Lipid-droplet-accumulating microglia represent a dysfunctional and proinflammatory state in the aging brain

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Microglia become progressively activated and seemingly dysfunctional with age, and genetic studies have linked these cells to the pathogenesis of a growing number of neurodegenerative diseases. Here we report a striking buildup of lipid droplets in microglia with aging in mouse and human brains. These cells, which we call ‘lipid-droplet-accumulating microglia’ (LDAM), are defective in phagocytosis, produce high levels of reactive oxygen species and secrete proinflammatory cytokines. RNA-sequencing analysis of LDAM revealed a transcriptional profile driven by innate inflammation that is distinct from previously reported microglial states. An unbiased CRISPR–Cas9 screen identified genetic modifiers of lipid droplet formation; surprisingly, variants of several of these genes, including progranulin (GRN), are causes of autosomal-dominant forms of human neurodegenerative diseases. We therefore propose that LDAM contribute to age-related and genetic forms of neurodegeneration.

Microglia are the resident immune cells of the CNS and play a pivotal role in the maintenance of brain homeostasis. In the aging brain and in neurodegeneration, microglia lose their homeostatic molecular signature and show profound functional impairments, such as increased production of proinflammatory cytokines, elevated generation of reactive oxygen species (ROS) and buildup of dysfunctional lysosomal deposits indicative of impaired phagocytosis. Recent single-cell transcriptomic studies have revealed several distinct microglia subpopulations and cellular states in aging and disease, including “disease-associated microglia” (DAM), a presumably protective phagocytic microglia population, and “neurodegenerative microglia” (MGNd), a dysfunctional microglia phenotype. Furthermore, proliferative-region-associated microglia arise during development and express genes that are also enriched in DAM.

Over 100 years before these technologically advanced genomic studies, Alois Alzheimer was one of the first to describe a unique microglial subset when he observed “many glial cells show[ing] adipose saccules” in brains of patients with dementia (Alzheimer, 1907). Although microglia had not been identified as a distinct cell type back then, Alzheimer’s description of these cells suggests that they were indeed microglia. Over the next few years, multiple studies confirmed this finding and regarded glial lipid accumulation as characteristic for senile dementia. However, after this initial excitement, lipid deposits in microglia had mostly been ignored for almost a century.

Cellular lipid accumulation became of interest in other myeloid cells in the 1970s when ‘foamy macrophages’ were discovered to contribute to atherosclerotic lesions. Since then, abnormal lipid accumulation has been recognized as a key aspect of immune dysfunction in myeloid cells. In particular, lipid droplets, which are lipid-storing organelles that contain neutral lipids such as glycerolipids and cholesterol, are increasingly being accepted as structural markers of inflammation. Myeloid cells form lipid droplets in response to inflammation and stress, including the aforementioned macrophages in atherosclerotic lesions, leukocytes in inflammatory arthritis and eosinophils in allergic inflammation. Here, lipid droplets are sites of production and storage for eicosanoids and inflammatory cytokines and are further involved in antigen presentation and pathogen clearance. Importantly, lipid-droplet-rich foam cells in atherosclerosis show hallmarks of senescent cells and they seem to be deleterious at all stages of disease.

Surprisingly, lipid droplets have not been studied functionally in brain myeloid cells in humans or vertebrates, and less than a handful of papers report the histological presence of lipid droplets in human brains. That they may have important functions in disease has recently been suggested in a Drosophila model whereby lipid droplet formation has been reported in glia at the onset of neurodegeneration. Oil-red-O-positive ‘lipid-laden’ cells, including neurons, astrocytes, ependymal cells and IBA1+ cells, have recently been reported in mice and shown to increase with age. Lipid droplets were also induced in lipopolysaccharide...
(LPS)-treated mouse hippocampal slice cultures and in the microglia N9 cell line.\textsuperscript{15,17}

Together, lipid droplets have been recognized for their role as inflammatory organelles in peripheral myeloid cells, and lipid droplets in glia have been rediscovered in the context of brain aging and disease. Yet little is known about the formation and role of lipid droplets in microglia in vivo, and whether they have a role in neuroinflammation, brain aging or neurodegeneration.

Here, we identify a novel state of microglia in the aging brain in which they accumulate lipid droplets. These LDAM exhibit a unique transcriptional signature, show defects in phagocytosis, produce increased levels of ROS and release elevated levels of proinflammatory cytokines. We identify SLC33A1, SNX17, VPS35, CLN3, NPC2 and GRN—genes that have variants that cause autosomal-dominant forms of neurodegeneration—as genetic regulators of microglial lipid-droplet formation. We further validate the accumulation of LDAM in mGFP\textsuperscript{+} mice, a model for frontotemporal dementia (FTD). Together, these findings indicate that LDAM represent a dysfunctional and proinflammatory microglia state in the aging brain, and further suggest that LDAM might contribute to neurodegenerative diseases.

Results
Microglia accumulate lipid droplets in the aging brain. In an attempt to discover structural differences between young and aged microglia, we analyzed their cytoplasmic content in 3-month-old and 20-month-old mice by transmission electron microscopy. Interestingly, we observed characteristic lipid droplets in aged microglia but rarely in young microglia, and these were frequently adjacent to dense lysosomal material (Fig. 1a). Histological staining of TMEM119\textsuperscript{+} microglia with BODIPY, a dye that specifically labels neutral lipids and is commonly used to detect lipid droplets,\textsuperscript{8} showed that lipid droplets were particularly abundant in the aged hippocampus (Extended Data Fig. 1), and we focused subsequent analyses on this region. Lipid droplets were primarily found in microglia but not in other cell types. The percentage of BODIPY\textsuperscript{+} TMEM119\textsuperscript{+} microglia in the hippocampus was more than fourfold higher in aged (51.95\%) compared with young (12.08\%) microglia, and lipid droplets were significantly larger in aged microglia (Fig. 1b–e). Microglial lipid droplets were also immunoreactive for the lipid droplet surface protein perilipin 3 (PLIN3) (Fig. 1f). Last, we detected PLIN2, a close paralog of PLIN3, in microglia in human hippocampal brain sections, and counted more PLIN2\textsuperscript{+} IBA1\textsuperscript{+} microglia in aged than in young individuals (Fig. 1g).

To corroborate our finding of lipid droplet accumulation in aged microglia, we used coherent anti-Stokes Raman scattering (CARS) microscopy, which is a label-free and nonlinear optical technique that enables the identification of molecules based on their specific vibrational energy. We performed CARS laser-scanning microscopy at 2,845 cm\(^{-1}\), which corresponds to the CH\(_2\) stretching frequency for neutral lipids and specifically identifies neutral lipids and lipid droplets,\textsuperscript{19} on TMEM119\textsuperscript{+} immunostained brain sections from young and aged mice. Consistent with our previous data, we found that the numbers of CAR5\textsuperscript{+} lipid-storing microglia were significantly higher in aged than in young mice (50.76\% versus 18.93\%) (Fig. 1h,i).

Lipid droplets are composed of neutral lipids such as glycerolipids (triacylglycerols (TAGs), diacylglycerols (DAGs) and monoacylglycerols (MAGs)) and cholesteryl esters (CEs), yet their content can vary greatly between cell types. We isolated lipid droplets from whole hippocampi of aged mice and, to more specifically determine the content of microglial lipid droplets, from cells sorted via fluorescently activated cell sorting (FACS) from aged hippocampal microglia (Fig. 1j). Lipidomics analysis revealed that lipid droplets from the whole hippocampus and from aged microglia show a nearly identical lipid distribution and are mainly composed of glycerolipids (41.3\% in the hippocampus, 44.4\% in microglia), while CEs were almost absent (1.1\% in the hippocampus, 0.7\% in microglia) (Fig. 1k).

Lipid-droplet-rich microglia have a unique transcriptome signature that is associated with cellular dysfunctions. To determine the transcriptional phenotype of lipid-droplet-containing microglia in the aged brain, we isolated CD11b\textsuperscript{+}CD45\textsuperscript{+} microglia from the hippocampus of 18-month-old mice based on their BODIPY\textsuperscript{+} mean fluorescence intensity and analyzed lipid-droplet-low (BODIPY\textsuperscript{−}; LD-low) and lipid-droplet-rich (BODIPY\textsuperscript{+}; LD-high) microglia by RNA sequencing (RNA-seq) (Fig. 2a,b). Of note, we used an optimized microglia isolation strategy that uses mechanical tissue homogenization instead of enzymatic digestion, which keeps microglia largely in a nonactivated state and therefore prevents unwanted bias toward an activated proinflammatory signature.\textsuperscript{18} Unsupervised cluster analysis segregated LD-low from LD-high microglia and revealed prominent differences between their transcriptome, with 692 significantly differentially expressed genes (Fig. 2c,d).

