Microglial BIN1 deficiency elicits enhanced microglial inflammatory responses that mimic early AD pathology

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Research Article

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Microglial BIN1 deficiency elicits enhanced microglial inflammatory responses that mimic early AD pathology

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Abstract

Bridging Integrator 1 (*Bin1*) has been identified as the second most important risk locus for developing late-onset Alzheimer's Disease (AD), after *Apoe*. BIN1 is an adaptor protein implicated in cell membrane dynamics and neuronal BIN1 has been linked to tau pathology and cellular transport mechanisms' defects; however, the contribution of microglial BIN1 to AD remains underexplored. To address the role of microglial BIN1 in homeostasis and neuroinflammation, we performed single-nucleus RNA sequencing and further phenotypic analysis in microglia-specific BIN1 conditional knockout (cKO) mouse cortices. Our findings indicate that deleting microglial BIN1 is not sufficient to cause significant changes at the transcriptional and cellular level under homeostatic conditions. Nevertheless, it is sufficient to alter the expression of key genes regulating microglial proliferation and proinflammatory activation in response to systemic inflammation, mostly through the enhancement of the microglial IFN-type I-mediated inflammatory response. Interestingly, our data also indicate that microglial BIN1cKO exerts a non-cell autonomous effect on other brain cell populations, particularly astrocytes, eliciting transcriptional changes in astrocytic reactivity genes in response to inflammation.

Introduction

Alzheimer's disease (AD) is the most prevalent type of dementia that causes progressive loss of cognition, for which there is no effective treatment or cure. In addition to environmental factors, such as aging and inflammation, AD pathogenesis has a strong genetic component. Genome-wide association studies (GWASs) have revealed that single nucleotide polymorphisms (SNPs) are strongly associated with an increased risk of developing AD. SNPs in the locus harboring the Bridging Integrator 1 (*Bin1*) gene show the strongest association with AD, following Apolipoprotein E (*Apoe*) [1]. BIN1 is a membrane adaptor protein implicated in cell membrane modeling dynamics and membrane-mediated endocytosis [2]. *Bin1* undergoes alternative splicing, generating several cell type-specific isoforms that are expressed in neurons, astrocytes, oligodendrocytes and microglia [3,4]. The contribution of neuronal BIN1 to AD risk has been shown to be related to a reduction in neuronal excitability due to a decrease in neuronal BIN1 [5]. Moreover, decreased expression of neuronal as well as astrocytic BIN1 isoforms contributes to greater accumulation of tau tangles and cognitive decline [3]. Similarly, recent evidence from human induced pluripotent stem cell (hiPSC)-derived neurons lacking BIN1 suggests that neuronal BIN1 is sufficient to induce alterations in the endocytic pathway and calcium homeostasis, leading to severe neural network dysfunctions [5]. BIN1 has also been shown to be involved in neuron–microglia cross-talk

in AD-related tau pathology by mediating the release of extracellular vesicles carrying tau from microglia and spreading into the brain parenchyma [6]. However, the impact of microglial-specific BIN1 on brain function and dysfunction and its possible association with the progression of AD remain underexplored.

Given the limited success of neuron-focused approaches in the search for an AD cure, recent research has shifted toward investigating glia-mediated mechanisms as potential drivers of neuronal dysfunction and contributors to AD pathogenesis. Microglia play critical roles in brain homeostasis and development, as well as in the response to injury and disease, and many distinctive states, defined by unique markers localized within the brain, change over time [7]. Alterations in microglial functionality have recently been recognized to play crucial roles in the progression of AD [8], while GWASs have identified many genetic risk factors enriched in microglia and astrocytes in AD [9]. Similarly, the less-studied microglial isoform of BIN1, which has been reported to be differentially expressed in the brains of AD patients, could be a potential genetic mediator of AD progression.

As the primary source of proinflammatory cytokines, microglia are pivotal mediators of neuroinflammation and can induce or modulate a broad spectrum of cellular responses. Moreover, systemic inflammation, which severely affects microglial function, has been shown to have a significant effect on AD pathology [10]. Microglial BIN1 has recently been related to the regulation of the brain inflammatory response in mice [11]; however, further studies are needed to reveal the transcriptional signatures elicited by microglial BIN1 deletion both in microglia per se and in all other brain cell types. This type of investigation is essential, as during AD progression,

human microglia acquire several distinct inflammatory states, each of which exhibit either elevated or decreased BIN1 levels [12], indicating a relationship between the reactive state of microglia and the levels of microglial BIN1 and AD progression.

Given the involvement of neuroinflammation and accompanying microglial dysfunction in AD pathogenesis, we aimed to investigate how microglial BIN1 contributes to the presence of distinct microglial signatures and how they modulate the other brain cell types under both homeostatic and systemic inflammatory conditions. Our data were derived from single-nucleus transcriptome analysis of the cortex of conditional double transgenic mice (Cx3CR1 Cre-ERT2//Bin1$^{fl/fl}$), in which BIN1 has been specifically knocked out in microglia, revealing that under inflammatory conditions, BIN1 deletion results in the enrichment of microglial cell subpopulations exhibiting increased proliferative capacity and an IFN-type I-mediated proinflammatory response. Importantly, these transcriptional changes are sufficient to drive BIN1-deficient microglia toward an enhanced reactive proinflammatory phenotype in response to systemic inflammation. Interestingly, BIN1 deletion in microglia can also elicit transcriptional changes in astrocytes, suggesting a non-cell autonomous role of BIN1 in the brain’s response to systemic inflammation.

Materials and methods

Mice

All mouse strains were maintained in the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. The experimental procedures were performed in compliance with European and National

legislation for Laboratory Animal Use (Guideline 2010/63/EE and Greek Law 56/2013) according to the FELASA recommendations for euthanasia and the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the Hellenic Pasteur Institute (Animal House Establishment Code: EL 25 BIO 013), and License No 193912/08-03-2022 for experimentation was issued by the Greek authorities (Veterinary Department of Athens Prefecture). B6.129S6-Bin1^{tm2Gcp}/J (*Bin1^{flox}* JAX#021145) mice were purchased from The Jackson Laboratory. Cx3cr1$^{tm2.1(cre/ERT2)Litt}$/WganJ mice (JAX# 021160, heterozygous mice, here referred as **Cx3cr1^{CreER}**) were provided to us by Dr. Vasiliki Kyrargyri from the Laboratory of Molecular Genetics of the Hellenic Pasteur Institute. *Bin1$^{fl/fl}$* mice were crossed with *Cx3cr1$^{CreER+/+}$* homozygous mice to generate double heterozygous *Cx3cr1^{CreER}//Bin1$^{fl/+}$* animals (F1 generation). F1 generation animals were then crossed with *Bin1$^{fl/fl}$* to generate *Cx3cr1^{CreER}:Bin1$^{fl/fl}$* (Bin1cKO) experimental animals. *Cx3cr1^{CreER}* heterozygous control animals were generated after crossing *Cx3cr1$^{CreER+/+}$* homozygous mice with C57BL6/J mice provided by the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. Adult male mice that were 8–12 week old were included in all of the experimental procedures. Food and water were available *ad libitum.*

Tamoxifen and LPS administration protocol

Tamoxifen (Sigma) was dissolved in 10% ethanol and 90% sunflower seed oil solution (Sigma) after being vortexed and placed in a water bath at 37°C (stock: 20 mg/ml), after which it was intraperitoneally injected in control and experimental animals (100 mg/kg) for 4 consecutive days. After three

weeks, lipopolysaccharides from *E. coli* 055:B5 (LPS) (Sigma) were dissolved in sterile saline (stock: 1 mg/ml) and intraperitoneally injected (2 mg/kg) to induce neuroinflammation. Control mice for neuroinflammation (homeostatic conditions) were intraperitoneally injected with sterile saline. All of our analyses were performed 48 hours after LPS injection.

