cells using unsupervised Uniform Manifold Approximation and Projection (UMAP) analysis, a dimension reduction method, which separated aged white matter from aged grey matter microglia (Figure S1K). This white matter microglia specific cluster was defined by a specific gene signature (see methods, Figure S1K and Table S2). Using this white matter microglia gene signature, we distinguished four distinct populations: two white matter specific cluster, which we called White-matter Associated Microglia (WAM) and activated microglia; and two additional clusters, found in both grey and white matter, which we named homeostatic microglia 1 and 2 (Figure 1B-E; Figure S2A; Table S2). Comparing wild-type with *Trem2*$^{-/-}$ mice revealed that both WAM and activated microglia are TREM2-dependent (Figure 1E; Figure S2A). WAM are characterized by the down-regulation of homeostatic genes (set 4 genes, Figure 1C; Figure S3A), such as the purinergic receptors (*P2ry12* and *P2ry13*) and checkpoint genes (*Csfr1r, Cx3cr1, Hexb, Tmem119*) and by the up-regulation of DAM associated genes (set 1 genes, Figure 1C; Figure S2A) such as lipid metabolism and phagosome related genes (*ApoE, Cst7, Bm2, Lyz2*, *Cd63*, *Clec7a*; cathepsins: *Ctsb, Ctss, Ctsz*, and the MHC class II-related genes: *H2-D1*, *H2-K1*). Activated microglia are marked by the up-regulation of genes encoding many metabolic genes, mostly ribosomal subunits and mitochondrial genes (set 2 genes), many of which are part of the microglial responses to aging (Ximerakis et al., 2019) (set 2 genes, Figure 1C and D; Figure S2A). Homeostatic microglia 1 and 2 were detected in both white and grey matter, and differed from WAM by the higher expression of homeostatic genes such as *Tmem119*, *Csf1r, Cx3cr1, Hexb, Tmem119* (set 4 genes, Figure 1C; Figure S2A) and lower expression of genes linked to the DAM signature such as *ApoE, Cst7, Bm2, Lyz2, Cd63, Clec7a, Ctsb, Ctss, Ctsz, H2-D1, H2-K1* (set 1 genes, Figure 1C; Figure S2A).

To validate our results above, we performed Drop-seq on 24 months old mice (21,197 high-quality cells from 17 mice used in five Drop-seq runs: Figure 2A-B; Figure S2B and C; Table S1-Excel-tab “Current Study- 10X”). We partitioned cells into major cell types (Figure 2B), revealing the white and grey matter cellular landscapes (Figure 2C). The ciliated and secretary ependymal cells' distributions between white and grey matter validate our microdissection, as these cells are located in the corpus callosum's surface. Using the white matter signature genes, we again identified a continuous range of microglia that reproduced the four microglia clusters of the SS2 scRNA-seq dataset (Figure 2D). The gene expression profiles of all four microglia clusters were very similar in both scRNA-seq datasets. None of the 5,991 microglia from frontal cortex, was identified as WAM, validating the allocation of WAM to the aged white matter (Figure 2E-G). The activated microglia population was enriched in the white matter, but was also detected in the grey matter. The difference in activated microglia distribution in SS2 and Drop-seq datasets might be due to different age-points analyzed (18-20 vs 24 months old) or the higher cell number in the Drop-seq dataset (Figure 2G). Using the Drop-seq dataset, we compared the activated microglia to WAM (Figure 2H and I) and identified 428 transcripts that were differentially expressed (Figure 2H). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the upregulated genes showed that ribosomes were the top enriched pathway in activated microglia. The most enriched pathway in WAM were hypoxia-inducible factor (HIF-1) signaling, lysosomal and cholesterol pathways (Figure 2I).

In order to address whether the WAM signature is aging dependent, we isolated microglia and other myeloid cells from the corpus callosum (white matter) and the frontal cortex (grey matter) of 4- and 21-month-old wild-type mice using CD11b microbeads and performed bulk RNA-seq analysis. Pathway analysis showed that genes involved in immune cell functions were among the most up-regulated pathways in the aged white matter (Figure S2E). Microglia in the aging grey matter did not show these changes in immune cell function, but instead exhibited alterations in pathways involved in ion channel activity (Figure S2F). When comparing the

genes differentially expressed in aged white matter as compared to young white matter or aged grey matter CD11b$^+$ cells, 39 transcripts were identified, which overlapped with the WAM signature genes such as *Lgals3*, *Spp1*, *Cst7*, *Lpl*, *Clec7a*, *Itgax* and *ApoE* (Figure S2G and H; Table S3), suggesting that WAM marker genes increase by aging in the white matter. Since recent scRNA-seq studies have analyzed microglia during normal brain aging without reporting WAM, we re-analyzed these existing datasets (Hammond et al., 2019; Sala Frigerio et al., 2019). We first analyzed the microglia scRNA-seq by Frigerio *et al.* 2019, which used cold dissociation to avoid *ex-vivo* induced transcriptional activity artifacts with SS2 protocol. We identified microglia with nearly identical gene expression pattern to WAM (Figure 3A). Already at 3 months age, microglia with WAM expression patterns can be identified, and the number of these microglia with WAM gene signature increase with aging (Figure 3B; Figure S3A). Furthermore, when we integrated our dataset with Frigerio *et al.* 2019, we found that the microglial states cluster together (Figure S3B). Similarly, we identified microglia with WAM gene signature in Drop-seq dataset by Hammond *et al.* 2019*,* which also used cold dissociated animals to collect cells (Figure 3A-D; STAR Methods). In both datasets, we observed an increase in the ratio of microglia with WAM gene signature during aging (Figure 3B). These microglia with WAM gene signature were characterized by the down-regulation of homeostatic genes (set 4 genes; *P2ry12*), and the up-regulation of DAM associated genes (set 1 genes; *Cd63*). In all three datasets, the activated microglia were marked by an up-regulation of translation related gene set 2 (*Rpl37, Rpl41*) (Figure 3A, C and D). Overall, WAM appear to be present in different datasets with very similar expression profile.

**White-matter associated microglia cluster in nodules in a TREM2-dependent pathway**

To determine the localization of reactive microglia in the brain, we co-stained IBA1$^+$ microglia with activated microglia and WAM markers (Figure S3C) using antibodies against CRYBA4, CLEC7A, AXL and LGALS3 (Galectin-3), and RNA *in situ* probes against *Itgax* (Figure 4 and

Figure S4A and B). Consistent with the scRNA-seq data, we found that antibodies against the activated microglia marker, CRYBA4 (Figure S4B), marked IBA1$^+$ cells in both young (2 months) and old (24 months) white matter tracts (~15-20% of IBA$^+$ cells; Figure S4C). This was in contrast to WAM markers, CLEC7A, AXL, LGALS3 (Galectin-3), and *Itgax*, which labeled IBA1$^+$ cells in the old, but not in the young white matter (Fig. 4A; Figure S4A). Quantification revealed that ~10-30% of the IBA1$^+$ cells within the corpus callosum were positive for the respective WAM markers in 24-month-old mice (20.75%±9.67 CLEC7A$^+$IBA1$^+$ cells/area, 14.44%±5.05 AXL$^+$IBA1$^+$ cells/area, 31.64%±11.68 LGALS3$^+$IBA1$^+$ cells/area, and 9.64%±6.46 *Itgax*$^+$IBA1$^+$ cells/area) (Figure 4A). Notably, and in agreement with our RNA-seq data, we found that these cells were almost absent from cortical areas of the aged brain (Figure 4B). Analysis of the localization of double positive microglia in the white matter showed that they were not evenly distributed, but often found in clusters consisting of 3 to 5 cells with large cell bodies and thick processes (Figure 4C; Figure S4E). Microglia clusters were only found in the white matter, where they increased with age (Figure 4C). Such clusters of activated microglia have previously been termed microglial 'nodules', and are found in a number of different brain diseases including brain trauma, multiple sclerosis and viral encephalitis (Rock et al., 2004). To determine the ultrastructure of these nodules, we performed correlated light and electron microscopy using an antibody against IBA1. We found myelin debris inside microglia nodules and degenerated myelin in the direct vicinity of the cells, suggesting that microglia nodules are engaged in myelin phagocytosis (Figure 4D; Figure S4F). We performed immunohistochemistry using antibodies against myelin basic protein (MBP), the main structural protein of myelin, to quantify the amount of microglia containing MBP and found that almost half of the IBA1$^+$ cells with internalized MBP$^+$ particles localized to nodules (Figure 4E; Figure S4G). Microglia within nodules were positive for WAM markers, and we did not detect any activated microglia defined as CRYBA4$^+$/Galectin3$^-$/IBA1$^+$ cells in nodules (Figure S4D). These data suggest that WAM are actively digesting myelin debris in the aging

white matter, possibly deriving from degenerated myelin sheaths that accumulate over time during aging. If this is the case, triggering myelin degeneration should induce the clustering of microglia into nodules prematurely. To test this predicition, we used a mouse model for Pelizaeus-Merzbacher disease, a leukodystrophy with extra copies of the proteolipid protein gene (PMD mice) (Readhead et al., 1994). These mice develop initially relatively normal myelin, but within weeks, myelin sheaths are gradually broken-down (Readhead et al., 1994). We co-stained $IBA1^+$ microglia using antibodies against CLEC7A, AXL and LGALS3 together with antibodies against MBP to detect myelin particles within microglia. Strikingly, already at 2 months of age, when the demyelinating phenotype of these mice starts, microglia nodules appeared in the corpus callosum (Figure S5A). These $IBA1^+$ cells in nodules containted $MBP^+$ intracellular particles and increased in number with time (Figure S5B-E). Such nodules were not observed in cortical areas. Quantification revealed that ~9-40% of the $IBA1^+$ cells within the corpus callosum were positive for CLEC7A, AXL or LGALS3 already in 2-month-old PMD mice. Similar results were obtained in 7- and 10-month-old PMD mice (Figure S5F-H).

Together, these data provide evidence that WAM within nodules are engaged in clearing degenerated myelin in the aging or diseased white matter. As our scRNA-seq data indicated a function for TREM2 in this process, we used antibodies and *in situ* probes to visualize microglia in *Trem2*$^{-/-}$ mice. Consistent with our scRNA-seq data, we failed to detect CLEC7A, AXL, *Itgax*, or LGALS3 (Galectin-3) in the corpus callosum of aged *Trem2*$^{-/-}$ mice (Figure 5A). Next, we determined the number of homeostatic microglia using antibodies against TMEM119 and P2RY12, and found that the number of $TMEM119^+IBA1^+$ or $P2RY12^+IBA1^+$ decreased with age in the white matter of wild-type mice (Figure 5B-D; Figure S4H). This was in contrast to *Trem2*$^{-/-}$ mice, in which the numbers remained unchanged in 6, 12 and 18 months old mice (Figure 5B-D; Figure S4H). In addition, and consistent with previous results (Poliani et al., 2015; Kleinberger et al., 2017), we found that the increase in the number of microglia that occurs during normal aging in wild-type white matter was abolished in *Trem2*$^{-/-}$ mice (Figure

S4I). Whereas nodules increased during aging in the white matter of wild-type mice, they were almost undetectable in *Trem2*$^{-/-}$ mice at all the time points analyzed (Figure 5E). We also examined microglia in the cortex of *Trem2*$^{-/-}$ and wild-type mice, but were unable to detect changes in cell density (Figure S4I) or in the proportion of homeostatic IBA1$^{+}$ cells (Figure 5B-D; Figure S4H), further suggesting that age-related and DAM-associated changes are mainly confined to the white matter of the brain.