A pathway analysis of differentially expressed genes revealed that ‘phagosome maturation’ and ‘production of nitric oxide and ROS’, two key functions of microglia that become dysregulated with age, as the most significant pathways associated with LD-high microglia (Fig. 2e). Regulated genes in the phagosome maturation pathway included lysosomal genes (CD63, ATP6V1A, ATP6V1C1, ATP6V1G1 and TUBA1), genes involved in vesicular transport (RAB5B and RAB8), and CD22, a negative regulator of phagocytosis in microglia.\textsuperscript{19} Interestingly, most genes linked to nitric oxide and ROS generation (for example, CAT, KL, PPP1CB, JAK and RAP1B) were upregulated in LD-high microglia (Fig. 2c). In addition, LD-high microglia were enriched in lipid-related genes, including PLIN3 and the ATP citrate synthase ACLY, which is involved in lipogenesis (Supplementary Table 1), and ‘fatty acid ß-oxidation’ was one of the top enriched pathways (Extended Data Fig. 2). Intriguingly, annotated functional transcriptomics predicted the proinflammatory endotoxin LPS as the most significant upstream regulator, which suggests that there is a link between innate inflammation and lipid droplets in microglia (Fig. 2f).

Fig. 1 | Microglia in the aged brain accumulate lipid droplets. a, Electron microscopic images of microglia from young (3-month-old) and aged (20-month-old) male mice. b, Hippocampi from aged mice stained for BODIPY\textsuperscript{+} lipid droplets and TMEM119\textsuperscript{+} microglia. The right-most panels show 3D reconstructions of BODIPY\textsuperscript{+} TMEM119\textsuperscript{+} microglia. Arrows indicate lipid droplets. c–e, Quantification of BODIPY\textsuperscript{+} lipid droplet numbers (c), percentage of BODIPY\textsuperscript{+} TMEM119\textsuperscript{+} cells (d) and average BODIPY\textsuperscript{+} lipid droplet size (e) in the hippocampus (dentate gyrus). n = 6 mice per group. f, Representative image of PLIN3\textsuperscript{+} lipid droplets and TMEM119\textsuperscript{+} microglia in aged mice. g, Confocal images of PLIN2\textsuperscript{+} lipid droplets and IBA1\textsuperscript{+} microglia (in the human hippocampus of a 22-year-old individual and a 67-year-old individual. Arrows indicate PLIN2\textsuperscript{+} IBA1\textsuperscript{+} cells. h,i, Representative images (h) and quantification (i; P = 0.01) of CAR5\textsuperscript{+} signals (2,845 cm\(^{-1}\)) in TMEM119\textsuperscript{+} microglia in the hippocampus of young and aged mice. n = 5 mice per group. j,k, Experimental schematic of the lipidomics analysis of lipid droplets isolated from whole hippocampus and from FACS-sorted microglia from 20-month-old mice (j), and pie charts (values in percentages; each pie chart represents 100\%) showing the composition of lipid droplets (k). n = 4 mice per group. Statistical tests: two-sided Student’s t-test. Data represent the mean ± s.d. **P < 0.01, ***P < 0.001. Data in f and g were replicated in at least two independent experiments. Scale bars, 1\(\mu\)m (a), 20\(\mu\)m (b,g,h), 10\(\mu\)m (f). Ly, lysosome; n, nucleus.
Finally, we compared the transcriptional profile of LD-high microglia with that of microglia in aging, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease, and of microglia subpopulations recently identified in development, aging and disease, including DAM, MGN and other microglia clusters reported in previous studies. We found a moderate overlap between genes...
Flow sorting scheme for the isolation of BODIPYlo (LD-low) and BODIPYhi (LD-high) CD11b+CD45+ cells from the hippocampi of 18-month-old male mice. The percentage overlap denotes the fraction of genes in each gene list that are upregulated or downregulated in LD-high microglia.

RNA-seq of LD-low and LD-high microglia from aged mice reveals transcriptional changes linked to phagocytosis and ROS production.

- **Fig. 2**  RNA-seq of LD-low and LD-high microglia from aged mice reveals transcriptional changes linked to phagocytosis and ROS production.
  - **a.** Flow sorting scheme for the isolation of BODIPYlo (LD-low) and BODIPYhi (LD-high) CD11b+CD45+ cells from the hippocampi of 18-month-old male mice. n = 3 samples per group. Each sample is a pool of microglia from the hippocampi of three mice.
  - **b.** Representative images of microglia after brain homogenization and marker staining before (top) and after (bottom) FACS sorting. Scale bars, 5 μm.
  - **c.** Volcano plot showing differentially expressed genes in LD-high versus LD-low microglia. The dashed line represents the q < 0.05 cutoff (two-sided Student’s t-test, Benjamini–Hochberg FDR).
  - **d.** Heatmap showing the top 50 differentially expressed genes ranked by log2(FC).
  - **e.** LD-high vs LD-low pathway enrichment.
  - **f.** Upstream regulators.
  - **g.** Gene set enrichment analysis.

Overlap between gene lists (%)

Overlapping genes in phagosome maturation (red) and ROS production (purple) are highlighted. Upstream regulator analysis of top 200 differentially expressed genes between LD-low and LD-high microglia (Fisher’s exact test, Benjamini–Hochberg FDR). Overlap between genes changing in microglia in aging and neurodegeneration (aging, Alzheimer’s disease (AD), ALS, DAM and MGnD), and genes upregulated (yellow) or downregulated (blue) in LD-high microglia. The percentage overlap denotes the fraction of genes in each gene list that are upregulated or downregulated in LD-high microglia.
differentially expressed in LD-high microglia and the cluster 3 microglia identified from a previous study⁹, a microglia subset mainly detected in embryonic day 14.5 brains with a transcriptional signature linked to inflammation and metabolic pathways (Extended Data Fig. 2c–i). Furthermore, genes downregulated in LD-high microglia partially overlapped with published gene sets of microglia in aging and neurodegeneration (Fig. 2g). However, the downregulated genes of LD-high microglia primarily matched with genes that were upregulated in microglia in aging and in DAM (for example, AXL, CD74, CLEC7A and CYBB) (Extended Data Fig. 2).

Overall, these data suggest that lipid-droplet-containing aged microglia show transcriptional changes of genes related to key microglia functions such as phagocytosis, ROS production and immune signaling, yet they have a unique transcripts signature that is distinct from previously described microglia states observed in aging and neurodegeneration. We therefore designate this microglia state as LDAM and use this term henceforth.

The innate TLR4 ligand LPS induces lipid droplet formation in microglia. LPS is the main upstream regulator of genes differentially expressed between LD-low and LD-high microglia (Fig. 2f), and immune cells such as macrophages, neutrophils and eosinophils accumulate lipid droplets in response to inflammatory conditions⁹. To determine whether inflammation triggers lipid droplet formation in microglia, we treated the mouse microglia-derived BV2 cell line with LPS and found a fivefold increase in the number of BODIPY⁺ cells and BODIPY mean fluorescence compared with control cells (Fig. 3a–d). To confirm the identity of these BODIPY-labeled droplets, we used triacsin C, which is an inhibitor of long-chain acyl-CoA synthetase that inhibits de novo synthesis of glycerolipids and prevents lipid droplet formation¹⁰. Indeed, treatment with triacsin C abolished the LPS-induced increase in BODIPY⁺ cell number and fluorescence (Fig. 3a–d).

To explore whether LPS-induced lipid droplets in BV2 cells resemble those in microglia from aged mice, we compared their lipid composition using mass spectrometry. Interestingly, they had a highly similar overall lipid content and a similar composition of neutral lipids, with low amounts of CEs but high levels of glycerolipids (MAG and TAG). In contrast, lipid droplets from the liver contained high levels of CEs (Fig. 3e; Extended Data Fig. 3). In addition, lipid droplets from BV2 cells and from aged microglia showed a similar chain-length distribution of TAG-associated fatty acids, with peaks at 38:2 and 44:5, while liver lipid droplets had a significantly less zymosan uptake (Extended Data Fig. 4).

To determine whether lipid-droplet-containing microglia had altered phagocytic activity, we induced lipid droplets in BV2 cells and exposed the cells to zymosan nanoparticles, which serves as a fluorescent indicator of cellular uptake to acid compartments and lysosomes. In line with previous reports¹¹, LPS increased phagocytosis (Fig. 4f–i) yet, interestingly, zymosan particles were mainly found in the BODIPY⁻ cell population and to a significantly lesser extent in lipid-droplet-rich BODIPY⁺ cells (Fig. 4f,g). Furthermore, triacsin C treatment increased zymosan phagocytosis in LPS-treated cells (Fig. 4h,i). Of note, to test lipid droplet formation in BV2 cells and its effects on phagocytosis in a system that mimics the aging environment, we treated BV2 cells with aged plasma, which has been shown to activate microglia and to trigger brain aging²⁰. Indeed, aged plasma induced lipid droplet formation, and cells with high numbers of lipid droplets showed significantly less zymosan uptake (Extended Data Fig. 4).