Single-nucleus isolation and single-nucleus RNA sequencing

For single-nuclei isolation from liquid nitrogen snap-frozen mouse brains for the single-nucleus RNA sequencing (snRNA-seq) experiment, 4 brains were processed at a time. For the preparation of single-nucleus suspensions, a small portion of the somatosensory cortex (~40-60 mg) was dissected from each brain and added to a 1.5 ml tube containing 300 µl of lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40) supplemented with RNase OUT (40 U/µL, 1:1000 dilution) (Thermo Fisher Scientific). The tissue was homogenized via a plastic pestle, then an extra 200 µl of lysis buffer was added (total 500 µl), and the homogenate was incubated for 5 min, triturated 10 times with a pipette and further incubated for 5 min. Next, 500 µl of wash buffer was added (1% BSA in 1X PBS) supplemented with 1:1000 RNase OUT, gently mixed 5 times with a pipette, followed by filtering through a 70 µm Flowmi strainer (Sigma) into a new 1.5 ml tube and centrifugation at 500 × g for 5 min at 4°C. Next, the supernatant was removed carefully, and the pellet was resuspended in 1 ml of wash buffer containing 1:1000 RNase OUT and then filtered with a 40 µm Flowmi strainer (Sigma) into a new 15 ml tube. The nuclear suspension was further diluted with 1 ml of wash buffer containing 1:1000 RNAse OUT and brought to a final volume of 2 ml. The nuclei were counted via a hemocytometer at a 1:10 dilution.

After the nuclei were counted, a volume containing 1.5×10^6 nuclei (approx. 500-700 µl) was collected and centrifuged at 500 × g for 5 min at 4°C. The supernatant was then carefully removed, and the nuclear pellet was resuspended in 500 µl of staining buffer (2% BSA in 1X PBS) supplemented with 1:1000 RNase OUT. Next, 2.5 µl of Fc receptor blocking solution (TruStain FcX™ PLUS - anti-mouse CD16/32 -, BioLegend) was added to the nuclear suspension, followed by incubation for 10 min at room temperature. For the labeling of neuronal nuclei, we added 1 µg of Anti-NeuN Antibody, clone A60, Alexa Fluor 488 conjugated (1 mg/ml, 1:500 dilution) (Merck Millipore, MAB377X) and 1 µl of a different per sample TotalSeq™-A Hashtag antibody (BioLegend) containing a different barcoded oligo to ensure multiplexing of samples (TotalSeq DNA-Barcoded Oligonucleotide), followed by incubation for 10 min at 4°C. Next, the nuclei were washed, resuspended in 500 µl of wash buffer with 1:500 RNAse OUT and stained with 1 µl of Sytox Orange nucleic acid stain (1:500 dilution) (Thermo Fisher Scientific) for 10 min at room temperature, followed by centrifugation at 500 × g for 5 min at 4°C and resuspension in 500 µl of wash buffer with 1:500 RNAse OUT. This last staining with SYTOX Orange was performed just prior to fluorescence-activated cell sorting (FACS) sorting. Next, we performed FACS sorting using the Cytek Aurora™ CS system, to separate NeuN+ and NeuN- nuclei. We sorted approximately 40,000 NeuN- non-neuronal nuclei and 70,000 NeuN+ neuronal nuclei from each sample. Sorted nuclei from each fraction were centrifuged at 500 × g for 5 min at 4°C and then resuspended in 100 µl of wash buffer containing 1:1000 RNAse OUT. Finally, nuclei from each fraction for each sample were counted with a hemocytometer, and mixes of 5,000 NeuN+ and 20,000 NeuN- nuclei (1:4 ratio) were prepared for each sample. Each mixture of nuclei was centrifuged at 500 × g for 5

min at 4°C, the supernatant was collected very carefully, and the pellet was resuspended in 30 µl of wash buffer containing 1:1000 RNAse OUT. A final mixture was prepared by adding 10 µl from each sample to a total of 40 µl containing approximately 40,000 nuclei from 4 samples.

For the preparation of single-nuclei RNA libraries, we loaded isolated nuclei on a Chromium 10X genomics controller following the manufacturer's protocol using chromium single-cell v3 chemistry and single indexing and the adapted protocol by BioLegend for HTO library preparation. The resulting libraries were pooled at equimolar proportions with a 9:1 ratio for the gene expression library and HTO library. Finally, the pool was sequenced via 100 bp paired-end reads using the NOVAseq 6000 system following the manufacturer's recommendations (Illumina).

Single-Nuclei RNA Sequencing Analysis

All samples were processed with simpleaf (v0.15.1) [13], using the mm10 reference mouse genome and the Ensembl Mus_musculus mm10.98 reference annotation. Ambient RNA was removed via CellBender (v0.3.0). Pooled samples were then demultiplexed using HTODemux function in Seurat. We identified supplemental putative doublets using scDblFinder (version 1.4.0) [14], which was applied to each sample separately (multiSampleMode = "split"), with all other parameters default. After removing doublets, we removed poor-quality cells by excluding cells whose percentage of exonic reads was greater than 75% [15,16] or whose percentage of mitochondrial reads was greater than 10%. This resulted in 18,678 nuclei across 16 samples passing the QC. We identified 2,000 highly variable genes via the FindVariableFeatures function in Seurat (v5.0.1) [17], calculated 50 principal components, and used these as inputs to Harmony

(v1.2.0) [18], with the parameter theta set to 0.1 and the other parameters set to default values. To identify clusters, we used the FindClusters function in Seurat applied to the Harmony outputs, with the resolution set to 0.5. The major cell type identities of the clusters were annotated on the basis of the expression of cell type-specific markers **(Figure 1E, G and Suppl. Figure 1A, B)**. Differentially expressed gene (DEG) analysis was performed via the nonparametric Wilcoxon rank sum test, and the absolute average $\log_2$-fold change (avg $\log_2$FC)>0.25 and p adj value<0.05 were used as thresholds for significance. Gene ontology analysis was performed via gprofiler v0.2.3 [19]. The classification of microglial subtypes/states was performed via CellID v1.12.0 [20].

For comparison with human brain microglia, we used data obtained from the Seattle Alzheimer's Disease Brain Cell Atlas (SEA-AD) consortium

https://cellxgene.cziscience.com/collections/1ca90a2d-2943-483d-b678-b809bf464c30). Microglia isolated from the middle temporal gyrus (MTG) were annotated as previously described [21]. DEGs were identified via the Wilcoxon rank sum test (avg $\log_2$FC >0.25 and p adj value <0.05) to compare microglia obtained from brain samples pathologically classified at the "mid" (III and IV) and "low" (0 to II) Braak stages.

Microglia isolation protocol and flow cytometry assay

The mice were terminally anesthetized with an overdose of isoflurane and perfused with ice-cold 1X PBS (Life Technologies). The cortical area was dissected from the brain, homogenized on ice and passed through a sterile 100 µm cell strainer (Fischer Scientific) with ice-cold 1X PBS. The cell suspension was centrifuged for 5 min at 420 × g at 4°C. The cell pellet was

subsequently resuspended in 70% Percoll (Sigma) solution, and a Percoll gradient was formed as follows: the bottom layer consisted of 70% gradient solution (8 ml), the middle layer consisted of 30% gradient solution (8 ml), and the top layer consisted of 1X PBS (8 ml). The samples were centrifuged for 25 min at 600 × g at RT, with no break and minimum acceleration. After centrifugation, demyelinated microglia were located on the border between the 30% Percoll gradient and 70% Percoll gradient, while myelin and debris were located at the top of the tube in 1X PBS. Approximately 6 ml of demyelinated cells were collected from the 30% and 70% gradient interface, diluted with 25 ml of sterile 1X PBS to remove the remaining Percoll and centrifuged for 10 min at 200 × g at 4°C with full break and acceleration. The cell pellet was washed with 1 ml of cold FACS buffer (2% BSA - Applichem -, 2 mM EDTA - Merck - in 1X PBS, sterile and filtered) and centrifuged again for 6 min at 600 × g at 4°C.

The cells were stained as follows: the cell pellet was resuspended in 150 µl FACS buffer containing 1:100 purified rat anti-mouse CD16/CD32 (Fc blocker, BD) and incubated for a maximum of 20 min on ice. Then, the mixture of the antibodies for cell labeling was added. The mixture contained PE-conjugated anti-mouse/human CD11b (BioLegend, 1:150), APC/Cyanine7-conjugated anti-mouse CD45 (BioLegend, 1:150) and APC-conjugated anti-mouse CD11c (BioLegend, 1:50) in 150 µl of FACS buffer. The samples were incubated on ice. After 30 min, 1 ml of FACS buffer was added to terminate the staining procedure, and the sample was centrifuged for 6 min at 600 × g at 4°C. The supernatant was removed, and the cells were resuspended in 600 µl of FACS buffer and passed through a 70 µm cell strainer to a FACS tube. As a final step, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000 diluted in FACS buffer) (Biotium).