**White-matter associated microglia are engaged in clearing myelin debris**

As the WAM response and the generation of nodules is abolished in *Trem2*$^{-/-}$ mice, this mouse model provides a means to investigate the biological function of WAM. We used electron microscopy to visualize possible differences in the ultrastucture of aged white matter in wild-type and *Trem2*$^{-/-}$ mice. We found an enhanced accumulation of myelin whorls (not associated to axons) in the corpus callosum of aged *Trem2*$^{-/-}$ as compared to wild-type control mice (Figure 5F). In addition, an increased number of cells with electron dense intracellular inclusions were detected in *Trem2*$^{-/-}$ mice, and by correlated light-electron microscopy, we found that these cells were microglia (Figure 5G). By light microscopy MBP$^{+}$, PLP$^{+}$ and fluoromyelin$^{+}$ intracellular particles were noticed within microglia of aged *Trem2*$^{-/-}$ in the white matter to the same extent as in wild-type mice (Figure S4J-L). Furthermore, using light microscopy, microglia with irregular processes were detected, as previously described in the context of human brain aging and particularly in neurodegenerative human diseases (Streit et al., 2004). By double immunostainings using antibodies against IBA1 and CD68 to visualize microglia process morphology (Tischer et al., 2016), we found a dramatic increase in the number of microglia with irregular processes in the corpus callosum of 18-month-old *Trem2*$^{-/-}$ compared to wild-type mice. Strikingly, microglia with irregular processes appeared to be restricted to white matter regions as microglia were of ramified morphology within cortical areas (Figure 5H). Thus,

TREM2-dependent WAM activity may represent a protective response against damaged myelin, possibly to enhance its uptake and breakdown during white matter aging.

To directly assess whether TREM2 is required for the uptake and/or breakdown of myelin debris, we performed *in vitro* experiments with cultured primary microglia. $CD11b^+$ cells were prepared from wild-type and *Trem2*$^{-/-}$ mice by magnetic-activated cell sorting (MACS), and myelin debris binding, uptake and degradation were examined. First, we explored the interaction of myelin debris to the cell surface, by performing cell surface binding assays at 4°C, which revealed that similar amounts of myelin debris attached to the surface of wild-type and *Trem2*$^{-/-}$ microglia (Figure 6A). In addition, phagocytic uptake of myelin debris occurred to the same extent in wild-type and *Trem2*$^{-/-}$ microglia (Figure 6B). However, differences were detected when cells were chased for 24 hours and immunostained for PLP to determine the degradation of the ingested myelin (Figure 6B). These experiments demonstrated that myelin debris degradation occurs less efficiently in *Trem2*$^{-/-}$ microglia as compared to wild-type, similar as it occurs when microglia are treated with inhibitors of lysosomal functions (leupeptin or bafilomycin A; Figure S6A). We used RT-qPCR to examine whether the impaired degradation, was due to the inability to induce the expression of lysosomal enzymes, and found that the expression of *cathepsinL*, *beta-galactosidase1*, *N-acetylglucosamine-6-sulfatase*, but not *hexoaminidase* were upregulated upon myelin debris uptake in wild-type, but not in *Trem2*$^{-/-}$ microglia (Figure 6C). The failure of *Trem2*$^{-/-}$ microglia to upregulate Cathepsin L upon myelin debris treatment was confirmed on protein level by Western blotting (Figure 6D). To determine whether, myelin debris degradation was also impaired *in vivo* in *Trem2*$^{-/-}$ mice, we employed a toxin-induced model, in which a single injection of lysolecithin is injected to the corpus callosum to induce a focal demyelinating lesion in the white matter. Removal of damaged myelin debris occurs mainly by microglia, as only relatively few monocyte-derived macrophages enter lesions from the periphery (Lloyd et al., 2019; Plemel et al., 2020). We used

the fluorescent myelin staining (Fluoromyelin) to determine the amount of myelin debris within LAMP1$^+$ degradative compartments in IBA1$^+$ cells, and found that the clearance of internalized myelin debris was delayed in *Trem2*$^{-/-}$ mice (Figure 6E).

Next, we established a cell culture-based uptake assay to systematically explore the requirements for myelin debris phagocytosis. We used HeLa cells for this assay, as only small amounts of myelin debris are taken up by these cells. This allowed us to transiently express a number of different phagocytic receptors and to determine their role in mediating myelin debris uptake (Figure S6B and C). First, we confirmed that the expression of TREM2 together with TYROBP (TYRO protein tyrosine kinase-binding protein) induced phagocytosis of myelin debris in HeLa cells (Figure S6D). However, this assay also revealed that a number of different phagocytic receptors were able to mediate myelin debris phagocytosis (Figure S6D-F). Notably, all receptors known to depend on direct phosphatidylserine binding or indirect binding using bridging molecules, promoted myelin debris uptake. Some of the receptors such as *Axl* are part of the WAM response, while others such as *Mertk* are highly expressed in homeostatic microglia. To confirm that phosphatidylserine is required for myelin debris uptake, we masked phosphatidylserine binding sites by pre-incubating myelin debris with Annexin V, which markedly reduced myelin debris phagocytosis in primary cultures of microglia (Figure S6G and H). Thus, the binding of myelin debris to multiple receptors, which are also expressed in homeostatic microglia, suggest that WAM have essential function in myelin debris degradation, rather than in the phagocytotic uptake.

**Microglia with WAM and DAM gene signatures co-exist and are generated in an APOE-dependent pathway in models of Alzheimer's disease**

Next, we explored whether a WAM-like population can be found in mouse models of AD, in which DAM have previously been described (Keren-Shaul et al., 2017; Krasemann et al., 2017). We generated scRNA-seq data with our optimized protocol from the 6-month-old 5xFAD

mouse model of AD. We identified DAM clusters as reported in Keren-Shaul *et al.* 2017, but also microglia with WAM gene signature (Figure 7A and B). We repeated the same clustering approach on a larger microglial scRNA-seq dataset from Frigerio *et al.* (Sala Frigerio et al., 2019), which used two mouse models of AD, an *App* knock-in mouse model ($App^{NL\text{-}G\text{-}F}$) (Masuda et al., 2016; Sasaguri et al., 2017) and transgenic APP/PS1 mice (Borchelt et al., 1997), also in combination with an $Apoe^{-/-}$ strain (APP/PS1- $Apoe^{-/-}$) (Sala Frigerio et al., 2019). Our analysis of microglia from AD models distinguishes two further microglial subtypes, in addition to the four we identified in aged mice. These two additional subtypes match the expression profiles of DAM1 and DAM2 microglia, previously reported in Keren-Shaul *et al.* 2017. In AD models, microglia with WAM gene expression profile appear very early, but with nearly identical signatures compared to the normal aging induced profiles. The DAM1 and DAM2 showed a strong gradual upregulation of set 1-3 and down regulation of the homeostatic genes in set 4 (Figure 1C, 2E and 7B). Next, we visualized the regulation of DAM program in our aged microglia dataset by ploting top 500 genes altered in DAM (Keren-Shaul et al., 2017). Interestingly, we observed that activated microglia and WAM up-regulated distinct parts of the DAM signature (Figure S7A). Overall, WAM appear to represent a microglial state displaying a partial activation of the DAM gene signature, suggesting that the DAM program consists of multiple transcriptional modules. WAM and DAM states are likely to be continuous cell identities that can blend with each other. In aging, the microglia activation appears to be shifted towards the WAM state, while in AD the shift is towards DAM.

Thus, in models of AD, microglia with WAM and DAM gene signatures, co-exist, raising the question of when and in which temporal sequence they form. In 3-month-old $App^{NL\text{-}G\text{-}F}$, we did not detect DAM2, while microglia with WAM gene signature were present (Figure 7C and D). The proportion of cells in both DAM and WAM clusters increased with the age until 12 months, after which DAM showed a decrease between 12 to 21 months.

How do WAM differ from DAM states? To address this question, we took advantage of one main characteristics of WAM, namely their involvement in myelin debris clearance in the white matter. While it is well established that activated microglia are distributed throughout the brain and cluster around amyloid plaques (Ulrich et al., 2017), it is not clear whether microglia nodules containing myelin debris are formed. We used immunohistochemistry to determine whether microglia form nodules in the white matter of mouse models of AD. We co-stained 6-month-old *App*$^{NL\text{-}G\text{-}F}$ with antibodies against IBA1, MBP and amyloid, and detected nodules with IBA1$^{+}$ cells containing MBP$^{+}$ intracellular particles in the corpus callosum, that were not associated with amyloid plaques (Figure S7B). Similar findings were obtained in 5xFAD mice, in which nodules containing IBA1$^{+}$ cells with MBP$^{+}$ intracellular particles were detected in the corpus callosum of 6-month-old mice (Figure S7C and D). A large fraction of IBA1$^{+}$ cells were also positive for CLEC7A and LGALS3 (Figure S7E and F). In 2-month-old 5xFAD mice, only a relatively small number of microglia nodules were detected (Figure S7E and F). When *App*$^{NL\text{-}G\text{-}F}$ were crossed with *Trem2*$^{-/-}$ mice, we found that white matter microglia nodules were completely absent, showing that their formation depends on TREM2 as in normal aging (Figure S7G). These data show that microglia in the white matter of mouse models of AD, show a response that is associated with white matter aging, suggesting that myelin degeneration and debris clearance starts much earlier in presence of Aβ pathology. To distinguish DAM clustering around amyoid plaques from microglia nodules in the white matter, we determined the differentially expressed genes between WAM and DAM2 (Figure 7E). Interestingly, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that DAM2 upregulated genes involved in multiple disease pathways including AD, Parkinson's and Huntington diseases, while WAM upregulated genes linked to atherosclerosis, cytokine signaling and apoptosis (Figure 7F). Next, we searched for genes with different expression levels in WAM compared to DAM (Figure S7H). *ApoE* and *Tyrobp*/*Dap12*, which are required for signaling functions in AD (Wang et al., 2015; Krasemann et al., 2017), were found to be

upregulated in DAM. In addition, the inflammatory cytokine, macrophage migration inhibitory factor (*Mif*), was expressed at higher levels in DAM. *Srgap2*, *Cd33* and *Abca1* are genes that were elevated in WAM, while *Serinc3*, *Lyz2* and *Clec7a* are examples of gene that were expressed at similar levels in both WAM and DAM (Figure S7H). Next, we asked whether we could spatially separate the different microglia states in brain sections of models of AD. We used antibodies against MIF, a gene which was found to be expressed at higher levels in DAM compared to WAM, and found that virtually all microglia associated with amyloid plaques stained positive for MIF, while this was not the case for microglia forming nodules in the white matter (Figure S7I). In contrast, antibodies against CLEC7A immunolabeled both populations of microglia (Figure S7J). Next, we compared the number of CLEC7A and the MIF positive cells in the white matter during aging of wild-type mice. We found hardly any MIF positive cells until 18 months of age, only at 24 months ~4% of $IBA^+$ were immunolabeled for MIF. This was in contrast to CLEC7A, which was expressed in cells in the white matter already earlier and to a much higher extent (20.75%±9.67 $CLEC7A^+IBA1^+$ cells/area at 24 months) (Figure S7K and L).

