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Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating the impact of viral encephalitis on amyloid neuropathology
in a mouse model of Alzheimer's disease

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Dominic Ibarra Javonillo

Dissertation Committee:
Professor Thomas E. Lane, Chair
Professor Kim N. Green
Professor Mathew Blurton-Jones
Professor Elizabeth Head
Professor Melissa B. Lodoen

2026

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Chapter 4 © 2026 Alzheimer's Association

Chapter 5 © 2026 Alzheimer's Association

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DEDICATION

To the healthcare workers that led the frontlines of the COVID-19 pandemic
at the cost of their health and sometimes their own lives.

To the survivors who now face the uncertainties of what may lie ahead of them.

To my Philippine ancestors who now have another one of their own pursuing
scientific exploration and biomedical discovery.

To my grandparents, who were all educators in the Philippines and helped me realize the value
of sharing knowledge with those around us.

To my parents Roy and Cynthia Javonillo, who are a constant inspiration and reminder of the
obstacles of immigrating to this country for better opportunities for their children.

To my siblings Ethan and Kayla, who I hoped to inspire by taking these opportunities and
paving my own path for myself.

To the extensive village of supporters, family, friends, and colleagues that brought me
moments of joy, peace, and safety in times of vulnerability.

To Andrew Juarez, a constant anchor that provided unwavering emotional support, strength,
and a beautiful life outside to endure momentary obstacles scattered throughout this journey.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
APP	amyloid precursor protein
ARMs	activated response microglia
A β	amyloid beta
BBB	blood-brain-barrier
BMDM	bone marrow-derived monocyte/macrophages
CNS	central nervous system
DAM	disease associated microglia
DD	differentially down-regulated
DEG	differentially expressed genes
DGE	differential gene expression
DU	differentially up-regulated
FOV	field of view
FTD	Frontotemporal Dementia
GFAP	glial fibrillary acidic protein
GO	gene ontology
GWAS	genome-wide association studies
HBSS	Hanks' Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
i.c.	intracranial
i.p.	intraperitoneal
ISH	<i>in situ</i> hybridization
JHMV	John Howard Mueller Virus

LFB	Luxol Fast Blue
LOAD	late-onset Alzheimer's Disease
MHV	Mouse Hepatitis Virus
MAC2	Galectin-3
MC	monocyte-derived cells
MGnD	microglia neurodegenerative phenotype
MHC	major histocompatibility complex
NBF	10% Neutral Buffered Formalin
NfL	Neurofilament Light
NFTs	neurofibrillary tau tangles
OCT	optimum cutting temperature compound
p.i.	post-infection
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PFU	plaque forming units
PSEN	presenilin
qPCR	quantitative real-time polymerase chain reaction
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SDS	sodium dodecyl sulfate
SS CTX	somatosensory cortex
SSC	saline-sodium citrate
SUB	subiculum
UMAP	Uniform manifold Approximation and Projection
VZV	varicella zoster virus
WT	wildtype

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CHAPTER 2: ABLATION OF MICROGLIA FOLLOWING INFECTION OF THE CENTRAL NERVOUS SYSTEM WITH A NEUROTROPIC MURINE CORONAVIRUS INFECTION LEADS TO INCREASED DEMYELINATION AND IMPAIRED REMYELINATION

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Vita

DOMINIC IBARRA JAVONILLO

EDUCATION

UNIVERSITY OF CALIFORNIA, IRVINE, CA
5th Year PhD Candidate
Department of Neurobiology & Behavior

SEPTEMBER 2021-PRESENT
EXPECTED DATE: JUNE 2026

UNIVERSITY OF CALIFORNIA, IRVINE, CA
M.S., Neurobiology & Behavior

JUNE 2024

UNIVERSITY OF CALIFORNIA, IRVINE, CA
B.S., Biological Sciences, Honors
Minor in Comparative Literature

JUNE 2018

UCI School of Biological Sciences Excellence in Research Thesis (2018): *Neuroprotective Effects of a PGC-1 α Enhancer Against MPTP-Induced Neurotoxicity in a model of Parkinson's Disease (PD)*

RESEARCH EXPERIENCE

UNIVERSITY OF CALIFORNIA, IRVINE, CA

Graduate Student Researcher

APRIL 2022 – PRESENT

Advisor: Dr. Thomas E. Lane

My graduate thesis project broadly investigates the interaction of infiltrating immune cells with Alzheimer's Disease (AD) pathology following murine coronavirus infection. I work specifically with JHMV, a neurotropic strain of mouse hepatitis virus that produces robust viral replication and antiviral immune responses within the central nervous system. Using the 5xFAD mouse model of AD, I used the following techniques:

- **Work under BSL-2 safety cabinet protocols** using proper mouse husbandry and care
- **Flow Cytometry** with 4-5 antibody panels for cellular surface markers
- **Cryopreserving & sectioning fixed brain tissue** with a cryostat
- **Immuno-staining & Confocal Microscopy** of fixed brain, spinal cord, and lymph nodes
- **Batch image analysis of confocal microscopy** using Bitplane IMARIS software
- **Biochemical analysis & ELISA assays** to measure amyloid beta protein concentrations
- **Tissue *In situ* hybridization & qPCR assays of extracted RNA** to assess viral replication
- **Neutralization antibody & viral titer assay** to measure viral load in plasma & brain tissue
- **Compiling data and perform statistical analysis** via GraphPad Prism
- **Spatial Transcriptomic Imaging & RNA sequencing analysis (R studio, Seurat, MAST)** to identify differentially expressed genes across discrete cell types with single-cell resolution

Rotating Graduate Student

JANUARY – MARCH 2022

Advisor: Dr. Thomas E. Lane

During this second rotation in the Interdepartmental Neuroscience Program, I infected Dp16 mouse model of Down syndrome (DS) with the JHM-mouse coronavirus. Our interest focused on investigating differences in neuroimmunological responses to viral infection that may be driven by

a homologous Trisomy 21 condition in mice. Due to this trisomy, immunological-relevant genes such as interferon receptors (I.e., *Ifnar1*, *Ifnar2*, *Ifngr2*) are triplicated, which may contribute to increased cytokine production by immune cells and increased risk of downstream pathology in response to viral infection. I pursued my hypothesis using the same techniques as described above.

Rotating Graduate Student

SEPTEMBER – DECEMBER 2021

Advisor: Dr. Elizabeth Head

During this first rotation in the Interdepartmental Neuroscience Program, I worked closely with Dr. Alessandra Martini to characterize morphological differences in oligodendrocytes—the primary glia type responsible for myelination of axons in the central nervous system. Our interest was investigating differences mediated by Down syndrome (DS) patients, who typically display Alzheimer's Disease (AD) symptoms due to a triplication of chromosome 21. In brain tissue of DS patients with AD, I utilized the following techniques:

- **Immuno-staining & Confocal Microscopy** of fixed, human brain tissue from patient donors
- **Communicating updates and experimental results** for new neuropathology of interest
- **Recording detailed protocols & comprehensive notes** of completed experiments & data

Lab Assistant II at Model Organism Development & Evaluation of Late-Onset

Alzheimer's Disease (MODEL-AD/UCI)

AUGUST 2018 – SEPTEMBER 2021

Advisor: Dr. Kim Green

This NIA-funded consortium across Indiana University, The Jackson Laboratory, and UCI is focused on developing the next generation of *in vivo* mouse models for late-onset Alzheimer's Disease (AD) based on human genetic data. I worked under UCI's Disease Model Development and Phenotyping (DMP) Core directed by Dr. Kim Green and Dr. Grant MacGregor, for developing standardized deep phenotyping assessments of pathophysiological features of new potential AD mouse models generated by MODEL-AD. In this role, I was responsible for the following skills:

- **Perform mouse husbandry, animal care, and tissue collection** (brains, liver, colons)
- **Cryopreserving & sectioning fixed brains** with a sliding microtome
- **Immuno-staining brain tissue** with 3-4 antibody panels for AD neuropathology
- **Obtaining whole images of immuno-stained tissue** via Zeiss Axio Scan.Z1 Slide Scanner
- **Capturing immunofluorescent images using confocal microscopy** (Leica DSM 2500/Leica 550) and Leica Application Suite X software)
- **Batch image analysis of confocal microscopy** using Bitplane IMARIS software
- **Compile data and perform statistical analysis** via GraphPad Prism
- **Micro-dissecting brain regions & tissue** for biochemical and histological assays
- **Providing regular updates & communicating results** during weekly lab meetings
- **Recording detailed protocols & comprehensive notes** of completed experiments & data
- **Training new students & research staff** in operating lab equipment, protocols, safety practices, and general lab management

Undergraduate Research Assistant at Translational Laboratory and

Biorepository (TLaB)

JANUARY 2017 – JUNE 2018

PI: Dr. Sudhakar R. Subramaniam, Dr. Massimo Fiandaca, and Dr. Howard Federoff

This research lab focused on translational research, specifically with the use of mouse models. Under the supervision of Dr. Subramaniam, our project focused on the neuroprotective effects of a confidential drug injected into neurotoxic models of Parkinson's Disease. As an undergraduate researcher, I independently performed the following techniques for my lab mentor:

- **Cryopreserving & sectioning fixed brains** with a sliding microtome
- **Immuno-staining brain tissue (e.g., DAB staining)** for neurodegeneration markers

- **Using ImageJ software to analyzing bright field images** for neuron survival and damage
- **Passaging & maintaining propagated cell cultures** of primary mouse microglia

PUBLICATIONS

JAVONILLO D.I., Furman S., Le L, Fernandez K, Mulford J.N., Singla V., Jha R., Tsourmas K.I., Kwang N.E., Green K.N., Lane T.E. Characterization of effects of a neurotropic murine coronavirus infection on Alzheimer's disease neuropathology of 5xFAD mice. *In review in Alzheimer's & Dementia*.

Furman S., Zayou L., Tsourmas K.I., **JAVONILLO D.I.**, Olivarria G.M., Cheng Y., Pachow C., Fernandez K., Le L., Edwards R.A., Nicholas D.A., Sanchez G.P., Baric R.S., Green K.N., Lane T.E. Infection of 5xFAD mice with a mouse-adapted SARS-CoV-2 does not alter Alzheimer's disease neuropathology yet induces wide-spread changes in gene expression across diverse cell types. *Accepted in Alzheimer's & Dementia*.

Lioudyno, M.I., E.A. Sevrioukov, G.M. Olivarria, L. Hitchcock, **JAVONILLO D.I.**, S.M. Schapansky, I. Rivera, S.T. Wright, E. Head, J. Fortea, T. Wisniewski, A.C. Cuellar, S. DoCarmo, T.E. Lane, J. Busciglio. (2025). Susceptibility to SARS-CoV-2 infection in human cortical cells is influenced by interaction between aneuploidy and biological sex in Down's syndrome. *Acta Neuropathologica*, May 30;149(1):54. doi: [10.1007/s00401-025-02895-2](https://doi.org/10.1007/s00401-025-02895-2).

Butler CA, Arvilla AM, Milinkeviciute G, Da Canche C, Kawauchi S, Rezaie N, Liang HY, **JAVONILLO D.I.**, Thach A, Wang S, Collins S, Walker A, Shi K, Neumann J, Gomez-Arboledas A, Henningfield CM, Hohsfield LA, Mapstone M, Tenner AH, LaFerla FM, Mortazavi A, Macgregor GR, Green KN. (2024) The Abca7^{V1613M} variant reduces A β generation, plaque load, and neuronal damage. *Alzheimer's and Dementia* 2024:1-21. <https://doi.org/10.1002/alz.13783>

Soni N, Hohsfield LA, Tran KM, Kawauchi S, Walker A, **JAVONILLO D.I.**, Phan J, Matheos D, Da Cunha C, Uyar A, Milinkeviciute G, Gomez-Arboledas A, Tran K, Kaczorowski CC, Wood MA, Tenner AJ, LaFerla FM, Carter GW, Mortazavi A, Swarup V, MacGregor GR, Green KN. (2024) Genetic diversity promotes resilience in a mouse model of Alzheimer's disease. *Alzheimer's and Dementia* 2024:1-23. <https://doi.org/10.1002/alz.13753>

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Trujillo-Estrada L., Vanderklisch P.W., Nguyen M.M.T., Kuang R.R., Nguyen C., Hyunh E., Cunha C.d., **JAVONILLO D.I.**, Forner S., Martini A.C., Sarraf S.T., Vincent S.F., Baglietto-Vargas D., LaFerla F.M. (2021) SPG302 reverses synaptic and cognitive deficits without altering amyloid or tau pathology in a transgenic model of Alzheimer's disease. *Neurotherapeutics*. <https://doi.org/10.1007/s13311-021-01143-1>

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JAVONILLO D., Subramaniam S.R. 2018. Neuroprotective Effects of a PGC-1 α Enhancer Against MPTP-Induced Neurotoxicity in a model of Parkinson's Disease (PD). *Journal of Undergraduate Research in Biological Sciences*.

CONFERENCE PRESENTATIONS

JAVONILLO D.I., Lane TE. (2025) Spatial transcriptomic imaging reveals an attenuated myeloid response to amyloid plaque pathology following viral encephalitis in 5XFAD mice. Poster presentation and invited short talk at Society for Neuroscience Annual Meeting 2025 & UC Irvine Immunology Fair.

JAVONILLO D.I., Fruman SM, Fernandez K, Tsourmas K, Manlapaz C, Green KN, Lane TE. (2024) Assessing the subacute impact of coronavirus-induced neuroinflammation on amyloid-beta pathology in 5XFAD mice. Invited short talk and poster presented at FASEB Science Research Conferences: Translational Neuroimmunology 2024, Keystone Symposia Neuroimmune Interactions, and Society for Neuroscience Annual Meeting 2024.

JAVONILLO D.I., Fruman SM, Fernandez K, Tsourmas K, Manlapaz C, Green KN, Lane TE. (2023) Infiltrating monocytes may attenuate amyloid-beta plaques in 5xFAD mice following neurotropic murine coronavirus infection. Poster presented at So Cal Alzheimer's Disease Centers Research Symposium, UCI REMIND Emerging Scientists Symposium.

JAVONILLO D.I., *et al.* (2021) ABCA7*V1599M^{em1Aduci} variant in 5xFAD mice mediates differences in amyloid-beta pathology and reactive gliosis. Virtual poster presentation at the

Society for Neuroscience (SfN) 2021 Annual Meeting, Minority Science Program Research Symposium.

Forner S, **JAVONILLO D.I.**, Tran K.M., Baglietto-Vargas D, Phan J., Forner S., Da Cunha C., Kawauchi S., Tenner A.J., La Ferla F.M., MacGregor G.R., Green K.N., MODEL-AD. Accumulation of periodic-acid schiff granules are increased in the presence of humanized amyloid beta. Poster presentation to the 2021 Alzheimer's Association International Conference (AAIC).

JAVONILLO D.I., Tran K.M., Baglietto-Vargas D, Phan J., Forner S., Da Cunha C., Kawauchi S., Tenner A.J., La Ferla F.M., MacGregor G.R., Green K.N., Humanized amyloid-beta expression drives accumulation of Periodic Acid-Schiff granules in the hippocampus of a knock-in mouse strain. Poster presentation to the 2021 Society for Neuroscience (SfN) Global Connectome & Alzheimer's/Parkinson's Disease (ADPD) Conference.

JAVONILLO D.I., Subramaniam S.R. 2018. Neuroprotective Effects of a PGC-1 α Enhancer Against MPTP-Induced Neurotoxicity in a model of Parkinson's Disease (PD). Short talk and poster presented at UCI School of Biological Sciences Undergraduate Research Symposium 2018; Irvine, CA.

JAVONILLO D.I. 2018. The Utility of MDMA for Treatment-Resistant PTSD. Public talk presented at UCI Reclaim Mental Health Conference 2018.

HONORS AND AWARDS

Allergan Foundation Graduate Student Award Recipient	MAY 2026
UCLA Bio-Launch Competitive Postdoc Recruitment Program	FEBRUARY 2026
Translational Neuroimmunology Scholarship Award, FASEB Research Conference	JULY 2024
NIH Neuroimmunology T32 Training Grant Fellowship	JUNE 2024
NIH Initiative for Maximizing Student Development (IMSD) Fellow	JUNE 2021
Honors in Biological Sciences, University of California Irvine	JUNE 2018
UCI School of Biological Sciences Excellence in Research	JUNE 2018
Dean's Honors List Recipient	2015–2018
Undergraduate Research Opportunities Program (UROP) Award Recipient	JUNE 2015

TECHNICAL SKILLS

Laboratory Techniques:

Flow Cytometry
RNA Extraction
Viral Titers (via Plaque Assay)
Tissue Homogenization
Protein Concentration (Nanodrop)
Intraperitoneal/Intracranial Injections

Animal Husbandry/Perfusion (Rodent)
Neutralization Antibody Assay
qPCR Assays
ELISA/Mesoscale Discovery Multi-Plex Assay
Protein Extraction
Sterile bench techniques (BSL-1 to BSL-3)
Organ Collection & Fixation

Data & Bioinformatic Analysis:

FIJI, Image J
FlowJo
Single-Cell Sequencing Analysis

GraphPad Prism
Bitplane IMARIS
R Studio Programming
Diff. Gene Expression & GO Pathway Analysis

Project & Office Management:

Electronic notebook record keeping
Data visualization & Publication Figures
Lab Organization/Maintenance
Microsoft Office Programs

Scientific writing
Mentoring undergraduate researchers
Lab Inventory
Adobe Illustrator, Photoshop, Audition

TEACHING EXPERIENCE

UNIVERSITY OF CALIFORNIA, IRVINE, CA

SEPTEMBER 2022–JUNE 2023

Teaching Assistant for: **Introduction to Biology** (*Biological Sciences 93*) **Fall 2022**
Neurobiology Lab (*Biological Sciences 113L*) **Spring 2023**
Visiting Lecturer for: **Neuroimmunology** (*Neurobiology 217*) **Winter 2025, 2026**

- Lead and facilitate individual class discussions of 83 students to review lecture material and retain learned content through interactive teaching activities, group work, and summarized lightning talks
- Maintain a safe lab environment for students learning key laboratory techniques in neurobiology including brain dissections, electrophysiology recordings, neuropharmacological manipulations, PCRs, and CRISPR

VALLEY HIGH SCHOOL, SANTA ANA, CA

Math Tutor at Gaining Early Awareness & Readiness for Undergraduate Programs (GEAR UP)

SEPTEMBER 2014–JUNE 2015

The GEAR UP partnership grant from the US Department of Education aimed to elevate academic achievement, college attendance, college retention, and graduation rates of first-generation, low-income, underrepresented, and underserved high school students. UCI GEAR-UP employed me as a Math Tutor to actively aid struggling juniors and seniors enrolled in remedial math classes.

Primary Responsibilities:

- Employ pedagogical approaches in tutoring students in Algebra I and Algebra II topics
- Actively assisting the high school instructor in assessing tests, delivering group work, and facilitating discussion with the class over relevant class materials
- Work individually with students who show evidence of a consistent lack of understanding

ACADEMIC & PROFESSIONAL DEVELOPMENT

NIH Neuroimmunology T32 Training Grant Fellowship

JUNE 2024

NIH Initiative for Maximizing Student Development (IMSD) Fellowship

SUMMER 2021

Visualizing Science Workshop, UCI GPS-STEM, University of California, Irvine

2020

Reclaiming STEM Workshop, University of California, Irvine

2019, 2020, 2021

- A science communication and science policy workshop for underrepresented groups in STEM, including BIPOCs, first-generation, minorities, women, LGBTQIA+, people with disabilities, etc.

Confronting Extremism Talks, University of California, Irvine

November 2019

UCI Addiction Symposium, Irvine Center for Addiction Neuroscience

October 2019

SoCal Science Writing Symposium, University of Southern California

April 2019

American Association for the Advancement of Science (AAAS) Annual Meeting,

Washington, D.C.

February 2019

Confronting Extremism Symposium: American Association for the Advancement of Science (AAAS) Communicating Science Workshop, University of California, Irvine

January 2019

Confronting Extremism Symposium: Denial of Science, Sue & Bill Gross Stem Cell Research Center,

University of California, Irvine

January 2019

UCI Reclaim Mental Health Conference, University of California, Irvine

2016–2019

LEADERSHIP & COMMUNITY OUTREACH

Lead Editor, *Loh Down on Science (LDOS)*

April 2025 – Present

LDOS is a daily science minute radio segment hosted by Sandra Tsing Loh, a former scientist turned actor who now helps trainees bring science to wider audiences through humor and insight. Episodes are written by UC Irvine graduate students to provide an opportunity to communicate

emerging scientific research to broader audiences through short form writing for radio. UCI graduate student writers find their work widely broadcasted and shared Southern California and the country through both LAist 89.3 and NPR, providing students with millions of listeners.

- **Manage the team of *Loh Down* editors & writers** to ensure deadlines & expectations are met
- **Educate & train new writers** on communication skills that enhance clarity & engagement
- **Collaborate with production team & host** to ensure each script is delivered on time

Editor, *Loh Down on Science*

November 2024 – April 2025

- **Review submitted pitches** each month to determine its suitability as a *Loh Down* episode
- **Collaborate & provide constructive feedback** to writers over rounds of script revisions
- **Perform fact checking** on scientific accuracy of each script

Writer, *Loh Down on Science*

January 2023 – November 2024

- **Searching & highlighting recent scientific research papers** that interest lay listeners
- **Submit pitches** to editors highlighting the innovation & broader impacts of the research paper
- **Craft a 130-word radio script** efficiently reporting research findings for a 90-second episode

Co-Chair, UCI Center for Neurobiology and Learning (CNLM) Ambassadors 2023

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Present

The CNLM aims to **advance public understanding of brain science** through outreach & educational activities. Over 100 UC Irvine undergraduate students, graduate students and postdoctoral fellows **design, execute & evaluate** meaningful activities on campus and in the community throughout the year. The program provides meaningful leadership opportunities for UCI brain scientists as well as professional development training in scientific communication, educational program outcome design, & evaluation.

- **Assist in community outreach and event programming** led by UCI CNLM such as the USA Brain Bee, public lectures, philanthropy social dinners, & week-long “brain camps”
- **Engage with community leaders, parents, and visiting students** from K-12 schools across the Orange County area to learn about neuroscience and the types of research occurring at UCI to learn about learning, memory, and neurological conditions through lab demonstrations, tours, and teaching activities

Co-Chair, UCI REMIND

June 2023 – Present

Research and Education in Memory Impairments and Neurological Disorders (REMIND) is a campus organization led by predoctoral and postdoctoral trainees within UCI MIND, UCI’s research center on aging and dementia research.

- **Develop outreach and education programming** for underrepresented minority high school students in the Orange County area to advance interest and retention in undergraduate biology programs, research, and education pertaining to dementia and neurodegenerative diseases such as Alzheimer’s Disease
- **Present comprehensive overview of dementia research** to deliver engaging talks to community leaders & stakeholders across broad demographics and levels of expertise through webinars & talks
- **Facilitate research symposia** to connect dementia scientists across diverse disciplines & institutions

Co-Executive Director, Queer, Trans, & Allies in Biological Sciences (QTABS) 2023

–

Present

QTABS is a trainee-led organization supported by the Inclusive Excellence Leadership Award from the UCI School of Biological Sciences Office of DEI. We are a collective of LGBTQIA+ and allied graduate, post-doctoral, and staff researchers within the biological and biomedical fields at UC

Irvine. QTABS event programming centers around the unique needs and interests of LGBTQIA+ trainees and will be held regularly throughout the quarter! We believe that building an affirming research community spanning the many disciplines that interact with biology, health, and medicine will provide much needed support for LGBTQIA+ researchers to succeed and thrive.