Flow cytometry was performed on a BD FACS Melody cell sorter. Live cells were gated by DAPI-negative staining. Mononuclear cells were gated by FSC-A/SSC-A, and single cells were gated by FSC-A/FSC-H. Microglia were isolated as $CD11b^{+}$ and $CD45^{nonhigh}$ populations, and the percentage of $CD11c^{+}$ microglia was calculated. All analyses were performed with FlowJo 10.0.8 analysis software.

Immunohistochemistry protocol

The mice were terminally anesthetized with an overdose of isoflurane and perfused with ice-cold 1X PBS and 4% paraformaldehyde (PFA) through the left cardiac ventricle. Mouse brains were postfixed in 4% PFA overnight at 4°C and then transferred to 30% sucrose solution (diluted in PBS) for cryoprotection. For maintenance, the brain tissues were then placed in isopentane solution for 10 min and stored at -80°C until sectioning. For our analyses, the tissues were sectioned into 20 µM coronal slices with a cryostat (Leica).

The sections were blocked in 1X PBS with 0.01% Triton X-100 and 5% normal donkey serum (NDS) for 1 h at RT and incubated with primary antibodies diluted in 0.3% Triton X-100 and 2% NDS at 4°C overnight. The following day, the sections were washed with 1X PBS and incubated with secondary antibodies for 2 h at RT. All the secondary antibodies were diluted in 0.3% Triton X-100 and 2% NDS (1:600). Finally, the sections were washed in 1X PBS and covered with EverBrite™ Hardset Mounting Medium with DAPI (Biotium) for nuclear staining. Images were acquired with a 20x, 40x or 63x

objective using a Leica TCS SP8 microscope (Leica Microsystems). When antigen retrieval was performed, as a first step, the slides were placed in a preheated solution of 10 mM sodium citrate (pH=6) and incubated at 70°C for 30 min. The antibodies and dilutions used are listed in **Table 1**.

Table 1

Epitope	Species	Dilution	Company
Iba1	Guinea-pig	1:1000	Synaptic Systems
Iba1	Rabbit	1:500	Wako
Ki67	Mouse	1:500	BD
Cd68	Rat	1:150	Bio-Rad
Ifi204	Rabbit	1:700	Abcam
BrdU	Rat	1:200	Abcam

5-Bromo-2'-deoxyuridine (BrdU) assay and immunostaining protocol

BrdU (5-bromo-2'-deoxyuridine) (Sigma) was dissolved in saline (stock: 10 mg/ml) and intraperitoneally injected into control and experimental animals (50 mg/kg) 24 h, 32 h and 40 h after LPS administration. Forty-eight hours after LPS injection, the animals were terminally anesthetized with an isoflurane overdose and perfused with ice-cold 1X PBS and 4% PFA. The same processing procedure was used for all the other immunohistochemical analyses.

For BrdU staining, brain slices were subjected to an additional chemical procedure: first, they were incubated with 0.1% Triton X-100 in 2 N HCl for 10 min at 37°C, and then they were incubated with 0.1 M sodium borate, pH=8.5, for 30 min at RT. These extra steps allow DNA hydrolysis. Then, the sections were washed with 1X PBS and incubated with blocking buffer and primary and secondary antibodies as described above.

RNA/protein extraction, cDNA synthesis and quantitative real-time PCR (qPCR)

Brains were removed and somatosensory cortex was isolated under a stereoscope. The isolated cortices were acutely freezed in liquid nitrogen and then stored at -80°C until RNA extraction. Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel). Total RNA was quantified using NanoDrop (Thermo) and cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was then performed using SYBR Select Master Mix (Thermo) in a Step-One Plus Real-Time PCR System (Applied Biosystems). Relative fold changes in the expression of target genes were calculated using the comparative 2^−ΔΔCt (Ct: cycle threshold) method with actin as the reference gene. The forward and reverse primers for each gene of interest are displayed in **Table 2**.

Table 2

Gene	Forward (5′-3′)	Reverse (5′-3′)
Actin	CCCAGGATTGCTGACAGG	TGGAAGGTGGACAGTGAGGC
Stat1	GCCGAGAACATACCAGAGAATC	GATGTATCCAGTTCGCTTAGGG
Irf7	TTGATCCGCATAAGGTGTACG	TTCCCTATTTTCCGTGGCTG

Ifi204	ACCTCTTCTGCTTTCACCTG	CATCACTTGTTTGGGACCATG
Ifi27l2a	AATGGAGGTGGAGTTGCAG	GAAGTGTCATCTCCTAAGCTCAG
Ifitm3	GGTCTGGTCCCTGTTCAATAC	CTCCAGTCACATCACCCAC
Ifi30	GGAGTGTAGACTGAACATGGTG	GTGACACCTCAGGAGCATAC
Oasl2	ATCATTGTCCTTACCCACAGAG	TGCTGGTTTTGAGTCTCTGG
C3	GGGCTGTTAAATGGTTGATTCTG	GATGAGGACGAAGGCTGTG
C1qa	CTGAAGATGTCTGCCGAGC	CCCCTGGGTCTCCTTTAAAAC
Mki67	TGCCCGACCCTACAAAATG	GAGCCTGTATCACTCATCTGC
Top2a	AGTCAGACGTGAGCAGTAATG	CTTCATCCTCATCCTTCTCATCC
IL1a	GCACCTTACACCTACCAGAGT	AAACTTCTGCCTGACGAGCTT
IL1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT

Proteome Profiler Mouse Cytokine/Chemokine Array

For every experimental condition (sample), cortical tissues from 4 different animals were combined. Tissue lysates were prepared in 1% Triton X-100 in 1X PBS containing a protease inhibitor cocktail (Cell Signaling) at 4°C. The lysates were frozen, thawed, and centrifuged at 10,000 × g for 5 min to remove cellular debris. Protein concentrations were quantified via a total protein assay. Cytokines/chemokines were analyzed via proteome profilers (R&D Systems) loaded with 200 µg of each protein sample following the manufacturer’s instructions. Each sample was incubated with a separate array precoated with 40 cytokines/chemokines in duplicate. The intensity of

the antibodies was analyzed via ImageJ software. Duplicates were averaged, and the background was subtracted to calculate the pixel density for each protein. Finally, the results were normalized using Cx3cR1^{CreER} + LPS as the baseline condition.

Confocal microscopy and image analysis

Immunofluorescence staining was performed with a Leica TCS-SP8 inverted confocal microscope at the Light Microscopy Unit at the Hellenic Pasteur Institute. For the processing and analysis of the images obtained, ImageJ and Imaris V. 9.3.1 were used. For quantification of the number of cells, at least 3 non-overlapping images from at least 3 sections from each animal were analyzed.

Morphometric analysis of individual microglia stained with IBA1 was performed with the Imaris Filament Tracer module. Initially, the filament tracer locates a sphere (cell body) as the beginning point and reconstructs the processes as either main or secondary branches. The cell bodies were located after an 8 μm sphere, which was set as the beginning point. The settings used, including thresholding and adjusting the remaining parameters, were the same for all the cells analyzed. For each individual cell analyzed, the morphological properties of the microglia were the total dendrite length (μm), which is the sum of the length of every process (main and secondary branches) per cell, the total dendrite volume (μm^3), which is the sum of the volume of every process (main and secondary branches), the mean dendrite diameter (μm), and the convex hull volume (μm^3). Furthermore, microglial complexity and the degree of ramification were assessed via Sholl analysis [22]. The number of total intersections of

microglial processes with concentric Sholl spheres was calculated at increasing distances with an increment of 1 μm from the cell body.