Next, to analyze phagocytosis in LDAM, we prepared acute organotypic brain slices from 12-month-old mice and treated them with pHrodo Red Zymosan Particles. Remarkably, the percentage of zymosan⁺ BODIPY⁺ microglia was tenfold lower compared with zymosan⁺ BODIPY⁻ microglia (2.1% versus 24.3%), which indicates
that LDAM have severe defects in phagocytosing zymosan (Fig. 4j,k). In addition, we assessed the phagocytic activity of LDAM in vivo by injecting myelin debris, a phagocytic substrate that accumulates in the aging brain, into the hippocampus of aged mice (Fig. 4l). We labeled the myelin debris with a constitutively fluorescent dye (Alexa Fluor 555, A555) and found that LDAM...
phagocytosed significantly fewer A555+ myelin particles compared with microglia without lipid droplets (Fig. 4m,n).

Collectively, these data suggest that lipid-droplet-rich microglia exhibit phagocytosis deficits and that increased lipid storage is associated with impaired phagocytosis.

**LDAM produce high levels of ROS and show excessive release of proinflammatory cytokines.** Aged microglia are one of the main sources of increased ROS levels in the aging brain3, and excessive microglial ROS production might contribute to age-related CNS dysfunctions. ‘Production of NO and ROS’ was the second most significantly regulated pathway in LDAM (Fig. 2e), with over 90% of the differentially expressed genes increased (Fig. 5a). Consistent with these transcriptional changes, aged hippocampal microglia showed higher fluorescence after treatment with CellRox, a dye that is nonfluorescent in a reduced state but exhibits bright fluorescence after oxidation by ROS, than young microglia (Fig. 5b,c). Moreover, the CellRox signal was increased by twofold in LD-high microglia compared with LD-low microglia (Fig. 5d,e), which suggests that the elevated ROS levels in aged microglia might be specifically driven by the increased ROS generation of LDAM. LPS-treatment-induced elevated ROS generation in BV2 cells, and inhibition of lipid droplets with triacsin C was sufficient to significantly reduce ROS levels (Fig. 5f,h). Cells with high CellRox fluorescence were often loaded with lipid droplets (Fig. 5g). Together, these findings demonstrate that LDAM have elevated concentrations of ROS and suggest that lipid droplets mediate LPS-induced ROS generation.

Another characteristic of aged microglia is the increased production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 under baseline conditions and excessive cytokine release following immune challenge. To determine the cytokine expression profile of LDAM, we acutely isolated hippocampal LD-low and LD-high microglia from aged mice and measured cytokine concentrations in the supernatant using a multiplex array 8 h after stimulation with LPS or saline (as the control). We found that under baseline conditions (saline treatment), LD-high microglia released increased levels of multiple cytokines, including CCL3, CXCL10 and IL-6, compared with LD-low microglia. In addition, LD-high microglia showed a strongly exaggerated release of multiple cytokines, such as IL-10, CCL3, CCL4, IL-6, CXCL5, TNF-α, IL-1β, IL-1α, CXCL1 and CXCL10, after LPS treatment compared with LD-low microglia (Fig. 5i,j). These findings suggest that LDAM are in a primed activation state that becomes hyperactivated after stimulation with LPS.

**CRISPR–Cas9 screen identifies genes linked to neurodegeneration as genetic regulators of lipid droplet formation.** To investigate the genetic regulators of microglial lipid-droplet formation, we performed pooled clustered regularly interspaced short palindromic repeats (CRISPR–Cas9) screens. Informed by our RNA-seq data of LDAM (Fig. 2c,e), we chose to screen with a single guide RNA (sgRNA) library targeting ~2,000 genes involved in the lysosomal pathway and protein degradation and in cellular stress, with ten distinct sgRNAs targeting each gene along with ~1,000 negative control sgRNAs. To probe the role of these genes in lipid droplet formation, we used the microglial BV2 cell line to generate a pooled population of targeted BV2 cells for every gene represented in the sgRNA library. LPS was used to induce lipid droplets in these cells.

To identify sgRNAs that inhibited or promoted the formation of lipid droplets, we developed a photoirradiation selection strategy in which BV2 cells are separated on the basis of their capacity to form lipid droplets. By adding iodine atoms to the lipid droplet marker BODIPY (iodo-BODIPY (iBP)), we transformed this molecule into a photosensitizer that induces cell death in iBP+ cells after photoexcitation52 (Fig. 6a). To prove the efficacy of this photoblation approach, we used Calcein live-cell imaging and found that irradiation of iBP+ BV2 cells selectively killed lipid-droplet-rich cells (Fig. 6b,c).

After three rounds of selection against lipid-droplet-rich cells, we sequenced the sgRNA composition of the selected lipid-droplet-negative BV2 cells. We found 112 genes that were significant positive or negative regulators of lipid droplet formation (P < 0.05; false discovery rate (FDR) < 5%) (Fig. 6e). Unexpectedly, the top hits included various genes that have been previously linked to neurodegeneration, including Slc33a1, Snx17, Vps35 and Grn3,31, which hints at a possible relationship between lipid storage in microglia and neurodegeneration.

We generated individual BV2 cell lines with CRISPR deletions of selected hits that were detected as negative (Slc33a1, Grn and Vps35) or positive (Snx17) regulators of lipid droplet formation in the screen. Indeed, BV2 cells with sgRNAs targeting Grn, Slc33a1 and Vps35 had significantly more lipid droplets than BV2 cells with control sgRNAs, and sgRNAs targeting Snx17 inhibited lipid droplet formation after LPS treatment (Fig. 6f–g). We used flow cytometry to measure ROS levels and found significantly increased ROS generation in BV2 cells with sgRNAs targeting Grn and Slc33a1 cells under baseline conditions and in Slc33a1 sgRNA expressing cells after LPS treatment (Fig. 6h). Next, we assessed phagocytosis by analyzing pHrodo Red Zymosan Bioparticle uptake and found that cells with sgRNAs targeting Grn and Slc33a1 had significant deficits in zymosan uptake compared with control cells. In cells expressing Vps35 sgRNA, phagocytosis was specifically compromised after LPS treatment, but not under baseline conditions (Fig. 6i).

Grn−/− mice contain high numbers of lipid-droplet-rich microglia that have functional impairments and a transcriptional signature similar to LDAM. To confirm the findings from our screen in vivo,
we analyzed lipid droplet numbers in microglia in Grn<sup>−/−</sup> mice. GRN mutations are linked to the development of FTD<sup>35</sup>, and Grn<sup>−/−</sup> mice are used as a model for FTD and are characterized by microglial changes, neuroinflammation and cognitive deficits<sup>37</sup>. Given the results of our CRISPR–Cas9 knockout screen, we hypothesized that the GRN knockout would promote lipid droplet formation. We used middle-aged (9–10 months) mice, because Grn<sup>−/−</sup> mice at this age already show behavioral changes and brain impairments<sup>37</sup>, while wild-type mice present only minor signs of neuroinflammation and contain low numbers of LDAM. We found that microglia in the
hippocampus of Grn−/− mice contained high numbers of lipid droplets, resulting in a twofold higher percentage of BODIPY+ microglia and twice as many lipid droplets per cell in Grn−/− mice compared with wild-type littermates (Fig. 7a–c). Moreover, we frequently detected BODIPY+ IBA1+ microglia in the thalamus and occasionally in the cortex and corpus callosum in Grn−/− mice (Extended Data Fig. 5).

Compared with LD-low Grn−/− microglia, LD-high microglia showed decreased uptake of pHrodo zymosan in an in situ slice culture phagocytosis assay (Fig. 7d,e), increased levels of CellROX fluorescence in the ROS assay, and elevated secretion of proinflammatory cytokines after stimulation with LPS (Fig. 7f,g). Almost 2,000 genes were differentially expressed between LD-high and LD-low microglia (Fig. 7h), and these were linked to metabolic processes and cellular responses to stress (Fig. 7i), pathways that are also enriched in LDAM (Fig. 2e; Extended Data Fig. 2b). Based on both the differences in gene expression (Fig. 7j) and the significance of these changes (Fig. 7k), LD-high Grn−/− microglia are highly similar to LDAM.