- **Assess and survey the needs of LGBTQIA+ researchers** (i.e. graduate students, post-doctoral scholars, and staff) by creating, distributing, and analyzing survey and poll materials to guide directed QTABS programming
- **Coordinate with QTABS leadership to plan and execute successful events programming** including professional development, community engagement, cross-organizational collaborations, and health justice and advocacy

Intercollegiate Neuroscience Research Journal Club (INRJC)

June – August 2020

Founders: Jeffrey Sagun, Meg Smith, John Albanese

The INRJC is a student initiative created by Trinity College–Hartford neuroscience students who wanted to provide individuals with the opportunity to have a productive and engaging summer in the field of neuroscience in lieu of the summer internships and jobs that were cancelled due to the pandemic. For students interested in continuing research in their undergraduate or graduate careers, reading research articles is a critical part of any research program. The INRJC is a way for these students interested in neuroscience, research, and medicine to enhance their engagement within the field of science. The journal club has garnered interest from approximately 1,400 students from 500+ U.S. colleges, including Trinity, Columbia, Harvard, Grinnell, Northwestern, Middlebury, University of Chicago, and more.

Primary Responsibilities:

- Read a weekly-assigned and primary research article or literature review on a variety of neuroscience topics
- Summarize the article to include key findings from the primary research or key points from the literature review
- Post the summary on a weekly basis on a LinkedIn discussion board and engage with other members' posts
- Attend weekly Zoom meetings with Group Leaders & other members to discuss assigned sections of the article
- Group Leaders will coordinate and manage Zoom call, while facilitating & leading discussions between members
- Group Leaders will report weekly summaries of the Zoom call & provide any feedback to INRJC executive board

Post-Baccalaureate Life Science Liaison for STEMbase.org

August – December 2020

Board Chair, Founder: Paulo Figueroa-Delgado

STEMbase.org is a digital platform, developed for trainees by trainees, that amplifies and promotes available STEM opportunities and resources to aid readiness for STEM careers. Their mission is to facilitate equal access to information and serve as a centralized database for trainees to navigate available opportunities and resources (i.e. internships, fellowships, outreach platforms) to increase accessibility to information and programs in STEM. This is to engage, expose and prepare trainees for their future careers in STEM. It also aims to aid personal and professional development with a strong focus on serving underrepresented and marginalized communities to bridge opportunity gaps and maximize retention and diverse representation in the STEM workforce. *(CONTINUED ON NEXT PAGE)*

Primary Responsibilities:

- Generate content on post-baccalaureate opportunities and resources in life sciences in a timely manner.
- Engage through social media platforms advocating and advertising the opportunities available in life sciences.
- Generate and curate content for a life sciences newsletter for members that sign-up to receive updates on new research and opportunities they should be aware of
- Maintain transparent communications with peer liaisons, leaders, and board members to complete assignments
- Encouraged to invite others to become a part of the STEMbase.org team by sharing position applications

Content Writer for Morning Sign-Out (MSO)

2017–2019

Supervisor: Andrew Lee, MD

Morning Sign Out is a blog network composed of short, digestible articles from writers across the campuses in the University of California. The articles focus on a range of topics relevant to biomedical research, healthcare, public health, and wellness. The aim is to bridge the divide between the world of medicine and science to the greater public. By turning science and medicine into something understandable for readers with enough critical information, MSO envisions leaving readers more knowledgeable than before.

Primary Responsibilities:

- Read, summarize, and translate primary scientific literature and review articles into accessible language
- Write engaging, well-researched articles of recent, primary science research directed to lay audiences
- Perform effective science communication with understandable analogies and limited jargon

ABSTRACT OF THE DISSERTATION

Investigating the impact of viral encephalitis on amyloid neuropathology

in a mouse model of Alzheimer's disease

By

Dominic Ibarra Javonillo

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2026

Professor Thomas E. Lane, Chair

Alzheimer's Disease (AD) is the most common cause of dementia impacting almost 7 million people in the United States with cases projected to increase over 13 million by 2050. Numerous large genome-wide association studies (GWAS) have repeatedly identified AD risk genes associated with immunity and specifically expressed in myeloid cells, implicating neuroimmune processes and neuroinflammation as a major factor of AD etiology. Microbial infection has been broadly explored as a mechanism through which both genetic and environmental contributions interact together to influence AD risk and neuropathology. Pathogenic microbes that invade the CNS can cause significant neuroinflammation through CNS-resident immune cells and infiltration of peripheral immune cells such as monocyte/macrophages and T cells. Recent epidemiological studies using human datasets have demonstrated several viral pathogens tightly associated with different neurological diseases, while the strongest association was between AD and viral encephalitis. While the field's current understanding of the contributions of viruses and their resulting inflammation on AD remains nuanced and complex, ongoing investigations are precisely identifying direct and indirect viral mechanisms leading to

CNS damage and associated cognitive impairments like dementia. This is further highlighted by the increasing frequency of emerging viral pathogens like SARS-CoV-2 in which the long-term neurologic sequelae are unknown.

To address this, several experiments were carried out to better understand the role of brain-resident microglia and infiltrating peripheral immune cells in mediating neurological damage following infection with a neurotropic strain of murine coronavirus (JHMV). Following intracranial inoculation and the development of immune-mediated demyelination, JHMV-infected mice were treated with the small molecular inhibitor of colony stimulating factor 1 receptor (CSF1R), PLX5622, to deplete microglia. Treatment with PLX5622 did not affect viral replication within the CNS yet the severity of demyelination was increased and remyelination impaired compared to control mice. Gene expression analysis revealed that targeting microglia resulted in altered expression of genes associated with immune cell activation and phagocytosis of myelin debris. These findings indicate that microglia are not critical in viral surveillance in persistently JHMV-infected mice yet restrict white matter damage and remyelination, in part, by influencing phagocytosis of myelin debris.

Furthermore, utilizing a tetracycline-inducible CXCL1 mouse line, we sustained neutrophil infiltration in the CNS of JHMV-infected mice to determine their contributions to increased demyelinating damage. Single-cell RNA sequencing revealed upregulation of neutrophil transcriptional programs associated with neutrophil activation, cytotoxicity, and degranulation. Flow cytometry confirmed increased numbers of CD11b⁺ and CD63⁺ neutrophils in DTg spinal cords, consistent with enhanced degranulation. Treatment with Nexinhib20 (N20), a small-molecule degranulation inhibitor reduced spinal cord demyelination in DTg mice without substantially altering neutrophil infiltration or surface expression of degranulation markers,

indicating that limiting granule release—rather than immune cell recruitment—is sufficient to attenuate tissue damage. Lastly, spatial transcriptomic analysis, via the CosMx platform, revealed that N20 treatment reprogrammed the lesion microenvironment, downregulating inflammatory mediators *Ccl2*, *Cxcl9*, *B2m*, *Ptpnc*, *Srgn*, and upregulating a disease-associated myeloid (DAM) signature, *ie. Apoe*, *Trem2*, *Lpl*, *Gpnmb*, *Ctsd*, and *Spp1*, consistent with enhanced debris removal and tissue repair programs. Together, these findings signal neutrophil degranulation as a contributor to white matter pathology during chronic viral-induced demyelination and suggest that targeting granule exocytosis represents a viable strategy to limit immune-mediated CNS injury.

To understand whether systemic SARS-CoV-2 could induce effects on AD neuropathology, aged 5xFAD and wildtype (WT) mice were infected with a mouse-adapted SARS-CoV-2 (MA10) and extensive characterization of molecular and cellular changes within the brains was performed. We found that while MA10 infection induced acute viral pneumonia, viral RNA was not detectable in the brains of infected mice nor induced glial activation and neuroinflammation. Furthermore, MA10 infection did not affect Ab plaque volume or numbers in 5xFAD mice compared to uninfected mice. However, spatial transcriptomics did reveal altered expression of genes associated with homeostatic function in neurons, glia, and vascular endothelial cells. Although MA10 infection did not affect AD neuropathology, systemic infection did result in numerous down-stream effects on gene expression associated with resident CNS cell functions that may impact neurologic disease.

Finally, to investigate how viral encephalitis may impacted AD neuropathology, we infected aged 5xFAD and WT mice with JHMV. We observed that JHMV-induced encephalitis at 12 days post-infection resulted in minimal changes to overall A β protein, despite increased CD4⁺ and CD8⁺ T cell infiltration and *Lgals3*/MAC2-expressing macrophages surrounding more

compact A β plaques in the brain. Spatial transcriptomic imaging and pathway analysis of differentially expressed genes (DEGs) within myeloid cells demonstrate down-regulated disease-associated (DAM) pathways involving A β clearance, response to lipids, and macrophage activation within infected 5xFAD brains. These findings reveal that viral encephalitis and its resulting inflammatory mechanisms impacts the expression of key genes within myeloid cells responding to A β plaque pathology, which has implications to the role of viral encephalitis as a risk factor for Alzheimer's disease.

CHAPTER 1

BACKGROUND AND INTRODUCTION

1. ALZHEIMER'S DISEASE

1.1. Overview of Alzheimer's Disease

Alzheimer's Disease (AD) is the most common cause of dementia impacting almost 7 million people in the United States with cases projected to increase over 13 million by 2050^{1,2}. As a progressive neurodegenerative disease, AD is defined by its substantial synaptic dysfunction and neuronal loss leading to brain atrophy, cognitive decline, and severe memory impairment^{3,4}. Two characteristic hallmarks of AD pathology are the widespread deposition of *neuritic beta-amyloid (A β) plaques* and aggregation of *neurofibrillary tau tangles (NFTs)*, which are proposed to occur consecutively or work synergistically towards neuronal damage and loss associated with AD^{5,6}. As neurodegeneration progresses, cognitive impairments begin to manifest through subtle problems with memory, language, and thinking before escalating to significant impairments to behavior, motor, and executive functions¹.

AD is typically categorized as either *early-onset (EOAD)* or *late-onset (LOAD)* based on an arbitrary age threshold of either 30–60 or 60–65 years, respectively⁷. The prevalence of EOAD makes up between 5-10% of all AD cases, yet patients with EOAD will often experience rapid progression of clinical symptoms^{8,9}. Even within EOAD cases, there are distinct observed patterns of inheritance. Mendelian EOAD usually arises from mutations in *amyloid precursor protein (APP)* or *presenilin genes (PSEN1, PSEN2)* and are fully penetrant with an autosomal dominant inheritance pattern^{7,10}. As a result, mendelian EOAD is also referred to as familial EOAD or *autosomal dominant AD (ADAD)*. However, EOAD can also occur through non-mendelian inheritance, which is often sporadic or inconsistent with autosomal dominant inheritance^{7,9}. This sporadic form of EOAD may be a result of polygenic effects from other rare genetic variants and mutations such as trisomy 21 that confer increased risk for EOAD¹¹. Similarly, LOAD is

understood to also be multifactorial with potential contributions from age, modifiable environmental factors, or a greater magnitude of potential genetic polymorphic variants inducing polygenic effects that may impact the age of onset¹².

1.2. Neuropathological Characteristics of Alzheimer's Disease

Neuritic Plaques: Extracellular deposition of aggregated A β protein can initially appear in the hippocampus and cortex before progressively spreading throughout the AD brain^{13–15}. A β protein arises from APP, a transmembrane protein highly abundant along neuronal synapses that undergoes intramembrane proteolysis through the *non-amyloidogenic* or *amyloidogenic pathway*¹⁶. Through the non-amyloidogenic pathway, APP is first cleaved by *α -secretase* to produce an 89-amino acid carboxy-terminal fragment (α -CTF), which is also cleaved by *γ -secretase* (a protein complex comprised of presenilin proteins) to release p3 into the extracellular space and *APP intracellular domain (AICD)* within the cytoplasm¹⁷. Importantly, A β production depends on the APP proteolytic processing via β - and γ -secretase enzymes through the amyloidogenic pathway¹⁷. Here, APP is initially cleaved by *β -secretase* into a 99-amino acid carboxy-terminal fragment (β -CTF), which is further cleaved by γ -secretase at various sites to produce A β before it is secreted out of the cell and into the extracellular matrix as a monomer¹⁸. The heterogeneity of γ -secretase cleavage produces multiple A β species of varying sizes, usually 27-43 amino acids in length with A β_{40} and A β_{42} as the most abundant species in the brain¹⁹. Neuritic A β plaques are typically comprised of A β_{42} , which has a higher hydrophobic propensity to aggregate in high concentrations¹⁷.

These plaques are initially assembled when A β monomers aggregate to form soluble *oligomeric A β (oA β)*, which are considered neurotoxic by inducing abnormal glutamate levels, NMDA-mediated excitotoxicity, and synapse loss^{17,20,21}. The continued production of oA β will

induce the formation of large soluble protofibril aggregates, which can further dysregulate synaptic plasticity and accumulate within inflammatory glial cells^{21,22}. If left unmanaged through impaired clearance and degradation mechanisms, A β protofibrils will continue to aggregate and assemble to form cross- β -sheets with variable polymorphic structures further stabilized by hydrogen bonds, which accumulate together into the characteristic extracellular neuritic plaque²¹.

Although tau NFT aggregation more closely correlates with neuronal loss and clinical symptoms of AD, genetic studies following autosomal-dominant inheritance of familial mutations (i.e. ADAD) in APP or enzymes processing APP into A β implicate plaque deposition occurs earlier in clinical disease as a key initiator of EOAD^{10,23,24}. This has been supported in animal studies demonstrating A β plaque deposition induces tau hyperphosphorylation and increased tau levels in the *cerebrospinal fluid (CSF)* of transgenic APP models without expressing a human tau transgene^{25,26}. In contrast to EOAD attributed with dysregulated APP processing, LOAD is proposed to be tied to impaired protein homeostasis such as degradation and clearance, further supporting the importance of A β aggregation in the AD brain^{27–29}. These findings illustrate A β -dependent pathways provoking AD onset while A β -independent pathways such as spreading tau aggregates impact the AD progression towards advanced neurodegeneration^{5,21,30}. This is corroborated by the failure of most anti-A β /amyloid therapies to mediate therapeutic benefits to AD patients during the late stages of clinical disease³¹.

Neurofibrillary Tangles: NFTs are primarily comprised of tau protein encoded by *microtubule-associated protein tau (MAPT)*, which acts as a microtubule binding component to promote the polymerization and stability of microtubules localized within axonal projections of neurons^{17,32}. The distribution of tau and its role in synaptic function is primarily regulated through post-translational modifications such as phosphorylation and proteolytic cleavage¹⁷. With 47

potential phosphorylation sites where protein kinases and phosphatases can act, *tau hyperphosphorylation* can impact binding between tau and microtubules, resulting with tau dissociation from microtubules and further enhancing its aggregation. Consequentially, intraneuronal accumulation of hyperphosphorylated tau monomers can further aggregate into oligomers and fibrils within cell bodies and dendrites before organizing into *paired helical filaments (PHF)*, the main component of NFTs³³.

The first appearance of NFTs occur in the entorhinal cortex where it will progressively spread to interconnected brain regions such as the CA1 and subiculum region of the hippocampus before reaching the neocortex^{15,34}. Notably, the widespread distribution of tau pathology strongly correlates with the progression of cognitive impairments in AD patients, resulting with the Braak stage system to classify the severity of AD onset using histological staining³⁵. While similar tauopathies may occur within the medial temporal lobe in other neurodegenerative conditions such as FTD, the progression of NFTs outside the medial temporal lobe seems to occur in the presence of elevated A β ³⁶. As such, numerous studies on pre-clinical animal models have proposed that tau fibrils seeded through intracranial injections can propagate and spread to other brain regions³⁷. The proposed mechanism describes the secretion and release of tau from donor cells, which are then internalized via endocytosis by neighboring cells where soluble tau will also begin to aggregate and accumulate³⁸.

Neuroinflammation: In response to the aggregated A β and tau pathology, microglia and astrocytes become activated and reactive to mount a neuroinflammatory response through gliosis³⁹. However, this area of research is quickly expanding to determine whether these neuroinflammation responses act to trigger and sustain A β and tau pathology, respond to pathology to limit its spread, or a combination of both during different stages of AD.

Microglia are derived from yolk sac primitive macrophages, which enter and colonize the brain parenchyma before *blood-brain-barrier (BBB)* formation restricts further myeloid cell infiltration into the CNS. As the principal myeloid cells of the CNS, microglia perform immunological roles such as responding to neuronal injury, clearance of cellular debris via phagocytosis, and combatting pathogens. During neurodegenerative diseases like AD, microglia transition from homeostatic states into activated inflammatory states, which correlate with cognitive decline and neurodegeneration. Activated microglia physically surround plaques in a sphere-like manner and are reported to uptake A β , providing evidence that microglia play a role in plaque maintenance. In response to A β , activated microglia can also polarize into two phenotypes dependent on the release of both pro- and anti-inflammatory cytokines. The M1 phenotype is responsible for producing pro-inflammatory responses with cytokines such as *interleukin (IL)-1 β* , *IL-6*, and *tumor necrosis factor-alpha (TNF- α)*, which can enhance oxidative stress, impair synaptic function through excessive pruning, and further stimulate neurotoxicity^{40,41}. Meanwhile, the M2 phenotype is closely associated with anti-inflammatory and neuroprotective responses with cytokines like IL-10, IL-4, and *transforming growth factor-beta (TGF- β)* which can mediate microglial A β and tau clearance^{40,42}. However, chronic microglial activation can also lead to dysregulated responses that may also trigger further seeding of misfolded protein aggregates like A β and tau³⁹. Indeed, the contributions of microglia to both the onset and progression of AD pathology is extensive and Section A.7 “*Alzheimer’s Disease and Tissue-Resident Immunity*” will further expand on how microglia have quickly emerged as a significant player in AD through its role as the tissue-resident myeloid cell of the CNS.

In addition to microglia, astrocytes also play a significant role in regulating inflammation within the CNS. As the most prominent glial cell in the CNS, astrocytes help to provide structural

support at CNS borders such as the BBB, create glial scars in response to brain injury, and maintain ion homeostasis and neurotransmission^{43–45}. Importantly, their roles along borders and scars act as functional barriers to limit entry of peripheral immune cells into the brain parenchyma, thus regulating key aspects of inflammation in the CNS⁴³. Following chronic A β -mediated activation of microglia, secreted pro-inflammatory cytokines can also lead to astrogliosis⁴⁶. Once reactive, astrocytes can also switch from a neurotoxic, pro-inflammatory A1 phenotype or an anti-inflammatory, neuroprotective A2 phenotype^{47,48}. Indeed, A1-reactive astrocytes have been found throughout the brains of AD patients and pre-clinical animal models surrounding A β plaques where they have exhibited impaired phagocytosis and neuroprotective mechanisms supporting neuronal survival⁴⁶. During this pro-inflammatory state, astrocytes release complement proteins and more pro-inflammatory cytokines, further amplifying the inflammatory environment within the CNS in response to A β ⁴⁹. Astrocytes exposed to pro-inflammatory cytokines IL-1, IL-6, TNF- α , and *interferon-gamma (IFN- γ)* have also demonstrated upregulation of amyloidogenic APP processing and thus increase A β production⁵⁰.

Neurodegeneration and Atrophy: Post-mortem analysis of AD brains broadly reveal characteristic regions where neurodegeneration typically appear. The most extensive brain atrophy appear to be in frontal and temporal cortices, where its gyri have reduced tissue corresponding with enlarged sulci⁵¹. These impacted brain regions also include the medial temporal lobe and hippocampus regions, which demonstrate atrophy through *structural magnetic resonance imaging (MRI)* to measure grey matter volume and cortical thickness^{15,52}. In ADAD, EOAD, and LOAD, *in vivo* brain imaging reveal similar patterns of neurodegeneration occurring in medial temporal lobes, parietal, and frontal cortices^{53,54}. As a result of regional atrophy in the AD brain, overall brain weight is decreased and ventricles containing CSF are also enlarged, specifically within the

frontal and temporal regions^{52,55}. One mechanism contributing to neuronal cell death is A β - and tau-mediated mitochondrial damage and energy metabolism. A β has been found in the mitochondria of both AD patients and pre-clinical AD animal models, which correlate with abnormal mitochondrial structure and impaired ATP production, which increases oxidative stress and the production of *reactive oxidative species (ROS)*⁵⁶. The resulting A β -induced production of ROS within mitochondria can exacerbate the ongoing mitochondrial dysfunction, ultimately leading to apoptosis and neuronal cell death⁵⁷.

While decreased brain volume is largely attributed with neuronal loss, synaptic loss appears to precede neuronal loss as a result of ongoing amyloid and tau pathology and is considered the strongest driver of cognitive decline in AD⁵⁸. Surrounding A β plaques in AD brains, there is a loss of structural proteins from both the pre-synaptic (i.e., synaptophysin, VAMP2, and SNAP25) and post-synaptic terminal (i.e., PSD95 and Shank1)⁵⁹. Notably, synapse loss appears to be disease-specific as these synaptic integrity appears to be preserved in the brain during healthy aging without the presence of AD pathology⁶⁰. Additionally, synaptic dysfunction may also contribute to the pathogenesis of synaptic and subsequent neuronal loss in AD. In a transgenic rodent A β models, soluble A β production resulted in hyperactive neuronal excitation due to synaptic calcium influx, which was rescued by inhibiting APP processing⁶¹. Additionally, secreted A β oligomers can elevate glutamate levels outside the synapse, resulting in NMDA receptor excitotoxicity which inhibit long-term potentiation (LTP)^{20,62}. Transgenic tau models also reveal impairments in electrophysiological properties of pyramidal neurons within the neocortex through altered firing rates and firing patterns⁶³. Additionally, soluble tau production in mice expressing A β and human tau appears to suppress neural activity within the parietal cortex, which was rescued by specifically reducing tau expression⁶⁴.

Autophagy mechanisms also have a role in exacerbating neuronal damage leading to cell death. Healthy autophagy is triggered by metabolic stress, resulting in the reprocessing of non-essential proteins or targeting of misfolded protein aggregates and damaged organelle components⁶⁵. However, ongoing mitochondrial dysfunction, ROS production, and APP products during disease-states leads to excessive burden of lysosomal functions responsible for protein degradation and clearance⁶⁵⁻⁶⁷. As a result, cellular waste and debris progressively accumulate within neurons in the form of waste-filled vacuoles which engorge neuronal branches into dystrophic neurites^{19,66,68}. These neurites have a high density of products, metabolites, and enzymes involved in APP processing, contributing to continued damage to lysosomal membranes leading up to the release of cathepsin proteins that trigger apoptosis pathways and subsequent neuronal cell death^{68,69}.