For measuring IFI204 and CD68, the Imaris V. 9.3.1 surface module was used. Specifically, the measurement of IFI204 fluorescence intensity was performed inside the nuclei of the cells after different surfaces were created in the images. To measure the volume of CD68 inside microglia, we quantified the total volume of the signal within the volume of IBA1. At least 3 non-overlapping images from at least 3 sections from each animal were analyzed. For each marker, the threshold value used was kept stable for all images, and $BIN1^{+}$, $Ki67^{+}$ and $BrdU^{+}$ cells were manually counted. For all the analyses, 40x images were used.

Statistical analysis

All the statistical analyses were performed via GraphPad Prism 8.4.3 software. After confirming that the data followed a normal distribution (Shapiro–Wilk test), Student's t-test and one-way ANOVA followed by post hoc tests were applied for comparisons of two or multiple groups, respectively. The p values are represented as follows: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. A p value of >0.05 was considered statistically non-significant. The data are presented as the mean ± standard error of the mean (SEM). For the data that did not follow a normal distribution, we performed Mann–Whitney and Kruskal–Wallis nonparametric tests. Our analyses included the following groups: $Bin1^{fl/fl}$, $Bin1^{fl/fl}$ + LPS, $Cx3cR1^{CreER}$, $Cx3cR1^{CreER}$ + LPS, Bin1cKO, and Bin1cKO + LPS.

RESULTS

Single-cell transcriptome analysis of the cell autonomous and non-cell autonomous effects of microglial BIN1 deletion in the mouse cortex

To investigate the process through which microglial BIN1 influences brain homeostasis and neuroinflammation, we utilized the Cx3cr1CreER//Bin1$^{fl/fl}$ (Bin1cKO) double transgenic mouse model. In this model, *Bin1* was conditionally knocked out specifically in microglia of the adult brain via intraperitoneal (IP) tamoxifen administration **(Figure 1A)**. Successful microglial BIN1 deletion was confirmed by immunohistochemical analysis three weeks after tamoxifen administration **(Figures 1C, D)**. To assess the impact of microglial BIN1 deletion under proinflammatory conditions, we induced mild neuroinflammation in both Bin1$^{fl/fll}$ (control) and Bin1cKO mice through a single IP injection of 2 mg/kg lipopolysaccharide (LPS). Subsequent analyses were conducted two days after LPS administration (Figure 1A), to investigate the microglia changes under the early stages of mild neuroinflammation [23].

To comprehensively examine the cell autonomous and non-cell autonomous effects of *Bin1* KO in microglia on gene expression, we performed single-nucleus RNA sequencing (snRNA-Seq) on somatosensory cortices from Bin$^{fl/fl}$ and Bin1cKO mice under both homeostatic and neuroinflammatory conditions (n=2 animals and 4 sequencing libraries per condition), employing 10x Genomics technology combined with the cell hashing multiplexing method [24] **(Figure 1B)**. Given our primary interest in microglia and their low abundance in the mouse cortex, we enriched their representation in our samples. This was achieved by staining isolated nuclei with the neuronal marker NeuN, followed by FACS sorting into NeuN+

(approx. 60%) and NeuN- (approx. 40%) fractions. The final nuclear sample for sequencing was prepared at a ratio of 20% NeuN+ versus 80% NeuN- nuclei (1:4) (**Figure 1B**). Following quality control (see Materials and Methods), we recovered 18,678 nuclei, which clustered into the major cell types of the adult brain on the basis of cell type-specific gene expression **(Figures 1E, G and Suppl. Figures 1A, B)**. The proportions of different cell types were consistent across genotypes and treatments **(Figure 1F and Suppl. Figure 1C)**, with over 60% of the cells being non-neuronal **(Figure 1F)**. These findings confirmed the effectiveness of our strategy for enriching glial populations in our single-nuclei mRNA libraries. Furthermore, we validated the enrichment of *Bin1* expression in oligodendrocytes, microglia and glutamatergic neurons **(Suppl. Figure 1D)**, which was consistent with previous findings [4].

Next, we employed the Wilcoxon rank sum test to identify differentially expressed genes (DEGs) within each major cell type of the brain across various conditions. Interestingly, a substantial number of DEGs ($log_2FC>0.25$ and p adj value<0.05) were found not only in microglia, where Bin1 was deleted, but also in all other major brain cell types, such as astrocytes, oligodendrocytes, glutamatergic and GABAergic neurons **(Figure 2A, Suppl. Figure 2A, and Suppl. File 1)**, indicating a non-cell autonomous effect of microglial *Bin1* deletion on the gene expression of other brain cell types. Notably, we observed that *Bin1* deletion led to an increase in the transcriptional response of 436 DEGs to LPS in microglia **(Figure 2A, Suppl. File 1)** and 99 DEGs in astrocytes **(Suppl. Figure 2A, Suppl. File 1)**.

Among the genes upregulated in microglia after Bin1cKO and LPS administration **(Figure 2A, right panels)**, several genes associated with proliferative capacity (*Mki67, Top2a, Knl1, Cenpa, and Kif11*) were identified **(Figures 2A, B)**. We also observed increased expression of genes related to the inflammatory response and type I interferon response (*Stat1, Ifi204, Ifi30, Itm2b, Ptprc, and Ezh2*) [25] and complement subunits (*C1qa and C1qb*) **(Figures 2A, B)**. Accordingly, Gene Ontology (GO) analysis of biological process (BP) terms confirmed that DEGs in both control and Bin1cKO microglia treated with LPS were enriched primarily for terms related to inflammation, such as the immune response, cytokine production, and the inflammatory response, as well as endocytosis and phagocytosis. Importantly, the enrichment of these terms in response to LPS was significantly greater in Bin1cKO microglia (Bin1cKO+LPS vs Bin1cKO - red bars) than in control microglia ($Bin1^{fl/fll}$+ LPS vs $Bin1^{fl/fll}$ - purple bars), implying a stronger inflammatory microglial response to LPS in the absence of Bin1 **(Figure 2C)**. Furthermore, it is worth noting that only DEGs observed in the Bin1cKO + LPS condition (compared with Bin1cKO – red bars or Bin1fl/fl + LPS – green bars) were highly enriched for terms related to cell proliferation, type I and type II interferon response and IL-1 production **(Figure 2C)**. Accordingly, GO analysis of cellular component (CC) terms revealed an enrichment of DEGs for the complement component C1q complex and MHC class I complex in Bin1cKO + LPS microglia (compared with Bin1cKO microglia, red bars or $Bin1^{fl/fl}$ + LPS microglia, green bars) **(Figure 2C)**.

As a next step, we further analyzed our snRNA-seq dataset to investigate the potential non-cell autonomous effects of microglial *Bin1* deletion. Our analysis revealed noteworthy changes in the astrocytic population. Among

the DEGs upregulated in astrocytes under Bin1cKO + LPS conditions **(Suppl. Figure 2A, right panels)**, we identified genes related to the response to inflammatory stimuli (the interferon-responsive gene *Ifi27* [25] and the MHC class I receptor *H2-K1*) **(Suppl. Figures 2A, B)** as well as genes with unique or increased expression associated with astrocytic reactivity (*Lgals3bp*, *C4b*, *Gfap*, *S1pr1*, *Celsr2*, *Phyhd1*, *Nat8f1* and *Ednrb)* [26–29] **(Suppl. Figures 2A, B)** and lipid metabolism, such as *Lrp1b* and *Scd2* **(Suppl. Figure 2A)**. In accordance with the above observations, GO analysis of BP revealed that the DEGs of astrocytes in Bin1cKO animals (Bin1cKO + LPS vs Bin1cKO - red bars) were enriched for terms related to the regulation of cell communication, response to stimulus, and regulation of immune system processes, indicative of an enhanced response to neuroinflammatory signals **(Suppl. Figure 2D)**. In parallel, in Bin1cKO animals both in the presence and absence of LPS (Bin1cKO + LPS vs Bin1cKO - red bars or Bin1cKO vs $Bin1^{fl/fl}$ - blue bars), DEGs in astrocytes were enriched for GO terms related to amyloid precursor protein metabolism and neurofibrillary tangles **(Suppl. Figure 2D)**. Notably, in the absence of neuroinflammation (Bin1cKO vs $Bin1^{fl/fl}$ – blue bars), we observed that astrocytes presented increased expression of genes related to AD pathology (*Apoe, Clu, Cst3, Itm2b, Pde10a and Zbtb16)* [30,31] and lipid metabolism (*Scd2, Acls3* and *Ttyh1*) [32] **(Suppl. Figures 2A, C)**. Taken together, these findings indicate that selective *Bin1* deletion in adult brain microglia impacts microglial proliferation and the neuroinflammatory response to LPS exposure, functioning in both cell autonomous and non-cell autonomous manners.