Next, we determined whether WAM and DAM formation depend on similar signal pathway in the aged brain as compared to AD. As previous work has shown that the APOE-TREM2 pathway triggers DAM conversion in AD models (Krasemann et al., 2017), and because *ApoE* and *Tyrobp*/*Dap12* were found to be upregulated in DAM, we tested the requirement of APOE in aging and AD. In the APP/PS1 AD model from Frigerio *et al.* (Sala Frigerio et al., 2019), we identified an ~8-fold reduction of the DAM populations in the $Apoe^{-/-}$ genotype, whereas microglia with WAM signature were reduced by ~4-fold (Figure 7G and H).

To determine whether WAM are also generated in an APOE-dependent pathway in aged mice, we used droplet-based scRNA-seq on white matter of 18 months old wild-type and $Apoe^{-/-}$ mice (13,954 high-quality cells from 8 mice used in four independent Drop-seq runs: Figure 8A;

Figure S8A-C). Tissues were prepared as described above for the Drop-seq dataset, and cells were partitioned into major cell types (Figure 8A). We focused our analysis on microglia using the white matter signature genes. These analyses identified a continuous range of microglia that reproduced the four microglial states in the previous scRNA-seq datasets (Figure 8B). Furthermore, wild-type and *Apoe*$^{-/-}$ mice white matter had similar ratios of WAM and activated microglia (Figure 8C). This result suggest that WAM formation is not APOE-dependent in aging. To validate scRNA-seq results, we co-stained IBA1$^{+}$ microglia with antibodies against CLEC7A, AXL and LGALS3 in 21-month-old wild-type and *Apoe*$^{-/-}$ mice. Quantification revealed that a similar number of IBA1$^{+}$ cells were positive for CLEC7A, AXL and LGALS3 (Figure 8D). Notably, nodules containing IBA1$^{+}$ cells with MBP$^{+}$ intracellular particles were observed to the same extent in 21 month-old wild-type and *Apoe*$^{-/-}$ mice (Figure 8E). Thus, WAM appear to be generated in an APOE-independent pathway in aged mice, while in models of AD, generation of microglia with DAM and WAM gene signature require APOE function.

## DISCUSSION

In this study, we identified, white-matter associated microglia, WAM, which are age-dependent, require TREM2 for their formation, and are defined by the activation of genes implicated in phagocytic activity and lipid metabolism. WAM localize in nodules that are engaged in clearing myelin debris within the white matter. They display a partial activation of the DAM program, and form in the absence of APOE in normal aging. WAM identification was possible by performing scRNA-seq on microglia purified from white and grey matter separately, and by integrating this spatial information in the gene clustering. Using this WAM signature of differentially expressed genes in white and grey matter microglia, we also identified WAM in previous scRNA-seq datasets (Hammond et al., 2019; Sala Frigerio et al., 2019), providing evidence for the robustness and the reproducibility of our findings.

Why does specifically white matter aging shape microglial identity? While aging is known to result in both grey and white matter damage, it is possible that the extent of myelin sheath degeneration and/or the nature of the lipid-rich membrane are responsible for the TREM2-dependent microglial responses in the white matter. Indeed, when we analyzed microglia in a mouse model for Pelizaeus-Merzbacher disease, in which myelin is gradually broken down due to a mutation in an oligodendrocyte-specific gene, *Plp1*, we observed that microglia nodules containing myelin debris were already formed after a few months in the white, but not the grey matter. Grey matter consists predominantly of neuronal cell bodies and dendrites, but is also composed of myelinated axons. Possibly, the extent of myelin degeneration is not sufficient to trigger WAM responses in the grey matter. However, our data does not exclude that microglia with WAM signature can occur outside of the white matter, for example, in diseases such as AD, where amyloid plaques trigger cell death in the grey matter. WAM and DAM states are likely to be continuous cell identities that can blend with each other in a continuum. In aging, microglia activation appears to be dominated by the WAM state, while it is shifted towards DAM in AD. Proliferative-region-associated microglia subset (PAM) that shares characteristics

with disease-associated microglia have previously been identified in the postnatal white matter at a time point that correlates with oligodendrocyte precursor cell apoptosis (Li et al., 2019). However, in contrast to the cells that we identified in this study, PAM do not require TREM2 for their formation (Li et al., 2019).

TREM2 is a V-type immunoglobulin (Ig) domain-containing receptor that binds to a variety of different ligands such as various apolipoprotein and anionic lipid species (Ulrich et al., 2017). TREM2 can also function as a receptor for myelin debris uptake (Cantoni et al., 2015; Poliani et al., 2015; Wang et al., 2015), and our results concur with these studies, but show that TREM2 is dispensable for myelin phagocytosis. We find that myelin debris uptake occurs by a wide range of phosphatidylserine receptors, that are most likely able to compensate for the loss of TREM2 receptor activity. Thus, our data provide evidence for an essential function of TREM2 not in phagocytic uptake, but rather in mediating the genetic response required to degrade and metabolize lipid-rich myelin debris once internalization has occurred. For other cargo that do not depend on phosphatidylserine interactions such as beads or bacteria, the function of TREM2 in phagocytosis appears to be more critical (Linnartz-Gerlach et al., 2019).

Complete loss of TREM2 function cause polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL or Nasu-Hakola disease), a disease characterized by progressive presenile dementia and bone cysts, and associated with the accumulation of lipomembranous structures in adipose tissues, bone marrow and the brain (Bianchin et al., 2010). An overarching pathogenic mechanisms could be the inability to initiate metabolic pathways required for the degradation of lipid-rich membrane structures in tissue-resident macrophages. Loss of TREM2 in mice results in a much more subtle myelin phenotype, with only a modest increase of degenerated myelin profiles in aged mice. Possibly the short life span of mice is not sufficient to induce a leukodystrophy as seen in humans. Compensatory functions of astrocytes, taking over myelin debris clearance, when microglia become dysfunctional, is another possible explanation. Rare variants in *Trem2* gene have also been shown to increase an

individual's risk of developing AD, which is associated with misfolded and aggregated proteins (Guerreiro et al., 2013; Jonsson et al., 2013). Several studies have shown that TREM2 is required to induce the DAM program in models of AD (Keren-Shaul et al., 2017; Krasemann et al., 2017; Mathys et al., 2017). DAM increase in number with progression of amyloid deposition, and accumulate close to amyloid plaque, where they are involved in the Aβ phagocytosis and plaque compaction (Condello et al., 2015; Wang et al., 2016; Keren-Shaul et al., 2017). The different functional requirements of microglia in aging and in neurodegenerative diseases raise the question of how these microglial populations differ. In aging, we find that the distinct genetic modules of reactive microglia are segregated in subpopulations, with activated microglia displaying up-regulation of translation-associated genes, and WAM exhibiting the activation of lysosomal, phagocytic and lipid metabolism pathways. While WAM represent the predominant microglial subpopulation induced by aging, our bioinformatics analyses reveal that microglia with WAM and DAM signatures coexist in models of AD. In the context of these disease models, microglia with WAM signature appear to be generated earlier than DAM. Interestingly, the proportion of DAM showed a decrease between 12 to 21 months, possibly due to microglial cell death in the DAM populations and repopulation with homeostatic microglia or due to DAM returning back to the homeostatic state. Previously, we developed methods to distinguish discrete versus continuous heterogeneity in scRNA-seq data (Stanley et al., 2020), and WAM and DAM identities are good examples of continuous cell identities without clear separation. Our data show that microglia in the white matter of mouse models of AD form nodules containing $MBP^{+}$ intracellular particles relatively early in the disease, suggesting that myelin degeneration and debris clearance starts prematurely in AD. Strikingly, in the Dominantly Inherited Alzheimer's Network (DIAN), white matter alterations were detected by MRI in individuals with autosomal dominant, fully penetrant mutations for AD up to 20 years before the expected onset of symptoms (Lee et al., 2016). The nature of these

alterations are not fully resolved, but possibly reflect demyelination and axonal damage (Brun and Englund, 1986; Prins and Scheltens, 2015; Nasrabady et al., 2018).

One interesting distinguishing feature of DAM and WAM responses appear to be their differential dependence on APOE. Previous work has shown that the APOE pathway triggers DAM conversion in models of neurodegeneration (Krasemann et al., 2017). Notably, our analyses reveal that in the absence of APOE also less microglia with WAM gene signature are generated in AD mouse models, whereas microglia with features of WAM are detected in similar numbers in the aged white matter of *Apoe*$^{-/-}$ and wild-type mice, suggesting that the activity of APOE depends on the brain environment. How APOE triggers WAM/DAM conversion in AD models but not in aging is an open question. APOE, which is produced in excess in AD could act directly on microglia, possibly by binding to TREM2 and by activating downstream signaling (Atagi et al., 2015; Bailey et al., 2015; Yeh et al., 2016). Because APOE is critical for Aβ deposition and for its subsequent fibrillization into amyloid deposits, amyloid-dependent indirect effects on microglia is another possible explanation for APOE-dependent microglial activation in AD (for review see Chen et al., 2020).

One important future question will be to understand the relationship between activated microglia, WAM and DAM. One possibility is that they are all generated from homeostatic microglia, but is also conceivable that they represent progressive activation states. While such transformations are in general regarded as a protective response (Deczkowska et al., 2018), precocious DAM conversion may also cause harm by exhausting DAM function or by inducing pro-inflammatory damage. Recently, lipid droplet-accumulating microglia have been identified in the aging brain (Marschallinger et al., 2020). These cells were defective in phagocytosis, produced high levels of reactive oxygen species and pro-inflammatory cytokines, representing a dysfunctional or dystrophic microglial state.