1.3. Risk Factors of Alzheimer's Disease

Age is the greatest risk factor for AD, yet it is not a normal part of aging. In the aged population, the prevalence of people with AD dramatically increases with age from 5% to 13.1% to 33% in people within 65–74, 75–85, and 85+ years of age, respectively^{1,2}. Notably, as the baby-boomer generation continues to age, the current prevalence of AD is predicted to significantly increase in the United States⁷⁰. Yet, age alone is not the sole etiological factor for AD. While many prevailing theories have been proposed to illustrate causal factors and mechanisms underlying AD pathogenesis, no single theory has gained universal acceptance. As a result, AD is widely accepted as a complex, multifactorial disease where multiple risk factors can contribute to the onset, progression, and severity of AD pathology⁷¹. The following section briefly describes the role of different risk and protective factors in impacting AD pathogenesis.

Genetic Risk Factors: Early twin studies have demonstrated that AD has a fairly high heritability factor ranging from 60-80%, yet an updated report presents genetic contribution estimates to about 71%^{72,73}. As described previously, AD can be subdivided into LOAD or EOAD, which can be further distinguished into ADAD or sporadic, non-mendelian forms of EOAD^{7,9,10}. Although rare, cases of ADAD are associated with highly-penetrant mutations in *APP* (which encodes the precursor to A β protein) and in presenilin genes *PSEN1* and *PSEN2* (which encode proteins that activate the γ -secretase complex for A β processing)⁷⁴⁻⁷⁶. While these rare ADAD mutations account for a small fraction of EOAD cases, polygenic effects from mutations and rare genetic variants can result in the sporadic form of EOAD⁷. One important consideration is that *APP* is located in chromosome 21, which becomes triplicated in trisomy 21 or *Down syndrome (DS)* and leads to overexpression of APP and accelerated A β deposition within the brains of patients with DS by 40-years of age⁷⁷.

While rare, highly penetrant mutations are defining characteristics of ADAD, the polygenic effects of more common, yet incompletely penetrant genetic variations contributing to sporadic EOAD and LOAD susceptibility are extensive and less defined^{7,12,19}. The strongest genetic factor contributing to LOAD is *APOE*, the gene within chromosome 19 that encodes *apolipoprotein E (ApoE)*^{78,79}. Within the CNS, ApoE is predominantly produced by astrocytes, activated microglia, and neurons under stress⁸⁰. ApoE proteins bind with cholesterol and phospholipids to form lipoprotein particles through astrocytic ABCA1 activity^{19,49,81}. These lipidated ApoE binds with A β monomers and the ApoE-A β complex interacts with LDLR and LRP1 lipoprotein receptors, which transports A β into perivascular spaces around the brain vasculature for clearance via glymphatic pathways^{19,81}. There are three *APOE* allelic variants relevant to AD: *APOE* ϵ 2, *APOE* ϵ 3, and *APOE* ϵ 4, all of which have varying impacts on A β clearance and seeding⁸¹. Relative to

the common *APOE* ϵ 3 allele, *APOE* ϵ 2 has been associated with protective effects against A β accumulation and AD while *APOE* ϵ 4 has been associated with increased risk to both EOAD and LOAD^{82–85}. In fact, APOE ϵ 4 risk is dose-dependent: carriers with one *APOE* ϵ 4 allele increases the LOAD risk by 3–4-fold, whereas carrying two *APOE* ϵ 4 alleles increase LOAD risk by 12–15-fold⁸⁵. ApoE efficiency of A β clearance also appears to be dependent on the APOE isoform where APOE4 performs less efficiently than APOE2 and APOE3⁸⁶. Indeed, amyloid PET imaging across different *APOE* genotypes identify APOE4 as a strong driver of A β deposition and likely increases LOAD risk by causing earlier and more abundant A β pathology in the CNS⁸⁷.

Over the past decade, *genome-wide association studies (GWAS)* have been a critical tool to determine polymorphic variants within genes that increase genetic risk and identify physiological processes contributing to AD pathogenesis in LOAD⁸⁸. For example, GWAS has identified variants within the *triggering receptor expressed on myeloid cells 2 (TREM2)* gene to significantly increase AD risk by 2–4-fold, specifically the common *TREM2*^{R47H} variant⁸⁹. TREM2 is a surface receptor abundantly primarily expressed in myeloid cells (i.e. microglia) that can mediate microglial responses to A β oligomers, which are internalized and degraded^{90–92}. A β can also enhance TREM2 interaction with neighboring DAP12 proteins to recruit tyrosine kinases and initiate phosphorylation cascades of downstream pathways⁹¹. Interestingly, preclinical AD mice expressing the *TREM2*^{R47H} risk variants appears to impair microglial interaction with plaques, suppressed neuroinflammatory responses, and increased A β burden during late-stage pathology⁹³. Another LOAD risk gene identified through GWAS encodes for *clusterin (CLU)*, an extracellular chaperon with a complex role in altering A β aggregation and mediating its clearance^{94,95}. Indeed, four *CLU* genetic variants have been associated with A β deposition through amyloid PET and one *CLU* variant was linked with hippocampal atrophy, yet no associations were observed in CSF or

FDG PET markers⁹⁶. Other LOAD risk genes identified through GWAS include *SORL1* and *ABCA7*^{88,97}. Beyond interactions with A β , variants to genes encoding tau-binding proteins have also been reported in large-scale GWAS. For example, *bridging integrator 1 (BIN1)* is measured to be upregulated in the AD brain and has functional roles in regulating microglial inflammation and membrane endocytosis with isoforms expressed within white matter in the CNS^{98,99}. Additionally, BIN1 isoforms are upregulated with significant positive correlations with NFT pathology and tau-dependent excitability as opposed to A β pathology^{100–102}. With the identification of hundreds of LOAD susceptibility variants, GWAS have been critical in revealing novel disease and cell-specific mechanisms underlying polygenic contributions to AD onset and pathogenesis.

Acquired Risk Factors: While twin studies have revealed that genetics largely contribute to Alzheimer's disease risk, there are still environmental and modifiable risk factors unique to each twin that account for 29% of AD risk⁷³. Even then, gene-environment interactions may also drive estimates of these measured genetic influences¹⁰³. While *cardiovascular disease (CVD)* has emerged to be a significant risk factor for dementia, its relationship with AD pathology is less defined. Yet, the presence of amyloid deposits in cerebral blood vessels in the form of CAA, microhemorrhages, and infarcts have been observed in a large proportion of post-mortem AD brains¹⁰⁴. Additionally, early vascular damage as a result of CAA can further disrupt vascular drainage, inflammation, and homeostasis within the CNS, which can exacerbate existing impairments in A β clearance and worsen AD neuropathologies^{105,106}. However, because both CVD and AD share many overlapping genetic risk factors, it is difficult to discern whether a directional relationship exists between the two diseases or if they both interact concurrently. Other overlapping risk factors between CVD and AD also include hypertension and Type 2 Diabetes Mellitus, which

has been associated with increased risk of developing AD dementia and worsening cognitive impairments^{107,108}.

Microbial Infection as a Risk Factor: Many LOAD risk genes identified through GWAS have functions within immunity and are highly expressed in myeloid cells^{109–111}. Therefore, microbial infection has been broadly explored as a mechanisms through which both genetic and environmental contributions interact together to influence risk of developing AD and impact ongoing AD neuropathology¹¹². Pathogenic microbes that invade the CNS can cause significant neuroinflammation through CNS-resident immune cells and infiltration of peripheral immune cells such as monocyte/macrophages and T cells¹¹³. Bacterial, parasitic, and viral pathogens that have been identified in post-mortem brains and associated with AD pathology include *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Salmonella typhimurium*, Herpes Simplex Virus-1, Human Immunodeficiency Virus, Epstein-Barr Virus, and Human Herpes Virus 6A and 7¹¹². Periodontitis have been associated with increased risk of developing AD, in which its etiological agent *P. gingivalis* has also been found in AD brains with levels correlating with AD pathology^{114,115}. Wildtype mice infected with *P. gingivalis* have also exhibited increased expression of pro-inflammatory cytokines, APP and β -secretase, tau phosphorylation, and neurodegeneration, which suggests a potential mechanism between periodontal disease and AD pathology¹¹⁶.

Other bacterial agents such as *C. pneumoniae* may also promote tau, A β deposition, and cognitive dysfunction. Specifically, *C. pneumoniae*-infected astrocytes *in vitro* decreased activity of α -secretase (involved in non-amyloidogenic APP processing) and increased expression of β -secretase and γ -secretase (involved in amyloidogenic APP processing and A β production)¹¹⁷. A higher burden of *C. pneumoniae* has been identified in the retina of AD patients, correlating with APOE ϵ 4 risk, disease stage, and cognitive deficits and elevated NLRP3-inflammasome

pathways¹¹⁸. Furthermore, *C. pneumoniae* infection in neuronal cultures and APP_{SWE}/PS1_{ΔE9} induced Aβ, inflammasome activation, and neurotoxicity, while chronic infection worsened cognition¹¹⁸. While the role of multi-pathogen infection in amplifying Alzheimer's disease pathologies requires further investigation, the utility of attenuating NLRP3-inflammasome pathways and anti-microbial therapies may prove to be promising interventional approaches.

Parasitic infections such as *Toxoplasma gondii* also have potential in influencing cognitive decline and neurodegenerative disorders like Alzheimer's disease¹¹⁹. As a neurotropic protozoan parasite, its latent infection in the central nervous system can modulate neurotransmission and induce chronic neuroinflammation¹¹⁹. A meta-analysis identified a significant association between *T. gondii* infection and Alzheimer's disease and associated impairments in cognition, language, and memory¹²⁰. However, *T. gondii* infection also leads to a robust neuroimmune response, leading to increased phagocytosis of amyloid plaques due to the infiltration of Ly6C⁺ and MAC2⁺ monocytes and proliferating microglia^{121,122}. In addition to infiltrating monocytes recruited towards amyloid plaques, chronic *T. gondii* infection also induces recruitment of CD4⁺ and CD8⁺ T cells interacting with IBA1⁺ myeloid cells in plaque-laden areas¹²³. While the role of *T. gondii* and Alzheimer's disease provides important insight on the association of infiltrating immune cells and reduced amyloid burden, chronic infection still yields significant concern over its induction of neuronal damage and neuronal death, as well as the potential for reactivation of latent infection¹²⁴.

A recent study probed medical records of two large biobanks for viral associations with different neurodegenerative diseases and found that the strongest association was between AD and viral encephalitis, the resulting neuroinflammation response to viral infection¹²⁵. While the brain is usually protected from viral entry into the CNS, many identified viruses associated with neurodegenerative disease are considered neurotropic^{125,126}. An extensive description of the

associations between viral pathogens, the resulting neuroinflammation response (e.g., viral encephalitis), and AD is provided in Section B.2 “*Viral Encephalitis, AD, and Dementia.*” Further investigations exploring the interaction between microbial infections and AD will help define whether these infectious pathogens itself, anti-microbial inflammatory pathways, or its interaction with risk genes contribute to AD risk and ongoing pathology.

1.4. Alzheimer’s Disease and Tissue-Resident Immunity

Numerous large GWAS have repeatedly identified AD risk genes associated with immunity and specifically expressed in myeloid cells, implicating neuroimmune processes contributing to altered neuroinflammation as a major factor of AD etiology. Myeloid cells within the CNS are primarily comprised of two distinct populations: CNS-resident microglia and monocyte-derived macrophages. Many studies have demonstrated a close association between A β plaques and microglia, which are considered the principal immune cell within the CNS. During normal conditions, microglia assume a homeostatic, quiescent state characterized by small soma and complex, ramified branching morphology that enables their surveillance of the CNS for inflammatory stimuli¹²⁷. During this homeostatic state, microglia can respond to a range of activation signals using surface receptors to initiate a pro-inflammatory response¹²⁸. These receptors may be specific for exogenous factors from pathogens such as bacterial LPS, microbial genetic material, or *pathogen-associated molecular patterns (PAMPs)*. Additionally, microglia are enriched with receptors for endogenous factors such as damage-associated molecular patterns (DAMPs), misfolded protein aggregates, cytokines, and other released products of nearby cellular stress¹²⁷. In AD, soluble A β oligomers and insoluble A β fibrils can trigger microglial activation and cytokine production through binding with microglial surface receptors including *Toll-like*

receptors 2 and 4 (TLR2 and TLR4), cell surface cluster of differentiation markers (CD) (i.e., CD36, CD14, CD47), and class A1 scavenger receptor (SCARA1)^{127,128}.

Once activated, microglia undergo a morphological and functional change into rounder, amoeboid shapes with short, thick processes that extend out to enable migration towards the inflammatory stimulus, cytokine secretion, and phagocytosis^{127,129}. In AD mice, microglia rapidly respond to plaque formation, migrate, and accumulate around amyloid plaques to prevent spread of toxic A β fibrils¹³⁰. Indeed, activated microglia seem to also be responsible for compacting A β fibrils to form dense-core plaques¹³¹. However, as progressive A β deposition continues, the sustained activation of microglia can further induce more A β secretion¹³². Microglia can also modulate tau pathogenesis by directly promoting tau clearance upon internalizing and degrading tau species¹³³. CX3CR1 may also play a role in mediating microglial-mediated tau internalization once bound to tau¹³⁴. However, depleting microglia also suppressed tau propagation by limiting spread through microglial secretion of exosomes containing phosphorylated tau, suggesting that microglia may also contribute to the progression of tau pathology as well¹³⁵.

Single-cell transcriptomic and proteomic studies have revealed activated microglia further assume functionally distinct microglial cell populations in during different diseased states within the CNS^{136,137}. In AD, the prominent transcriptional signature within microglia are *disease-associated microglia (DAMs)*, *microglia neurodegenerative phenotype (MGnD)*, and *activated response microglia (ARMs)*^{127,137}. Genes upregulated during DAM states are familiar genetic risk factors associated with AD such as *APOE*, *TREM2*, and *SPPI*. The functional role of many upregulated DAM genes are also implicated in phagocytosis, chemotaxis, damage-mediated cytokine release, while downregulated genes are typical of microglial homeostasis (i.e. CX3CR1 and P2RY12)¹³⁷. Interestingly, it is an open question whether the DAM state is beneficial or

detrimental, which has been suggested to depend on the timing of microglial activation. During early, asymptomatic stages, increased microglia activation into DAM states can promote A β clearance and confer neuroprotection, which can shift to a neurodegenerative, proinflammatory state reminiscent of the MGnD phenotype as disease progresses further¹³⁸. The TREM2-ApoE signaling pathway seems to underlie this transition from homeostatic to MGnD in which *APOE* is initially upregulated before increased *CLEC7A* expression and continued suppression of homeostatic genes¹³⁹. Here, MGnD can be triggered by microglial phagocytosis of apoptotic neurons and often found in proximity to neuritic plaques^{137,139}. *CD33* also seems to drive the proinflammatory transition from homeostatic microglia to MGnD through opposing modulation of microglial activation in synchrony with *TREM2*, which primarily promotes phagocytosis and A β clearance^{140,141}. In the context of tau pathology, MGnD in brains of APP^{NL-G-F} mice overproduce extracellular vesicles containing phosphorylated-tau while compacting A β into plaques, suggesting the involvement of MGnD in mechanistically linking A β plaque deposition and aggregated tau propagation in this AD mouse model¹⁴².

1.5. Alzheimer's Disease and Peripheral Immunity

Other myeloid cells contributing to neuroinflammation are non-parenchymal macrophages, which undergo similar development from *bone marrow (BM)-derived monocytes (BMDM)* and represent functionally distinct cell populations from microglia under steady-state conditions^{143–145}. However, studies have reported that inflammatory and diseased conditions (e.g., AD) result with robust infiltration of peripheral-derived monocytes into the brain, which differentiate into macrophages upon immune activation with similar transcriptional and phenotype changes as microglia^{146–150}. In post-mortem AD tissue, circulating monocyte/macrophages have been identified infiltrating into brain parenchyma and localizing in proximity with amyloid plaques¹⁴⁸.

In pre-clinical AD models, infiltration of *monocyte-derived macrophages (MDM)* has also been demonstrated to be closely associated with A β plaques^{150–152}. Notably, BM-derived macrophages appear to be more efficient than resident microglia in phagocytosis and clearance of A β within brain parenchyma, while protecting synapses from loss^{152–154}. The recruitment of MDMs into the CNS have also conferred improved cognitive performance in addition to clearance of parenchymal A β ^{155,156}. Notably, a similar mechanism of MDM recruitment helped modulate inflammatory responses and mitigate cognitive impairments in a tau mouse model as well¹⁵⁷. The ability of BM-derived macrophages to reduce A β is further illustrated in selective ablation utilizing either CC chemokine receptor 2 (CCR2)-deficiency or diphtheria toxin in AD mice resulted in exacerbated cortical and hippocampal A β pathology^{158,159}. Conversely, disease stage may impact the phagocytic capacity of peripheral myeloid cells in clearing A β during the late-stage disease¹⁶⁰. Further supporting the importance of timing monocyte and macrophage infiltration, glatiramer acetate used to recruit immunomodulatory BMDMs only modestly reduced A β burden in 5xFAD mice during the development stage of pathology and increased A β levels during later stages of neuropathology¹⁶¹. These results provide additional evidence of the importance of myeloid cells in regulating neuroinflammation and shaping AD pathology, specifically highlighting the nuanced disease-modifying role of peripheral MDMs recruited into the CNS.

Although largely understudied, recent evidence has also implicated components of adaptive immunity in regulating neuroinflammation in the AD brain^{162,163}. Notably, genetic ablation of adaptive immune cells in pre-clinical AD models accelerates amyloid pathogenesis and worsens neuroinflammation¹⁶⁴. However, increased infiltration of T cells have been detected in post-mortem AD brains and correlated with neuronal loss^{165,166}. Although contradictory, these findings suggest adaptive immune responses in AD are heavily nuanced and multifaceted in

modifying neuroinflammation within the AD brain. With growing evidence identifying how A β -dependent pathways, GWAS-identified immunity genes, and nuanced innate/adaptive immune responses may impact AD onset, microbial infection and its resulting peripheral inflammation has been put forward as a major environmental risk factor for AD onset.

2. VIRAL ENCEPHALITIS

2.1. Overview of viral encephalitis

To illustrate the association of viral infection with several neurodegenerative diseases, Levine et al. mined medical record data from both the Finnish biobank (FinnGen) and UK Biobank. Their findings demonstrate several viral pathogens that are tightly associated with different disease diagnoses, but the strongest association was between AD and viral encephalitis¹²⁵. Viral encephalitis is defined as viral-mediated inflammation of the brain parenchyma. While the BBB prevents most viral pathogens from invading from the periphery, neurotropic viruses can circumvent these barriers to infect and replicate within the CNS¹¹². The resulting inflammation via indirect neuroimmune mechanisms may induce acute and chronic neurologic damage with potential consequences in the onset or severity of neurodegenerative disease^{113,167,168}. Therefore, it is unsurprising that majority of identified viral pathogens associated with neurodegenerative disease and dementia are neurotropic (i.e., herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein-Barr Virus, and flaviviruses) and/or systemic infections leading to viral encephalitis (i.e., human immunodeficiency virus (HIV) and severe acute respiratory syndrome-Coronavirus 2 (SARS-CoV-2)). Given the diversity of viral pathogens that can replicate within the CNS or instigate encephalitis, there is an equally diverse inflammatory response that may contribute to the onset or severity of AD pathology. The following sections summarize the current

understanding of how each viral pathogen and its distinct viral-mediated inflammation potentially interacts with AD pathogenesis.

2.2. Viral encephalitis, AD, and dementia

Herpes Simplex Virus (HSV): *Herpes simplex virus-1 (HSV-1)* and *Herpes simplex virus-2 (HSV-2)* are both double-stranded DNA viruses with enveloped capsids. Viral surface proteins attach to host cell receptors, which facilitate endocytosis into the cell where replication occurs within the nucleus¹⁶⁹. HSV is primarily lysogenic and exits the host cell by nuclear egress, budding, and microtubular transport¹⁶⁹. Following infection and replication in epithelial cells, immunological changes can initiate HSV to migrate to the dorsal root ganglia through retrograde axonal transport, after which it will establish latency within neurons¹⁶⁹. HSV encephalitis is among the most common causes of viral encephalitis, which appears to be exclusively driven by HSV-1¹⁷⁰. While being both highly prevalent and contagious, HSV-1 can produce damage to different brain regions following neuronal infection and microglial inflammatory responses^{170,171}.

Given its impact on the CNS, HSV-1 has been explored as a potential risk factor for AD¹⁷². Post-mortem analysis has identified colocalization of HSV-1 viral DNA with A β plaques in AD brains¹⁷³. This is also supported with a study reporting that HSV-1 infection led to seeding of surface glycoproteins within A β oligomers and accelerated A β deposition in the 5xFAD mouse model of familial AD, suggesting A β playing a potentially protective role in anti-viral immunity¹⁷⁴. However, recent studies have also complicated this prevailing theory in which unique post-mortem AD brain tissue with concurrent HSV encephalitis revealed HSV-infected cells localized close to A β plaques neurofibrillary tangles, but not directly associated with worsening AD pathology¹⁷⁵. Additionally, 5xFAD nor combined LOAD mutations (e.g., humanized A β , *APOE4*, and *TREM2*^{R47H} transgenes) in mice did not significantly protect against HSV-1 exposure nor initiate

A β aggregation following HSV-1 infection, respectively^{176,177}. Interestingly, the researchers did observe limited viral invasion in regions with high A β plaque densities attributed with phagocytic activity of reactive microglia¹⁷⁶. Additionally, HSV-1 infection of LOAD mice resulted in extensive infiltration of peripheral leukocytes and phagocytic activity of myeloid cells¹⁷⁷. Rather than a direct cause, HSV-1 infection may be a modulating risk factor for AD with a combination of both environmental and genetic factors contributing to neurological damage.

Chickenpox and Shingles: *Varicella Zoster Virus (VZV)* is another DNA virus within the *Herpesviridae* family and impacts humans through its primary infection of T cells, epithelial cells, and ganglia in the form of varicella or chickenpox. Upon primary infection, VZV infects T cells, which circulate and disseminate virus to the skin where innate immunity will attempt to control replication¹⁷⁸. However, VZV can enter nerve endings in the skin and travel up axons via retrograde transport towards the neuronal cell body, where it can establish latency within the neuron and spread to other innervating neurons¹⁷⁸. VZV can remain latent for many years while cellular immunity declines before reactivating to cause *herpes zoster (HZ)* or shingles¹⁷⁸. An important risk factor for shingles is age and its highest incidence occurs in individuals over 50¹⁷⁸. Additionally, VZV is observed to replicate in cerebral arteries and contribute to vascular damage, which can further exacerbate neuronal damage¹⁷⁸. With its high incidence among older populations and impact on vascular integrity, the relationship between VZV infection, dementia, and AD have been explored in various epidemiological studies.