Importantly, by analyzing a recent snRNA-seq dataset from postmortem brains of healthy individuals and patients diagnosed with AD [33] we

discovered that *Bin1* expression in human microglia is significantly reduced during disease progression, as indicated by the Braak stages **(Suppl. Figure 3A and Suppl. File 2)**, and in individuals clinically diagnosed with dementia **(Suppl. Figure 3B and Suppl. File 2)**. We also found a significant overlap between genes differentially expressed in human microglia during the early stages of AD progression (mid vs low Braak stages) [21] and those expressed in our animal model after Bin1cKO and LPS administration (Bin1cKO + LPS vs Bin1cKO or $Bin1^{fl/fll}$ + LPS) (**Suppl. Figure 3C and Suppl. File 3)**. This overlap includes known AD risk factors such as ABCA1, C1QA/B/C, CST3, CTSB, CTSS, FKBP5, ITM2B and SORL1 [34–37]. No overlap was observed when gene expression changes in microglia in our animal model were compared with those found in human microglia at late pathological stages (high vs mid Braak stages). Therefore, reduced *Bin1* expression and systemic inflammation in mice appear to mimic early microglial responses to AD pathology.

Cortical Bin1cKO microglia exhibit an altered response to LPS-mediated systemic inflammation

To further elucidate the effects of LPS and Bin1cKO on microglia, we independently reanalyzed these cells and identified seven main cell clusters across the control and experimental conditions (**Figure 3A and Suppl. File 4**). The proportion of cells in clusters 1 and 3 was significantly greater in non-treated animals, whereas clusters 0 and 2 represented a significantly greater proportion in LPS-treated animals. Notably, clusters 4 and 5 were largely absent in non-treated $Bin1^{fl/fl}$ and Bin1cKO animals and only minimally increased in $Bin1^{fl/fl}$ + LPS animals. However, these clusters were

overrepresented in the Bin1cKO + LPS animals, suggesting that these two clusters were more abundant in the Bin1cKO microglia than in the control microglia in response to LPS. Specifically, while the proportions of cells in clusters 4 and 5 in $Bin1^{fl/fl}$ + LPS animals were 2.60 ± 1.89% and 2.22 ± 1.79%, respectively, they increased to 14.80 ± 1.77% **(cluster 4)** and 5.84 ± 0.50% **(cluster 5)** in Bin1cKO + LPS animals **(Figure 3B).** Using the Wilcoxon rank sum test to identify DEGs for each cluster compared with all other clusters, we found that cluster 0 expressed significantly higher levels of genes associated with vesicle-mediated transport, including *Sik3, Neat1, Sorl1* and *Abca1*. Conversely, the cells in cluster 2 expressed higher levels of genes associated with complement activation, such as *C1qa, C1qb* and *C1qc* (**Figures 3C, D**). Importantly, the cells in cluster 4 expressed a large set of genes associated with the regulation of cell proliferation, such as *Top2a, Mki67, Kif11* and *Aspm*, whereas the cells in cluster 5 expressed significantly greater levels of genes associated with the inflammatory response, including type I and II interferon responses, such as *Ifi204, Irf7, Stat1* and *Stat2* (**Figures 3C, D**). Among the two clusters that were more common in both $Bin1^{fl/fl}$ and Bin1cKO non-treated animals (clusters 1 and 3), the cells expressed significantly greater levels of genes associated with homeostatic microglia, such as *Cx3cr1, P2ry12* and *Selpig* (**Figures 3C, D**). Finally, cells in cluster 6 constituted less than 3% across all groups and expressed significantly higher levels of *Cd163*, a marker for perivascular macrophages (PVMs) [38].

To further characterize the microglial states observed under our experimental conditions, we employed CellID to extract gene signatures previously identified under well-defined conditions [39,40]. We used CellID to calculate the gene signatures for each microglial cluster and perform

hypergeometric tests against a list of genes previously identified as homeostatic (HOM; n=484 genes), disease-associated microglia (DAM; n=306 genes), plaque-associated microglia (PAM; n=57 genes), interferon-responsive microglia (IRM; n=34 genes), cycling (n=27 genes) and activated responsive microglia (ARM; n=131 genes) **(Figures 3E, F, Suppl. File 5)**. We observed that cells in cluster 1 (more abundant in non treated animals) were significantly enriched for HOM gene signatures, that were also present at a lower percent in cluster 2. Moreover, cells within clusters 0, 2 and 4 (more abundant after LPS treatment) were significantly enriched for the ARM, DAM and IRM gene signatures. Finally, approximately 90% of the cells in cluster 4 were significantly enriched for genes associated with the cell cycle, whereas 15% of the cells in cluster 3 were enriched for HOM gene signatures **(Figure 3F)**.

Collectively, these data suggest that the conditional deletion of *Bin1* in microglia of the adult mouse brain enhances proliferation, the interferon response and differentiation into an activated and disease-associated state following stimulation with a low dose of LPS.

Cortical Bin1cKO microglia exhibit increased proliferation potential following LPS-induced systemic inflammation

The notable increase in cell proliferation-related genes within microglial cluster 4 led us to investigate, in vivo, whether conditionally deleting Bin1 in adult cortical microglia would be sufficient to increase cell proliferation after LPS-induced neuroinflammation. To account for any potential influence of Cx3cr1 haploinsufficiency on this phenotype, we analyzed cell cycle-related gene and protein expression across three groups: $Bin1^{fl/fl}$ mice, Bin1cKO mice, and an additional control group carrying one $Cx3cr1^{CreER}$

allele with two wild-type Bin1 alleles ($Bin1^{wt/wt}$). Our real-time RT–PCR analysis confirmed that both the *Mki67* and *Top2a* genes presented minimal expression under homeostatic conditions. While their expression was slightly upregulated after LPS administration in both genetic backgrounds, we observed significant upregulation following microglial Bin1cKO **(Figure 4A)**.

To further investigate whether microglial proliferative capacity is increased, we performed double immunofluorescence labeling for IBA1 (microglial marker) and KI67 (marker for proliferating cells). Our measurements revealed that Ki67+ microglia were nearly absent under homeostatic conditions. However, their numbers significantly increased following LPS administration, regardless of genetic background, and exhibited a statistically significant further increase in the Bin1cKO + LPS condition **(Figures 4B-C)**. To assess the effect of microglial *Bin1* deletion on microglial proliferation in response to neuroinflammation more comprehensively, we administered IP BrdU (50 mg/kg) at 24 h, 32 h, and 40 h after LPS administration. We subsequently performed IBA1/BrdU immunohistochemical analysis. The percentage of BrdU+ microglia was greater in Bin1cKO microglia than in $Cx3cr1^{CreER}$ control microglia after LPS treatment **(Figures 4D-E).**

Cortical BIN1cKO microglia adopt a hyper-ramified state and stimulate an altered inflammatory response to systemic inflammation

To assess how *Bin1* deletion affects microglial phenotypic responses to neuroinflammation, we conducted a morphometric analysis of IBA1+ cortical microglia under $Cx3cr1^{CreER}$ + LPS and Bin1cKO + LPS conditions.

We used non-LPS-treated Cx3cr1^{CreER} as the homeostatic negative control. Our measurements revealed that microglial process length, the number of process intersections and total microglial cell convex hull volume increased following LPS administration. Notably, all these parameters were further significantly increased in the Bin1cKO + LPS condition. **(Figures 5A-D)**. These morphological characteristics indicated that Bin1cKO microglia exhibited a more pronounced hyper-ramified morphology, indicative of an intermediate activation state linked to both acute and chronic stress, in response to LPS [41,42].

To better understand how Bin1cKO affects microglial reactivity in response to neuroinflammation, we isolated mouse cortices and we performed FACS isolation and analysis of the CD11b^{+}/CD45^{nonhigh} microglial population **(Figure 5E)**. Our findings revealed a significant increase in the proportion of microglial cells that expressed the reactivity marker CD11c, with an even more pronounced increase after BIN1cKO **(Figure 5F)**.