In summary, we identify WAM as a novel microglial state associated with white matter aging. We propose that WAM represent a protective metabolic response, required to clear the increasing amounts of myelin debris that accumulate during aging. Because WAM signature is also associated with phagosome as well as antigen processing and presentation, harmful functions such as immune reactivity cannot be excluded. In addition, we speculate that attrition of WAM function could contribute to the development of neurodegenerative disease by the accumulation of toxic protein or lipid species within the brain. If this is the case, enhancing the formation of WAM could be used therapeutically, for example to help to combat age associated decline of white matter function, and possibly also dementia resulting from white matter involvement. Transcriptional signatures differ between mouse and human microglia (Masuda et al., 2019; Zhou et al., 2020), but interesting differences between human grey and white matter microglia have been detected (Sankowski et al., 2019), raising the possibility that WAM also exist in human.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

M.S., O.G. conceived and supervised the project. S.S., S.B.-G., T.K., M.Sch., L.L., N.K., O.G., H.J., F.U., LC, MJR, RP, GG, MB, DF, XX performed experiments and analyzed the data, S.B.-G., T.K. developed software, curate and visualize the scRNA-seq data, O.G., M.R., M.S. analyzed the data or supervised data acquisition, M.S., O.G. wrote the manuscript with input from all authors.

## COMPETING INTERESTS

The authors declare no competing interests.

## MAIN FIGURE TITLES AND LEGENDS

### Figure 1

**White matter associated microglia (WAM) are specific to white matter and exhibit a differential gene expression signature.**

(A) Experimental design from dissection to cell sorting for the Smart-seq2 pipeline (Methods).

(B) UMAP of microglia, colored by populations after identification of the white matter activated microglia signature. Smart-seq2 dataset, GM and WM, WT and *Trem2*$^{-/-}$, 18-20 months old (Table S1).

(C) Heatmap of the average expression of the differentially expressed genes comparing the 4 populations of microglia (WAM signature). Values are normalized per gene, showing the gene expression across the populations. The gene sets (1 to 4) were identified by hierarchical clustering of the differentially expressed genes of each population (Methods). Gene ontology analysis pathways are below each set of genes. A single-cell version of this heatmap is available in Figure S2A. List of differentially expressed genes (WAM signature) is in Table S2.

(D) UMAP of microglia in the WAM signature genes (Table S2). Gene expression is the scaled value.

(E) Bar plot showing the relative distribution of each microglia population in wild-type grey matter, *Trem2*$^{-/-}$ grey matter, wild-type white matter, *Trem2*$^{-/-}$ white matter.

### Figure 2

**Drop-seq validates that WAM are specific to white matter.**

(A) Experimental design from dissection to cell loading for Drop-seq pipeline (Methods).

(B) UMAP of 21,197 single-cell transcriptomes coloured by major cell-type clusters.

(C) Grey and white matter proportions in major cell type clusters.

(D) UMAP of microglia, colored by populations after identification of the white matter microglia signature. Drop-seq dataset, GM and WM, 24 months old (Table S1).

(E) Heatmap of the average expression of the differentially expressed genes comparing the 4 populations of microglia (WAM Signature). Values are normalized per gene, showing the gene expression across the populations. Gene ontology analysis pathways are below each set of genes.

(F) UMAP colored by tissue annotation and expression of selected marker genes. Gene expression is the scaled value.

(G) Bar plot showing the relative distribution of each microglia population in wild-type grey matter and wild-type white matter.

(H) Activated and WAM populations analysed separately. Heatmap with top 40 differentially expressed genes for Activated and WAM populations. Each row is a gene and each column is a single cell.
(I) KEGG pathway enrichment for Activated and WAM up-regulated DEGs.

**Figure 3**

**WAM signature is consistent across multiple datasets.**

(A) Heatmaps of the average expression of WAM signature gene sets (Set 1-2-3-4) for each population, aligned to the four populations identified in data from Hammond *et al.* and Frigerio *et al.*. Values are normalised per gene. Note that the first heatmap is identical to Figure 1C and is reported here for easier comparison. All age groups are pooled within the respective datasets (Table S1).

(B) Cell population ratios by age groups. 1-18 months old wild-type mice data and 3-6-12-21 months old wild-type mice data, from Hammond *et al.* and Frigerio *et al.*, respectively.

(C) UMAPs showing the 4 populations identified and the average scaled expression of each gene set per cell. The first row is data from Hammond *et al.*, young and old mice data combined, 9558 microglia. The second row is data from Frigerio *et al.*, all age groups combined, 5093 microglia (Table S1).

(D) PCAs based on the WAM signature genes. Overlay with Slingshot plots and colored by microglia populations (left), Homestatic marker *P2ry12*, Activated markers *Rpl37* and *Rpl41*

(center), WAM markers *Fth1* and *Cd63* (right). Rows 1 to 3 correspond to data from this study, Hammond *et al.* and Frigerio *et al.*, respectively. Gene expression is the scaled value.

**Figure 4**

**WAM are localized in microglia nodules and contain myelin fragments.**

(A) Confocal images of CLEC7A, AXL, Galectin3 and *Itgax* (red) co-localization with IBA1$^+$ microglia (green) in 24-month-old mice using immunohistochemistry and RNAScope *in situ* hybridization. Scale bars, 100µm (overview), 20µm (zoom in). Quantification of CLEC7A, AXL, Galectin3 and *Itgax* expression in 2, 6, 12, 18 and 24 months old mice in white matter (corpus callosum; n= 4-5 mice per group, mean +/- s.d., one-way ANOVA followed by Bonferroni's *post hoc* test, AXL: ****$P$<0.0001, Galectin3: ****$P$<0.0001, CLEC7A: **$P$=0.0013, *Itgax*: **$P$=0.0039).

(B) Bar plots show the fraction of IBA1$^+$ microglia found as single cells or in nodules co-localizing with Galectin3, CLEC7A, AXL and *Itgax* in white matter (corpus callosum) and grey matter (Cortical layers I-V) of 24-month-old wild-type mice (n=5 mice per group, each fraction represents the mean value of 5 mice).

(C) Confocal image of IBA1$^+$ microglia nodules (green) co-labeled with PU.1 (red) in the corpus callosum and cortex of 24-month-old wild-type mice. Scale bars, 50µm (overview), 15µm (zoom in). Quantification of number of microglia nodules in 6, 12, 18 and 24-month-old wild-type male mice (n= 4-5 mice per group, mean +/- s.d., one-way ANOVA , ****$P$<0.0001).

(D) Correlative light and electron microscopy of microglia in corpus callosum. Upper left: confocal image of a whole section as a guide map for correlation. Lower left: SEM serial section (100×100×100nm) overlaid with confocal image (transparent, IBA1 in green, DAPI in blue). Two microglia nodules are relocated on this SEM serial section (Yellow boxes A, B). A and B: Confocal and SEM image of microglia nodules. Right column: higher resolution images (20×20×100nm) shows degenerated myelin around microglia nodules (orange arrows) and intracellular myelin fragments (blue arrows). Scale bars, 100µm (overview, IHC), 100µm (CLEM overlay), and 10µm (high magnification IHC and SEM).

(E) Confocal image of co-localization of MBP (green) with IBA1$^+$ microglia (red) in nodules in the corpus callosum of a 24-month-old wild-type mouse. Clipped 3D images show MBP$^+$

myelin particles (green) within microglia nodule from different angles. Scale bars, 10µm. Bar plot showing the faction of IBA1$^+$ single cells and IBA1$^+$ cells in nodules with internalized myelin fragments in 2 and 24 months old wild-type male mice (n=5 mice per group, each fraction represents the mean value of 5 mice, 2 brain sections were analyzed per mouse).

**Figure 5**

**TREM2 is required for WAM states and myelin debris clearance.**

(A) Quantification of IBA1$^+$ cells also positive for AXL, CLEC7A and Galectin3 in the corpus callosum of 18-month-old *Trem2*$^{-/-}$ and control mice (n=4 mice per group, mean +/- s.d., AXL: ***P*=0.009, CLEC7A: ***P*=0.0022, Galectin3: ***P*=0.0013, Student's two-tailed *t* test).

(B) Confocal images showing expression of P2RY12 (red) in IBA1 (green) in 18-month-old *Trem2*$^{-/-}$ and age-matched control mice. The white arrows point to P2RY12$^-$ microglia and the yellow arrows point to P2RY12$^+$ microglia. Scale bars, 50µm (overview), 20µm (zoom in).

(C) Quantification of IBA1$^+$ microglia expressing P2RY12 in white and grey matter in 6, 12 and 18-month-old *Trem2*$^{-/-}$ and age-matched control mice (n=4 mice per group, mean +/- s.d., one-way ANOVA, control, white matter: **P*=0.0299; *Trem2*$^{-/-}$, white matter: *P*=0.1491; 18 months: control vs *Trem2*$^{-/-}$, ****P*=0.0003, Student's two-tailed *t* test).

(D) Quantification of IBA1$^+$ microglia expressing TMEM119 in white and grey matter in 6, 12 and 18-month-old *Trem2*$^{-/-}$ and control mice (n=4 mice, mean +/- s.d., one way ANOVA, control, white matter:, ****P*=0.0002; *Trem2*$^{-/-}$, white matter: *P*=0.1149; 18 months, control vs *Trem2*$^{-/-}$, ***P*=0.0058, Student's two-tailed *t* test).

(E) Confocal image showing abundant microglia nodules in the corpus callosum of 18-month-old control compared to age-matched *Trem2*-/- mice. Scale bars, 50µm (overview), 20µm (zoom in). Quantification of number of microglia nodules in 6-, 12- and 18-month-old *Trem2*-/- and control male mice (n=4 mice per group, mean +/- s.d., one-way ANOVA, **P*=0.0161).

(F) Representative TEM micrograph of corpus callosum in 18-month-old *Trem2*$^{-/-}$ and control mice showing myelinated axons and myelin fragments (arrows). Scale bar, 0.2µm. Quantification of myelin fragments in 6, 12 and 18-month-old *Trem2*$^{-/-}$ compared to control mice (n=3-5 mice per group, mean +/- s.d., two-way ANOVA, followed by Bonferroni's *post hoc* test, 6 months old, *P*>0.9999, 12 months old, *P*>0.9999, 18 months old, **P*=0.0186).

(G) Correlative light and electron microscopy of 18-month-old *Trem2*$^{-/-}$ corpus callosum region. Upper left: confocal image as a guide for correlation. Upper right: overlaid confocal image (transparent, Iba1 in green, DAPI in blue) onto SEM serial section. Relocation of an IBA1$^{+}$ cell on SEM section (red box). Lower right: confocal and SEM images of IBA1$^{+}$ cell. 3 images of SEM serial sections (10×10×100nm) reveal intracellular electron-dense inclusions (black arrows). Scale bar, 50µm (overview, IHC), 20µm (CLEM overlay), 10µm (high magnification SEM). Quantification of number of cells with electron dense inclusions (n=3-4 per group, mean +/- s.d., control, **P*=0.0245, *Trem2*$^{-/-}$, **P*=0.0371, Student's two-tailed *t* test).