Patients diagnosed with ophthalmic zoster (blisters within the eyelids as a result of HZ) were followed up five years after their initial diagnosis¹⁷⁹. Almost 5% of patients were diagnosed with dementia within the follow-up period compared to 1.6% of patients without ophthalmic zoster, demonstrating that HZ may significantly increase risk for dementia¹⁷⁹. Additionally,

researchers found that diagnosis with a herpesvirus (either HSV or HZ) significantly increased risk of AD by 30% in individuals over 65¹⁸⁰. Other studies have similarly reported a slightly increased risk of AD and incidence of dementia in patients diagnosed with untreated HZ¹⁸¹. However, a direct link between HZ and AD has yet to be identified. Instead, researchers have proposed an indirect effect through HZ-mediated neuroinflammation. HSV-1 infected human-induced neural stem cells demonstrated A β and tau accumulation, while VZV infection increased both gliosis and pro-inflammatory cytokine levels, which can indirectly impact AD¹⁸². Interestingly, VZV infection of cells with latent HSV-1 also caused reactivation of HSV-1 and resulted with AD-like pathological changes¹⁸². While more studies evaluating the contribution of VZV infection on AD are required, its potential role in driving reactivation of latent HSV-1 could lead to downstream effects on AD pathology.

Epstein Barr Virus (EBV): Characterized by Epstein, Barr, and Achong in 1964, *Epstein Barr Virus (EBV)* is another double-stranded DNA virus within the *Herpesviridae* family¹⁸³. EBV primarily infects epithelial cells and B cells either through binding of viral gp350 and host CD21 proteins or viral gp42 and host HLA Class II molecules¹⁸⁴. Upon entry into the host cell, EBV can undergo lytic viral replication where infected B cells may differentiate into memory B cells and circulate throughout the periphery¹⁸⁴. However, EBV can also persist in memory B cells without active viral replication through latency¹⁸⁴. As a result, these latently infected B cells can be immunologically stimulated to reactive EBV and reinfect new B cells and epithelial cells, but more research is required to understand what triggers reactivation.¹⁸⁴ EBV is transmitted mainly through saliva and is clinically associated with over half of mononucleosis cases in young adults¹⁸⁵. Additionally, EBV has demonstrated an ability to compromise the integrity of the BBB and replicate within the CNS, leading to different neuropathological features such as neural damage

and neuroinflammation¹⁸⁶. Indeed, EBV has demonstrated potential associations with neurodegenerative diseases, specifically Parkinson's disease and Multiple Sclerosis.

Many studies have highlighted latent EBV as a risk factor for AD development. In attempting to detect EBV presence in peripheral leukocytes in AD patients, researchers found that 45% of AD patients were affected by EBV compared to only 31% of non-AD patients¹⁸⁷. Additionally, they also found that APOE4 isoforms was associated with EBV positivity in controls while IgG levels for EBV antigens was increased in those diagnosed with AD¹⁸⁷. Supporting these findings, another group found that patients with higher anti-EBV IgG levels were more prone to develop mild cognitive impairments and dementia¹⁸⁸. Moreover, a recent study also demonstrated a significant association between EBV-associated mononucleosis and AD risk¹⁸⁹. However, other epidemiological studies have also demonstrated a lack of association between EBV infection, IgG levels, and cognitive decline¹⁹⁰. Given these mixed conclusions, researchers have suggested a constantly active immune system following primary EBV infection, which may lead to a chronic inflammatory state that has been theorized to induce AD-like pathologies through aging¹⁸⁷. However, more mechanistic studies are required to expand on whether a clear relationship between EBV infection and AD exists.

Flaviviruses: *Zika Virus (ZIKV)* is an arbovirus in the family *Flaviviridae* transmitted through bites of infected mosquitos, but can also be transmitted through other means such as blood/organ transfusion and from mother to child¹⁹¹. It contains a single-stranded RNA genome enclosed by a capsid and has potential to infect epidermal cells and dendritic cells, although more research is required to determine its exact mechanism of infection¹⁹¹. Many ZIKV outbreaks have been reported around the world, especially in the Global South¹⁹¹. While most clinical disease occur in the periphery, ZIKV can lead to neurological complications such as Guillain-Barré

Syndrome and microencephaly¹⁹¹. Additionally, ZIKV may potentially interact with AD pathology. Recently, ZIKV was found to block BACE1-binding necessary for APP cleavage, upregulating expression of APP¹⁹². Another study found that ZIKV infection could induce A β accumulation in human organoids by increasing BACE1 abundance and enhance endoplasmic reticulum stress through overactivation of PERK pathways¹⁹³. However, APP may also interact with ZIKV through protective roles. For example, APP may negatively regulate ZIKV replication in human iPSCs¹⁹². Therefore, while the relationship between APP and ZIKV may appear protective, the impact of ZIKV on accumulated protein aggregates such as A β warrant further investigation.

Another positive single-stranded RNA flavivirus transmitted through mosquitos and blood transfusions is *Dengue Virus (DENV)*¹⁹⁴. Its primary targets are dendritic cells and monocytes within the periphery, which will induce host pattern recognition receptors to sense and recognize viral antigen¹⁹⁴. These pattern recognition receptors will upregulate interferon and NK- κ B signaling to produce proinflammatory cytokines to establish an anti-viral immune response¹⁹⁴. Importantly, DENV has been implicated with several neurological conditions such as dengue encephalopathy, encephalitis, and even dementia¹⁸⁶. Epidemiological studies have found that elderly individuals diagnosed with dengue fever had an increased risk of developing dementia^{195,196}. The relationship with DENV and AD may be modulated through neuroinflammation through indirect upregulation of proinflammatory cytokines or directly through DENV-infected monocytes and macrophages infiltrating into the CNS through compromised BBB integrity¹⁹⁷. Another indirect mechanism for DENV to interact with AD pathology is through reducing lipoprotein receptor-related protein 1 (LRP-1), which has been identified as an ApoE receptor facilitating A β clearance in the vasculature with impacts on cerebral amyloid angiopathy (CAA)¹⁹⁸.

Finally, another neurotropic mosquito-borne Flavivirus is *West Nile Virus (WNV)*, which is a positive-sense single-stranded RNA virus resulting with meningitis, encephalitis, and cognitive impairments such as memory loss, confusion, and fatigue¹⁹⁹. WNV can access the CNS through passive transport through the BBB, infection-mediated BBB permeability, trafficking of WNV-infected leukocytes, or through retrograde axonal transport of infected peripheral neurons¹⁹⁹. Once inside the CNS, it can replicate within astrocytes, microglia, and neurons, initiating a cascade of proinflammatory cytokines to produce an anti-viral response²⁰⁰. The neurological consequences of WNV infection have been explored in both epidemiological studies reporting an increased risk of dementia following WNV encephalitis and brain imaging studies that illustrate cortical thinning in brain regions implicated in AD²⁰¹. Furthermore, WNV may directly exacerbate AD pathology through microglial engulfment of synapses following viral clearance. While complement activation is key to limiting WNV replication in the CNS, microglial activation and complement-mediated pathways following WNV infection resulted in neurodegeneration, specifically in CA3 regions of hippocampus in both mice and human brain tissue²⁰². WNV-mediated microglial and astrocyte activation has also persisted long after WNV clearance within the CNS, potentially contributing to the subsequent neurodegeneration²⁰³. Even anti-viral CD8+ T cells appear to persist within the CNS, which is an important source of IFN- γ to initially control viral replication but can mediate further loss of synaptic terminals in the hippocampus, leading to defects in spatial learning²⁰⁰.

Many of the described neuroinvasive mosquito-borne Flaviviruses share common mechanisms through which the resulting encephalitis can result with not only neuronal damage, but also neuroinflammation and associated cognitive deficits that may exacerbate AD pathology. These shared mechanisms include BBB permeability, peripheral inflammation, direct

neuroinvasive infection of resident CNS cells, or infiltration via virus-infected immune cells. However, more studies are required to deepen the mechanistic understanding of how neurotropic flavivirus infection can modulate AD.

Human Immunodeficiency Virus (HIV): HIV collectively refers to two retrovirus strains (HIV-1 and HIV-2) that are both single-stranded, positive-sense RNA lentiviruses enveloped by a protein capsid surrounded by a fluid matrix and lipid membrane¹⁸⁶. The virus is primarily infectious to humans and primarily targets CD4+ T cells through interactions between CD4 and CCR5 surface receptors, but it has can also replicate within macrophages and dendritic cells resulting in inactivation or even destruction of infected cell populations through apoptosis²⁰⁴. This depletion of lymphocytes leads to the development of Acquired Human Immunodeficiency Syndrome (AIDS), which is characterized by impaired immune responses to potentially fatal opportunistic microbial pathogens²⁰⁴. Many available *antiretroviral therapies (ART)* are capable of successfully reducing the infection rate of HIV to the point where it is undetectable and untransmittable²⁰⁵. While ART therapies can reduce the progression into AIDS, the ongoing HIV reservoir within infected immune cells results in a chronic condition²⁰⁶. Moreover, despite HIV infection occurring in the periphery, epidemiological research has associated infection with cognitive, motor, and behavioral impairments as part of *HIV-associated neurological disorders (HAND)*²⁰⁷.

One of the first studies investigating the occurrence of amyloid plaques in AIDS patients was reported in 1998. While there was a general trend for both AIDS and non-HIV-infected individuals to contain amyloid plaques in their brain, AIDS patients had a significantly greater prevalence of plaques²⁰⁸. This finding has been reproduced recently in both HIV-1 infected patients with HAND symptoms and in HIV-1 transgenic rats, which show intraneuronal A β aggregation in

the prefrontal cortex and hippocampus, but no extracellular amyloid plaques as seen in AD²⁰⁹. One of the highest genetic risk factors for AD is APOE, which occur in different isoforms that may influence viral production and infectivity in macrophages overexpressing APOE^{210,211}. Additionally, viral proteins may play a direct in modulating protein processing, aggregation, and clearance. For example, supernatant from HIV-infected human macrophages was used to treat primary cultures of rat cortical neurons resulting in reduced expression of ADAM10, a protein involved in non-amyloidogenic α -secretase cleavage of APP. Another study found that HIV-tat (a viral transcriptional regulator) can directly contact A β polymers to stimulate their accumulation into neurotoxic A β fibrils, which could not be detected for phagocytosis or clearance²¹². Additionally, HIV surface glycoproteins may stimulate tau phosphorylation and enhance its pathogenic effect²¹³. However, more studies are required to disentangle the mechanistic contribution of persistent HIV-induced neuroinflammation to outline the similarities and differential neurological impairments attributed to HAND and AD.

Severe Acute Respiratory Syndrome-Coronavirus 2 (SARS-CoV-2): SARS-CoV-2 is a viral strain identified in 2020 to be the etiologic agent for *Coronavirus Disease 2019 (COVID-19)*, an ongoing global pandemic affecting hundreds of millions of people around the world. This positive-sense single-stranded RNA virus is part of the *Coronaviridae* family with a relatively large genome of 30 kB long²¹⁴. It's mechanism of entry is through the ACE2 receptor, which is highly expressed in the periphery, mainly in alveolar epithelial cells in the lungs and enterocytes along the small intestine²¹⁴. However, 36% cases of SARS-CoV-2 infection develop neurological symptoms such as cognitive impairments, confusion, dizziness, and encephalitis²¹⁵. Given its novelty, global relevance, and impact on the CNS, an increasing number of studies have put

forward cautionary warnings of the impact of SARS-CoV-2 on neurodegeneration and dementia, including AD pathogenesis.

While APOE4 is recognized as the strongest genetic risk factor for AD, it has also been associated with risk of SARS-CoV-2 infection, COVID-19 disease severity, and mortality. Specifically, homozygous carriers of APOE4 have a two-fold increase in SARS-CoV-2 infection and COVID-19 death, even after controlling for existing comorbidities such as pre-existing dementia²¹⁶. Additionally, ACE2 expression was found to be upregulated in the brains of AD patients, which may facilitate higher viral entry into the CNS thereby increasing the risk of neurological impairments and inflammation associated with SARS-CoV-2²¹⁷. In the reverse direction, epidemiological data on individuals treated for COVID-19 reveal that SARS-CoV-2 infection increased the risk of a new AD diagnosis within 1 year of infection, especially in elderly populations²¹⁸

Mechanistically, more research is required to determine how SARS-CoV-2 and its resulting inflammation impacts accumulation of A β and tau pathologies. However, early studies of organoid models exposed to SARS-CoV-2 have demonstrated hyperphosphorylation and altered distribution of tau²¹⁹. Interestingly, clinical studies have also observed enhanced A β toxicity in the brain, impaired clearance in blood serum, and accumulation of amyloid proteins in CSF²²⁰. Additionally, post-mortem analysis of brains from individuals who died from COVID-19 reveal gliosis and immune cell accumulation associated with axonal damage and breakdown of BBB integrity²²¹. These features are likely to further exacerbate existing AD pathology as the resulting systemic inflammation and pro-inflammatory cytokine production can contribute to production of protein aggregates such as A β and hyperphosphorylated tau. Indeed, the release of pro-inflammatory

cytokines has been observed in severe COVID-19 cases, including cytokines previously associated with impairments in neurogenesis and hippocampal-dependent memory^{203,222}.

Given the novel nature of SARS-CoV-2, further experimental and clinical investigations are necessary to further elaborate the relationship between COVID-19 infection and the development and/or established AD pathologies within the CNS. With the manifestations of neurological sequelae following SARS-CoV-2 infection getting characterized, mechanistic experiments are required to elucidate how these two diseases impact one another and their potential synergistic effects on neurodegeneration.

2.3. The utility of vaccines and anti-microbial therapies

As ongoing research is performed identifying how infectious viral agents may contribute or interact with AD pathology, an increasing number of studies have demonstrated potentially protective effects of anti-viral therapies on AD pathogenesis. Notably, data from US medical surveys demonstrated a reduced risk for dementia and cognitive impairments following vaccination against HZ²²³. This relationship between HZ vaccination and reduced dementia incidence was also observed in a population in Wales²²⁴. Even repeated vaccination against influenza seemed to confer a slight reduction to the risk of developing dementia^{225,226}. More recently, the prevalence of dementia risk in people with HIV has declined in the ART era compared to people living with HIV without ART²²⁷. Yet despite ART treatment for HIV, people with HIV have a much higher risk of developing dementia compared to people without HIV infection²²⁸. Unfortunately, while these observation studies demonstrate an association between dementia risk and anti-viral treatments and vaccination, it may not completely mitigate the onset of dementia or neurodegenerative diseases like AD. Exploring the direct relationship between anti-viral

immunity, AD pathogenesis, and dementia symptoms is necessary to consider utilizing these treatments as means to prevent or mitigate subsequent neurodegeneration.

4. JHMV, a neurotropic murine coronavirus

Mouse Hepatitis Virus (MHV) is an enveloped positive-strand RNA virus that is a member of the *Coronaviridae* family of viruses within the genus *Betacoronaviridae*, which include SARS-CoV, *Middle Eastern Respiratory Syndrome (MERS)*, and SARS-CoV-2. Upon infection, respiratory, gastro-intestinal, and neurological disease may occur in both humans and animals²⁸⁵. MHV is a natural pathogen of mice and pathogenesis is determined by a number of factors, including the viral strain, inoculation route, and background strain of the mouse^{286,287}. The *John Howard Mueller strain of MHV (JHMV)* is a neuroadapted strain of MHV^{288,289}. *Intracranial inoculation (i.c.)* of susceptible mice with JHMV results in acute encephalomyelitis characterized by viral replication within astrocytes, microglia, and oligodendrocytes with relative sparing of neurons^{290–292}. MHV spike protein recognizes and binds to the surface receptor *carcinoembryonic antigen-cell adhesion molecule (CEACAM-1)* of the host cell, enabling viral entry and resulting with a host immune response and disease pathogenesis^{286,293–296}. Animals that survive the acute stage of disease develop chronic demyelinating disease through which virus will persist primarily in oligodendrocytes and astrocytes and lead to chronic neuroinflammation consisting of both T cells and macrophages^{293,297–300}. JHMV replication is controlled within astrocytes and microglia through CD8+ T cell perforin/granzyme-mediated cytolytic activity^{298,300}. In oligodendrocytes, viral replication is primarily controlled by IFN- γ ^{297,299}. JHMV will eventually be cleared below detectable levels, however sterilizing immunity is not achieved and viral antigen/RNA will persist within oligodendrocytes³⁰¹. Potential reasons for persistent viral presence with the CNS include waning expression of MHC class I and II on oligodendrocytes and *antigen-presenting cells*

(APCs), CD8⁺ T cell exhaustion, and viral RNA evolution within the CNS, producing viral quasispecies of spike deletion variants^{302–305}.

Early during MHV infection, virus will replicate within astrocytes, which subsequently become reactive and secrete macrophage chemoattractants CCL2 and CCL5³⁰⁶. Once secreted, they bind to their corresponding receptors CCR2 and CCR5 on the surface of macrophages, enabling infiltration into the CNS^{307–310}. Astrocytes also secrete CXCL9 and CXCL10, which recruit virus-specific T cells into the CNS through the CXCR3 receptor^{311–314}. Notably, CCR5 is also necessary for CD4⁺ T cell migration into the CNS, but not for CD8⁺ T cells which depends on CCL3 for effective CD8⁺ T cell activation and CNS infiltration^{315–317}. By 14 dpi, overall immune cell infiltration declines, but virus-specific T cells and macrophages continue to reside within the CNS up to three months following infection^{318–321}.

5. JHMV-induced Neurological Disease

Our lab has employed experimental infection mice with the neurotropic JHM strain of murine hepatitis virus (JHMV, a member of the *Betacoronaviridae* genus) to model molecular and cellular mechanisms of host defense and disease in response to viral encephalitis within the CNS^{285,322}. Intracranial inoculation of susceptible mice with JHMV results in an acute encephalomyelitis characterized by wide-spread replication of virus in glia with relative sparing of neurons. Innate immune responses characterized by the expression of type I interferon are important in limiting the spread of virus early following infection. Ultimately, virus-specific CD4⁺ and CD8⁺ T cells are attracted into the CNS in response to T cell chemoattractant chemokines and control viral replication via expression of anti-viral cytokines including IFN- γ and cytolytic activity^{298,299}. However, sterile immunity is not achieved and virus will persist within white matter tracts, where immune-mediated demyelination will occur due to chronic infiltration of

macrophages and T cells^{309,320}. Demyelination is associated with the sustained presence of both viral antigen and inflammatory CD8+ and CD4+ T cells as opposed to apoptosis-mediated death of myelinating oligodendrocytes^{323,324}. CD4+ T cells secrete IFN- γ , which upregulates MHC class II in microglia and MHC class I in oligodendrocytes and enables CD8+ T cell survival^{325–327}. CD4+ T cells contribute to demyelination by secreting CCL5, which recruits more macrophages into the CNS, while CD8+ directly contributes to demyelination through cytolytic activity and IFN- γ secretion, which is directly damaging to oligodendrocytes and *oligodendrocyte precursor cells (OPCs)*^{328–333}. Secretion of TNF α and ROS by microglia and macrophages can also trigger oligodendrocyte cell death^{334–336}. However, IFN- γ is not solely responsible for demyelination since it still continued in mice with IFN- γ R1 ablation in oligodendrocytes²⁹⁷. Indeed, myelin-laden macrophages have been observed to directly strip and engulf myelin from axons and neutralizing antibody against macrophage attractant CCL5 diminished demyelination^{309,337}. Even genetic silencing CCR5 specifically in macrophages impaired viral clearance³⁰⁸.

Recent evidence has also implicated demyelination in the etiology of pre-clinical AD, demonstrating that lack of myelin integrity exacerbates A β plaque deposition in both AD brains and in AD mouse models³³⁸. During acute disease, JHMV replicates throughout discrete anatomic areas of the brain, but the frontal cortex and hippocampus are areas of enriched viral replication. Based upon evidence that neuroinflammation impacts AD-associated neuropathology and that viral encephalitis is now recognized as a major risk factor associated with AD, the overall objective of this proposal is to determine the molecular and cellular mechanisms by which viral-induced inflammation impacts AD pathology and neurodegeneration after the onset of plaque deposition. Moreover, this proposal will identify if acute viral-induced encephalitis affects late-stage AD pathology by defining long-term consequences on synapse loss and neuronal damage following

recovery of early JHNV infection in 5xFAD mice. We will accomplish these experimental goals through a series of interrelated experiments employing state-of-the-art techniques such as single-cell and spatial transcriptomics to gain mechanistic insights that regulate A β plaque deposition and removal in response to viral-induced inflammation with the long-term goal of identifying potential therapeutic pathways to control and eliminate plaque deposition and limit the severity of neurologic disease in AD patients.

3. ADDRESSING THE GAPS IN KNOWLEDGE

Immunity is a key characteristic in the pathogenesis of AD with numerous genetic risk alleles that are both highly expressed in myeloid cells and involved in inflammation pathways. Therefore, it is unsurprising that viral exposure has also been identified as a key risk factor associated with the development of numerous neurodegenerative diseases. These observational datasets have identified a strong association between viral encephalitis and AD. Indeed, many viruses have acute and long-lasting impacts on the CNS through direct infection demonstrated with neurotropic viruses (i.e. HSV, VZV, EBV, and Flaviviruses) or by dysregulating the peripheral immune system through systemic infection, constant immune activation, and inflammation (i.e. HIV and SARS-CoV-2). While the field's current understanding of the contributions of these viruses and their resulting inflammation on AD remains nuanced and complex, ongoing investigations are precisely identifying direct and indirect viral mechanisms leading to CNS damage and associated cognitive impairments like dementia. Thus far, these findings have presented evidence of direct interactions with protein aggregates such as A β or tau, while others have argued its resulting neuroinflammation response as the key mediator of AD pathology. Conversely, new insight on the effects of vaccines and novel anti-viral therapies have demonstrated significant reductions to risk of developing dementia and worsening deterioration.

This is further highlighted by the increasing frequency of emerging viral pathogens like SARS-CoV-2 in which the long-term neurologic sequelae are unknown. Committing to defining these post-infectious neuroimmune mechanisms and their interaction with AD pathogenesis holds promise, with potential to offer both treatments for current patients and preventative interventions to improve public health outcomes and reducing AD risk in an aging population.

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CHAPTER 2

ABLATION OF MICROGLIA FOLLOWING INFECTION OF THE CENTRAL NERVOUS SYSTEM WITH A NEUROTROPIC MURINE CORONAVIRUS LEADS TO INCREASED DEMYELINATION AND IMPAIRED REMYELINATION

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Ablation of microglia following infection of the central nervous system with a neurotropic murine coronavirus infection leads to increased demyelination and impaired remyelination

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ABSTRACT

Intracranial inoculation of susceptible mice with a glial-tropic strain of mouse hepatitis virus (JHMV), a murine coronavirus, results in an acute encephalomyelitis followed by viral persistence in white matter tracts accompanied by chronic neuroinflammation and demyelination. Microglia are the resident immune cell of the central nervous system (CNS) and are considered important in regulating events associated with neuroinflammation as well as influencing both white matter damage and remyelination. To better understand mechanisms by which microglia contribute to these immune-mediated events, JHMV-infected mice with established demyelination were treated with the small molecular inhibitor of colony stimulating factor 1 receptor (CSF1R), PLX5622, to deplete microglia. Treatment with PLX5622 did not affect viral replication within the CNS yet the severity of demyelination was increased and remyelination impaired compared to control mice. Gene expression analysis revealed that targeting microglia resulted in altered expression of genes associated with immune cell activation and phagocytosis of myelin debris. These findings indicate that microglia are not critical in viral surveillance in persistently JHMV-infected mice yet restrict white matter damage and remyelination, in part, by influencing phagocytosis of myelin debris.