Finally, to characterize the overall inflammatory state of the brain cortex after microglial Bin1cKO, we conducted two key analyses. First, we performed real-time RT−PCR analysis to measure the expression of several proinflammatory genes (*Il-1a and Il-1b)* and complement components (*C1qa and C3*) **(Figure 5G).** Second, we used a proteome array to assess the levels of various cytokines, chemokines and acute-phase proteins **(Figures 5H-I)**. Our real-time RT−PCR results indicated that under inflammatory conditions, microglial Bin1 deletion drove a proinflammatory response by increasing the expression of *Il-1a* and the complement components *C1qa* and *C3* **(Figure 5G)**. The proteome array supported this finding further by showing elevated protein levels of proinflammatory cytokines, such as IL-12p70, IL-6, IL-1a, IFN-γ and IL-17, after microglial BIN1cKO **(Figures 5H-**

I). We also noted an increase in various chemokines such as CCL1, CCL2, CCL3, CCL4, CXCL9, CXCL2, and CCL12 that act as proinflammatory mediators by recruiting T cells, monocytes and neutrophils [43,44]. Interestingly, we also observed an increase in the anti-inflammatory cytokines IL-4 and IL-10 **(Figure 5H-I)**. This could indicate a disrupted regulatory balance after microglial Bin1cKO, possibly representing the system's attempt to restore equilibrium and/or reflect the heterogeneity of microglial states.

Systemic inflammation enhances the proinflammatory response in Bin1cKO cortical microglia via the IFN type I pathway

Our snRNA-Seq analysis revealed that the most prominent transcriptional signature of Bin1cKO microglia following LPS-induced inflammation was associated with genes involved in the type I interferon (IFN-I) inflammatory response pathway. Notably, transcription factors such as *Stat1* and *Irf7* were significantly upregulated in microglial subtypes that were overrepresented in Bin1cKO + LPS animals **(Figure 3C)**. Consistently, the IFN-I response mediator gene *Ifi204* was substantially upregulated after microglial Bin1cKO during neuroinflammation (comparing Bin1cKO + LPS to $Bin1^{fl/fl}$ + LPS) **(Figure 3C)**.

To explore the role of microglial *Bin1* deletion in the IFN-I response in greater depth, we performed real-time RT–PCR in the somatosensory cortex. We focused on the transcription factors *Stat1* and *Irf7*, which are master regulators of type I interferon-dependent immune responses. Our results indicated that LPS positively regulated both genes, with microglial BIN1 deletion leading to an even greater upregulation **(Figure 6A)**. Furthermore, we analyzed the expression levels of the IFN-I-stimulated

genes *Ifi204, Ifi30 and Ifitm3.* All three genes were upregulated after LPS administration, with a significant additional increase observed in the Bin1cKO + LPS group. Notably, the IFN-I mediator gene *Ifi204* showed the most pronounced change among all the genes analyzed **(Figure 6B)**.

Next, we investigated *Ifi27l2a* and *Oasl2*, genes known to be upregulated in interferon-responsive microglia (IRMs) alongside *Ifi204* [45]. LPS stimulation led to the upregulation of all these genes in the Cx3cr1^{CreER} + LPS group, with a further increase in the Bin1cKO + LPS group **(Figure 6C)**. This comprehensive analysis of IFN-I response-related genes strongly supports our observation of an enhanced microglial proinflammatory response when BIN1 is deleted.

To further investigate the enhanced microglial IFN-I proinflammatory response at the protein level, we focused on IFI204, a key mediator of the IFN-I response that was most strongly upregulated in the Bin1cKO + LPS group. Double immunofluorescence labeling for IBA1 and IFI204 revealed that LPS induced IFI204 expression, which was almost absent under homeostatic conditions, in all microglial cells across both Cx3cr1^{CreER} and Bin1cKO conditions **(Figures 6D-E)**. However, IFI204 expression in microglia was significantly greater in Bin1cKO + LPS microglia than in Cx3cr1^{CreER} + LPS microglia **(Figure 6F)**. These findings further support our hypothesis that microglial *Bin1* deletion promotes the establishment of a proinflammatory IRM phenotype.

Finally, we aimed to assess whether the phagocytic capacity of microglia is altered [45]. Immunohistochemical analysis of CD68 (lysosomal phagocytic marker) volume within microglia revealed that, compared with the Cx3cr1^{CreER} control, Bin1 deletion increased microglial CD68 expression

(Figures 6G-H). These findings suggest that Bin1cKO microglia transition to a state with elevated phagocytic potential.

Discussion

In this study we aimed to elucidate the role of the microglial-specific BIN1 protein in brain homeostasis and systemic inflammation, which constitutes both a recognized hallmark and a risk factor for AD progression [1]. To this end, we performed, for the first time, single-cell transcriptome analysis of microglial BIN1-deficient mouse cortices to dissect the transcriptional signatures of cortical cell populations and their responses to LPS-induced inflammation.

Initially, our findings indicate that simply deleting microglial BIN1 is not sufficient to cause significant changes at the transcriptional and cellular levels in microglia under homeostatic conditions, which is in line with previously published data [11]. However, an inflammatory trigger through LPS-induced systemic inflammation in the presence of BIN1 deletion results in changes in the microglial state. The two most prominent characteristics of BIN1-deficient microglia in response to systemic inflammation are marked activation of their proliferative potential and pronounced enhancement of the microglial IFN-type I-mediated inflammatory response.

Specifically, gene signature analysis revealed that the microglial clusters highly represented in BIN1cKO cortices following LPS stimulation were enriched for disease-associated microglia (DAMs) and interferon-response microglia (IRMs). The predominant presence of this IRM transcriptional signature in the BIN1cKO + LPS group was further confirmed by real-time RT−PCR, which revealed significant upregulation of master regulators of the

interferon immune response, such as *Irf7*, along with interferon-modulating factors, such as *Ifi204,* and IFN type I-responsive genes, including *Ifi27l2a* and *Oasl2* [46]. Given that *Ifi204* exhibited the greatest transcriptional increase, we confirmed a corresponding increase in Ifi204 protein expression levels, underscoring its central role in modulating the INF-I response in *Bin1*-deficient microglia

Further analysis of cytokine and chemokine protein levels revealed elevated levels of proinflammatory mediators, such as IL-1a and IL-12p70 and CCL3 supporting an enhanced response to inflammation, after microglial BIN1cKO [47,48]. Interestingly, CCL2, CCL4, CXCL9, CXCL11, IL-6, and IFN-γ, all of which are known to be induced by type I and type II interferon (IFN) response [39,49], were also elevated. Notably, the increase in the levels of few known anti-inflammatory cytokines, such as IL-10 and IL-4, might indicate the occurrence of immunoregulatory feedback to control inflammation and/or reflect the heterogeneity of microglial states [50].

Molecular phenotypic and morphometric analysis corroborated these findings by demonstrating that BIN1cKO microglia adopt a proinflammatory phenotype characterized by elevated CD11c expression and a hyper-ramified morphology indicative of an intermediate activation state linked to both acute and chronic stress [41,42]. Additionally, the increased CD68 protein expression observed in Bin1cKO + LPS microglia in our model may reflect transient IFN-I-responsive signaling, which has been reported to drive IRM microglial phagocytic activity in the cortex [51]. Moreover, we detected increased transcription of the complement subunits *C1qa and C1qb*, which is consistent with previous studies showing that sustained IFN-I expression induces an inflammatory microglial phenotype and stimulates

the complement cascade to mediate synapse loss during AD progression through engulfment of C1q-tagged post-synaptic terminals [46].

In AD animal models, IFN-I signaling is activated early in microglia and then triggers the response of other brain cell types, particularly astrocytes, which also become IFN-I-responsive in an amyloid-beta pathology-dependent manner [45,52]. Our observation that microglial BIN1 deletion leads to the upregulation of both interferon-responsive and reactive genes in astrocytes points to a BIN1-mediated microglia-to-astrocyte communication mechanism that is potentially mediated by proinflammatory cytokines [29], whose levels are increased in our system, and possibly other yet unidentified mediators.