Finally, we sought to explore the transcriptional commonalities of lipid-droplet-rich microglia in aging mice, in Grn−/− mice and in LPS-treated young mice. We found that although only nine common genes were differentially expressed in all three data sets (one of them was the lipid-droplet-specific gene PLIN3), the directionality of expression changes of LDAM genes was remarkably similar for all groups and almost identical for LDAM and LD-high Grn−/− microglia (Fig. 7j,m; Extended Data Fig. 6). To identify enriched pathways for genes shared between lipid-droplet-rich microglia, we used the nine genes shared between all datasets and additionally included the top 20 common genes between LDAM and LD-high Grn−/− microglia because of their high transcriptional similarity. Interestingly, significant pathways included ‘endosome lumen’, ‘ROS production’ and ‘lysosome’, pathways linked to the dysfunctional phenotype observed in LDAM and in LD-high Grn−/− microglia (Fig. 7n,o).

Together, these findings validate the in vivo relevance of our in vitro CRISPR screen and confirm that microglia from Grn−/− mice contain lipid droplets. Remarkably, LD-high microglia from Grn−/− mice and LDAM in aging mice have similar functional and transcriptional phenotypes, which suggests that lipid-droplet-containing Grn−/− microglia share the LDAM state.

Discussion

In this study, we identified a novel microglia state in the aging brain and showed that LDAM are characterized by a unique transcriptional signature, severe functional deficits and a proinflammatory phenotype.

What is the role of LDAM in the aging brain? During aging, microglia undergo profound transcriptional and functional changes. They are considered to be in a primed state and show an increased baseline production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6, and become hyperactivated following immune challenge.

Whether lipid-droplet-containing cells are beneficial or detrimental remains an open question.

What causes lipid droplet formation in LDAM? Lipid droplets can form due to various environmental and cellular conditions, including elevated concentrations of extracellular lipids, inflammatory events, increased ROS levels and intracellular metabolic changes.

This is in line with findings that have shown that lipid-droplet-containing immune cells in the periphery are detrimental, including foam macrophages in atherosclerosis or lipid-droplet-rich eosinophils in experimental models of allergy. However, lipid droplets have also been correlated with beneficial functions, such as improved host defense and antigen cross-presentation in myeloid cells.

In the brain, a protective role has been reported for lipid droplets in glia in a Drosophila model for neurodegeneration.

Whether lipid-droplet-containing cells are beneficial or detrimental may depend on the cell type, the environmental context and the composition of lipid droplets. Ultimately, pharmacological and genetic ablation experiments will be required to define the role of LDAM in brain aging and disease.

Fig. 5 | LDAM and lipid-droplet-rich BV2 cells show increased ROS production, and LDAM secrete elevated levels of inflammatory cytokines.

**a** Pathway map of genes related to ROS production that are differentially expressed in LDAM (see Fig. 2). **b** Schematic of the ROS analysis in primary microglia from young (3-month-old) and aged (20 month-old) male mice. **c** Representative flow cytometry histogram (left) and quantification (right) of CellROX fluorescence in primary microglia from young and aged mice (P < 0.0013). **d** Gating scheme for BODIPY+ (LD-low) and BODIPY+ (LD-high) microglia from aged mice (P < 0.0025). **f** CellROX fluorescence in BV2 cells treated with PBS or LPS (5 μg ml−1) for 18 h, co-treated with triacsin C (1 μM) or saline. Representative images of CellROX+ signal in BV2 cells (f). Confocal images showing BODIPY+ and CellROX+ in LPS-treated BV2 cells (g). Flow cytometry histogram and quantification of CellROX fluorescence in BV2 cells (h). **i** Acutely isolated LD-low and LD-high primary microglia from aged mice were treated with LPS (100 ng ml−1) for 8 h, and cytokine concentrations in the media were measured using a multiplex array. n = 4 biologically independent samples, pooled from two independent experiments (n = 3 mice per experiment); each sample corresponds to microglia isolated from the hippocampus of one mouse. Heatmap showing changes in cytokine secretion under baseline conditions and after LPS treatment (i) and individual dot plots of selected cytokines (j).

Experiments were performed three times (BV2 cells) or two (primary cells) times in technical triplicates. Primary cells were isolated from three mice per group per experiment. Statistical tests: two-sided Student’s t-test (**c,e**), one-way ANOVA (h,j) followed by Tukey’s post hoc test. Horizontal lines in the box plots indicate medians, box limits indicate first and third quartiles, and vertical whisker lines indicate minimum and maximum values (**c,e,h**). Data represent the mean ± s.d. (**c,e,j**) and individual dot plots of selected cytokines (j). Scale bars, 20 μm (**f**), 5 μm (**g**).
buildup of lipid droplets in microglia, and it is tempting to speculate that age-related neuroinflammation provokes the formation of LDAM.

Besides inflammation, metabolic changes toward increased fatty acid production have been reported to cause lipid droplet formation in immune cells and in cancer cells. Interestingly, genes...
KO promotes a key enzyme in the shift from the tricarboxylic acid (TCA) cycle to lipid formation and has been shown to cause lipid droplet accumulation, was significantly higher in LDAM than in microglia without lipid droplets. Similarly, RNA-seq analysis of lipid-droplet-rich...
microglia in LPS-treated young mice and in Grn−/− mice revealed significant enrichment of pathways related to metabolism, including TCA cycle and fatty acid β-oxidation. Moreover, LDAM showed a significantly higher NAD+/NADH ratio compared with microglia without lipid droplets, which implies that there are metabolic alterations in these cells (Extended Data Fig. 7). These findings suggest that there is increased lipid synthesis in LDAM, yet it remains to be shown to which extent metabolic changes are involved in microglial lipid-droplet formation in aging.

Cholesterol accumulation has recently been observed in phagocytes in a model of experimental autoimmune encephalomyelitis demyelination in aged mice as a result of excessive uptake of myelin debris45,46. Because demyelination is a characteristic of brain aging, we analyzed the cholesterol content of lipid droplets in LDAM. We did not find ultrastructural evidence for cholesterol crystals, and lipidomics analysis revealed that these lipid droplets contained mainly glycerolipids and only low amounts of CE. Moreover, the lipid composition of lipid droplets from young microglia and from old microglia was almost identical (Extended Data Fig. 2). Thus, we suggest that demyelination does not contribute to lipid droplet formation in LDAM.

In a Drosophila model of neurodegeneration45,46, lipid droplets in glia form due to an APOE-dependent transfer of lipids from neurons to glia. Given that transcription of APOE and other lipid transporters was either downregulated or not significantly regulated in LDAM (Supplementary Table 1), it is unlikely that this mechanism leads to lipid droplet formation in aged microglia.

Role of lipid droplets in the LDAM functional phenotype. Increased ROS generation is a main characteristic of LDAM and was observed in peripheral lipid-droplet-containing immune cells. Reports about whether ROS is a cause or consequence of lipid droplet formation are contradictory47,48. Interestingly, our in vitro results demonstrated that pharmacological inhibition of lipid droplet formation with triacsin C prevented ROS generation in BV2 cells, which supports the idea that triglycerides and lipid droplets have a causal role in the LPS-induced generation of ROS. But it is possible that elevated ROS initially trigger lipid droplet formation, and subsequently lipid droplets induce ROS formation and exacerbate intracellular ROS load. LDAM showed severe phagocytosis deficits compared with microglia without lipid droplets in the aging brain. This finding is in line with a previous report which observed that at the sites of atherosclerotic lesions, lipid-droplet-rich foamy macrophages show decreased phagocytosis activity compared with macrophages without lipid droplets49. Again, defective phagocytosis could be a cause or consequence of lipid droplet accumulation. Our in vitro findings showed that pharmacological inhibition of lipid droplet formation significantly increased phagocytosis in BV2 cells, which suggests that lipid droplets have a detrimental role for phagocytosis. The exact mechanism of how lipid droplets might interfere with phagocytosis remains to be shown. In this context, a study of macrophages has shown that engulfment of cellular debris relies on the availability of free fatty acids, which are released following the degradation of lipid droplets, thus linking effective lipid droplets with successful phagocytosis50. Notably, our RNA-seq analysis revealed that ADRB1 and ADRB2, two key enzymes in the process of lipid degradation, were significantly downregulated in LDAM. This suggests that there is impaired lysis of lipid droplets in LDAM, thus the subsequent lack of free fatty acids might be an underlying reason for impaired phagocytosis in these cells.