(H) Confocal image of microglia co-labeled with IBA1 (green) and CD68 (red) showing microglia with irregular processes in *Trem2*$^{-/-}$ and microglia in control mice (18-month-old). Normal processes are defined by a CD68-positive connection between two IBA1-positive structures (white arrow in image A), whereas fragmented processes have no CD68-positive connection (white arrows in image B). Scale bar, 20µm (overview), 5µm (zoom in). Quantification of number of microglia with irregular processes in the corpus callosum and cortex (Layers I-IV) of 18-month-old *Trem2*$^{-/-}$ and control male mice (n=4 mice per group, mean +/- s.d., two-way ANOVA, followed by Bonferroni's *post hoc* test, grey matter vs. white matter: control, **P*=0.0156, *Trem2*$^{-/-}$, *****P*<0.0001, white matter: control vs. *Trem2*-/-, *****P*<0.0001).

**Figure 6**

**TREM2 is required for lysosomal degradation of internalized myelin debris in microglia.**

(A) Immunocytochemistry showing PKH67-labelled myelin particles (green) bound onto *Trem2*$^{-/-}$ and wild-type primary microglia (red) in a cell surface binding assay. Scale bar, 20µm. Quantification of number of primary microglia with attached myelin particles 2 and 4 hours after myelin treatment (n=3 independent experiment, mean +/- s.d., each dot shows percentage of microglia bound to myelin particle in the area of 0.1mm$^2$; two-way ANOVA followed by Bonferroni's *post hoc* test, control vs. *Trem2*$^{-/-}$: 2h, *P*=0.2828, 4h: *P*>0.9999).

(B) Immunocytochemistry showing uptake of PLP-labeled myelin particles (green) by Isolectin-labeled microglia (red) isolated from *Trem2*$^{-/-}$ and wild-type mice. Scale bar, 25µm. Quantification of the amount of myelin within microglia cells by measuring the average PLP

fluorescence intensity per cell (n=3 independent experiment, mean +/- s.d., two-way ANOVA, followed by Sidak's *post hoc* test, control vs. *Trem2*-/-: 2h, *P*=0.9970, 24h, **P*=0.0322).

(C) Quantification of expression of lysosomal enzymes, *cathepsinL*, *beta-galactosidase1*, *N-acetylglucosamine-6-sulfatase*, and *hexoaminidase* in *Trem2*$^{-/-}$ and wild type microglia treated with myelin or beads (as control) compared to *Trem2*$^{-/-}$ and wild type untreated cells. Data are expressed as fold changed compared to the untreated cells 24 hours after myelin debris treatment (two-way ANOVA followed by Sidak's *post hoc* test, *cathepsinL,* *****P*<0.0001, *beta-galactosidase,* ****P*<0.0004, *acetylglucosamine-6-sulfatase:* ****P*=0.0007, *hexoaminidase, P*=0.7427).

(D) Western blot analysis of Cathepsin L level in control and *Trem2*$^{-/-}$ microglia culture lysates, 4 and 24 hours after myelin treatment. Quantification of expression level of full length and cleaved Cathepsine L compared to Tubulin in each condition. (mean +/- s.d, error bar represents 4 indipendent experiments, Student's two-tailed *t* test, cleaved Cat L: ***P*=0.0053, ; full length Cat L: **P*=0.0312).

(E) Confocal images of deymelinating lesions (lysolecithin model) showing myelin debris accumulation (fluoromyelin in green) in lysosomes (LAMP1 in red) of 3-month-old *Trem2*-/- and control microglia. Scale bar, 100μm. Quantification of number of myelin particles within *Trem2*$^{-/-}$ and control microglia in the deymelinating lesions (n=6 lesion taken from 3 animals per group, mean +/- s.d., **P*=0.0108, Student's two-tailed *t* test).

**Figure 7**

**Microglia with WAM-like signature co-exist with DAM in AD models.**

(A) Heatmap of microglia from 5xFAD and wild-type mice, 6-months old. Each row is a single cell and each column is a gene. Bars on the left correspond to color annotation for population and genotype.

(B) Heatmaps of the average expression of WAM signature gene sets (Set 1-2-3-4) for each population, along with the six populations of Frigerio *et al.* dataset. Values are normalised per gene.

(C) UMAPs showing the populations identified on Frigerio *et al.* $App^{NL\text{-}G\text{-}F}$ data. Each age group is emphasized separately on the right column.

(D) Cell population ratios by age groups. 3-6-12-21 months old $App^{NL\text{-}G\text{-}F}$ mice data from Frigerio *et al.* (Table S1).

(E) WAM and DAM2 populations analyzed separately. Heatmap with top 20 DEGs for DAM2 and WAM. Each row is a gene and each column is a single cell. 947 cells in total of which 644 are WAM and 303 are DAM2.

(F) KEGG pathway enrichment for WAM and DAM2 upregulated DEGs.

(G) Bar plot showing the relative distribution of each microglia population in APP/PS1, and APP/PS1 $Apoe^{-/-}$.

(H) UMAP of microglia from APP/PS1 and APP/PS1 $Apoe^{-/-}$ combined, using the WAM signature genes.

**Figure 8**

**WAM response is induced in an APOE-independent pathway in aged mice.**

(A) UMAP of 13,954 single-cell transcriptomes coloured by major cell-type clusters. 18 months old, white matter only, WT and $Apoe^{-/-}$.

(B) UMAP of microglia (7,666 cells) depicting the identified populations, genotype annotation and expression of representative marker genes.

(C) Bar plot showing the relative distribution of each microglia population in wild-type white matter and $Apoe^{-/-}$ white matter.

(D) Confocal images of CLEC7A, AXL, and Galectin3 co-localized with $IBA1^{+}$ microglia (green) in corpus callosum in 21-month-old $Apoe^{-/-}$ and control mice. Scale bar, 20µm. Quantification of percentage of $IBA1^{+}$ microglia expressing CLEC7A, AXL and Galectin3 in 21-month-old $Apoe^{-/-}$ and control mice in the white matter (corpus callosum; n=4 mice per group, mean +/- s.d., AXL: $P$=0.2348, CLEC7A: $P$=0.5373, Galectin3: $P$=0.9962, Student's two-tailed $t$ test).

(E) Confocal images of microglia nodules (red) containing internalized MBP-labeled myelin particles (green) in the corpus callosum of a 21-month-old *Apoe*$^{-/-}$ (arrows). Clipped 3D reconstruction shows myelin within microglia. Scale bars, 20µm (overview), 8µm (zoom in), 5µm (Clipped 3D). Quantification of microglia with internalized myelin particles in the corpus callosum of 21-month-old Apoe$^{-/-}$ and age matched control mice (corpus callosum, n=4 mice per group, mean +/- s.d., $P$=0.0753, Student's two-tailed $t$ test).

## STAR METHODS

Detailed methods are provided in the online version of the paper and include the following:

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## RESOURCE AVAILABILTY

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mikael Simons (mikael.simons@dzne.de).

**Material Availability**

All unique reagents generated in this study are available from the Lead Contact with a completed Material Transfer Agreement

**Data and code availability**

All gene cell count matrices, metadata and raw data generated during this study (scRNA-seq and Bulk RNA-seq) are deposited at GEO (NCBI) with accession code XXXXXX (active upon publication). All other data that support findings are available upon request from the authors.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Mice:* All animal experiments were reviewed and overseen by the institutional animal use and care committee in German Center for Neurodegenerative Diseases (DZNE) in Munich. The following mouse lines were used in the study: Wild-type C57BL/6J mice from Janvier Labs; *Trem2*$^{-/-}$ mice in the C57BL/6J background (Turnbull et al., 2006, the mice were provided by Prof. Christian Haass, Laboratory of Neurodegenerative Disease Research, DZNE, Munich), ApoE KO mice (B6.129P2-Apoe$^{tm1Unc}$/J, Jackson Laboratory, Stock No. 002052, the brain sections were provided by Dr. Dirk Fitzner, Department of Neurology, University of Göttingen); 5xFAD mice (B6SJL-Tg (APPSwFILon, PSEN1*M146L*L286V) 6799 Vas/Mmjax, Jackson Laboratory, stock No. 034840-JAX, the brain sections were provided by Dr. Sabine Tahirovic, Laboratory of ex vivo models, DZNE, Munich); APP $^{NL-G-F}$ Knock-in mice (available through Takaomi Saido, Ph.D.Wako-shi, Saitama 351-0198, Japan, the brain sections were provided by Dr. Sabine Tahirovic (Laboratory of ex vivo models, DZNE, Munich); APP $^{NL-G-F}$ Trem2$^{-/-}$ mice (the brain sections were provided by Prof. Christian Haass, Laboratory of Neurodegenerative Disease Research, DZNE, Munich). The mice were kept in groups of three in Greenline IVC GM500 plastic cages and were housed in a temperature-controlled environment (21 ± 2°C) on a 12 h light/dark cycle with food and water available *ad libitum* in the animal facility in German Center for Neurodegenerative Diseases (DZNE) in Munich. The ApoE KO mice were kept under the same condition in the animal facility of Max Planck Institute of Experimental Medicine in Göttingen. Pups were bred in-house and kept with the adult female under standard light/dark conditions until P21, then they were weaned. Most

of the experiments including immunohistochemistry, scRNA sequencing and electron microscopy were performed on adult mice at the age of 2, 6, 12, 18 and 24 months. The exact age of mice used for each analysis is indicated in the figure legends. Mice were aged either in house or purchased from Janvier Labs. Both males and females were included in all analyses and we did not notice any influence of sex on our analyzed parameters in the study. For microglia isolation, P6-P8 C57BL/6J wild type or knockout mice and for myelin extraction from brain, 2 months old C57BL/6J wild type mice were used. lysolecithin injection was done on 9 to 15 weeks old mice. The mice and samples including brain sections were allocated into experimental groups randomly. *Cell line:* HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% FCS. For maintenance, these cells were re-suspended in the freezing media containing 50% FCS and 10% DMSO at concentration of $5\times10^6$ to $1\times10^7$ cells/mL. Aliquots in 1.5 mL cryo-tubes were frozen slowly at 1°C/min by placing tubes in a NALGENE cryo freezing container in –80°C freezer, then transferring to liquid nitrogen storage.