Collectively, our data show that microglial BIN1 deletion specifically triggers a distinct microglial inflammatory response characterized by elevated activation of IFN-I signaling and related cascades. However, the duration and functional consequences of this response remain unclear. Thus, discrepancies with a related recent study [11] reporting the attenuated ability of BIN1-deficient microglia to mount IFN-I responses could be attributed to variations in inflammation protocols, microglial harvesting methods or differential brain region-specific responses, as our analysis was restricted to the neocortex. We also acknowledge the dynamic and complex positive and negative regulation of the IFN-I pathway to maintain a balance between immune and hyper-inflammatory responses [53], suggesting that different mediators might be differentially expressed to precisely control microglial inflammatory states depending on the timing and brain region, potentially reflecting different snapshots of disease progression. In this context, IFI204, which is generally known to induce the production of type I

interferons and proinflammatory mediators, has also been reported to inhibit IRF7-mediated type I interferon production to avoid a hyper-inflammatory response [53].

Microglia, as key regulators of brain immune homeostasis, adopt diverse activation states characterized by distinct transcriptional signatures, such as the interferon-responsive microglia (IRM) phenotype observed across development, aging, and neurological diseases [7,54]. The IRM signature, identified in subsets of microglia in AD models and brain aging [7], has been linked to chronic IFN-I presence in the aged brain environment [55]. In this light, our findings might also suggest the emergence of an aging transcriptional signature within a specific microglial subpopulation. Future studies are needed to fully elucidate this hypothesis.

In addition, our analysis revealed a signature of cycling microglia in BIN1cKO microglia following LPS stimulation. Phenotypic analysis confirmed that a small but significant percentage of microglia exhibited increased proliferation potential in response to LPS. Microglial proliferation is a hallmark microglial response in AD-like pathologies [56], with proliferative microglia accumulating near amyloid-beta plaques. As BIN1, which possesses a MYC-binding domain, is a known MYC-interacting pro-apoptotic tumor suppressor [57], its absence in microglia could lead to MYC-mediated microglial proliferation, increasing the expression of proliferation markers.

An important aspect of our single-cell transcriptomic analysis is its concordance with recent transcriptomic data demonstrating that microglia from individuals clinically diagnosed with AD or dementia exhibit significantly reduced levels of BIN1 [33]. Together with the observation that BIN1 downregulation specifically in microglia carrying a single-nucleotide

polymorphism within the BIN1 locus is associated with an increased risk of developing AD [58], this could suggest that reduced BIN1 expression is associated with altered microglial responses in AD pathology [59].

Accordingly, we detected a significant overlap between genes differentially expressed in human microglia during the early stages of AD (mid- vs. low-Braak stages) [21] and those observed in our Bin1cKO + LPS mouse model, including known AD risk factors such as *Sorl1, Itm2b, C1qa/b/c, Cst3, Fkbp5* and *Abca1* [34–37]. These findings strongly suggest that both reduced Bin1 expression and systemic inflammation in mice collectively mimic early microglial responses to AD pathology. Further research is needed to determine whether this early microglial response is detrimental or beneficial for the progression of the disease.

Conclusions

Overall, our results support a feed-forward LPS–BIN1 loop in which microglial BIN1 deficiency stimulates factors that further exacerbate the microglial proinflammatory response. Additionally, we show for the first time that microglial BIN1 deletion also elicits non-cell autonomous changes in astrocytes, affecting genes related to astrocytic activation.

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FIGURE LEGENDS

Figure 1. Single-nuclei RNA Sequencing and Analysis of Different Cortical Brain Cell Types after Microglia-specific Bin1cKO and LPS treatment

(A) Experimental design of tamoxifen and LPS administration.

(B) Experimental design of single-nucleus RNA sequencing (snRNA-seq).

(C) Representative confocal images of the somatosensory cortex showing BIN1 expression (red) in IBA1^{+} (green) microglia. Left panel: Microglia in Bin1$^{fl/fl}$ (control) mice expressing BIN1 (colocalization in yellow - white arrows). Right panel: BIN1 is efficiently knocked out in IBA1^{+} cells of Bin1cKO mice (white arrows showing that there is no colocalization).

(D) Analysis of IBA1^{+}/BIN1^{+} cells indicated that there were almost any double-positive cells in the experimental sample. *Unpaired two-tailed t-test, ****p<0.0001, mean±SEM.*

(E) Uniform manifold approximation and projection (UMAP) plot of snRNA-seq data showing clustering of 18,678 cells from the four different experimental conditions into various cell types.

(F) Bar plot showing the proportions of different cell types across the four experimental conditions: Bin1 $^{fl/fl}$, Bin1 $^{fl/fl}$ + LPS, Bin1cKO, and Bin1cKO + LPS.

(G) Dot plot summarizing the expression of cell type-specific marker genes across the identified cell clusters. The size of each dot corresponds to the percentage of cells expressing the marker gene, and the color intensity indicates the average expression level.

Figure 2. Differential expression and Gene Ontology analysis of microglia.

(A) Volcano plots presenting differentially expressed genes (DEGs) (abs(avg_log2FC) > 0.25 & p_val_adj < 0.05) in microglia originating from the comparisons Bin1$^{fl/fl}$ + LPS vs Bin1$^{fl/fl}$ (top left panel), Bin1cKO + LPS vs Bin1cKO (top right panel), Bin1cKO vs Bin1$^{fl/fl}$ (bottom left panel) and Bin1cKO + LPS vs Bin1$^{fl/fl}$ + LPS (bottom right panel). The upregulated DEGs are presented in red, and the downregulated DEGs are presented in blue.

(B) Dot plot of selected DEGs associated with cell proliferation (*Mki67, Top2a, Knl1, Cenpa, and Kif11*), inflammation (*Ezh2, Stat1, Ifi204, Ifi30, Itm2b,* and *Ptprc*) and complement activation (*C1qa* and *C1qb*). The size of each dot corresponds to the percentage of cells expressing the marker gene, and the color intensity indicates the average expression level.

(C) Presentation of enriched Gene Ontology (GO) terms associated with various biological processes (BP) and CCs for the DEGs (abs(avg_log2FC) > 0.25 & p_val_adj < 0.05) identified in microglia from the following comparisons: Bin1cKO + LPS vs Bin1cKO (red bars), Bin1$^{fl/fl}$ + LPS vs Bin1fl/fl

(purple bars), Bin1cKO + LPS vs $Bin1^{fl/fl}$ + LPS (green bars). Notable processes include the regulation of the immune response, cytokine production, the response to type I interferon, the response to type II interferon, the lysosome, the complement component C1q complex, the mitotic cell cycle process, and phagocytosis.

Figure 3. Clustering and Analysis of Gene Expression in Microglia Under Different Conditions

(A) UMAP plots showing the Seurat clustering of microglia from the four different groups ($Bin1^{fl/fl}$, $Bin1^{fl/fl}$ + LPS, Bin1cKO, Bin1cKO + LPS). Each cluster is color-coded and numbered according to identified cell subpopulations (0-6). There are two microglial subpopulations (4,5) with minimal representation under non-inflammatory conditions, and their numbers are increased in Bin1cKO microglia following inflammation.

(B) Bar plot quantifying the percentage of cells in each cluster (0-6) across the four experimental conditions.

(C) Dot plot showing the expression levels of selected gene markers for each subcluster. Cluster 0 expressed higher levels of genes associated with vesicle-mediated transport (*Sik3, Neat1, Sorl1 and Abca1*), whereas cluster 2 expressed higher levels of genes associated with complement activation (*C1qa, C1qb, and C1qc*). Clusters 1 and 3 expressed higher levels of genes associated with homeostatic microglia (*Cx3cr1, P2ry12*, *Selpig*). Notably, cluster 4 expressed a large set of genes associated with the regulation of cell proliferation (*Top2a, Mki67, Kif11, Aspm*), whereas cluster 5 expressed significantly higher levels of genes associated with the inflammatory

response, including type I and II interferon responses (*Ifi204, Irf7, Stat1* and *Stat2*). Last, cluster 6 expressed significantly higher levels of *Cd163*.