In addition to enzymatic lysis, lipid droplets can be degraded by lysosomes, a process termed lipophagy. Intriguingly, we observed that LDAM contained high numbers of lysosomes, and these lysosomes accumulated in close contact to lipid droplets. It is possible that in LDAM, lysosomes are used for lipophagy of lipid droplets rather than for degrading phagocytosed material, resulting in impaired phagocytosis. Finally, there is emerging evidence to indicate that lysosomes become dysfunctional in aged microglia51. Hence, there is the possibility that in LDAM, lipid droplets accumulate due to defective degradation processes, and lipid droplet accumulation and impaired phagocytosis could be two co-existing yet independent processes that are the consequence of defective lysosomes.

LDAM in neurodegeneration. Recently, it has been shown that several subsets of microglia with unique molecular and functional phenotypes exist in the healthy, the aged and the degenerating brain52. The LDAM transcriptional signature showed almost no overlap with these signatures, and, surprisingly, typical aging genes were not expressed in LDAM.
**Gm** −/− LD-high vs LD-low pathway enrichment

- TCA cycle and respiratory electron transport
- Glycolysis/glucose metabolism
- TCA cycle
- Oxidative phosphorylation
- Glucose catabolic process to pyruvate
- Cellular response to oxidative stress
- Fatty acid degradation
- Fatty acid beta oxidation
- ApoE and miR-146 in inflammation and atherosclerosis
- Cholesterol metabolism

**Pathway enrichment (shared genes)**

- Integral component of plasma membrane
- Longevity regulating pathway
- Endosome lumen
- Neutral amino acid transmembrane transporter activity
- β-adrenergic receptor signaling pathway
- Prostaglandin synthesis and regulation
- ROS, RNS production in response to bacteria
- Lysosome
such as AXL, CLEC7A and CYBB, were regulated in a reciprocal direction in LDAM. Furthermore, TREM2 and APOE, two key genes involved in the progression of neurodegeneration that are upregulated in DAM and MGNd, were not regulated in LDAM. Likewise, LDAM showed only low overlap with the transcriptome of recently identified ‘lipid-associated macrophages’ from mouse and human adipose tissue, a cell population that controls metabolic homeostasis in a TREM2-dependent manner and is transcriptionally similar to DAM40. LDAM and DAM also show different functional phenotypes; while DAM are actively phagocytic populations, LDAM are severely impaired in this function. The specific dynamics of the LDAM state and the possible conversion between different microglia states during aging and disease remain to be investigated.

By using pooled CRISPR–Cas9-targeted screening, we discovered genes for which variants cause autosomal-dominant forms of neurodegeneration (SLC33A1, SNX17, GRN, VPS35, CLN3 and NPC2) as genetic modulators of lipid droplet formation in microglia13,15. Indeed, sgRNAs targeting these genes were able to increase (Slc33a1, Grn and Vps35) and decrease (Snx17) lipid droplet loads in microglial BV2 cells. In addition, sgRNAs targeting Grn and Slc33a1 induced significant defects in phagocytosis and increased ROS production in BV2 cells, hence recapitulating the functional impairments of LDAM. Excitingly, knockout of Grn, which causes FTD in people with loss-of-function mutations in this gene41, resulted in severe accumulation of lipid droplets in microglia in vivo. These lipid-droplet-rich microglia in Grn−/− mice showed a similar transcriptome signature and the same functional impairments as LDAM. The finding of lipid-droplet-containing microglia in Grn−/− mice is corroborated by a recent study that showed that cultured progranulin-deficient macrophages exhibited increased foam cell formation (that is, lipid droplet accumulation) compared with wild-type macrophages42. In addition, a recent study reported that loss of GRN leads to intracellular and intralysosomal accumulation of long polyunsaturated TAGs in the brains of humans and mice. Since TAGs are a major component of lipid droplets in LDAM, it is possible that lipid droplet accumulation in GRN-deficient microglia contributes to elevated TAG levels in brains lacking GRN. It has to be shown to what extent lipid-droplet-containing microglia contribute to disease pathology in FTD.

Together, we showed that LDAM demonstrated a novel state of microglia with a unique transcriptional signature and functional impairments in the aging brain, and we identified lipid-droplet-containing microglia in a Grn−/− mouse model of chronic neuroinflammation and in an LPS-induced acutely inflamed brain milieu. Future studies will show whether LDAM are a common characteristic of neuroinflammation and whether they have a role in neurodegenerative diseases. In the future, targeting LDAM might represent an attractive and druggable approach to decrease neuroinflammation and to restore brain homeostasis in aging and neurodegeneration, with the goal of improving cognitive functions.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-019-0566-1.

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Methods

Animals. Aged C57BL/6J male wild-type mice (18–20 months old) were obtained from the National Institute on Aging, and young C57BL/6J males (2–4 months of age) were purchased from The Jackson Laboratory. Grm2−/− mice (B6.129S4(FVB)-Grm2tm1(Jax)Har/Mmja) and wild-type littermates were bred and aged in-house, but were originally acquired from The Jackson Laboratory. Mice were housed under a 12-h light–dark cycle in pathogen-free conditions in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All animal procedures were approved by the VA Palo Alto Health Care System Institutional Animal Care and Use Committee (IACUC) and the Stanford University IACUC. Male mice were used for all experiments.

LPS injections. Male wild-type mice (3 months old) were intraperitoneally injected with LPS (from E. coli, Sigma) at a dosage of 1 mg per kg body weight once a day for four consecutive days. Control mice were injected with body-weight corresponding volume of PBS. After 24 h of the last LPS injection, mice were killed, brains were extracted and brain tissue was processed for immunohistochemistry staining (see “Perfusion and tissue processing” and “Immunohistochemistry and BODIPY staining” below) or for microglia isolation (see “Microglia isolation” below).

Electron microscopy. Three-month-old and 20-month-old male C57BL/6J wild-type mice (n = 3 per group) were anesthetized with 3.8% chloral hydrate (w/v) and transcardially perfused with 0.9% saline followed by 2% paraformaldehyde (PFA)/2.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and postfixed in the same fixative overnight at 4 °C and then stored in 0.1 M PB. Sagittal 200-µm sections were cut on a Leica VT1000S vibratome (Leica). The sections were incubated in 2% osmium (Electron Microscopy Sciences) for 2 h, rinsed in 0.1 M PB, dehydrated in a graded series of ethanol and embedded in Araldite (Durcupan, Electron Microscopy Sciences). Semi-thin (1.5 µm) and ultrathin (70–80 nm) sections of the dentate gyrus were cut using a Reischert Om-U 3 ultramicrotome (Leica). Ultrathin sections were mounted on Formvar-coated 75-mesh copper grids, contrasted with aqueous solutions of uranyl acetate (0.2%) and lead citrate (3%), and analyzed at 80 kV in a EM 910 transmission electron microscope (Zeiss) equipped with a Troendle sharp-eye 2k CCD camera.

To evaluate the ultrastructure of microglia within the dentate gyrus, ten ultrathin sections were analyzed per mouse. Microglia were identified using a combination of ultrastructural characteristics, including a highly electron-dense cytoplasm and nucleus, an often star-shaped cell morphology, an irregularly shaped nucleus with coarsely clumped chromatin, and a cytoplasm-rich in free ribosomes and vesicles. The cytoplasm and nucleus area were analyzed using the software ImageJ v.1.45s.

Perfusion and tissue processing. Mice were anesthetized using Avertin (tribromoethanol) and transcardially perfused with 0.9% NaCl solution. Brains were removed and postfixed in 10% formalin for 3 h at RT and then sectioned sagittally or coronally at 50 µm intervals using a freezing microtome (Leica). Sections were cut at 50 µm in cryoprotectant solution (ethylene glycol, glycerol, 0.1 M PB; pH 7.4; 1:1:2 by volume) until used for immunohistochemistry and CARS imaging.

Immunohistochemistry and BODIPY staining. Free-floating sections were washed three times in PBS followed by 1 h blocking in PBS with 10% donkey serum. Primary antibodies were used in PBS with 10% donkey serum and were followed by primary antibodies for 4 h at 4 °C: rabbit anti-TMEM119 (1:400; Abcam, ab209064); rat anti-CD68 (1:200; Bio-Rad, MCA1957GA); rabbit anti-IBA1 (1:500; Wako, 1:1,000; Wako, 019-19741); and guinea pig anti-PLIN2 (1:200; Fitzgerald, 20R-AP002). After primary antibody incubation, sections were washed three times in PBS and incubated in PBS with 10% donkey serum and the following secondary antibodies for 1 h at RT: donkey anti-rabbit Alexa Fluor 647, donkey anti-rabbit Alexa Fluor 405 (both at 1:500 dilution), and donkey anti-rabbit Alexa Fluor 543 (Jackson ImmunoResearch) (see the protocol in the section “Immunohistochemistry and BODIPY staining” below). Microscopy was performed using a Leica SP5 confocal microscope with spectral detection (Leica Microsystems) and a Leica HCX PL APO CSx63 numerical aperture 1.4 oil-immersion objective. Alexa Fluor 647 was excited at 633 nm and emission detected between 650 nm and 700 nm using a hybrid detector. Transmission images were simultaneously acquired. Label-free CARS microscopy was performed using a commercial setup consisting of an optical parametric oscillator pumped by a picosecond laser source (picoEmlad; APE) integrated into the Leica SP5 microscope. The CARS signal was detected using a 650/210 bandpass emission filter and a non-descanned detector with an emission filter. To detect the transmitted laser, the laser was tuned to 2,845 cm−1, thus enabling imaging of CH symmetric stretching vibrations. CARS and fluorescence/transmission images were sequentially acquired. For quantification of the CARS signal in microglia, 20 randomly selected microglia in the dentate gyrus per animal were imaged. The percentage of TIMM119+ microglia with CARS+ vesicles from the total TIMM119+ microglia was calculated.