## METHOD DETAILS

### Mice perfusion, cell isolation for Smart-seq2

The mice were deeply anesthetized and perfused with cold HBSS between 9am-11am (to decrease circadian fluctuations). Each brain was removed and under a dissection microscope individually micro-dissected; grey matter was isolated from the frontal cortex and white matter form optic tract, medial lemniscus and corpus callosum (attached grey matter and choroid plexus were removed carefully) isolated. We developed and established a microglia isolation protocol that prevents *ex-vivo* transcription and automatizes the mechanical isolation parts using GentleMacs with the Neural Tissue Dissociation Kit (Papain) (Miltenyi Biotec). We added actinomycin D (Act-D, Sigma, No. A1410) to a final concentration of 45 μM into the dissociation solution and enzyme mix to prevent *ex-vivo* transcription. The dissociated cell suspension was passed through a 70 μm cell strainer (Corning, 352350) before labeling. Subsequently, cells were blocked with mouse FcR-blocking reagent (CD16/CD32 Monoclonal Antibody, eBioscience cat:14-0161-82,1100) and then stained for 15 min using 7AAD (Thermo Fisher, A1310, 25 ug/mL) and the antibodies against CD45 (eFluor 450, 30-F11, eBioscience，Cat.:48-0451-82, 1:200) and CD11b (PE/Cy7,M1/70, eBioscience, Cat:48-0451-82,1:200) and after washed with PBS (Sigma, D8537). Viable (7AAD negative) single immune cells (CD45

and CD11b positive cells) were sorted by flow cytometry (SH800; Sony). For GFP positive microglia from CX3CR1$^{GFP/+}$ mice, cells were either dissociated with Act-D or without and labeled with DAPI (4',6-diamidino-2-phenylindole, 1:4000 dilution; Sigma) to label dead cells. After FSC-A/FSC-H selection of single-cells, DAPI negative and GFP positive cells were selected. Single immune cells (CD45 and CD11b positive cells) were sorted by flow cytometry (SH800; Sony). Flow cytometry data were analyzed using FlowJo v10. Single-cells were sorted into 96 well plates filled with 4 µL lysis buffer containing 0.05% Triton X-100 (Sigma) and, ERCC (External RNA Controls Consortium) RNA spike-in Mix (Ambion, Life Technologies) (1:24000000 dilution), 2.5 µM oligo-dT, 2.5 mM dNTP and 2 U/µL of recombinant RNase inhibitor (Clontech) then spun down and frozen at -80°C. Plates were thawed and libraries prepared as described below.

**Library preparation for Smart-seq2**

The 96-well plates containing the sorted single cells were first thawed and then incubated for 3 min at 72°C and thereafter immediately placed on ice. To perform reverse transcription (RT) we added each well a mix of 0.59 µL H2O, 0.5 µL SMARTScribe™ Reverse Transcriptase (Clontech), 2 µL 5x First Strand buffer, 0.25 µL Recombinant RNase Inhibitor (Clontech), 2 µL Betaine (5 M Sigma), 0.5 µL DTT (100 mM) 0.06 µL MgCl2 (1 M Sigma), 0.1 µL Template-switching oligos (TSO) (100 µM AAGCAGTGGTATCAACGCAGAGTACrGrG+G). Next RT reaction mixes were incubated at 42°C for 90 min followed by 70°C for 5 min and 10 cycles of 50°C 2 min, 42°C 2 min; finally ending with 70°C for 5 min for enzyme inactivation. Pre-amplification of cDNA was performed by adding 12.5 µL KAPA HiFi Hotstart 2x (KAPA Biosystems), 2.138 µL H2O, 0.25 µL ISPCR primers (10 µM, 5′ AAGCAGTGGTATCAACGCAGAGT-3), 0.1125 µL Lambda Exonuclease under the following conditions: 37°C for 30 min, 95°C for 3 min, 23 cycles of (98°C for 20 sec, 67°C for 15 sec, 72°C for 4 min), and a final extension at 72°C for 5 min. Libraries were then cleaned using AMPure bead (Beckman-Coulter) cleanup at a 0.7:1 ratio of beads to PCR product. Libraries were assessed by Bio-analyzer (Agilent 2100), using the High Sensitivity DNA analysis kit, and also fluorometrically using Qubit's DNA HS assay kits and a Qubit 4.0 Fluorometer (Invitrogen, LifeTechnologies) to measure the concentrations. Further selection of samples was performed via qPCR assay against ubiquitin transcripts Ubb77 (primer 1 5'-GGAGAGTCCATCGTGGTTATTT-3' primer 2 5'-ACCTCTAGGGTGATGGTCTT-3', probe 5'-/5Cy5/TGCAGATCTTCGTGAAGACCTGAC/3IAbRQSp/-3') measured on a LightCycler 480 Instrument II (Roche). Samples were normalized to 160 pg/µL. Sequencing libraries were constructed by using in-house produced Tn5 transposase (Picelli et al.,

2014). Libraries were barcoded and pooled then underwent three 3 rounds of AMPure bead (Beckman-Coulter) cleanup at a 0.8:1 ratio of beads to library. Libraries were sequenced 2x150 reads base pairs (bp) paired-end on Illumina HiSeq4000 to a depth of $3x10^5$–$6x10^5$ reads/sample.

**Processing and analyses of Smart-seq2 data**

BCL files were demultiplexed with the bcl2fastq software from Illumina. After quality-control with FastQC, reads were aligned using rnaSTAR (Dobin et al., 2013) to the GRCm38 (mm10) genome with ERCC synthetic RNA added. Read counts were collected using the parameter "quantMode GeneCounts" of rnaSTAR and using the unstranded values. From that point, Seurat R v.2.3.4 package was used (Butler et al., 2018). Low-quality samples were filtered out from the dataset based on a threshold for the number of genes detected (min 1000 unique genes/cell), percentage of mitochondrial genes (max 0.5%), percentage of ERCCs (5% max) and number of reads on a log10 scale (between 4 to 5.5) as shown in Figure S1G and H. 1038 single-cells passed the quality-control. Gene expressions were log normalized to 10,000 using the NormalizeData function of Seurat. Dataset were scaled and depth of sequencing was regressed using ScaleData function and using the percentage of ERCCs. The first 12 principal components were considered for the UMAP of all cell types. Non-microglia cells were removed from analysis using markers and threshold such as performed in Keren-Shaul et al. (Keren-Shaul et al., 2017) and shown in Figure S1I and J. The first 8 principal components were used for the UMAP of microglia only. To find the clusters/populations of microglial states, hierarchical clustering (Ward's method) was used on the differentially expressed genes specific to the WAM cluster (FindClusters on first 8 PCs). To refine the WAM signature, differential expression analysis was performed using DESeq2(Love et al., 2014) using FindAllMarkers. 212 genes were found significant at adjusted p-value < 0.05 (Table S2). Gene sets of 1, 2, 3 and 4 were defined using hierarchical clustering (Ward's method) on the genes of the WAM signature. For the DAM signature, the top 500 genes were grouped by set in a similar way as described above. Slingshot analyses are based on the first 2 components of the PCA using the WAM signature genes. The previously described 4 clusters of microglia were used as input for Slingshot (Street et al., 2018). All gene ontology analyses were performed using Metascape (http://metascape.org/).

**Mice perfusion, cell isolation for Drop-seq**

Briefly, the mice were deeply anesthetized and perfused with cold HBSS between 9am-11am (to decrease circadian fluctuations). Each brain was removed and under a dissection microscope

individually micro-dissected and dissociated same as described above. After dissociation, myelin debris is removed using Myelin Removal Beads II (Miltenyi Biotec). The cells were resuspended in 0.04% BSA+PBS and cell are counted using automated cell counter (TC20 Bio-Rad) before loading to Chromium Controller.

**Library preparation for Drop-seq**

Single-cell suspensions were loaded onto the Chromium Single Cell Controller using the Chromium Single Cell 3' Library & Gel Bead Kit v3.1 (10X Genomics) chemistry following the manufacturer's instructions. Sample processing and library preparation was performed according to manufacturer instructions using AMPure beads (Beckman Coulter). Libraries were sequenced on the DNBSEQ Sequencing System (BGI group).

**Processing and analyses of Drop-seq data**

Fastq files were processed with Cell Ranger v4 and aligned to the mm10 (Ensembl 93) genome. From that point, Seurat R v.3 package was used (Butler et al., 2018). Low-quality samples were filtered out from the dataset based on a threshold for the number of genes detected (min 200 unique genes/cell), percentage of mitochondrial genes (max 10%). 21197 out of 25719 and 13954 out of 17263 single-cells passed the quality-control for the aging and Apoe-KO datasets, respectively. Gene expressions were normalized using SCTransform function of Seurat. The first 30 principal components were considered for the UMAP of all cell types. Non-microglia cells were removed from analysis using markers genes as shown in Figure S2C and D. The first 15 principal components were used for the UMAP of microglia only. WAM populations were identified using the WAM signature gene set via hierarchical clustering (Ward's method). All gene ontology analyses were performed using Metascape (http://metascape.org/) and STRING (Szklarczyk et al., 2019). After identification of the novel microglial populations, WAM signature was used for generating the UMAPs, as described previously. For the *Apoe*$^{-/-}$ dataset, Apoe was omitted from the WAM signature while generating the UMAPs.

**Processing and analyses of external datasets**

External datasets from Hammond *et al*. and Frigerio *et al.* were analyzed with the same Seurat pipeline using Seurat 3 and MAST (Finak et al., 2015) for differential gene expression. For Hammond et al. datasets, raw data was downloaded from GSE121654. 4 samples for wild-type data; two replicates of each 1 month old (GSM3442024, GSM3442025) and 18 months old (GSM3442036, GSM3442037) were quality-controlled and processed as described above.

After the QC steps, 9558 cells were kept for downstream analyses (Table S2). For Frigerio et al. datasets, normalized datasets were available. Wild-Type and *App*$^{NL\text{-}G\text{-}F}$ data were downloaded from GSE127892 and analyzed separately. After filtering, 4856 and 5093 microglia were included in the downstream analyses for *App*$^{NL\text{-}G\text{-}F}$ dataset and WT dataset, respectively. The whole dataset acquired from GSE127892, consists of 32 experimental conditions, 2 mice per conditions as reported by the authors. Data for each condition were pooled (Table S1). Merging the current study and Frigerio *et al.* wild-type data (Figure S3B) was established by using the Seurat 3 data integration functions. APP/PS1 and APP/PS1-*Apoe*$^{-/-}$ data were downloaded from GSE127884. After filtering, 1143 microglia were kept for downstream analyses. For the average heatmap (Figure S7A) from Keren-Shaul *et al.* data, their Supplementary Table 2 was used to plot the average gene expression per microglial population for the 500 DAM signature genes.