Keywords: *Microglia, Coronavirus, Neuroinflammation, Demyelination, Remyelination*

INTRODUCTION

Intracranial inoculation of susceptible C57BL/6 mice with the neuroadapted JHM strain of mouse hepatitis virus (JHMV, a member of the *Coronaviridae* family) results in an acute encephalomyelitis characterized by wide-spread replication of virus in glia with relative sparing of neurons¹. Innate immune responses characterized by the expression of type I interferon is critical with regards to controlling viral replication². Virus-specific CD4⁺ and CD8⁺ T cells are attracted into the CNS in response to T cell chemoattractant chemokines³⁻⁶ and control viral replication via expression of anti-viral cytokines including IFN- γ and cytolytic activity^{7, 8}. Although T cells are efficient in controlling viral replication, sterile immunity is not achieved and virus persists primarily in spinal cord white matter tracts^{1, 9-11}. Persistent viral infection within glial cells leads to expression of chemokine gene expression resulting in chronic inflammation comprised primarily of inflammatory monocyte/macrophages and T cells that contribute to immune-mediated demyelination¹²⁻²².

While the contributions of inflammatory CD4⁺ and CD8⁺ T cells and monocyte/ macrophage in contributing to host defense and demyelination have been extensively studied, the contributions of glial cells in these responses remain to be better characterized. Under non-disease conditions, microglia function to maintain tissue homeostasis and have also been shown to be important in maintaining overall myelin health through regulation of myelin growth as well as preservation of myelin integrity²³⁻²⁵. Microglia serve various functions under neuroinflammatory conditions resulting from either infection, injury, or onset of disease by influencing antigen presentation, which either amplifies or restricts disease progression via secretion of regulatory factors that, in turn control the activation state of inflammatory immune cells^{26, 27}. Pharmacologic depletion of microglia via administration of PLX5622, a small molecule antagonist for colony stimulating

factor 1 receptor (CSF1R), prior to JHMV infection results in increased morbidity/mortality associated with impaired control of viral replication, muted anti-viral CD4⁺ T cell responses associated with reduced expression of MHC class II expression on inflammatory monocyte/macrophages²⁸⁻³⁰. Microglia depletion in JHMV-infected mice results in a significant increase in the severity of demyelination accompanied by a reduction in remyelination, indicating that microglia augment both host defense as well as restrict myelin damage and influence repair²⁹,³¹. Indeed, microglia have been shown to influence remyelination in response to CNS damage and may serve as therapeutic targets to promote remyelination^{32, 33}.

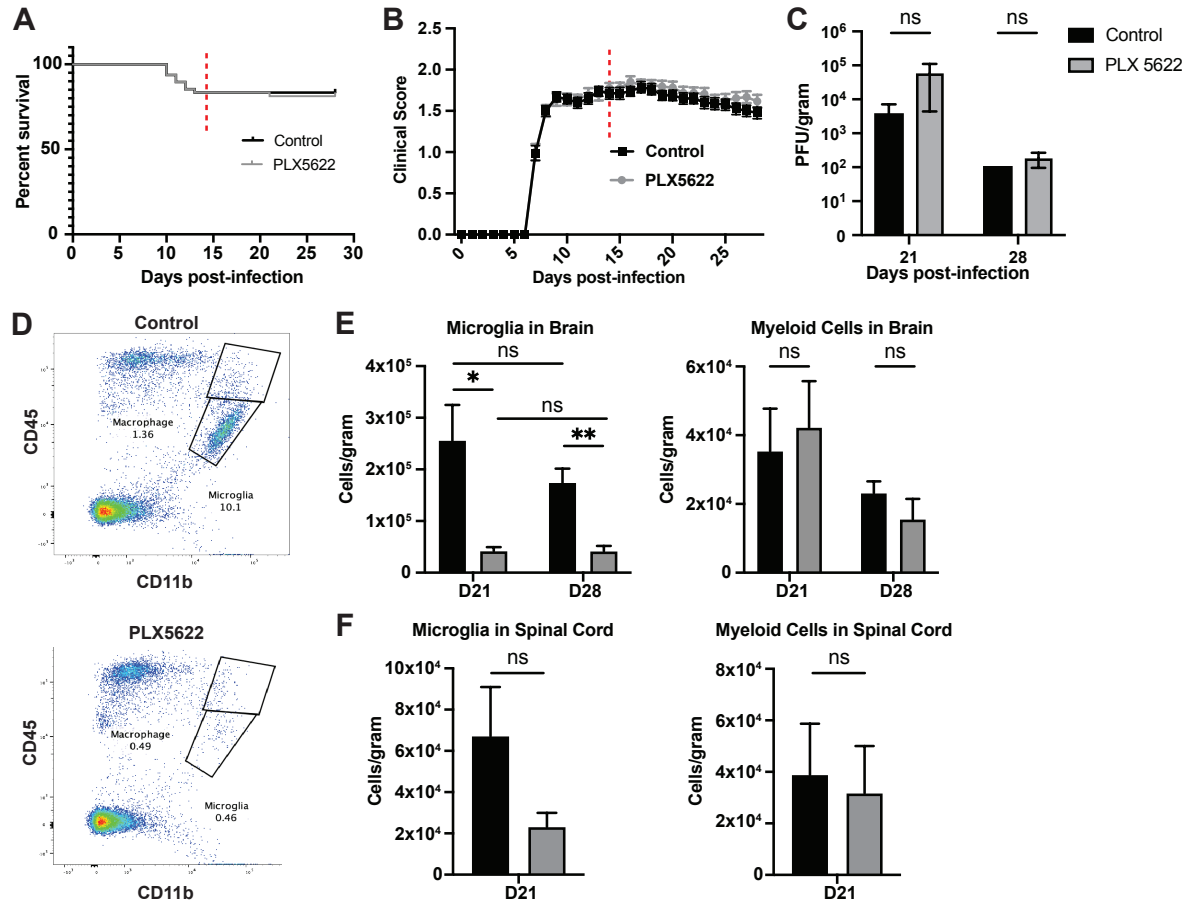
Sariol et al.³¹ have recently performed a series of studies evaluating how targeted ablation of microglia in JHMV-infected mice at defined times following infection affects host defense, demyelination and remyelination. Their findings argue for a defined time window in which PLX5622-mediated reduction in microglia affected clinical disease and demyelination. Depletion of microglia during clearance of virus increased the severity of clinical disease and demyelination while reducing remyelination whereas delayed microglia reduction did not affect either clinical disease or demyelination³¹. These findings argue that microglia are critical in limiting the severity of white matter damage and promoting repair early following JHMV infection yet are dispensable at later stages of disease in mice persistently infected with JHMV³¹. In the current study, we report that targeted reduction of microglia in mice persistently infected with JHMV with established demyelination does not result in viral recrudescence but there is increased spinal cord white matter damage and impaired remyelination. These findings suggest that microglia are dispensable in continuing to suppress viral replication within the CNS of persistently-infected mice yet continue to influence mechanisms that influence disease and repair.

RESULTS

Timed ablation of microglia in JHMV-infected mice does not affect viral replication.

JHMV-infected mice were fed formulated chow containing either PLX5622 (1,200 mg/kg of chow) or control chow beginning at day 14 p.i., Experimental mice were kept on either PLX5622 or control chow until sacrificed at days 21 and 28 p.i. at which point brains and spinal cords were collected to assess viral burden and immune cell infiltration. Notably, there were no differences in survival (**Figure 1A**) nor the severity of clinical disease (**Figure 1B**) between PLX5622 and control chow-treated mice. Pharmacologic targeting of microglia resulted in slightly higher viral titers within the brains at days 21 and 28 p.i. compared to control mice as determined by plaque assay yet these differences were not significant (**Figure 1C**). Using flow cytometry, we confirmed efficient microglia (CD45^{lo}CD11b⁺) depletion in PLX5622-treated mice within the brain at days 21 p.i. ($p < 0.05$) and 28 p.i. ($p < 0.005$) (**Figures 1D and E**). PLX5622 treatment did not affect numbers of myeloid cells (CD45^{hi}CD11b⁺) within brains of experimental mice at either days 21 and 28 p.i. (**Figures 1D and E**). PLX5622-treatment also reduced microglia numbers within the spinal cords (although this difference was not significant) while not affecting myeloid cell numbers at day 21 p.i. (**Figures 1F**).

FIGURE 1. Treatment of JHMV-infected mice with PLX5622 does not affect survival or control of viral replication. JHMV-infected mice were fed either PLX5622 or control chow beginning at day 14 p.i. through day 28 p.i. PLX5622 treatment did not affect either (A) survival or (B) clinical disease severity ($n = 44$ per group) compared to control mice. The red dash line indicates the time point that PLX5622 treatment was initiated. (C) PLX5622-treatment did not affect control of viral replication within the brains at days 21 and 28 p.i. (control $n = 4$ at day 21 p.i., $n = 9$ at day 28 p.i.; PLX5622 $n = 5$ at day 21 p.i., $n = 9$ at day 28 p.i.) compared to control mice as determined by plaque assay. (D) Representative flow cytometric data from JHMV-infected mice treated with either PLX5622 or control chow and gating on microglia (CD45^{lo}CD11b⁺ cells) or myeloid cells (CD45^{hi}CD11b⁺ cells). Quantification of microglia and myeloid cells in (E) brains at day 21 and 28 p.i. ($n = 6$ per group at day 21 p.i.; $n = 4$ per group at day 28 p.i.) and (F) spinal cords at day 21 p.i. ($n = 5$ per group). PLX5622-treatment resulted in reduced numbers of microglia in brains at day 21 p.i. and day 28 p.i. compared to control mice. Data in C, E, and F are presented as average $\pm$ SEM; * $p < 0.05$ and ** $p < 0.01$; ns, not significant.



Flow cytometry was performed to assess how targeting microglia in JHMV-infected mice with established disease affected T cell infiltration into the CNS. Initiating PLX5622 treatment at day 14 p.i. in JHMV-infected mice did not affect infiltration of either total CD4⁺ or virus-specific CD4⁺ T cells (**Figures 2A-C**) or total CD8⁺ T cells or virus-specific CD8⁺ T cells (**Figures 2D-F**) into the brain at day 21 p.i. Similarly, there were no differences in brain infiltration of total and virus-specific CD4⁺ (**Figures 2B and C**) and CD8⁺ T cells (**Figures 2E and F**) at day 28 p.i.

Targeting microglia impacts mRNA expression within spinal cords of JHMV-infected mice. Bulk tissue RNA sequencing was performed on spinal cords from experimental mice at day 28 p.i. to identify whether ablation of microglia affects specific gene pathways associated with

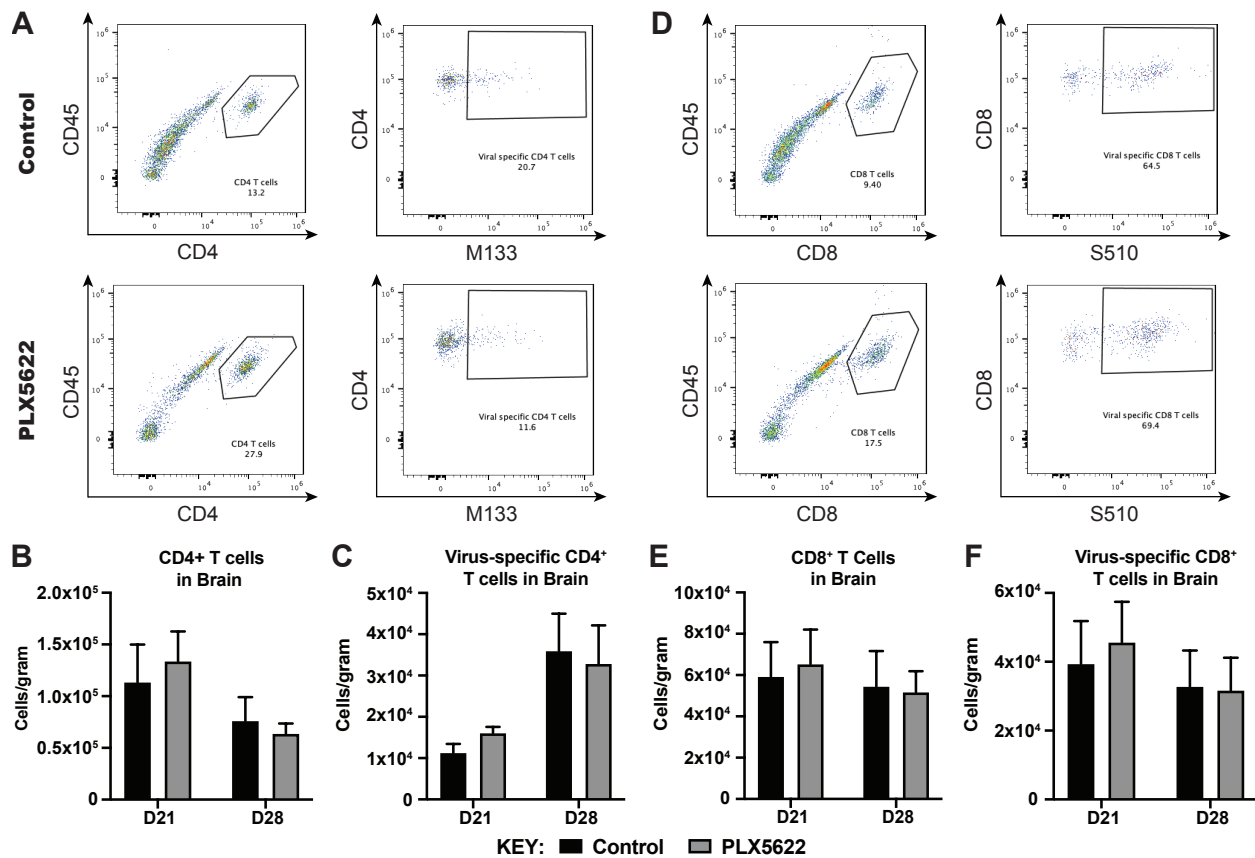


FIGURE 2. PLX5622 treatment does not affect T cell infiltration into the CNS.

Representative flow plots show (A) total CD4+ and virus-specific CD4+ T cells from control and PLX5622-treated mice at day 21 p.i. Quantification of (B) total and (C) virus-specific CD4+ T cells at days 21 and 28 indicate no differences between control mice and PLX5622-treated mice. Representative flow plots show (D) total CD8+ and virus-specific CD8+ T cells from control and PLX5622-treated mice at day 21 p.i. Quantification of (E) total and (F) virus-specific CD8+ T cells at days 21 and 28 indicate no differences between control mice and PLX5622-treated mice. PE-conjugated I-Ab/M133-147 tetramers and PE-conjugated Db/S510-518 were used to stain virus-specific CD4+ T cells and virus-specific CD8+ T cells, respectively. Data is derived from two independent experiments: n = 6 per group at day 21 p.i.; n = 4 per group at day 28 p.i. Data in B–D are presented as average ± SEM.

host defense and neurologic disease in mice following JHMV infection. Differentially expressed genes (DEGs) between JHMV-infected PLX5622-treated and control mice were identified and plotted as a volcano plot (Figure 3A) and a heatmap (Figure 3B). Down-regulated transcripts in PLX5622 versus control represented myeloid-expressed genes that included *Cx3cr1*, *P2ry12*, *Csfr1* and *Tmem119* (Figure 3A). Moreover, microglia targeting reduced expression of transcripts

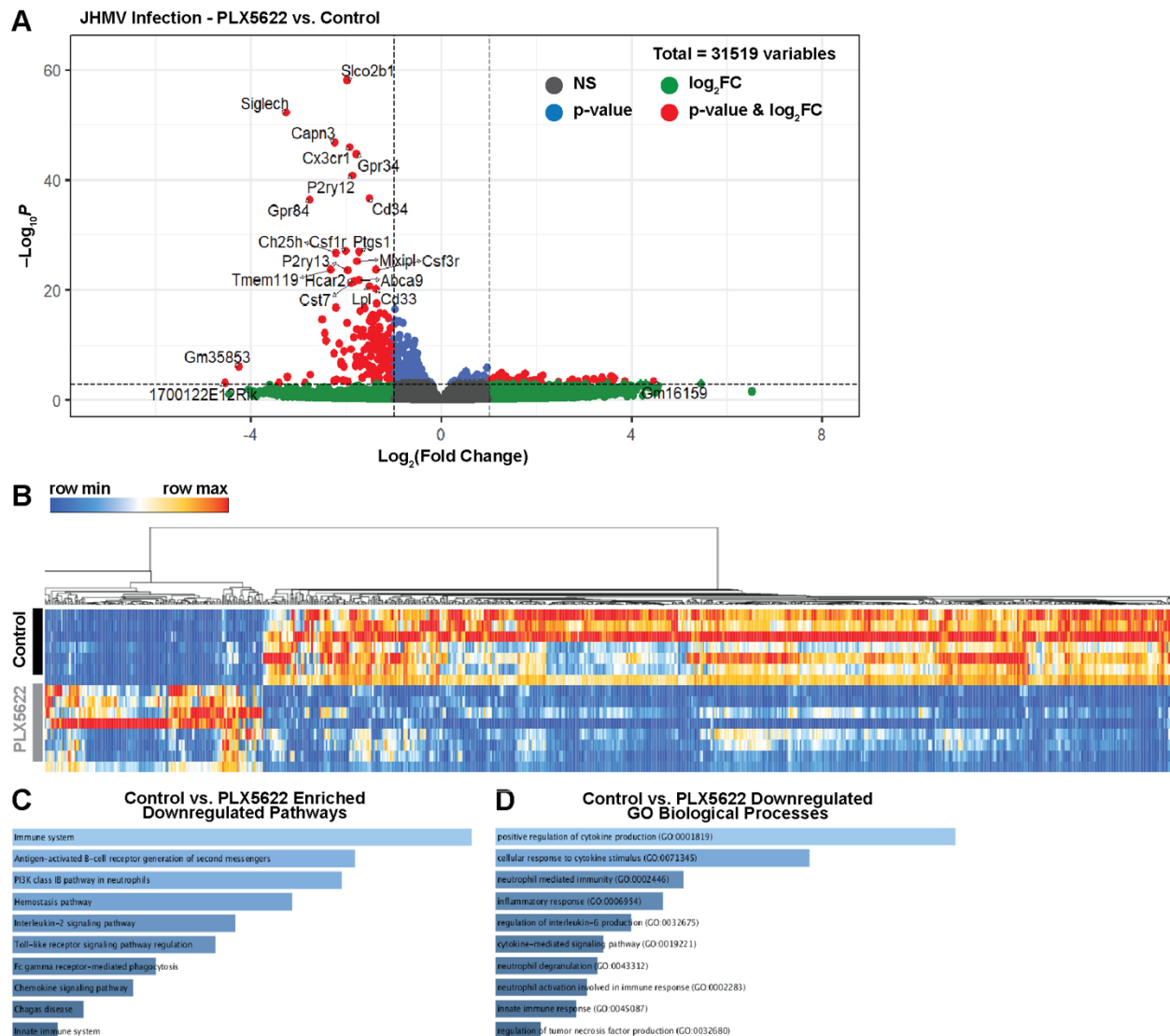
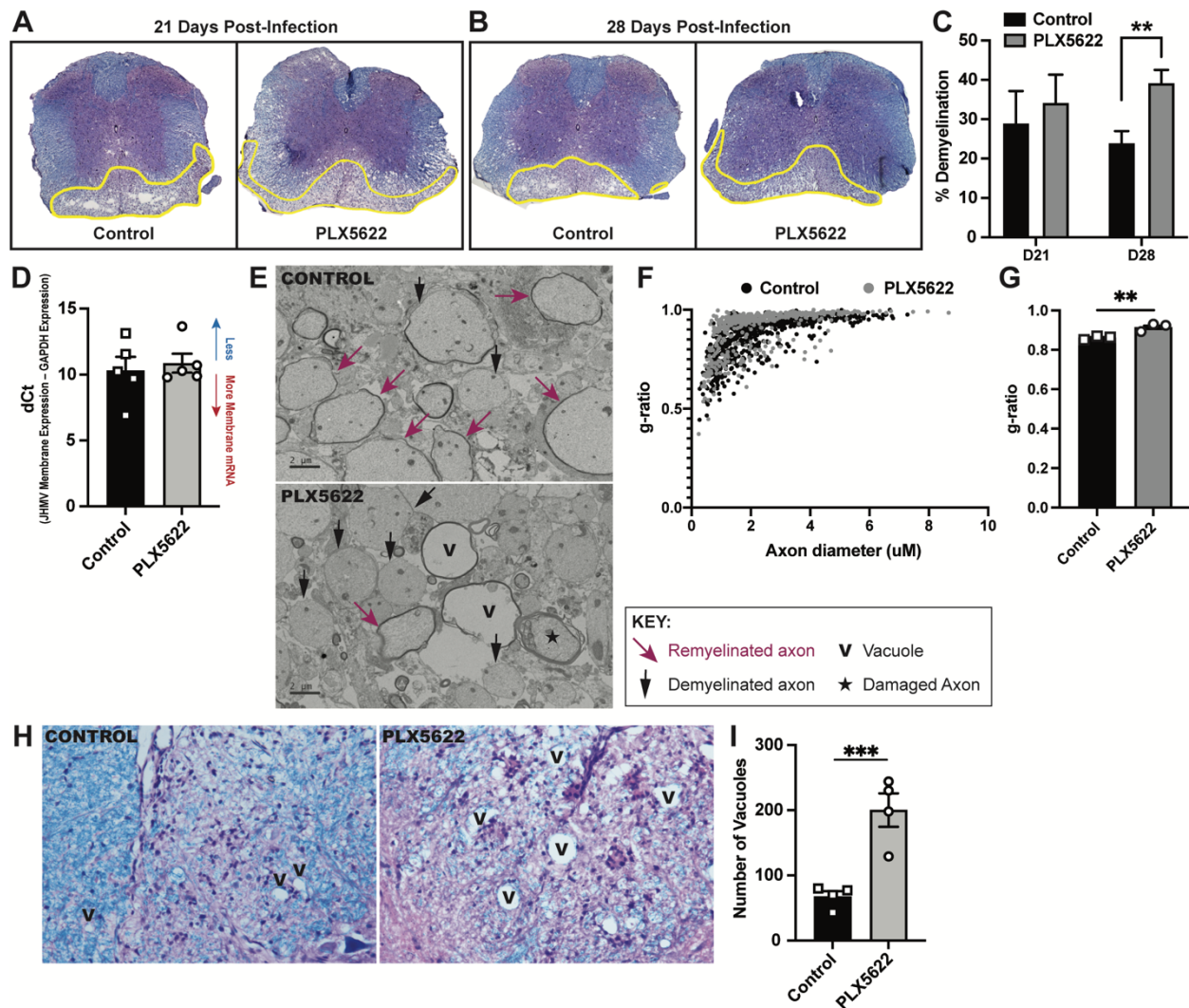


FIGURE 3. Targeting microglia impacts immune response gene expression. RNA sequencing was performed on spinal cords from JHMV-infected mice given either control chow (n=7) or PLX5622 (n=8) beginning at day 14 p.i. and subsequently sacrificed at day 28 p.i. (A) Volcano plot displaying fold change of genes (log2 scale) and P values ($-\log_{10}$ scale) between JHMV-infected control and PLX5622-treated mice. (B) Heatmap analysis showing differential gene expression in spinal cords from control versus PLX5622-treated mice. Biological pathways (C) and (D) examined via GO enrichment that are downregulated in PLX5622-treated mice compared to controls.