Lipidomics. Five-month-old and 20-month-old male wild-type mice (n = 4 mice per group) were perfused, and the hippocampus and the liver were extracted. Alternatively, hippocampal microglia were FACS-sorted from 20-month-old mice (see the section “Microglia isolation” below). BV2 cells were treated for 18 h with 5 µg/ml LPS (from E. coli, InvivoGen) or 5 µg/ml LPS (from E. coli, InvivoGen). LPS (5 µg/ml) was used to induce lipodroplet formation. Lipid droplets from liver, whole hippocampus, isolated microglia and LPS-treated BV2 cells were isolated using a lipodroplet isolation kit from Cell Biolabs according to the manufacturer’s instructions. Lipid droplets were stored at −70 °C until sample preparation and extraction according to a previously described protocol, but with modifications. Before extraction, 10 µl of a synthetic lipid standard mastermix (including 15 different lipids) was added to 90 µl of extraction buffer containing lipodroplet extract.

Extracted lipids were analyzed by flow injection analysis shotgun lipidomics using an eXpert MicroLC 200 system (Eksigent) connected to a TripleTOF 4600 System (AB SCIEX). Each sample was injected twice: one for measurement in the positive ionization mode and one for the negative ionization mode. Instrumental controlling and data acquisition were achieved using the software Analyst TF (v.1.7, AB SCIEX).

Data were processed using Lipid View (v.1.3 beta, AB SCIEX). Lipid identification was based on precursor ion and neutral loss scans specific for...
proposed lipid species. Internal standard correction for each lipid was carried out by normalization against the appropriate synthetic isotopically labeled lipid standard.

**Microglia isolation.** Primary microglia were isolated as previously described.**Mice were perfused with medium A (HBSS, 15 mM HEPES, 0.05% glucose and 1:500 DNase I), and hippocampi were dissected. Hippocampi were chopped and homogenized using a Dounce homogenizer in 2 ml of cold medium A, filtered through a 100-μm cell strainer, rinsed with 5 ml medium A and centrifuged at 340 × g for 5 min. For myelin removal, the precipitate was resuspended in 30% standard isotonic Percoll (30% Percoll in PBS, diluted with medium A) and centrifuged at 900 × g for 20 min. Precipitated cells were washed with HBBS and resuspended in FACS buffer (PBS, 1% BSA and 2 μg/mL EDTA). The samples were stained with 1,300 CD11b-PE and 1,300 CD45-APC for 30 min at RT, centrifuged at 400 × g for 5 min, resuspended in PBS with BODIPY 493/503 (1:2,000 from a 1 mg/mL stock solution in DMSO; Thermo Fisher) and incubated for 10 min at 37°C. Cells were washed two times with FACS buffer and resuspended in FACS buffer with DNase I and 5 μl/mL RNAase inhibitor (Clontech). Dead cells were excluded by staining with Sytox Blue dead cell stain (1:10,000; Invitrogen). Cells were isolated using an ARIA 3.1 (BD Biosciences) with FACSDiva (BD Biosciences), sorted into RLT lysis buffer (Qiagen) with 1% 2-mercaptoethanol and frozen at −80°C.

**RNA isolation and library preparation.** Frozen cells were thawed to RT and total RNA was isolated from cell pellets using a RNAasy Plus Micro kit (Qiagen, 74034). RNA quantities and RNA quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). All samples that passed a quality control threshold (RNA integrity number of ≥9.0) proceeded to library preparations and RNA-seq. Total mRNA was transcribed into full-length complementary DNA using a SMART-Seq v4 Ultra Low Input RNA kit (Clontech) according to the manufacturer's instructions. Samples were validated using the Agilent 2100 Bioanalyzer and an Agilent High Sensitivity DNA kit. Full-length cDNA (150 pg) was processed using a Nextera XT DNA library preparation kit (Illumina) according to the manufacturer's protocol. Library quality was verified using the Agilent 2100 Bioanalyzer and the Agilent High Sensitivity DNA kit. Sequencing of microglia isolated from aged wild-type mice was carried out using an Illumina HiSeq 2000/2500, paired end, 2 × 100 bp depth sequencer, and microglia from 10-month-old Env− mice and from 3-month-old LPS-treated mice were sequenced using an Illumina Novogene 6000, paired end, 2 × 100 bp depth sequencer. The quality of fastq files was assessed using FASTQC (v0.11.4). Reads were mapped to the mouse mm10 reference genome using STAR (v.2.5.1b). Raw read counts were generated with STAR using the GeneCounts function.

**RNA-seq differential expression.** Differential expression in RNA-seq was analyzed using the package DEseq2 (ref. 49). Read counts were used as input and normalized using built-in algorithms in DESeq2. Pairwise comparisons among the two groups (BODIPY+ and BODIPY− microglia) were done on all genes and 12,129 genes with calculable fold changes and FDRs were used for further analysis. The FDR was estimated using the Benjamini and Hochberg approach. R was used for RNA-seq data visualization and ingenuity pathway analysis (IPA), and Enrichr were used to analyze pathways and upstream regulators.

For comparison of microglia transcriptome changes in vivo with published datasets (Fig. 2g; Extended Data Fig. 3), we selected the following published RNA-seq datasets: homeostatic microglia (Keren-Shaul et al. 2017); MGnD versus homeostatic microglia (Grn et al., 2017); MGnD versus dense cell strainer, filtered with 5 ml medium A and centrifuged at 400 × g for 5 min at 37°C. Cells were washed twice with FACS buffer and resuspended in FACS buffer with DNase I and 5 μl/mL RNAase inhibitor (Clontech). Dead cells were excluded by staining with Sytox Blue dead cell stain (1:10,000; Invitrogen). Cells were isolated using an ARIA 3.1 (BD Biosciences) with FACSDiva (BD Biosciences), sorted into RLT lysis buffer (Qiagen) with 1% 2-mercaptoethanol and frozen at −80°C.

**Plasma collection.** Blood from young (2-month-old) and aged (18-month-old) wild-type mice was collected (with EDTA as the anticoagulant) via intracardial bleeding at the time of death. EDTA plasma was obtained from freshly collected blood by centrifugation (1,000 × g for 10 min at 4°C). Plasma was aliquoted and stored at −80°C until use. For BV-2 experiments, thawed plasma was dialyzed in PBS to remove EDTA, and then delipidized using a lipid removal adsorbent (Sigma). Briefly, plasma was mixed with lipid removal adsorbent (40 mg/mL) for 60 min at RT, centrifuged at 2,200 × g for 2 min, and the supernatant was collected. Dialyzed and delipidized plasma was diluted to a working concentration (5%) in DMEM + 5% FBS and incubated for 20 min at RT to allow to clot. The solution was filtered through a polyethersulfone 0.22-μm filter unit and used for cell culture assays.

**Plasma treatment.** BV-2 cells were treated for 18 h with 5% plasma (for preparation see the section "Plasma collection") in DMEM + 5% FBS. Controls received vehicle solution (PBS) only.

**BODIPY in vitro staining.** BV-2 cells were seeded at 5 × 105 cells on poly-l-lysine-coated glass coverslips in DMEM + 5% FBS. Following specific treatments, cells were fixed in 4% PFA for 10 min at 4°C, washed with PBS, permeabilized with 0.1% Triton X-100, and blocked using a blocking solution (5% FBS and 5% bovine serum albumin in PBS). Cells were stained with anti-IBA1 primary antibody (1:100; Wako) and Hoechst 33342 (1:2,000; Thermo Fisher) for 10 min at RT. Sections were washed twice in PBS and mounted on microscope slides with Vectorshield (Vector Laboratories, H-1000). Four randomly selected visual fields per well were acquired using a Nikon Eclipse Ti inverted microscope and 20× objective (×40 magnification) using a confocal scanning laser microscope (LSM 700, Zeiss) with LSM software (ZEN 2011). To analyze the percentage of lipid-droplet-containing BV-2 cells, the numbers of total HoCht+ cells and of HoCht− cells with BODIPY lipid droplets were counted, and the percentage of BODIPY− BV-2 cells was calculated.