(D) GO terms associated with various biological processes (BP) for different microglial clusters. Cluster 4 was linked to cell proliferation (mitotic cell cycle, organelle fission, and nuclear division), whereas cluster 5 was linked to the response to inflammation (response to virus, innate immune response, and inflammatory response).

(E) UMAP plots showing the enrichment of gene sets associated with specific microglial states. The top left plot shows microglial clusters, whereas the other 6 plots show cells labeled according to the Z score of gene set enrichment. PAM: plaque-associated microglia; DAM: disease-associated microglia; ARM: activated responsive microglia; IRM: interferon-responsive microglia.

(F) Bar plots showing the percentage of cells significantly enriched ($p<0.05$) for specific gene signatures within each microglial cluster.

Figure 4. BIN1 regulates microglial proliferation in the adult mouse brain after LPS treatment

(A) Real-time PCR analysis of selected genes involved in cell proliferation (*Mki67, Top2a*) in the somatosensory mouse cortex. Both genes are upregulated in microglia from Bin1cKO mice following neuroinflammation. No difference was detected between the $Bin1^{fl/fl}$ and $Cx3cr1^{CreER}$ control samples. *Ordinary One-way ANOVA, ns: non-significant, *p<0.05, **p<0.01, MEAN±SEM, n≥3/condition.*

(B) Representative confocal images of the somatosensory cortex of $Cx3cR1^{CreER}$ and Bin1cKO mice. mice, showing $Ki67^+$ (red) microglia ($IBA1^+$–green) (colocalized with white arrows) under various experimental conditions.

(C) Analysis of Ki67+ microglia. LPS increases the percentage of $KI67^+$ microglia in every genetic background. The percentage of Ki67+ microglia is further increased in microglial Bin1cKO mice during neuroinflammation compared with that in control mice. *Ordinary One-way ANOVA, ns: non-significant, *p<0.05, **p<0.01, MEAN±SEM, n≥3/condition.*

(D) Representative confocal images of the somatosensory cortex showing microglial $IBA1^+$ (IBA1–green) cells expressing $BrdU^+$ (red) (white arrows) under various experimental conditions.

(E) Analysis of $BrdU^+$ microglia under various experimental conditions. LPS increased the percentage of $BrdU^+$ cells, which further increased after Bin1cKO. *Ordinary One-way ANOVA, ns: non-significant, *p<0.05, MEAN±SEM, n≥3/condition.*

Figure 5. BIN1 regulates microglial reactivity in the mouse brain after LPS-induced inflammation

(A) Representative confocal images of individual microglia ($IBA1^+$ - green) from cryostat brain sections of the somatosensory cortex showing microglial morphology under various experimental conditions using Imaris (V. 9.3.1.) Filament Tracer module. Upper panel: The cell skeleton (red) is depicted. Lower panel: Cell volume (3D shape in green) is depicted.

(B-D) Analysis of microglial morphology using Imaris (V. 9.3.1.) for parameters such as the length of microglial processes, sholl intersections and convex hull volume. LPS administration significantly increased all the parameters. Bin1cKO resulted in further significant increases in all parameters (Bin1cKO + LPS vs $Cx3cr1^{CreER}$ + LPS). *Kruskal–Wallis nonparametric test for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, MEAN±SEM, n=50 cells/condition*.

(E) FACS gating strategy: single, mononuclear, and live cells were gated, and microglia were sorted into a $CD11b^{+}$//$CD45^{non\text{-}high}$ population. A representative flow cytometric image of $CD11c^{+}$ microglia in each condition is displayed.

(F) Flow cytometry analysis indicated that Bin1cKO caused an increase in the percentage of $CD11c^{+}$ microglia ($CD11b^{+}$//$CD45^{non\text{-}high}$) in LPS conditions. Notably, there was no difference due to Cx3cr1 haploinsufficiency ($Cx3cr1^{CreER}$ + LPS vs $Bin1^{fl/fl}$ + LPS). *Ordinary one-way ANOVA and unpaired two-tailed t-test (Cx3cr1CreER + LPS* vs *Bin1fl/fl + LPS), ns: non-significant, *p<0.05, ****p<0.0001, MEAN±SEM, n=4 samples/condition; each sample contained n=2 cortices.*

(G) Real-time PCR analysis of selected genes involved in systemic inflammation in the somatosensory mouse cortex. *Il-1a,* a proinflammatory cytokine, and *the* complement gene C3 were upregulated after LPS induction, and there was an increase in neuroinflammation after microglial Bin1cKO. *Il-1b,* a proinflammatory cytokine, seems to be positively regulated mainly by LPS, and the *C1qa* complement gene was upregulated after Bin1cKO in neuroinflammation. *Unpaired two-tailed t-test, ns: non-significant, *p<0.05, ****p<0.0001, MEAN±SEM, n≥3/condition.*

(H) Cytokine/chemokine profiling for tissue protein lysates was performed on one detection membrane per treatment group (from n=4 biological replicates), with the Cx3cr1^{CreER} + LPS group used as a control.

(I) Quantification of cytokines/chemokines from 4 mouse cortices per group. Heatmap of 40 mouse cytokine/chemokine arrays. The signals from the Cx3cr1^{CreER} + LPS mice were normalized to 1 and compared with those from the Bin1cKO + LPS mice (upregulated cytokines in red, downregulated in blue).

Figure 6. BIN1 regulates the interferon type I microglial proinflammatory response in the adult mouse brain after LPS treatment

(A) Real-time PCR analysis of selected transcription factors (*Stat1, Irf7*) involved in the IFN-I proinflammatory response in the somatosensory mouse cortex. shows their upregulation after microglia Bin1cKO and LPS-induced inflammation. *Ordinary One-way ANOVA, ns: non-significant, *p<0.05, **p<0.01, MEAN±SEM, n≥3/condition.*

(B) Real-time PCR analysis of selected IFN-I-stimulated genes in the somatosensory mouse cortex. *Ifi204, Ifi30* and *Ifitm3* were upregulated after LPS stimulation. There was an increase in neuroinflammation after microglial Bin1cKO, with *Ifi204* showing the most pronounced increase. *Ordinary One-way ANOVA, ns: non-significant, *p<0.05, **p<0.01, MEAN±SEM, n≥3/condition.*

(C) Real-time PCR analysis of selected genes indicative of the microglial IRM subpopulation in the somatosensory mouse cortex. *Ifi27l2a* and *Oasl2* were upregulated after LPS stimulation, and there was a further increase in

neuroinflammation after microglial Bin1cKO. *Unpaired two-tailed t-test, ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, MEAN±SEM, n≥3/condition.*

(D) Representative confocal images of the somatosensory cortex showing microglia (IBA1 - green) and IFI204^{+} cells (IFI204 - red) in Cx3cr1^{CreER} (white arrows), Cx3cr1^{CreER} + LPS and Bin1cKO + LPS conditions.

(E) Analysis of the percentage of IFI204^{+} microglia revealed that after LPS stimulation, almost all the microglia expressed IFI204. *Ordinary one-way ANOVA, ns: non-significant, ****p<0.0001, MEAN±SEM, n=3/condition.*

(F) Fluorescence intensity measurements of IFI204 in microglia revealed that LPS increased IFI204 protein expression, which was further enhanced after microglial Bin1cKO. *Kruskal–Wallis nonparametric test for multiple comparisons, ****adjusted p value<0.0001, MEAN±SEM, n=600 cells/condition*.

(G) Representative confocal images of the somatosensory cortex showing CD68^{+} (CD68- red) microglia (IBA1^{+}) in Cx3cR1^{CreER} and Bin1cKO mice under inflammatory conditions.

(H) Immunohistochemical analysis of the percentage of the volume of CD68 inside microglia (volume of IBA1) revealed an increase in CD68 in Bin1cKO microglia following neuroinflammation (Bin1cKO + LPS vs Cx3cr1^{CreER} + LPS). *Unpaired two-tailed t-test, *p<0.05, MEAN±SEM, n=3/condition.*

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.pdf
- FileS1AllDEGs.xlsx
- FileS2DEGsHumanMicrogliaWilcoxSEAAD.xlsx
- FileS3DEGsMidvsLowBraakMouseintersect.xlsx
- FileS4MicrogliaClustermarkers.xlsx
- FileS5Microgliagenesignatures.xlsx