Cd34, *Cst7* and *Lpl* that are expressed by reactive or activated microglia (**Figure 3A**). Gene ontology analysis indicated microglia depletion resulted in downregulation of pathways associated with immune cell activation and inflammation (**Figures 3C and D**).

Microglia restrict the severity of demyelination and enhance remyelination in mice persistently infected with JHMV. JHMV-infected mice were fed either PLX5622-formulated chow or control chow beginning at day 14 p.i. and were subsequently euthanized at days 21 and 28 p.i. to assess the severity of spinal cord demyelination by LFB staining. PLX5622-mediated ablation of microglia starting at day 14 p.i. did not affect the severity of spinal cord demyelination at day 21 p.i. between experimental groups (**Figures 4A and C**); however, by day 28 p.i. demyelination was significantly ($p < 0.01$) increased in PLX5622-treated mice when compared to control mice (**Figures 4B and C**). We did not detect any differences in viral RNA burden within the spinal cords of control mice compared to PLX5622-treated mice (**Figure 4D**). To further evaluate both demyelination and remyelination in control versus PLX5622-treated mice, EM analysis of spinal cord sections was performed. In contrast to control animals, spinal cords from PLX5622-treated mice exhibited an overall increase in spinal cord tissue damage as characterized by the presence of vacuoles as well as degenerating axons (**Figure 4E**). Assessment of the g-ratio, the ratio of the inner axonal diameter to the total outer fiber diameter, is commonly employed as a

FIGURE 4. The severity of spinal cord demyelination is increased in PLX5622-treated mice compared to control mice. JHMV-infected mice treated with either control chow or PLX5622 from day 14 p.i. were sacrificed at day 21 or 28 p.i. and spinal cords removed to evaluate the severity of demyelination. Representative images of H&E/LFB-stained spinal cord sections showing an increase in severity of demyelination (yellow line) in JHMV-infected mice treated with PLX5622 compared to control treated mice at day (A) 21 p.i. and (B) 28 p.i. (C) Quantification of spinal cord demyelination (control $n = 4$ at day 21 p.i., $n = 9$ at day 28 p.i.; PLX5622 $n = 5$ at day 21 p.i., $n = 12$ at day 28 p.i.) reveals a significant increase after PLX5622 treatment compared to control-treated animals at 28 p.i. (D) qPCR for viral Matrix protein RNA transcripts in spinal cords of control and PLX5622-treated mice at day 28 p.i. (E) Representative EM images with magnification $\times 1200$ from control and PLX5622 mice spinal cord with demyelinated and remyelinated axon, vacuoles and damaged axons depicted. Scatter plot (F) depicting individual g-ratio's and (G) overall calculation of g-ratio of control and PLX5622, from lateral and ventral white matter columns of control and PLX5622 treated mice as a function of axon diameter. Representative images of H&E/LFB stained spinal cords of control and PLX5622-treated mice at day 28 p.i. with vacuoles indicated (H) and quantified (I). Data in C, G, and I are presented as average $\pm$ SEM. ** $p < 0.01$ and *** $p < 0.001$.

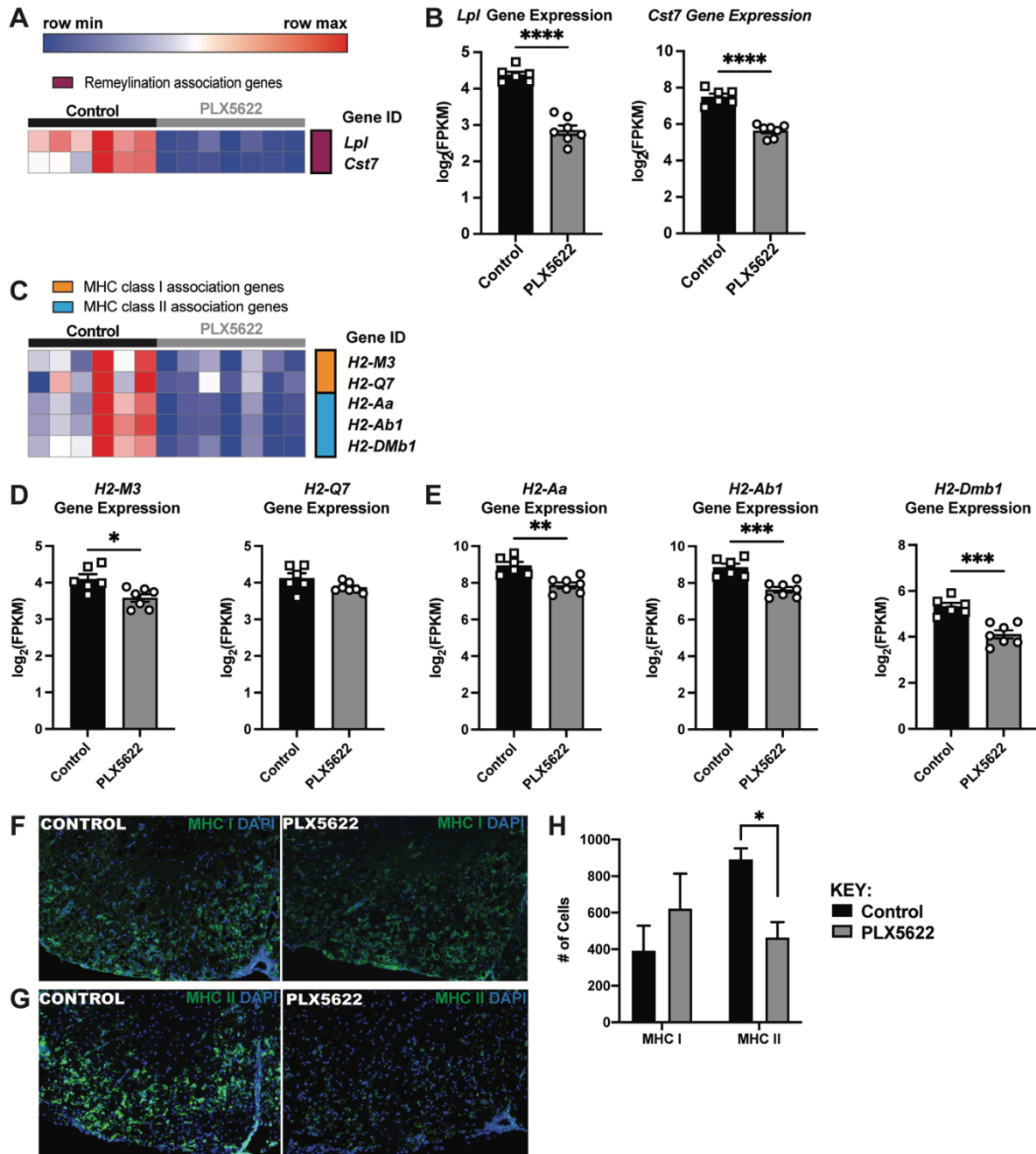


structural index of both demyelination and remyelination with lower ratios indicating myelination (29, 42). Analysis of spinal cords indicated a significant ($p < 0.01$) decrease in remyelination as determined by g-ratio's in PLX5622-treated mice compared to control animals (**Figures 4F and G**). Further examination of tissue damage in PLX5622-treated mice indicated that decreased remyelination was associated with fewer axons associated with large vacuoles. Quantification of vacuoles in LFB-stained spinal cords indicated a significant ($p < 0.0001$) increase in the number of vacuoles present in PLX5622-treated mice compared to control mice (**Figure 4H and I**). Overall, there was an increase in tissue damage within the spinal cords of PLX5622-treated mice compared

to controls, suggesting an impairment in the clearance of tissue and cellular debris, further contributing to remyelination deficiency in the absence of microglia.

PLX5622 treatment alters expression of genes associated with remyelination and host defense. We have previously suggested that one mechanism by which microglia aid in myelin repair is through regulating remyelination-associated genes including *Lpl* (Lipoprotein Lipase) and *Cst7* (Cystatin F) within microglia as well as macrophage populations^{15, 29}. Heatmap analysis of RNA sequencing indicated decreased ($p < 0.0001$) expression of both *Lpl* and *Cst7* within the spinal cords of PLX5622-treated mice compared to control mice (**Figures 5A and B**). With regards to controlling viral replication within the CNS, expression of transcripts encoding MHC class I molecule *H2-M3* was reduced ($p < 0.05$) in PLX5622-treated mice compared to controls yet there was no difference in transcripts encoding *H2-Q7* between PLX5622-treated mice and control mice (**Figures 5C and D**). Similarly, transcripts encoding MHC class II molecules *H2-Aa*, *H2-Ab1*, and *H2-Dmb1* were significantly ($p < 0.01$; $p < 0.001$) reduced in PLX5622-treated mice compared to control mice (**Figures 5C and E**). Immunohistochemical staining of spinal cords of experimental mice revealed no difference in expression of MHC class I proteins between control and PLX5622-treated mice (**Figures 5F and H**), yet there was a significant ($p < 0.05$) reduction in MHC class II staining of spinal cords from PLX5622-treated mice (**Figures 5G and H**).

FIGURE 5. PLX5622 treatment alters expression of genes associated with remyelination and host defense. JHMV-infected mice treated with either control chow or PLX5622 from day 14 p.i. were sacrificed at day 28 p.i. and spinal cords removed. Analysis of expression of transcripts *Lpl* and *Cst7* within spinal cords of control and PLX5622-treated mice was evaluated by (A) heatmap analysis and (B) quantification of expression levels of transcripts. Expression of MHC class I (*H2-M3*, *H2-Q7*) and MHC class II (*H2-Aa*, *H2Ab1*, *H2-Dmb1*) transcripts was assessed by (C) heatmap analysis as well as quantification of transcript levels for (D) MHC class I and (E) MHC class II genes. Representative immunofluorescent staining for (F) MHC class I and (G) MHC class II proteins in spinal cords of control and PLX5622-treated mice. (H) quantification of MHC class I and MHC class II positive cells in control ($n = 3$) and PLX5622 ($n = 3$) treated mice. Data in panels B, D, E, and H are presented as average $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.



DISCUSSION

Pharmacologic depletion of microglia prior to CNS infection with neurotropic strains of murine coronaviruses results in increased mortality associated with impaired control of viral replication that correlates with reduced expression of MHC class II on CNS myeloid cells and muted anti-viral CD4⁺ T cell responses^{28, 29}. Moreover, depletion of microglia prior to infection also increases the severity of demyelination while impairing remyelination, and this is associated with altered expression of genes previously associated with either white matter damage or repair²⁹. These findings support previous studies that implicate an important role for microglia that aid in host defense in response to infection with neurotropic viral pathogens⁴³⁻⁴⁷ as well as restricting viral-induced damage and promoting repair⁴⁸. In experimental autoimmune encephalomyelitis (EAE), an autoimmune model of neuroinflammation and demyelination, depletion of microglia has been shown to reduce the severity of demyelination^{49, 50} and this highlights differences between pre-clinical models of viral-induced demyelination and autoimmune-mediated demyelination. Microglia have been shown to contribute to demyelination through release of proinflammatory cytokines/chemokines that contribute to damage to the myelin sheath⁵¹ yet have also been shown to be protective through elimination of myelin debris that ultimately enhances myelin repair^{42, 52} as well as secreting regenerative factors that enhance the recruitment and differentiation of OPCs⁵³.

The current study was undertaken to better understand how targeted ablation of microglia in mice persistently infected with JHMV impacts viral replication within the CNS as well as determining its impact on both demyelination and remyelination. Using this approach, we have demonstrated that administration of PLX5622 in JHMV-infected mice beginning at 14 days p.i. did not affect either survival, clinical disease nor viral replication within the brain at days 21 p.i. and 28 p.i. as determined by both plaque assays and qPCR for viral RNA. This is in contrast to our

earlier study in which microglia were depleted prior to JHMV infection which resulted in increased mortality associated with impaired control of JHMV replication in both the brain and spinal cord that correlated with reduced expression of MHC class II transcripts and protein in both remaining microglia and inflammatory monocyte/macrophages²⁹. These findings emphasize the importance of MHC class II expression during acute disease with regards to presenting viral antigen to CNS infiltrating virus-specific CD4⁺ T cells which leads to secretion of IFN- γ that aids in host defense by both suppressing viral replication and increasing expression of MHC class II on CNS infiltrating myeloid cells. With regards to the current study, delaying administration of PLX5622 until after acute disease and when mice are persistently infected with JHMV did not significantly affect viral replication compared to control mice although there was reduced expression of transcripts encoding both MHC class I (*H2-M3*) and MHC class II (*H2-Aa*, *H2Ab-1*, and *H2-Dmb1*). Immunostaining of spinal cords from experimental mice depicted a slight increase in expression of MHC class I in spinal cords of PLX5622-treated mice compared to controls while MHC class II was reduced in PLX5622-treated mice and not control mice. Collectively, these results suggest that pharmacologic targeting of microglia impacts MHC expression within the CNS during both JHMV-induced acute and chronic disease. More importantly, these findings argue that in mice persistently infected with JHMV, inflammatory T cells may not be crucial in suppressing viral replication during chronic disease and further highlights the importance of antibody-secreting cells (ASCs) in preventing viral recrudescence as previously shown by Bergmann and colleagues⁵⁴⁻⁵⁶.

Targeted reduction of microglia in mice persistently infected with JHMV with established demyelination increased the severity of white matter damage that correlated with impaired remyelination. Recently, Sariol et al.³¹ reported that PLX5622-mediated depletion of microglia

beginning at day 7 p.i. with JHMV, representing the acute stage of disease, resulted in worsened clinical disease associated with increased demyelination and reduced remyelination, yet CNS viral titers were not affected when compared to control mice. However, PLX5622-treatment beginning at day 15 p.i. did not affect the severity of clinical disease nor increase demyelination³¹. These results argue that microglia are important in impacting the severity of white matter damage and myelin repair during early stages of JHMV infection of the CNS, yet this influence over these processes wanes over time³¹. Our findings in which microglia are depleted at day 14 p.i contrast with these results and argue for an extended time window in which microglia aid in restricting white matter damage, leading to a more conducive environment for promoting remyelination. These results also demonstrate the lack of redundancy of macrophages and microglia in the demyelinated CNS and are consistent with other studies describing distinct functions for microglia and macrophages in CNS pathologies^{42, 57-59}. Interestingly, the increase in the severity of demyelination when we depleted microglia beginning at day 14 p.i. did not result in an increase in clinical disease compared to control mice. These findings suggest that the increase in white matter damage in PLX5622-treated mice may not be associated with either extensive axonal damage or axonal dysfunction⁶⁰. Mechanisms potentially contributing to this include lack of an overwhelming increase in CNS viral titers in PLX5622-treated mice compared to control mice and this would reduce the activation state of resident glia and infiltrating immune cells thereby potentially limiting axonal damage/function. In addition, blocking CSF1R signaling may alter responses in other inflammatory cells e.g. monocyte/macrophages leading to preservation of axonal function. Collectively, these findings illustrate the complexity of evaluating CSF1R signaling on disease progression and repair in models of viral-induced neurologic disease.

We are continuing to explore the molecular and cellular mechanisms by which microglia modulate the CNS microenvironment that influence demyelination in mice persistently infected with JHMV. Microglia have been shown to enhance remyelination in a toxin-model of demyelination that is aided by microglial death and subsequent microglial repopulation^{33, 53}. Microglia may alter the microenvironment through secretion of growth factors and cytokines, which influence either the maturation of oligodendrocyte progenitor cells (OPCs) into mature myelin-producing oligodendrocytes or aid in clearance of myelin debris. Sariol et al.³¹ has demonstrated via RNA sequencing that reducing microglia had a global impact on dampening specific pathways associated with immunological responses which could influence OPC maturation in JHMV-infected mice. Our previous studies also show that PLX5622-mediated ablation of microglia prior to JHMV infection led to increased spinal cord demyelination and a reduction in remyelination. These changes correlated with reduced expression of transcripts encoding molecules associated with remyelination including Cystatin F (*Cst7*) and Lipoprotein lipase (*Lpl*) by inflammatory macrophages as determined by single cell RNA sequencing²⁹. Bulk RNA sequencing indicated that both *Cst7* and *Lpl* transcripts were reduced within the spinal cords of JHMV-infected mice when treatment with PLX5622 commenced at day 14 p.i., further supporting an important role for these molecules in promoting myelin repair. Cystatin F has been shown to regulate both demyelination and remyelination in animal models of neurodegeneration and this has been suggested to be due, in part, to regulation of neuroinflammation⁶¹⁻⁶⁶. In addition, recent studies have argued for an important role for lipoprotein lipase in contributing to phagocytosis of myelin debris, thereby promoting efficient remyelination in models of disease as well as in MS patients⁶⁷⁻⁷⁰. With this in mind, Perlman and colleagues³¹ clearly demonstrated that impaired remyelination in response to PLX5622-mediated ablation of microglia was strongly

associated with an inability to efficiently phagocytize myelin debris. Similarly, we also detected histological features associated with muted phagocytosis of myelin debris that would contribute to a reduction in efficient remyelination.

To summarize, our data indicate that microglia are important in host defense via controlling JHMV replication within the CNS during acute disease yet are dispensable during chronic disease. However, we argue that microglia are important in restricting the severity of demyelination and promoting remyelination during both acute and chronic disease. Importantly, the role for microglia in these processes are separate and distinct from inflammatory macrophages. Ongoing studies will continue to define the molecular mechanisms by which microglia tailor the microenvironment leading to altered disease status, which may lead to discovery of novel therapeutics that can be used for treatment of human demyelinating diseases.

MATERIALS AND METHODS

Mice and viral infection. C57BL/6 mice (6-8 weeks of age) were purchased from the Jackson Laboratory. Mice were inoculated via intracranial (i.c.) infection with 1000 plaque forming units (PFU) of JHMV in 30 μ L of sterile Hanks balanced sterile solution (HBSS). Inoculations were performed under deep anesthesia through an intraperitoneal injection of a mixture of ketamine and xylazine. To determine viral titers in the brains and spinal cords, experimental animals were sacrificed at defined times post-infection (p.i.) and tissues isolated, homogenized and plaque assays performed on the DBT astrocytoma cell line as described²⁹. All animal studies were reviewed and approved by the University of California, Irvine Animal Care and Use Committee.

PLX5622 treatment. AIN-76A (Research Diets, NJ) rodent chow formulated with CSF1R inhibitor- PLX5622 at a dose of 1,200 mg/kg of chow was kindly provided by Plexxikon, Inc (Berkeley, CA)²⁹. Mice were fed with either PLX5622 chow or control chow beginning at day 14 p.i., and chow was continued until the mice were sacrificed at day 28 p.i. to harvest brains and spinal cords.

Cell isolation and flow cytometry. Flow cytometry was performed by using established protocols^{34, 35}. In brief, single cell suspensions were generated from tissue samples by grinding, and immune cells were enriched via a two-step Percoll gradient (90% and 63%), and cells were collected at the interface of the two Percoll layers. Isolated cells were incubated with anti-CD16/32 Fc block (BD Biosciences, San Jose, CA) at a 1:200 dilution before staining with fluorescent antibodies. Immune cells were identified by staining with the following cell surface markers: CD4, CD8 (eBioscience, San Diego, CA), CD11b (Abcam, Boston, MA) and CD45 (BD Pharmingen, San Diego, CA). PE-conjugated I-A^b/M133–147 tetramers and PE-conjugated D^b/S510–518

(National Institutes of Health/National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility) were used to stain virus-specific CD4⁺ T cells and virus-specific CD8⁺ T cells, respectively^{34, 35}. Data were collected using an ACEA NovoCyte 2060 flow cytometer and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

RNA sequencing. Whole transcriptome RNAseq libraries were produced from spinal cords of control and PLX5622 treated mice sacrificed at day 28 p.i. RNA was isolated with a RNeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. Library preparation, RNA sequencing, and read mapping analysis were performed by Novogene (Beijing, China). Volcano plots were created using the EnhancedVolcano package on R (<https://bioconductor.org/packages/release/bioc/html/EnhancedVolcano.html>).

EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1.16.0, <https://github.com/kevinblighe/EnhancedVolcano>. Differentially expressed genes were filtered using false discovery rate (FDR) < 0.05 and log fold change (logFC) < -0.5 and > 0.5. Gene expression values were normalized to log₂FPKM (fragments per kilobase of transcripts per million mapped reads) and visualized onto heatmaps using Morpheus (Morpheus, <https://software.broadinstitute.org/morpheus/>). Pathway analysis and gene ontology analysis of downregulated genes (logFC < -0.5) were performed and visualized using Enrichr (Enrichr, <https://maayanlab.cloud/Enrichr/>) (36-38). Data derived from bulk RNAseq were used for data presented in Figures 3 and 5.

qPCR analysis. RNA from mice spinal cords was extracted via the RNeasy Mini Kit (Qiagen, Baltimore, MD). TRIzol was substituted for QIAzol, and Buffer RW1 was substituted for Buffer RWT. cDNA was made by following the "First Strand cDNA Synthesis" standard protocol provided by New England Biolabs with their AMV Reverse Transcriptase (New England

Biolabs, M0277L). Random hexamers (Invitrogen, Waltham, MA) were used for the reactions. qPCRs were performed using the Bio-Rad iQ5 and iTaQ™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA). Reactions were 10µL, and the machine was set to run for 1 cycle (95°C for 3 minutes), followed by 40 cycles (95°C for 10 seconds, then 55°C for 30 seconds). Ct values for each sample were normalized to an internal control (GAPDH), yielding the dCt values. The following primer sequences were used: GAPDH – forward sequence, AACTTTGGCATTGTGGAAGG; reverse sequence, GGATGCAGGGATGATGTTCT; JHMV Matrix glycoprotein– forward sequence, TCAACCCCGAAACAAACAACC; reverse sequence, GGCTGTTAGTGTATGG TAATCCTCA.

Histology and immunohistochemical staining. Experimental mice were euthanized at day 28 p.i. and spinal cords harvested according to IACUC-approved guidelines. Tissues were collected and placed in 4% PFA for fixation and subsequent histological analysis. Following fixation for 24-48 hr, spinal cords cut into 1-mm transverse blocks and processed so as to preserve the craniocaudal orientation and subsequently embedded in O.C.T. (VWR, Radnor, PA, USA) and 8 µm thick coronal sections were cut and stained with hematoxylin/eosin (H&E) in combination with Luxol fast blue (LFB) with between 4-8 sections/mouse analyzed. Areas of total white matter and demyelinated white matter were determined with Image J Software and demyelination was scored as a percentage of total demyelination from spinal cord sections analyzed^{29, 35, 39, 40}. For immunohistochemical staining, slides were rinsed with PBS to remove residual O.C.T., and antigen retrieval (incubation with 10mM sodium Citrate at 95°C for 15min) was performed if required for specific antigens at which point samples were incubated with 5% normal goat serum and 0.1% Triton-X, followed by overnight incubation at 4 °C with primary antibodies. Primary antibodies used including MHC class I (1:200 Abcam, Boston, MA) and MHC class II (1:200

Abcam). On the second day, slides were treated with appropriate secondary antibodies following PBS rinsing. Slides were then mounted with DAPI Fluoromount-G (Birmingham, AL) and high-resolution fluorescent images were obtained using Revolve-D75.