**Immunohistochemistry**

Animals were anesthetized by intraperitoneal injection of 14% chloral hydrate, perfused transcardially with 4% paraformaldehyde. Post fixation of brain tissue was done in 4% PFA overnight. Then the brain tissue was further cryo-protected in 30% sucrose in PBS for 24 h. After freezing the tissue on dry ice using Tissue-Tek O.C.T, 30 µm coronal sections were cut by cryostat Leica CM 1900. Free-floating sections were collected in a solution containing 25% glycerol and 25% ethylenglycol in PBS. The sections were rinsed with 1x PBS containing 0.2% Tween-20 and permeabilized in 0.5% Triton X-100 for 10 to 30 min depending on primary antibody. Fab fragment goat anti mouse IgG (1:100) (Dianova) was added for 1 h at room temperature to block endogenous mouse tissue immunoglobulins. After a brief wash the sections were blocked for 1 h at room temperature in a solution containing 2.5% FCS, 2.5% BSA and 2.5% fish gelatin in PBS. Primary antibodies, diluted in 10% blocking solution, were added and incubated overnight at 4°C. On the following day sections were incubated with secondary antibodies, diluted in 10% blocking solution, for 1 h at room temperature. The sections were washed with PBS followed by distilled $H_2O$ and mounted using fluorescence mounting medium (Dako) over superfrost plus slides. For AXL and CLEC7A staining, antigen retrieval protocol using citrate buffer (10 mM, pH 6) was performed on free-floating sections followed by staining protocol as mentioned above. The immunostaining of PU.1 was done as following. The free floating sections were permeabilized with 0.5% Triton X-100 for 30 min at room temperature. Then sections were treated with blocking solution containing 0.3% Triton X-100 for 24 hours at 4°C. The primary antibody in blocking solution without Triton X-100

was incubated for 65 hours at 4ºC. the sections were incubated with secondary antibody in blocking solution at 4ºC over night.

**RNAscope in Situ Hybridization**

RNAscope *in situ* hybridization assay was applied to detect mRNA of *Itgax* in the brain cryosections prepared from aged and young wild-type mice. The assay was performed using a commercially available kit, RNAscope Multiplex Fluorescent Detection Reagents v2 (Advanced Cell Diagnostics, ACD) and the manufacturer instruction was followed. Briefly, 30 µm cryosections were fixed on superfrost plus slides; they were pretreated with hydrogenproxide for 10 min at room temperature and then with antigen retrieval reagent (5min boiling) to unmask the target RNA. After applying Protease III on the sections for 30 min at 40°C, probe hybridization was done by incubating sections in mouse *Itgax* probe assigned to channel 1 (Cat. 311501), diluted 1:50 in probe diluent, for 2 h at 40°C. Positive control probes targeting housekeeping genes including *Polr2a*, *Ppib* and *Ubc*. (Advanced Cell Diagnostics, Hayward, CA) and 3-Plex negative control probes targeting the bacterial *DapB* gene (Advanced Cell Diagnostics, Hayward, CA) were used to test mRNA integrity in the tissue. Afterwards, signal amplification and detection were performed according to the instruction of the kit. Signal detection was done using Opal dyes (Opal520-green) diluted 1:3000 in TSA buffer. To visualize microglia, after *in situ* hybridization, immunohistochemistry assay was performed using Iba1 antibody (Wako, 1:1000). The nuclei of cells were counterstained with DAPI (4',6-diamidino-2-phenylindole) and then the slides were mounted over superfrost plus slides using prolong gold antifade reagent (Invitrogen).

**Bulk RNA Sequencing**

The isolated microglia were homogenized in RLT buffer using QIAShredder (QIAGEN) and the total RNA was extracted using RNeasy Micro Kit (QIAGEN) and cDNA was synthesized using Ovation RNA-Seq System V2 (NuGEN). 1 µg of cDNA was used as input for Ion Xpress™ Plus Fragment Library Kit (ThermoFisher Scientific) to generate barcoded libraries. Barcoded libraries were then quantified using qRT-PCR (KAPA Library Quantification Kit). Barcoded libraries were then pooled and clonally amplified on Ion Spheres (Ion One Touch 200 Template Kit v2, ThermoFisher Scientific) and were sequenced on an Ion Proton sequencer (ThermoFisher Scientific). Raw reads were sorted based on barcodes and were subjected to quality analysis using FASTQC. The sequences were subsequently aligned to the genome of *Mus musculus* (GRCm38/Mm10) using the TMAP aligner with default parameters. The reads mapping to unique locations were quantified using RefSeq Gene Annotations(v73) into genes.

Differential gene expression analysis and hypergeometric pathway analysis using KEGG genesets was performed using a commercial platform (Partek). Genes with fold change greater than 2 and p-values less than 0.05 were considered for further hypergeometric pathway enrichment analysis.

**Transmission Electron Microscopy**

Mice brains were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 after deep anesthesia (isoflurane) perfusion. Brains were vibratome sectioned and immersion fixed in the same buffer for 24 h at 4°C. After tissue trimming and washes in 0.1 M sodium cacodylate buffer, postfixation in reduced Osmium (2% Osmium, 2.5% potassium ferrocyanide in 0.1 M cacodylate buffer) was followed by en bloc uranyl acetate (1% aqueous uranylacetate) contrasting, graded dehydration in ethanol and embedding in epon resin (Serva). After ultrathin sectioning the grids (Leica UC7 ultramicrotome) were contrasted by 1% uranyl acetate and lead citrate (Ultrostain, Leica). Images were acquired with a JEOL JEM1400 plus TEM equipped with a Ruby 8Mpx CCD camera. For each analysis, randomly selected regions in three to five different animals were imaged. Data analysis was carried out using ImageJ 1.41.

**Correlative Light and Scanning Electron Microscopy**

The correlated workflow was adapted from Fang et al.(Fang et al., 2018). Mice were perfused and brains fixed for 24 h in 4% paraformaldehyde and 3% w/v sucrose in 0.1 M PBS (pH 7.4). The brain tissue was sectioned into 100µm thick vibrotome sections. After 24 h incubation in fixative, smaller regions containing the corpus callosum were trimmed to restrict the correlation area and prevent tissue curving during the en bloc staining. The immunostaining was performed as mentioned before with a slight change. The sections were incubated with Iba1 antibody (Wako, 1:1000) for 48 h and with secondary antibody (Alexa Fluor 488, Invitrogen) overnight. Sections were stained with DAPI (1:2000 in PBS) for 5 min, washed in PBS and mounted on glass slides using secure-seal spacer (13 mm, 0.12 mm thickness, Invitrogen). Tile scans of the whole section with a 20x air objective and regions of interest with a 63x oil objective were acquired on a Leica TCS SP5 confocal microscope. After careful unmounting, sections were post-fixed (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer) for 24 h. In order to keep a flat orientation corresponding to the confocal imaging plane, the sections were glued onto of Aclar spears (Science Services) using Cell-Tak adhesive (Corning)(Luckner and Wanner, 2018). We applied a rOTO (reduced osmium-thiocarbohydrazide-somium) staining procedure adopted from Tapia et al.(Tapia et al., 2012). Briefly, the tissue was initially postfixed in 2%

osmium tetroxide (EMS), 2% potassium ferricyanide (Sigma) in 0.1 M sodium cacodylate (Science Services) buffer (pH 7.4). After three washing steps in buffer and water the staining was enhanced by reaction with 1% thiocarbohydrazide (Sigma) for 45 min at 50°C. The tissue was washed in water and incubated in 2% aqueous osmium tetroxide. All osmium incubation steps were carried out over 90 min with substitution by fresh reagents after 45 min, respectively. To further intensify the staining, 2% aqueous uranyl acetate was applied overnight at 4°C and subsequently warmed to 50°C for 2 h. The samples were dehydrated in an ascending ethanol series and infiltrated with LX112 (LADD). The sample were flat embedded into gelatin capsules (Science Services) and cured for 48h. For SEM experiments without correlation samples were fixed as mentioned in the TEM protocol and subjected to the rOTO protocol described above. The block was trimmed by 200 µm at a 90° angle on each side using a TRIM90 diamond knife (Diatome) on an ATUMtome (Powertome, RMC). The front face was carefully trimmed to collect all sections beginning at the very surface areas that correspond to the confocal images (depth of 15-20 µm). Consecutive sections were taken with a 35° ultra-diamond knife (Diatome) at a nominal cutting thickness of 100nm and collected on freshly plasma-treated (custom-built, based on Pelco easiGlow, adopted from Mark Terasaki) CNT tape(Kubota et al., 2018). Starting from the block face with the complete tissue exposed we collected 300 ultrathin sections, covering a thickness of 30 µm in depth. Tape strips were mounted with adhesive carbon tape (Science Services) onto 4-inch silicon wafers (Siegert Wafer) and grounded by additional adhesive carbon tape strips (Science Services). EM micrographs were acquired on a Crossbeam Gemini 340 SEM (Zeiss) with a four-quadrant backscatter detector at 8 kV. In ATLAS5 Array Tomography (Fibics), the whole wafer area was scanned at 2000-4000 nm/pixel to generate an overview map. For correlation, sections were selected and the entire corpus callosum region (1356 x 491 $\mu m^2$) of 177 sections (one wafer, 17.7 µm in z) scanned at 200 × 200 $nm^2$. The images were aligned by a sequence of automatic and manual processing steps in Fiji TrakEM2(Cardona et al., 2012). The correlation was achieved by using nuclei and further anatomical landmarks (section border morphology, myelinated areas). Based on this correlation we selected an area in xy (544 × 324 × 13.9 $\mu m^3$) and the respective sections (corresponding to the ROI in z) for high resolution acquisition at 20 x 20 $nm^2$. After correlation of this dataset we imaged single images containing IBA1 positive cells at 4 × 4 $nm^2$.

**Myelin isolation and purification**

Myelin was isolated from 8-week-old C57BL/6 mice brains by sequential centrifugation on discontinuous sucrose gradient according to a protocol previously described(Norton and Poduslo, 1973) with some modifications. The ultracentrifugation was done using SW41 Ti rotor. The brain tissues were homogenized with a Dounce homogenizer in a solution containing 10 mM HEPES, 5mM EDTA, and 0.32 M sucrose. The homogenized tissue was layered on HEPES/EDTA buffer containing 0.85 M sucrose, centrifuged at 24600 rpm for 30 min with low deceleration and acceleration. The crude myelin fraction was removed from interface, resuspended in ice-cold distilled water, and centrifuged at 9500 rpm for 15 min. The hypo-osmotic shock was applied to the pellet two more times. The pellet from the last step was dissolved in HEPES/EDTA buffer containing 0.3 M sucrose, and placed over the 0.85 M sucrose; all the centrifugation steps and hypo-osmotic shocks were repeated as before. Eventually, the purified myelin pellet was resuspended in 1 mL PBS and stored at -20°C.

**Myelin uptake assay**

18mm coverslips were coated with fibronectin in PBS (20 µg/mL) in the 24-well plates for 1 h in the incubator (37°C, 5% $CO_2$). After changing PBS with the culture medium (DMEM plus 10% fetal calf serum, 1% Glutamax, and 0.5% antibiotics) Hela Cells were seeded at $4\times10^4$ cells/mL and cultured with 5% $CO_2$ at 37℃ for 18-24 h before transfection. 1 µg of expression plasmids were introduced into Hela cells by the calcium phosphate precipitation method. The level of gene expression was tested 24-48 h after incubation. Next, transfected cells were treated with myelin as following. Purified myelin isolated from 2-month-old wild-type mouse brains was labelled with PKH67 (Sigma), and then washed in PBS by centrifugation at 15000g. The final pellet was resuspended in culture medium and sonicated for 20 min in an ultrasound water bath. Transfected HeLa cells were treated with 4µg PKH76-labelled myelin and incubated at 37℃ in the presence of 5% $CO_2$ for 2 to 5 h. The cells were fixed in 4% PFA for 15 min and myelin uptake was assessed by immunocytochemistry.