**In vitro phagocytosis assay.** For in vitro phagocytosis assays, BV-2 cells were split into 96-well plates at 1,000 cells per well in DMEM + 5% FBS and treated with LPS, triasin C and vehicle solutions, or with 5% plasma and vehicle, for 18 h. Following specific treatments, 5 ng of pHRedo Red Zymosan Bioparticles (Thermo Fisher Scientific, P35364) in 100 μl of DMEM + 5% FBS was added per well. Four phase-contrast and red fluorescent images per well were acquired every 2 h for 16 h using an Incucyte S3 live cell analysis system (Essen Biosciences). For each time point, phagocytosis was calculated by normalizing the red fluorescent area to the phase fluorescence.

**Organotypic brain slices and in situ phagocytosis assay.** Twelve-month-old male wild-type mice (n = 3 mice) and 9-month-old male Env− mice were decapitated, and dissected brains were immediately put in precooled culture medium with serum (65% MEM (Sigma), 10% horse serum, 25% HBSS, 6.5 mg/mL glucose, 2 mM glutamine and 1% pen/strep).

The entire procedure was done on ice with precooled solutions until culturing. Coronal sections were prepared using a vibratome (Leica VT1000S) at 250-μm thickness and then transferred to insert wells (Millicell Cell Culture Insert, 30 mm, Millicell) on a 6-well plate with medium. Sections were incubated for 1 h in the incubator (37°C, 5% CO2), pHRedo Red Zymosan Bioparticles (Thermo Fisher Scientific) were opsonized (three washes in PBS, followed by incubation in plasma for 1 h and in PBS for 45 min at 37°C and three washes in PBS) and added at 0.5 mg/mL to cover the entire section (about 150 μl each). The plate was incubated for 1 h in the incubator (37°C, 5% CO2). After washes with PBS, sections were fixed with 4% PFA for 30 min at RT. Immunohistochemistry for IBA1 and BODIPY staining was performed as described above.

To measure phagocytosis in BODIPY− and BODIPY+ microglia, four randomly selected visual fields per section (three sections per mouse) were photographed using a confocal scanning laser microscope (LSM 700, Zeiss) with LSM software (ZEN 2011). The numbers of BODIPY− IBA1+ and BODIPY+ IBA1+ cells were quantified, and the percentage of zymosan-containing cells was calculated.

**In vivo phagocytosis assay.** Alexa-Fluor-555-labeled myelin (25 mg/mL in PBS) was injected into the hippocampus of 20-month-old male mice using a stereotaxic apparatus (Kopf Instruments). Mice were anesthetized using isofluorane, their skulls were exposed and a hole was drilled at the injection site using aseptic techniques. One microliter of the myelin solution was injected at +0.7-mm lateral, −1.7-mm anteroposterior and −2.04-mm dorsoventral relative to the intersection of the coronal and sagittal suture (bregma) at a rate of 200 nL/min. The needle was left in place for an additional 3 min to allow for diffusion, then slowly withdrawn. Mice received post-surgical buprenorphine and bayertil for pain and infection prevention, respectively. After 48 h, mice were anesthetized and transcardially perfused with 4% PFA. The entire injection site was sectioned (coronal, 40-μm thick) and stained for IBA1 and BODIPY as described above. Five to eight sections were quantified to assess myelin uptake of BODIPY+ IBA1+ cells and BODIPY− IBA1− cells.

**ROS assay.** To assess ROS generation in primary microglia, cell homogenates from 3-month-old and 20-month-old wild-type mice and from 9-month-old male mice...
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NAD+Casp9 assay. Primary microglia from 3-month-old (young) and 20-month-old (aged) wild-type mice were isolated as described above ("Microglia isolation"); hippocampi from three mice were pooled per group), and CD11b+CD45+ primary microglia sorted for BODIPY® and BODIPY® cells from aged mice, were sorted into 5% FBS-containing microglial culture medium (see "Cytokine assay" above). Cells were seeded into 96-well white-walled tissue culture plates at 5,000 cells per well in 50 μl microglial culture medium with 5% FBS and incubated for 30 min in the incubator (37°C, 5% CO2). A NAD+Casp9, Glucose oxidase/TK (Promega) was performed according to manufacturer’s instructions. Cell lysates were incubated for 2 h at RT, and luminescence was recorded using a luminometer (Lmax, Molecular Devices).

Statistical analyses. Data collection was randomized for all experiments. Experimenters were blinded for imaging and data analyses. No sample size calculations were performed. Sample sizes were determined to be adequate based on the magnitude and consistency of measurable differences between groups.

Table 2 for the gene list) at a multiplicity of infection of 10,000. A Cas9-expressing BV2 cells were subcloned to maintain a monoclonal knockout population.

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Author contributions
J.M. and T.W.-C. conceptualized and designed the study, interpreted and analyzed data, and wrote manuscript. J.M. and M.Z. conducted histology and organotypic slice culture experiments. J.M., T.I. and M.Z. performed cell culture experiments. J.M., T.I. and S.E.L. performed the RNA-seq experiments. J.V.P., M.Z. and J.M. performed the stereotactic procedures. V.M. conducted...
the in vivo LPS injections and provided Grn−/− mouse brain sections. J.M., M.S.H. and D.W.M. generated and analyzed the CRISPR-Cas9 screen data. J.M. and B.L. analyzed the RNA-seq data. J.T., T.K.F. and O.H. performed the mass spectrometry experiments. J.M. and H.W. performed the CARS imaging. J.K. and C.R.B. designed and produced the methylated BODIPY derivatives. M.C.B and L.A. reviewed the manuscript.

Competing interests
T.W.-C., J.M., C.R.B. and M.S.H. are co-inventors on a patent application related to the work published in this paper. All other authors have no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41593-019-0566-1. Supplementary information is available for this paper at https://doi.org/10.1038/s41593-019-0566-1.

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Extended Data Fig. 1 | Lipid droplet accumulating microglia are abundant in the hippocampus but rare in other brain regions of aged mice.

**a–d.** Representative confocal images of the cortex (**a**), thalamus (**b**), corpus callosum (**c**) and hippocampal dentate gyrus (**d**) from 20-month old male mice stained for BODIPY⁺ (lipid droplets) and Iba1⁺ (microglia). Scale bar: 20 μm. Arrows point towards BODIPY⁺ lipid droplets. 

**e.** Quantification of BODIPY⁺/Iba1⁺ cells. n = 4 mice per group. One-way ANOVA followed by Tukey’s post hoc test. Error bars represent mean ± SD. ***P < 0.001.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | LDAM have a unique transcriptional signature that minimally overlaps with published gene expression profiles of microglia in aging and neurodegeneration. 

**a,b.** IPA pathway analysis of genes that are significantly upregulated (a) or downregulated (b) in LD-hi microglia in aging. Analysis based on top 100 down- and up-regulated genes (Fisher’s exact test, Benjamini-Hochberg FDR).

**c-g.** Expression plots comparing RNA-Seq data of LDAM (see Fig. 2) with published RNA-Seq data of microglia in aging (**c**), AD (**d**), ALS (**e**), disease-associated microglia (DAM) (**f**) and neurodegenerative microglia (MGN) (**g**). Data are expressed as signed fdr, i.e the product of log2 FC and log10 fdr.

**h.** Paired dot plot showing FPKM values of LD-lo and LD-hi microglia for ApoE (paired Student’s t-test; $P = 0.423$). Dotted lines connect LD-lo and LD-hi microglia sorted from the same samples.