Electron microscopy and g-ratio analysis. For EM analysis of spinal cords, mice were sacrificed and underwent cardiac perfusion with 0.1 M cacodylate buffer containing 2% paraformaldehyde/2% glutaraldehyde. Serial ultrathin sections of spinal cords embedded in Epon epoxy resin were stained with uranyl acetate-lead citrate and analyzed as previously described⁴⁰. Images at 1200X magnification were analyzed for g-ratio using Image J software. In adult animals, there is a relationship between axon circumference and myelin sheath thickness expressed by the g-ratio (axon diameter/total fiber diameter); in remyelination this relationship changes such that myelin sheaths are abnormally thin for the axons they surround^{29, 35, 40, 41}. An abnormally thin myelin sheath, relative to axonal diameter, was used as the criterion for oligodendrocyte remyelination. Absence of a myelin sheath was used as the criterion for demyelination. For control and PLX5622-treated mice, three mice/group were used for analysis with >500 axons counted for each group.

Statistical analysis. GraphPad Prism v.9 (GraphPad, La Jolla, CA) was used to perform statistical analyses. Data for each experiment is presented as mean±standard error of mean (SEM). For flow cytometry analysis, unpaired Student's *t* test or two-way ANOVA was used to determine significance. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ was considered statistically significant. Holm-Šidák post-hoc tests were utilized to examine biological relevant interactions from the two-way ANOVA.

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AUTHOR AGREEMENT

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DECLARATION OF COMPETING INTEREST

None.

DATA AVAILABILITY

Data will be made available on request.

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CHAPTER 3

SMALL MOLECULE INHIBITOR NEXINHIB20 REDUCES THE SEVERITY OF DEMYELINATION IN A VIRAL-MODEL OF MULTIPLE SCLEROSIS

Small molecule inhibitor Nexinhib20 reduces the severity of demyelination in a viral-model of multiple sclerosis

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ABSTRACT

Neutrophils have been increasingly implicated in the pathology of demyelinating diseases like multiple sclerosis (MS), yet the mechanisms by which they can contribute to demyelination remain incompletely defined. JHMV infection of transgenic mice in which expression of the neutrophil chemoattractant chemokine CXCL1 is under the control of a tetracycline-inducible promoter active within GFAP-positive cells results in sustained neutrophil infiltration of the central nervous system (CNS) that correlates with an increase in spinal cord demyelination. Here, using single-cell RNA sequencing, flow cytometry, pharmacological inhibition, and spatial transcriptomics we analyze how neutrophils can contribute to demyelinating pathology. Single-cell RNA sequencing revealed upregulation of neutrophil transcriptional programs associated with neutrophil activation, cytotoxicity, and degranulation. Flow cytometry confirmed increased numbers of CD11b⁺ and CD63⁺ neutrophils in DTg spinal cords, consistent with enhanced degranulation. Treatment with Nexinhib20 (N20), a small-molecule degranulation inhibitor reduced spinal cord demyelination in DTg mice without substantially altering neutrophil infiltration or surface expression of degranulation markers, indicating that limiting granule release—rather than immune cell recruitment—is sufficient to attenuate tissue damage. Lastly, spatial transcriptomic analysis, via the CosMx platform, revealed that N20 treatment reprogrammed the lesion microenvironment, downregulating inflammatory mediators *Ccl2*, *Cxcl9*, *B2m*, *Ptprc*, *Srgn*, and upregulating a disease-associated myeloid (DAM) signature, *ie.* *Apoe*, *Trem2*, *Lpl*, *Gpnmb*, *Ctsd*, and *Spp1*, consistent with enhanced debris removal and tissue repair programs. Together, these findings signal neutrophil degranulation as a contributor to white matter pathology during chronic viral-induced demyelination and suggest that targeting granule exocytosis represents a viable strategy to limit immune-mediated CNS injury.

INTRODUCTION

Susceptible C57BL/6 mice intracranially infected with the neuroadapted JHM strain of mouse hepatitis virus (JHMV) mount a coordinated immune response that controls viral replication within the central nervous system (CNS), yet virus persists within white matter tracks which prompts immune-mediated demyelination with, both clinical and pathological, features similar to the human demyelinating disease multiple sclerosis (MS) (1,2). JHMV primarily infects glial cells – astrocytes, oligodendrocytes, and microglia – while relatively sparing neurons (1). Early following JHMV infection, both infected and uninfected resident cells of the CNS secrete pro-inflammatory cytokines and chemokines that drive infiltration by peripheral innate immune cells (3). Neutrophils infiltrate as early as 1 day post-infection (DPI) and contribute critically to JHMV pathogenesis by secreting matrix metalloproteases (MMP), such as MMP-9, to help permeabilize the blood-brain barrier (BBB) (4–6). In turn, this enables subsequent infiltration by monocytes, macrophages, CD4⁺ and CD8⁺ virus-specific T cells that aid in control of viral replication (7–9). Although viral titers in the brain are reduced below level of detection (as determined by plaque assay), viral antigen persists in spinal cord white matter tracts and results in demyelination mediated by chronic infiltration of activated T cells and macrophages (1). While cells of the adaptive immune response have been extensively studied as important drivers of autoimmune disorders, such as MS, accumulating evidence suggests that innate immune cells, like neutrophils, also play a critical and underappreciated role in disease pathogenesis. Neutrophils have been recognized as active players in the pathophysiology of various autoimmune disorders, including rheumatoid arthritis, psoriasis, and neuromyelitis optica (10–13). In MS, elevated neutrophil chemokines and neutrophil counts in serum and cerebrospinal fluid, together with post-mortem evidence of neutrophil accumulation in the choroid plexus, have suggested a potential contribution

of neutrophils to MS pathogenesis (14–18). However, the mechanisms underlying their involvement remain to be further examined.

Neutrophils are short-lived innate immune cells that act as the first responders of the immune system, mounting a rapid response in sterile injury as well as in defense against invading pathogens (19,20) (21). In mice, neutrophils predominantly express the chemokine receptor CXCR2, which mediates chemotaxis toward binding chemokine ligands CXCL1, CXCL2, and CXCL5 (2,21). Equipped with potent effector functions (EFs), neutrophils can target, incapacitate, and eliminate microbial pathogens via a potent arsenal of antimicrobial mechanisms. These include phagocytosis, degranulation, cytokine and chemokine secretion, and the release of neutrophil extracellular traps (NETs) which is a specialized process in which decondensed chromatin is extruded to ensnare and help dispose of pathogens (22,23). Of these EFs, degranulation, which is often accompanied by simultaneous release of reactive oxygen species (ROS), has the ability to greatly amplify the inflammatory cascade as well as one of the highest potentials to cause widespread collateral tissue damage (24,25). Degranulation is initiated through receptor-mediated signaling (e.g., Fc γ receptors and Toll-like receptors) and involves the hierarchical exocytosis of granules preloaded with cytotoxic enzymes and antimicrobial peptides (22)(26). These are released in response to corresponding stimuli strength and exocytose in order of least to most cytotoxic: secretory vesicles first, followed by tertiary, secondary, and lastly primary granules (22). In addition to these cytotoxic components, granules also carry adhesion molecules and surface receptors, which are transferred to the cell membrane upon vesicle fusion. For example, tertiary granules store the adhesion molecule CD11b (ITGAM); when these granules fuse with the neutrophil membrane, surface CD11b expression is upregulated, enhancing neutrophil adhesion and migration (27). Under physiological conditions, neutrophil degranulation functions as a

localized and tightly controlled response, however, when dysregulated or constantly stimulated, pathological degranulation can result in unintended and chronic tissue injury (11), (27). Understanding the mechanisms and contexts in which such aberrant degranulation occurs is therefore critical for elucidating its contribution to inflammatory and neurodegenerative pathology.

Using a mouse model developed in our laboratory in which tetracycline induces expression of CXCL1 from glial fibrillary acidic protein (GFAP)-positive astrocytes (C57BL/6^{pBI-CXCL1-rtTA}, referred to as “DTg”) we demonstrated that sustained CXCL1 expression derived from astrocytes results in sustained neutrophil infiltration that correlates with increased clinical disease severity and demyelination in both the JHMV model of demyelination (28) as well as experimental autoimmune encephalomyelitis (EAE) (29), the prototypic pre-clinical model of autoimmune-mediated demyelination. Conversely, blocking sustained neutrophil infiltration in both models ameliorated these effects, arguing that neutrophils contribute to immune-mediated demyelination (28,29). Mechanisms by which neutrophils may mediate white matter damage have not been well-defined given the paucity of these cells within the CNS in the majority of pre-clinical models of MS. Previously, we employed single cell RNA sequencing (scRNAseq) on immune cells infiltrating into the spinal cords of JHMV-infected DTg mice to show neutrophils have a distinct mRNA expression profile that impacts the immunological landscape during chronic disease. Neutrophils isolated from spinal cords of JHMV-infected DTg mice exhibited increased expression of transcripts *ASPRV1*, *CD63*, *MMP9*, *S100a8*, and *S100a9*, all of which have been associated with activation of neutrophil extravasation, chemotaxis, and degranulation (30). To better understand mechanisms by which increased neutrophil infiltration and degranulation amplifies white matter damage we administered Nexinhib20 (N20), a novel small molecule inhibitor that blocks neutrophil granule exocytosis, to JHMV-infected DTg and STg mice, which

resulted in a dramatic decrease in spinal cord demyelination in DTg mice as compared to controls. Analysis via spatial transcriptomics revealed important differences in the presence of disease-associated myeloid (DAM) cells present in DTg mice treated with N20 and flow cytometric analysis of blood taken from DTg mice treated with N20 showed a decrease in the total number of neutrophils found in circulation as compared to experimental controls, suggesting changes in DAM populations and the neutrophil uptake of N20 in the periphery could be involved in mediating the exhibited effects of N20 on pathology.

RESULTS

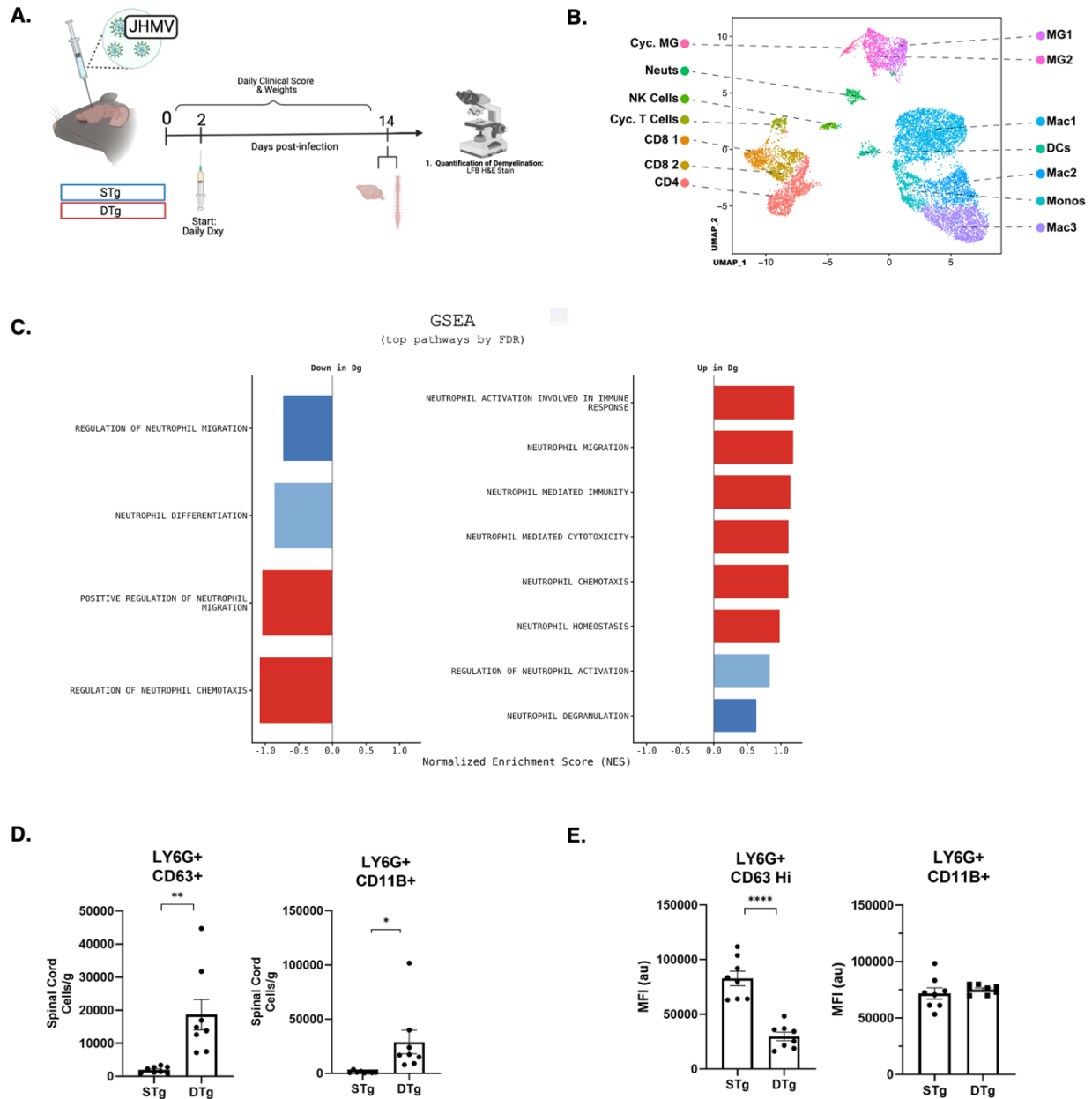
DTg mice exhibit upregulation of transcriptional pathways associated with degranulation and display significant increases in spinal cord degranulating neutrophils.

As previous scRNAseq analysis identified neutrophil differential expression of transcripts like ASPRV1, CD63, MMP9, S100a8, and S100a9 between DTg and STg conditions, we continued to probe this dataset to determine if overall changes in neutrophil profiles led to differential engagement of biological pathways or networks between the two conditions. The original dataset, obtained from the spinal cords of DTg and STg mice intracranially infected with JHMV and treated with doxycycline as previously described in Skinner et al., (Figure 1A), was generated from isolated CD45⁺ cells using the 10x Genomics scRNAseq platform (36248905). Data from DTg and STg mice was aggregated and analyzed using the Seurat R package, applying unbiased clustering based on transcriptional profiles to identify and group distinct cell populations. This analysis yielded 14 distinct immune cell clusters, including a discrete neutrophil cluster (Figure 1B). Gene set enrichment analysis (GSEA) conducted on this cluster identified differential engagement of various gene ontology biological pathways (GOBP) involved in DTg neutrophil function as compared to control, including upregulation of pathways related to neutrophil migration, antimicrobial immune response, cytotoxicity, chemotaxis, regulation of neutrophil activation, and degranulation. The analysis also noted a downregulation in DTg mice, as compared to controls, for pathways involved in neutrophil differentiation as well as for those involved in activation of regulons upstream of neutrophil migration and chemotaxis (Figure 1C).

Functional activation of neutrophil pathways involved in antimicrobial response and degranulation can prompt overall activation of the inflammatory cascade and promote further recruitment of peripheral immune cells (31)(27). The antimicrobial response pathway and

degranulation pathway are closely related and represent overlapping aspects of neutrophil effector activity, with substantial overlap existing between the transcriptional reference genes of the two pathways. Genes common to both pathways, as defined by the neutrophil-associated GOBP molecular signatures database (MSigDB) gene setlists (GSEA-MSigDB: Mouse Collections – M7), include transcripts such as *Anxa3*, *CD11b(Itgam)*, *Pram1*, *Spi1(PU.1)*, *Stxbp2/3*, *Syk*, and *Vamp7/8*. These transcripts are largely involved in membrane trafficking, exocytosis, cell adhesion, ROS production, granule docking, granule fusion, and azurophilic granule exocytosis. Given that most of the shared genes relate to various aspects of degranulation, we decided to assess for known neutrophil surface markers of degranulation, CD11b and CD63, via flow cytometric analysis in order to determine if degranulation might play a role as a contributing EF to the increase in demyelination seen in DTg mice (27)(32). Adhesion molecule CD11b and membrane marker CD63 reside originally in storage within neutrophil tertiary and primary granules, respectively, and are trafficked to the neutrophil cell surface upon degranulation (35). Flow cytometric analysis of CD45⁺ cells from the spinal cords of STg and DTg mice demonstrated that, as expected, DTg mice showed significantly higher numbers of infiltrating neutrophils, as well as a significantly

Figure 1: DTg mice exhibit upregulation of transcriptional pathways associated with degranulation and display significant increases in spinal cord degranulating neutrophils as compared to controls. (A) Schematic overview showing the experimental design. Single transgenic mouse controls (STg) and double transgenic mice (DTg) were infected intracranially (i.c) with 2300 PFU of JHMV and treated daily with doxycycline (50 mg/kg) via i.p. injection beginning at 2DPI. Tissue was collected and analyzed at defined times post-infection. (B) Uniform Manifold Approximation and Projection (UMAP) plot of scRNASeq data revealing 14 distinct cell clusters (aggregate data from JHMV-infected DTg (n=4) and STg mice (n=4) treated with doxycycline and processed at 14DPI). (C) Gene set enrichment analysis plot analyzing DTg neutrophil transcription from scRNAseq data, as compared to STg controls, using Molecular Signatures Database neutrophil associated-Immunological Signature Gene Sets - M7. (D) Flow cytometry graphs showing increased CD63⁺ and CD11b⁺ neutrophil presence (CD45⁺Ly6g⁺CD63⁺ and CD45⁺Ly6g⁺CD11b⁺, respectively) in spinal cords of DTg mice as compared to STg controls (n=8 per condition). cumulation in the blood, brain and spinal cords of double-tg mice compared to single-tg control. (E) Mean fluorescence intensity (MFI) graphs measuring CD63 and CD11b expression to assess rate of degranulation in DTg mice as compared to controls (n=8 per condition). Males and females are combined for all experiments. **p*<0.05, ***p*<0.01, *****p*<0.0001.



higher number of both, CD63⁺ ($p < 0.01$) and CD11b⁺ ($p < 0.05$) neutrophils, suggesting that DTg spinal cords have a higher amount of degranulating neutrophils in comparison to controls (Figure 1E). To determine if, in conjunction with the increased amount of degranulating neutrophils in DTg mice, the rate of degranulation also played a role in increasing pathology we assessed for mean fluorescence intensity (MFI) of CD11b and CD63. The MFI for CD11b on LY6g⁺ cells was similar across conditions while the MFI for CD63 was significantly higher in STg neutrophils

($p < 0.0001$) (Figure 1E), suggesting a potentially higher rate of degranulation for the primary granules of the few neutrophils found in STg spinal cords.

Treatment of DTg mice with small-molecule degranulation inhibitor Nexinhib20 ameliorates demyelination severity.

Despite DTg neutrophils exhibiting an equal to or lower rate of degranulation than STg neutrophils, the significantly higher number of neutrophils present in DTg mice that was positive for degranulation markers CD63 and CD11b prompted us to ask whether presence of degranulation on its own might be a significant contributing EF to the increase in demyelination seen in DTg mice. To test this, we employed the small molecule inhibitor N20. N20 is a degranulation inhibitor which prevents exocytosis of the most cytotoxic granules by blocking vesicular GTPase Rab27a from interacting with its effector JFC1 (synaptotagmin-like protein 1; SLP1) (33). JFC1 is responsible for the depolymerization of the actin surrounding these secretory granules and for the bridging connection between Rab27a-containing granules and the neutrophil plasma membrane (Figure 2A) (33,34). The Rab27a-JFC1 interaction mediates granule docking, fusion and exocytosis of primary granules, which contain marker CD63, as well as of a subset of JFC1-expressing tertiary granules, which contain marker CD11b. Notably, N20 does not play a role in other neutrophil EFs, allowing for normal function of neutrophil phagocytosis, NETs extrusion, and cytokine and chemokine production (33–35).