**Microglia-myelin binding assay**

Microglia were isolated from C57Bl/6, P6-P8 wild-type mice by MACS Technology. Brain tissue was dissociated using a Neural Tissue Dissociation Kit (Papain) (Miltenyi Biotec). Briefly, brain tissue was removed, cut into small pieces and dissociated by enzymatic digestion provided in the kit. The tissue suspension was applied to a 40 µm cell strainer, and washed twice with DMEM containing 1 mM sodium pyruvate. The final palette was resuspended in 10 volume of DMEM containing 10% FCS, 1 mM sodium pyruvate, 1% antibiotics (DMEM/FCS)

plus 1 volume of CD11b microbeads (Miltenyi Biotec) and incubated at 4℃ for 15 min. After washing with DMEM/FCS, the pellet was resuspended in 500 µL of the same medium, applied in a MACS column in the magnetic field, following three times wash, CD11b positive cells (microglia) were flushed out of the column, centrifuged at 400 x g for 10 min at 4°C. Isolated microglia were plated over 12 mm coverslips at $7\times10^4$ cell/mL, and incubated for 48-72 h. PKH67-labeled myelin was sonicated in an ultrasound water bath for 20 min. Primary microglia cultures were treated with 4 µg myelin and incubated on the ice for 2-4 h. Purified myelin was conjugated with Annexin V Alexa Fluor 555 (Thermo Fisher) as following. 4 µg of sonicated myelin was incubated with different concentration of Annexin V (13.5 M, 18 M and 22.5 M) in the binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) for 1 h at room temperature.

**Myelin clearance assay**

To analyze the rate of clearance of myelin debris, microglia cultures were pretreated with serum free DMEM, supplemented with 2 ng/mL TGF-β2 (Peprotech, 100-35B), 20 ng/mL CSF1 (M-CSF, Peprotech, 315-02) and 1.5 µg/mL cholesterol (Avanti Polar Lipids (Otto Nordwald), 700000P) (TCC medium). The cells were treated with 10 µg/mL myelin (or HEPES control) in the TCC medium for 2 h. After treatment, the cells were washed three times, and incubated with the TCC medium for 2 or 24 h. For the experiment with the inhibitors of lysosomal degradation, 100 nM Bafilomycin (Invivogen) or 10 µM leupeptin (Selleckchem) were administered 30 minutes before myelin administration and kept in the media until the end of the experiment. After fixation, the myelin in cells was stained using anti-PLP antibody; the cells were stained using DyLight 694 labeled tomato lectin (Vector Laboratories, DL-1178) and 2 µg/mL Hoechst 33342. The cells were imaged on a Leica SP5 confocal microscope with a 63x objective. For the quantification, the intensity of the PLP staining contained within the cell membrane was measured per cell.

**Lysolecithin-induced demyelination**

Stereotactic injection of lysolecithin in the spinal cord was performed in wt C57BL\6 and *Trem2*$^{-/-}$ mice that were 9 to 15 weeks old. 1% lysolecithin was prepared by dissolving L-α-Lysophosphatidylcholine from egg yolk (Sigma, L4129) in PBS, pH 7.4 (Gibco™, 10010056). 3% Monastral blue was prepared by dissolving Copper(II) phthalocyanine-tetrasulfonic acid tetrasodium salt (Aldrich, 274011) in milliq water, and the solution was sterilized by filtration through a 0.45-µm filter and autoclaving. Prior to injection, 1µl of 3% Monastral blue was mixed with 25µl of 1% lysolecithin. Glass Capillaries for Nanoliter 2010 (World Precision

Instruments, 504949 or 4878) were pulled using the P-1000 Next Generation Micropipette Puller (Sutter Instrument). The program had the following parameters: Heat 530, Pull 0, Vel 60, Time 250, Pressure 500, Ramp 520, Microinjection – BF100.50.10, Tip < 1 µm, Taper 6-8 mm. R ~40-80 Meg, Heat = Ramp, FB255B, 2.5mm Box. Before surgery, the animals were anesthetized by intraperitoneal injection of 0.5 mg/kg body weight medetomidine, 5.0 mg/kg midazolam and 0.05 mg/kg fentanyl (MMF). The anesthetized animals were kept on a heating pad at 37°C, and the anesthetic depth was monitored by checking the reflex between the toes and the corneal reflex. The surgery and intraspinal injection of lysolecithin was conducted using the digital mouse stereotaxic frame and Nanoliter 2010 Injector with MICRO4 controller (World Precision Instruments) as previously described (Cantuti-Castelvetri et al., 2018). After the spinal cord was exposed, the capillary was positioned 0.55 mm lateral to the dorsal artery, and lowered 1.15 mm into the tissue. At each injection site, 1µl of 1% lysolecithin containing 0.12% monastral blue was injected at a speed of 350 nl/min. 1 minute after the end of the delivery, the capillary was retracted. After injection, the skin was sutured, and the wound was sutured. After the operation, the animals were injected with 250µl of 0.9% NaCl (normal saline solution) to compensate for the loss of blood and with the analgesic buprenorphine at a dose of 0.1 mg/kg. When MMF was used for anesthesia, 2.5 mg/kg atipamezole, 0.5 mg/kg flumazenil and 1.2 mg/kg naloxone (AFN) was injected IP for the animals to antagonize the anesthesia and awaken the animal. The animals were injected SC with buprenorphine for two days after surgery.

**Gene expression analysis**

For the preparation of RNA from cell, primary microglia was treated with myelin or fluoresbright beads. At the end of the treatment, the cells were collected in RLT buffer and the RNA was isolated with the RNAeasy isolation kit (Qiagen, 74104). The RNA was retrotranscribed to cDNA with the Superscript III kit (Thermo Fisher Scientific, 18080051). For the quantitative PCR, the cDNA was quantified with the Power SYBR green PCR Master mix (4367659 Thermo Fisher Scientific) on a Applied Biosystem 7500 Fast Cycler, according to the PCR mix data sheet. The relative quantification of each gene was performed with the ΔΔCt method: each gene was quantified and its expression was normalized to the house keeper gene (Cytochrome C1, cyc1). The primers used for the analysis were:

hexosaminidase forward 5`-GCTGCAGAATCCTTTGCTTACGG-3´;

hexosaminidase reverse 5`-GGGTCACGTGAACGGGAGG-3´;

N-acetylglucosamine-6-sulfatase forward 5`-GAAAACCAAGGCCCTCATCG-3´;

N-acetylglucosamine-6-sulfatase reverse 5`-TGTTGTTAACGACGTGGTGG-3´;

Galactosidase Beta 1 forward 5`-CACTGCCTAACGGAGAGACC-3´;
Galactosidase Beta 1 reverse 5`-TCCCGAGATGTATCGGAATGG-3´;
Cathepsin L forward 5`-TCGGATTTCACCTCAGTGTCC-3´;
cathepsin L reverse 5`-CTTAAAAACTAGTGGGGCTGGC-3´.

**Western Blotting**

Following separation with SDS-PAGE, the proteins were transferred from the gel onto the nitrocellulose membrane using the mini Trans-Blot Module. The gel sandwich in blotting cassette was placed in the tank of the module containing transfer buffer (0.25 M Tris base, 1.92 M glycine, 20 % methanol) with 100 V for 1 hour. The membrane was washed in PBST (0.1 % Tween 20 in PBS) for 10 min, immersed in 4 % skim milk powder in PBST as a blocking solution for 30 min at room temperature and then incubated with primary antibody in PBST at 4°C overnight. After washing, it was incubated with horse radish peroxidase (HRP)-conjugated secondary antibody in PBST for one hour at room temperature. Then targeted protein was detected with enhanced chemiluminescence method using Luminol enhancer and peroxide solutions (Pierce/Thermo Scientific) and was visualized with an Odyssey Fc imager from LI-COR.

**Image processing and analysis**

Images were acquired via a Leica TCS SP5 confocal microscope and were processed and analyzed with Imaris (64x version 9.2.0) and ImageJ 1.41 image processing software. For quantification of microglia nodules, a cluster of three or more cells that were identified by their nuclei labeled with DAPI or PU.1 antibody.

## QUANTIFICATION AND STATISTICAL ANALYSIS

To compare more than two groups, one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test was applied. A two-tailed Student's t-test was performed for comparison of two groups. Two-way ANOVA followed by Bonferroni *post-hoc* test was used for analyzing the interaction of age and brain region or age and genotype. In all tests a p value of $<0.05$ was considered as significant. Statistical analyses were done using GraphPad Prism (GraphPad Software, Inc.). The type of statistical test and the exact p value for each experiment are included in the figure legends. All cell culture experiments using cell lines and primary cells were done at least three times independently to ensure reproducibility. Technical and biological

replicates were included. The average of at least 3 technical replicates was counted as one biological replicate which was then used for statistical analysis and comparison within the biological replicates. For all mouse experiments, 4 to 5 mice per genotype were analyzed. For histological analysis, 3 to 4 random region of interest (ROIs) per brain section were taken and three random brain sections per animal were quantified to account for variability within the biological sample. All values obtained from in vivo experiments were represented as mean ± sd of 3 brain sections per mouse for immunohistochemistry and 2 brain sections per mouse for RNAScope in situ hybridization. The values from in vitro experiments were reported as mean ± sd of the number of independent experiments. No power analyses were used to predetermine sample sizes. However, sample sizes were chosen based on prior literature using similar experimental paradigms. The value of n per group and what n represents in each experiment can be found in the figure legends. All data acquisition and analysis for wild type and knockout samples were done in a blinded manner and no data were excluded from any analysis. For the scRNA-seq statistical data analysis, the n represents a sequencing lane, containing then a defined tissue (GM or WM) for a defined mouse (SS2) or a pool of mice (Drop-seq). Differential expression analysis was performed with DESeq2 (Love et al., 2014) and MAST (Finak et al., 2015) with adjusted p-value below 0.05 considered as significant. For pathway enrichment analysis, Metascape (Zhou et al., 2019) and STRING (Szklarczyk et al., 2019) were used with default parameters.

**Table S1 Metadata sample information on scRNA-seq data** (related to Figure 1, Figure 2, Figure 3, Figure 7, Figure 8). Provided as an excel file (Table S1).

**Table S2 Summary of gene expression by all microglia states including disease associated microglia (DAM).** The mean expression value for the 212 differentially expressed genes between old white and grey matter microglia is shown (related to Figure 1, Figure 2, Figure 3, Figure 7, Figure 8). Provided as an excel file (Table S2).

**Table S3 Bulk RNA-seq data with the differentially expressed genes from 4 months (4m) and 21 months (21m) old white (WM) and grey (GM) matter** (related to STAR Methods section – bulk sequencing and Figure S2). Provided as an excel file (Table S3).

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