**i.** Heatmap showing expression changes of LDAM genes (genes differentially expressed in LD-hi microglia in aging) in LD-hi microglia from GRN$^{-/-}$ mice, from LPS treated mice, and in microglia clusters revealed by Li et al. (2019) and Hammond et al. (2019)\(^{15,16}\). Sample size in a,b,h: $n = 3$ samples per group. Each sample is a pool of microglia from the hippocampi of 3 mice. LD, lipid droplet.
Extended Data Fig. 3 | LPS treatment induces lipid droplet formation in microglia and in BV2 cells. **a, b**, 3-month-old male mice were given intraperitoneal (i.p.) injections of LPS (1 mg/kg BW) for four days. Representative confocal images of BODIPY⁺ and Tmem119⁺ in the hippocampus (a) and of BODIPY⁺ and Iba1 staining in the cortex, corpus callosum, and thalamus (b). **c-e**, Lipidome profiling of lipid droplets from LPS-treated BV2 cells, primary microglia, and liver tissue. **c**, Pie charts showing that the lipid composition of lipid droplets from young and aged microglia is highly similar, but differs between young and aged liver tissue. **d, e**, Distribution of MAG chain lengths (d) and TAG saturation levels (e) of lipid droplets isolated from LPS-treated BV2 cells and from microglia and liver tissue from aged mice. young = 5-month-old male mice, old = 20-month-old male mice; n = 4 mice per group. Data in **a-b** were replicated in at least two independent experiments. Error bars represent mean ± s.e.m. Scale bars, 20 μm.
Extended Data Fig. 4 | Aged plasma induces lipid droplet formation in BV2 cells. a, Representative micrographs of BODIPY⁺ staining and of phagocytosis of pHrodo red Zymosan in BV2 cells treated with 5% plasma from young (3-months) and aged (18-months) mice for 12 hours. Scale bars, 5 μm. b, Quantification of BODIPY⁺ staining in BV2 cells treated with young and aged plasma. c, d, Quantification of Zymosan uptake in BV2 cells treated with young and aged plasma (c), and in aged plasma treated BODIPY-low and BODIPY-high cells (d). Statistical tests: two-sided Student’s t-test. Error bars represent mean ± SD. *P < 0.05, ***P < 0.001.
Extended Data Fig. 5 | Lipid droplet containing microglia in the cortex, corpus callosum, and thalamus of GRN<sup>−/−</sup> mice. a-c, Representative confocal images of BODIPY<sup>+</sup> (lipid droplets) and Iba1<sup>+</sup> (microglia) in the cortex (a), corpus callosum (b), (c) and thalamus from 9-month-old male GRN<sup>−/−</sup> mice. BODIPY<sup>+</sup>/Iba1<sup>+</sup> cells were frequently found in the thalamus and were detected to a lesser extent in cortex and corpus callosum. Data were replicated in at least three independent experiments.
Extended Data Fig. 6 | Expression changes of LDAM genes in lipid droplet-rich microglia from normal aging, GRN<sup>−/−</sup> and LPS-treated mice. a, Heatmap showing expression changes of LDAM genes (genes differentially expressed in LD-hi microglia in aging; 692 genes) in LD-hi microglia from GRN<sup>−/−</sup> mice and from LPS treated mice.
Extended Data Fig. 7 | LDAM show signs of metabolic alterations. a, Paired dot plot showing FPKM values of LD-lo and LD-hi microglia for ACLY (data obtained from RNA-Seq analysis, see Fig. 2). Dotted lines connect LD-lo and LD-hi microglia sorted from the same samples. P=b, NAD colorimetric assay showing the NAD+/NADH ratio of primary hippocampal microglia from 3-month old mice (young) and of LD-lo and LD-hi primary microglia from 20-month old male mice. Experiments were performed two times in technical triplicates. n=3 mice per group per experiment. Statistical tests: paired two-sided Student's t-test (a) one-way ANOVA (b) followed by Tukey’s post hoc test. Horizontal lines in the box plots indicate medians, box limits indicate first and third quantiles, and vertical whisker lines indicate minimum and maximum values. *P < 0.05, ***P < 0.001.
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- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- Data collection
  - Primary microglia were isolated using an Aria3.1 with FACSDiva software (v6.0.0.485). Lipidomics data were collected using the Analyst TF Software (v 1.7) and processed with Lipid View Software (v1.3 beta). Further, LSM 700 laser scanning confocal microscope, ZEN Black 2010 SP1 (v.6.0.0.485) and Incucyte S3 (Essen) were used for image Acquisition.

- Data analysis
  - Flow cytometry data analyzed using FlowJo (v10), Confocal images were analyzed using ImageJ (v1.45s); 3D images were converted from confocal image stacks using Imaris BITPlane software (v7.6.1). Statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software), and R DESeq2 package was used for RNA-Seq analysis. Ingenuity Pathway Analysis (IPA) and EnrichR were used for pathway analysis of RNA-Seq data. For the CRISPR-Cas9 screen, sgRNA composition of selected cells was analyzed using the Software CasTLE (version 1.0^5; https://bitbucket.org/dmorgens/castle).

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- A description of any restrictions on data availability

Raw sequencing data and raw CRISPR screen data are available from the corresponding author upon request. CRISPR hits and RNA-seq results are provided (Supplementary Table 2).
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Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- **Sample size**: No sample-size calculations were performed. Sample size was determined to be adequate based on the magnitude and consistency of measurable differences between groups. Further, sample sizes were chosen based on prior literature using similar experimental paradigms.

- **Data exclusions**: For osmotic pump infusions, 3 mice were excluded and euthanized prior to experiment endpoint due to post-operative wound dehiscence. These exclusion criteria were predetermined as per relevant animal protocols.

- **Replication**: For in vitro experiments, technical triplicates were performed. For in vivo experiments, biological replicates as well as independent cohorts of mice were used. CRISPR screen data and RNA-seq data were not replicated in independent experiments due to resource restrictions. All other data were replicated in at least two independent experiments as stated in figure legends. All attempts for replication were successful.

- **Randomization**: Data collection was randomized for all experiments.

- **Blinding**: Experimenters were blinded for imaging and data analysis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | Antibodies |
| [ ] | Eukaryotic cell lines |
| [x] | Palaeontology |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |
| [x] | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| [x] | ChIP-seq |
| [ ] | Flow cytometry |
| [x] | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- **Immunohistochemistry:**
  - primary antibodies: rabbit anti-TMEM119 (1:400, Abcam, ab209064, 28-3), rat anti-CD68 (1:200, Bio-Rad, MCA1957GA, FA-11), rabbit anti-Iba1 (1:1000, Wako, 019-19741, NCNP24), goat anti-Iba1 (1:50, Abcam, ab5076), guinea pig anti-Adipophilin (Plin2) (1:200, Fitzgerald, 20R-AP002), guinea pig anti-Perilipin 3 (1:200, Progen, G37).
  - secondary antibodies: donkey anti-rabbit Alexa 555, (1:500, Invitrogen, A-11035), donkey anti-rabbit Alexa 647 (1:500, Invitrogen, A-31572), donkey anti-goat Alexa 405 (1:200, Abcam, ab175664), donkey anti-rat Cy3 (1:500, Jackson Immuno Research, 712-175-153), donkey anti-guinea pig Alexa 488 (1:500, Jackson Immuno Research, 706-545-148).

- **Flow cytometry:**
  - CD11b (clone M1/70, BioLegend, cat. no. 101205, 1:100), CD45 (clone 30-F11, Biolegend, cat. no. 147708, 1:100)

**Validation**

- All antibodies used were validated by manufacturers. Furthermore, antibodies were validated by adding 2nd Antibody controls (withdrawal of primary antibody), and by cellular localization and morphology of stained structures (CD68, Adipophilin, Perilipin3) and by the morphology of stained cells (Iba1, Plin2, Perilipin3).
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | BV-2 cell line; originally obtained from Banca Biologica e Cell Factory, IRCCS Azienda Ospedaliera Universitaria San Martino, Genua, Italy. |
| Authentication | The cell line was authenticated by the supplier, but not further validated by our lab. |
| Mycoplasma contamination | The cell line was tested and are free from mycoplasma contamination. |
| Commonly misidentified lines | No commonly misidentified cell lines were used. |

Animals and other organisms

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| Laboratory animals | Aged C57BL/6J male wild type mice (male, 18-20 months old) were obtained from the National Institute on Aging (NIA), and young C57BL/6J males (male, 2-4 months of age) were purchased from Jackson Laboratory. Grn-/- deficient mice (B6.129S4(FVB)-Gnrntm1.1Far/Mmja) and wild type littermates (male, 8-12 months) were bred and aged in-house but originally acquired from Jackson. |
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight | All animal procedures were approved by the V.A. Palo Alto Committee on Animal Research and the institutional administrative panel of laboratory animal care at Stanford University. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

- Confirm that:
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  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

| Sample preparation | Sample preparation listed in Methods |
| Instrument | FACS Aria 3.1 BD Biosciences |
| Software | FACSDiva software (BD Biosciences) was used to collect the data. Flow cytometry data were analyzed using FlowJo (v10). |
| Cell population abundance | For ROS Analysis, 20,000 BV-2 cells and 20,000 primary microglia, respectively, were analyzed per sample. Microglia were identified by CD11b+CD45-low antibody fluorescence intensity. Lipid droplet-low and lipid droplet-high cells were distinguished based on BODIPY+ fluorescence intensity. For sorting for RNA-seq, at least 10,000 microglia were collected per sample. |
| Gating strategy | Relevant gating strategies shown in Figure 2 |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.