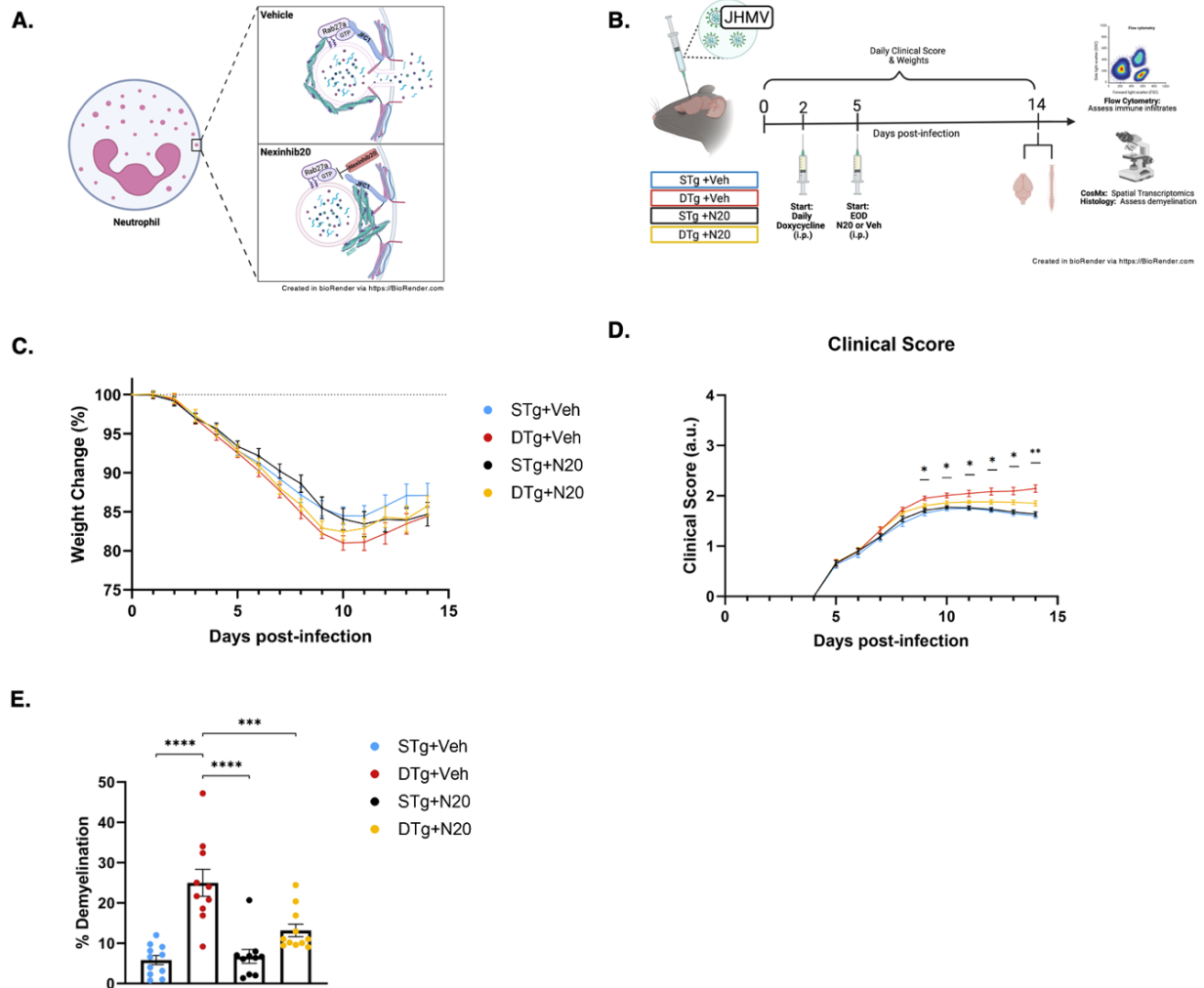


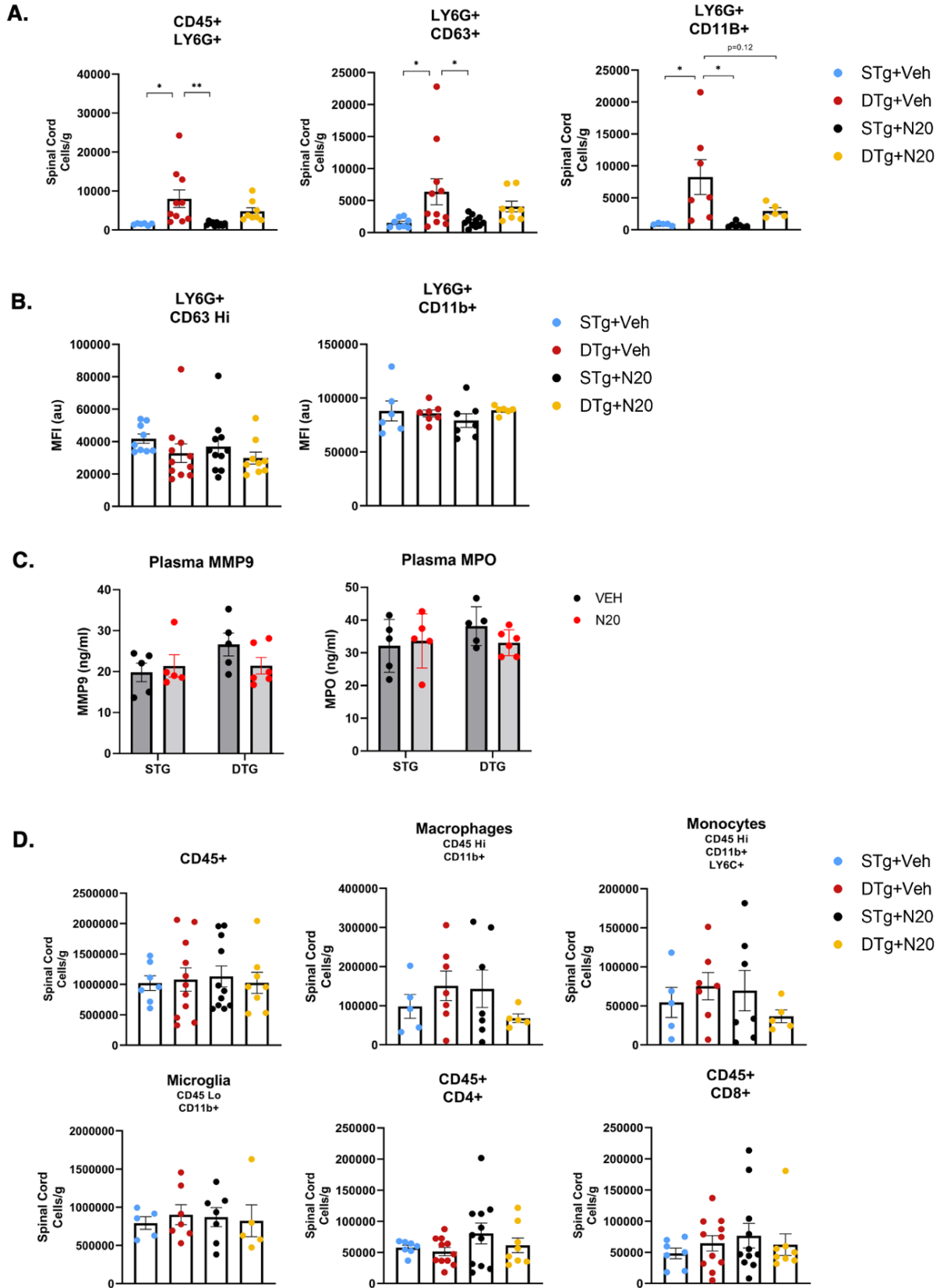
Figure 2: Treatment of DTg mice with small-molecule degranulation inhibitor Nexinhib20 ameliorates demyelination severity. (A) Illustration of Nexinhib20 mechanism of action in which the inhibitor prevents Rab27a-JFC1 interaction, thereby preventing granule docking, membrane fusion and exocytosis into extracellular space. (Created with BioRender). (B) Schematic of experimental design. Both, STg and DTg, mice are infected with 2300 PFU of JHMV and are treated with doxycycline beginning at 2DPI. Both conditions are treated with either N20 or vehicle beginning at 5DPI and are delivered treatment every other day (*ie.* 7, 9, 11, and 13 DPI), *i.p.*, contralateral to the site of doxycycline injection until tissue collection at 14DPI. (C) Percent weight change graph illustrating percent weight loss as compared to initial mouse weight at 0DPI; dotted line represents 100% of initial weight. ($n=20-24$ per condition). (D) Graph depicting daily clinical scoring until 14DPI in which mice are graded on a 4-point scale denoting clinical worsening of hindlimb paralysis: 1) hunched back/limp tail/ruffled fur, 2) mouse on its back takes >15 seconds to right itself, 3) mouse cannot right itself, but is still mobile and alert, 4) full hindlimb paralysis. ($n=20-24$ per condition). (E) Demyelination graph measured in percent of demyelinated area out of total white matter area as denoted by LFB H&E staining of 8 spinal cord coronal sections (T10-13, L1-4) per mouse ($n=10-12$ per condition). Males and females were combined for all experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

STg and DTg mice were administered N20 or vehicle (conditions: STg+Veh; STg+N20; DTg+Veh; DTg+N20) intraperitoneally, contralateral to the site of doxycycline injection every other day (EOD), beginning at 5DPI, and were then assessed for changes in weight and presentation of clinical symptoms until 14DPI when tissue was collected for analysis (Figure 2B). Blood and brain tissues, which were collected 24 hours after the last N20 injection, were subjected to mass spectrometric analysis to determine concentration of N20 in circulation and to evaluate ability of N20 in permeating the BBB. Blood samples tested positive for the presence of N20 with final concentrations measuring approximately 1nM (Table 3; Supplementary Table 1), similarly, brain tissue samples also tested positive for N20 with samples testing above blank controls but below the 1nM level of detection (Table 4; Supplementary Table 2). Change in percent weight trended towards significance between STg+Veh and DTg+Veh but showed no statistically significant difference between any of the conditions (Figure 2C). Clinical disease severity, however, showed a statistically significant difference between all conditions, except STg+Veh and STg+N20, by 11DPI (Figure 2C). Notably, DTg mice treated with N20 diverged in clinical severity from DTg mice treated with vehicle by 11DPI ($p<0.05$) and continued to display significantly lower disease severity until time of tissue collection at 14DPI ($p<0.01$) (Figure 2D). Assessment of spinal cord tissues at 14DPI, in correlation with clinical disease, revealed a significant decrease in demyelination severity ($p<0.001$) in DTg mice treated with N20 as compared to DTg+Veh, suggesting a potential role for degranulation in DTg pathology (Figure 2E-F).

DTg mice treated with N20 do not display significant decreases in spinal cord neutrophil infiltration, degranulating neutrophils or degranulation rate, neither do they display decreased degranulation in peripheral circulation or significant changes in overall immune cell infiltration.

Considering that treatment with N20 ameliorated demyelination in DTg mice, we next conducted flow cytometry in order to determine if N20 mediated these effects by either impacting the frequency of neutrophil infiltration into spinal cords or by reducing the rate of degranulation in infiltrating neutrophils. While, as expected, DTg+Veh mice showed an increased amount of infiltrating LY6g⁺ neutrophils as compared to STg+Veh ($p<0.05$) and STg+N20 ($p<0.01$), DTg mice treated with N20 did not show a statistically significant difference from DTg+Veh controls, suggesting there was similar rates of neutrophil infiltration between DTg+N20 mice and those treated with vehicle (Figure 3A). Similarly, when evaluated for changes in infiltrating neutrophils expressing degranulation markers CD11b and CD63, DTg+N20 mice did not exhibit a significantly lower frequency of infiltration as compared to DTg+Veh controls, although DTg+N20 LY6g⁺CD11b⁺ neutrophils did trend towards a lower rate of infiltration ($p=0.12$) as compared to controls. Neutrophils displayed similar levels of CD63 MFI across all conditions, as well as similar levels of CD11b MFI (Figure 3B), suggesting similar rates of degranulation across conditions. Overall, since DTg mice treated with N20 did not appear to show significantly lower levels of spinal cord neutrophil infiltration, degranulating neutrophils, or rate of degranulation we

Figure 3: DTg mice treated with N20 do not display significant decreases in spinal cord neutrophil infiltration, degranulating neutrophils or degranulation rate, neither do they display decreased degranulation in peripheral circulation or significant changes in overall immune cell infiltration. (A) Flow cytometry plots of LY6g⁺, LY6g⁺CD63⁺, and LY6g⁺CD11b⁺ neutrophils from spinal cords of mice collected at 14DPI (n=6-11 per condition). **(B)** Mean fluorescence intensity plots of LY6g⁺CD63⁺ and LY6g⁺CD11b⁺ neutrophils from spinal cords of mice collected at 14DPI (n=6-11 per condition). **(C)** Enzyme linked immunosorbent assay (ELISA), in nanograms per milliliter, of tertiary granule enzymes MMP-9 and MPO measured from the isolated plasma of mice sacrificed at 14DPI, ~24h after the last i.p. injection of N20 (n=5-6 per condition). **(D)** Flow cytometry plots of CD45⁺ cells, macrophages, monocytes, microglia, CD4⁺, and CD8⁺ T cells taken from the spinal cords of mice approximately 24h after the last i.p. injection of N20 (n=5-11 per condition). Males and females were combined for all experiments. * $p<0.05$, ** $p<0.01$.



decided to examine bloodstream neutrophils to assess if N20 mediated its primary effects outside of the CNS.

As N20 treatment is delivered i.p., it first enters the bloodstream prior to reaching the CNS. N20 is then accessible to neutrophils in circulation and can begin enacting its effects prior to neutrophil infiltration of the CNS. This in turn can prevent neutrophil degranulation in the bloodstream, as well as potentially neutrophil ability to extravasate as certain adhesion molecules, like CD11b, localize to neutrophil granules and are necessary for transmigration (33,34,36). To determine if bloodstream levels of N20 impacted neutrophil ability to degranulate, plasma samples were assessed via enzyme-linked immunosorbent assay (ELISA) for presence of neutrophil granule enzymes MMP-9 and myeloperoxidase (MPO), which reside in tertiary and primary granules, respectively. Plasma concentration for MMP-9 and MPO showed similar concentrations across conditions (Figure 3C), indicating that DTg+Veh bloodstream neutrophils do not degranulate at a higher rate than controls or than DTg+N20 neutrophils, thereby suggesting treatment with N20 should not significantly impact bloodstream degranulation.

In order to further dissect the effect of N20 on the immune landscape in demyelinating spinal cords we conducted flow cytometry on CD45⁺ cells across all conditions. No significant differences in infiltration were observed between any of the conditions for either macrophages, monocytes, microglia, or T cells (Figure 3D). While no evident changes in these broadly classified cell types were seen, we wondered if maybe changes in DTg pathology prompted by N20 treatment relied more so on transcriptional changes of the infiltrating immune cells rather than on the amount or frequency of the cells infiltrating.

CosMx spatial transcriptomic analysis reveals that DTg mice have increased presence of myeloid cells and DTg mice treated with N20 have increased presence of neutrophils and

disease associated myeloid cells which downregulate inflammatory transcripts and upregulate anti-inflammatory signatures.

To evaluate transcriptional changes in the infiltrating immune cells we conducted spatial transcriptomic analysis using the CosMx 1000-gene Mouse Neuroscience RNA panel multiplexed with a custom 50-gene immune panel that included probes for JHMV transcripts (Table 2). The tissue was processed and stained using the CosMx Fixed Frozen RNA Assay Protocol and imaged using the NanoString CosMx Spatial Molecular Imager (SMI) platform. This technique is particularly advantageous as it allows for *in situ* profiling of CNS and major immune cell types, enabling identification of cells and their transcriptional profiles within the spatial context of demyelinated areas in spinal cord tissue. All four conditions were assessed, with $n = 4$ per condition (with the exception of STg+N20, where $n = 3$), and each n was represented by four 10 μ m coronal spinal cord sections belonging to spinal vertebrae sections T1-T4. The CosMx SMI assessment yielded 185,804 cells for analysis, with individual cells identified by automated cell segmentation based off histone, GFAP, and DAPI staining, as exemplified in Figure 4A. Profiling detected 12 different broad categories of cells, including neurons, astrocytes, oligodendrocytes, vascular cells, fibroblasts, T cells and myeloid cells, and allowed for visualization of these in XY space (Figure 4B). We performed unsupervised clustering, visualized via Uniform Manifold Approximation and Projection (UMAP), and manually annotated clusters by evaluating highly expressed genes, using lineage markers chosen based on the genes available in each panel (Figure 4C). Cell type was defined as “other” for gene clusters whose top genes were not immediately identifiable as a singular or known cell type. Each of the 12 major cell types was then broken down by proportion of cells corresponding to each of the four conditions, with a majority of cell types showing roughly similar proportions of ~25% each between conditions with the exception of the myeloid cluster, which demonstrated unequal proportions between conditions. Notably, a smaller proportion of the

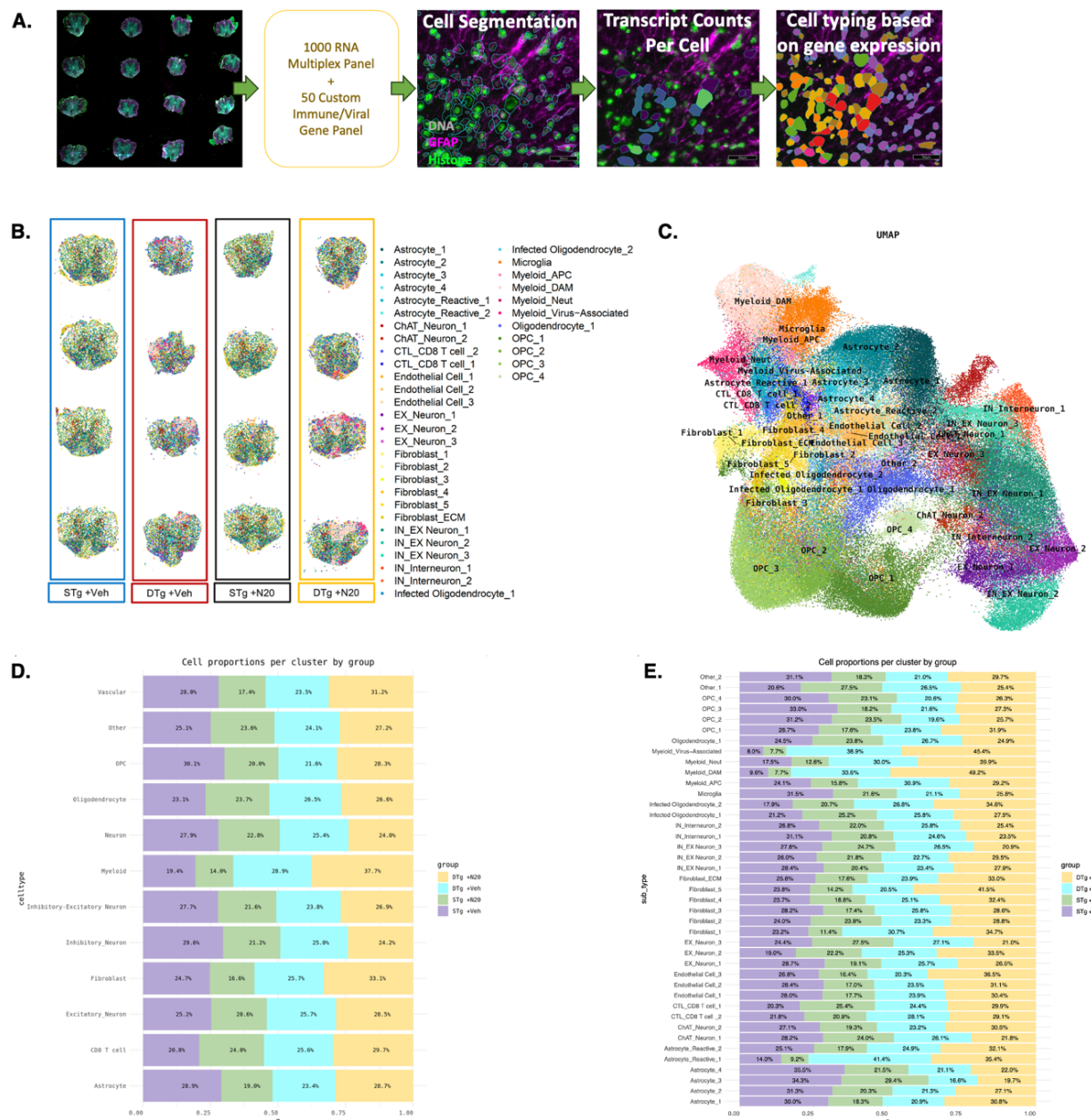
total myeloid cell count belonged to the STg conditions, with STg+Veh at 19.4% and STg+N20 at 14%, while a larger proportion of myeloid cells were instead attributed to DTg+Veh, at 28.9%, and DTg+N20, at 37.7% (Figure 4D).

Although T cells are important in viral control and overall progression of disease in JHMV, myeloid cells play a central role in pathogenesis as they also contribute to virus control, facilitate CNS immune cell infiltration and contribute to development of demyelination (1). Neutrophils belong to the myeloid lineage and are responsible for facilitating the entry of monocytes, and thereby monocyte-derived-macrophages (MDM's), past the BBB and into CNS parenchymal space (1). Both, MDM's and microglia, are abundantly present within regions of parenchymal demyelination in JHMV pathology and are thought to contribute to demyelination through

Figure 4: CosMx spatial transcriptomic analysis reveals that DTg mice have increased presence of myeloid cells and DTg mice treated with N20 have increased presence of neutrophils and disease associated myeloid cells which downregulate inflammatory transcripts and upregulate anti-inflammatory signatures. (A) Schematic illustrating the CosMx platform workflow. Processed tissue is stained with the CosMx proprietary staining protocol and 1000-gene Neuroscience Panel multiplexed with the Lane lab custom 50-gene immune panel (Table 2). This is followed with cell segmentation conducted by the CosMx imager which uses DNA, GFAP, and histone staining as cell-identification guides. Transcript counts per cell and cell typing based off the transcriptional readout per cell are then analyzed and aggregated to perform bioinformatic analyses. (B) (Right) List of 40 cell subtypes, identified via unbiased clustering, present in the spinal cord tissue processed for spatial transcriptomic analysis. (Left) Localization of the 40 identified cell types in XY space mapped onto the tissue; 1 representative mouse sample shown here per condition. In total, 32 coronal spinal cord cut tissues were analyzed, with 4 mice per condition and 4 samples per mouse (with the exception of STg+N20 which had 3 mice). (C) Uniform manifold approximation and projection plot illustrating the 40 identified subclusters and their location in relation to each other. (D) Stacked bar graph showing the breakdown of cell proportions by broad cluster by group condition, allowing comparison of how specific cell populations expand or contract relative to their abundance in other conditions. There are 12 broad categories that each of the 40 cell subtypes can be sorted into. (E) Stacked bar graph showing the breakdown of cell proportions by subtype cluster by group condition, for 40 subclusters overall. (F) Differential gene expression (DGE) analysis volcano plots of broad cluster cell types depicting upregulated and downregulated genes for DTg+Veh spinal cords as compared to STg+Veh. (G) DGE volcano plots of broad cluster cell types depicting upregulated and downregulated genes for DTg+N20 spinal cords as compared to DTg+Veh.

localized secretion of toxic mediators (e.g., iNOS, TNF- α) as well as via myelin phagocytosis (37–40).

In order to further probe myeloid dynamics within these DTg mice we looked at the proportion of cells within the cell subtypes of the myeloid cluster. When parsed out by subtype, cells associated with the myeloid cluster consistently showed a higher proportion of cells as belonging to the DTg condition when compared to STg, with the exception of homeostatic



microglia (designated as only “Microglia”) which showed a more even proportional distribution between conditions (Figure 4D). Interestingly, as probes for the JHNV membrane, nucleus and spike (Table 2) were included in the custom panel, CosMx analysis identified cells whose highest upregulated transcripts were JHNV-associated transcripts and grouped these cells as clusters. One of these clusters demonstrated JHNV-associated transcripts as the first and/or second highest transcripts expressed in these cells and were then followed up by characteristic myeloid gene transcripts, as such this cluster was labeled myeloid_virus-associated. The rest of the myeloid cell subtypes included a neutrophil, DAM, and antigen presenting cell (APC) subcluster. Each of these subclusters comprised their makeup of primarily DTg cells, with ~30-40% coming from DTg+Veh cells and ~40-50% from DTg+N20 cells (Figure 4E). Notably, the virus-associated and APC clusters had a more even distribution between the two conditions, with the virus-associated cluster being comprised of ~40% in the case of each DTg condition and the APC cluster of ~30% for each of the DTg conditions, while the neutrophil and DAM subclusters demonstrated an increased disparity between conditions in which DTg+N20 demonstrated ~20% increased presence as compared to DTg+Veh (Figure 4E). This increase in neutrophils and DAM’s in the DTg+N20 condition as compared to DTg controls was surprising as we had hypothesized that DTg treatment with N20 would decrease chronic neutrophil activation, thereby leading to decreased inflammation and recruitment of neutrophils and macrophages.

For this reason we conducted differential gene expression (DGE) analysis on all of the broad clusters, including the myeloid cluster. This yielded a downregulation of transcripts associated with inflammation, like *B2m*, *CCL2*, *CXCL9*, *Srgn*, *Samhd1*, and *Ptpnc* and an upregulation of transcripts characteristic of a classic DAM signature, including genes like *Apoe*,

Gpnmb, *Ctsd*, *Spp1*, *Trem2*, and *Lpl*, which have previously been associated with anti-inflammatory and tissue protective programs (Figures 5A-B).

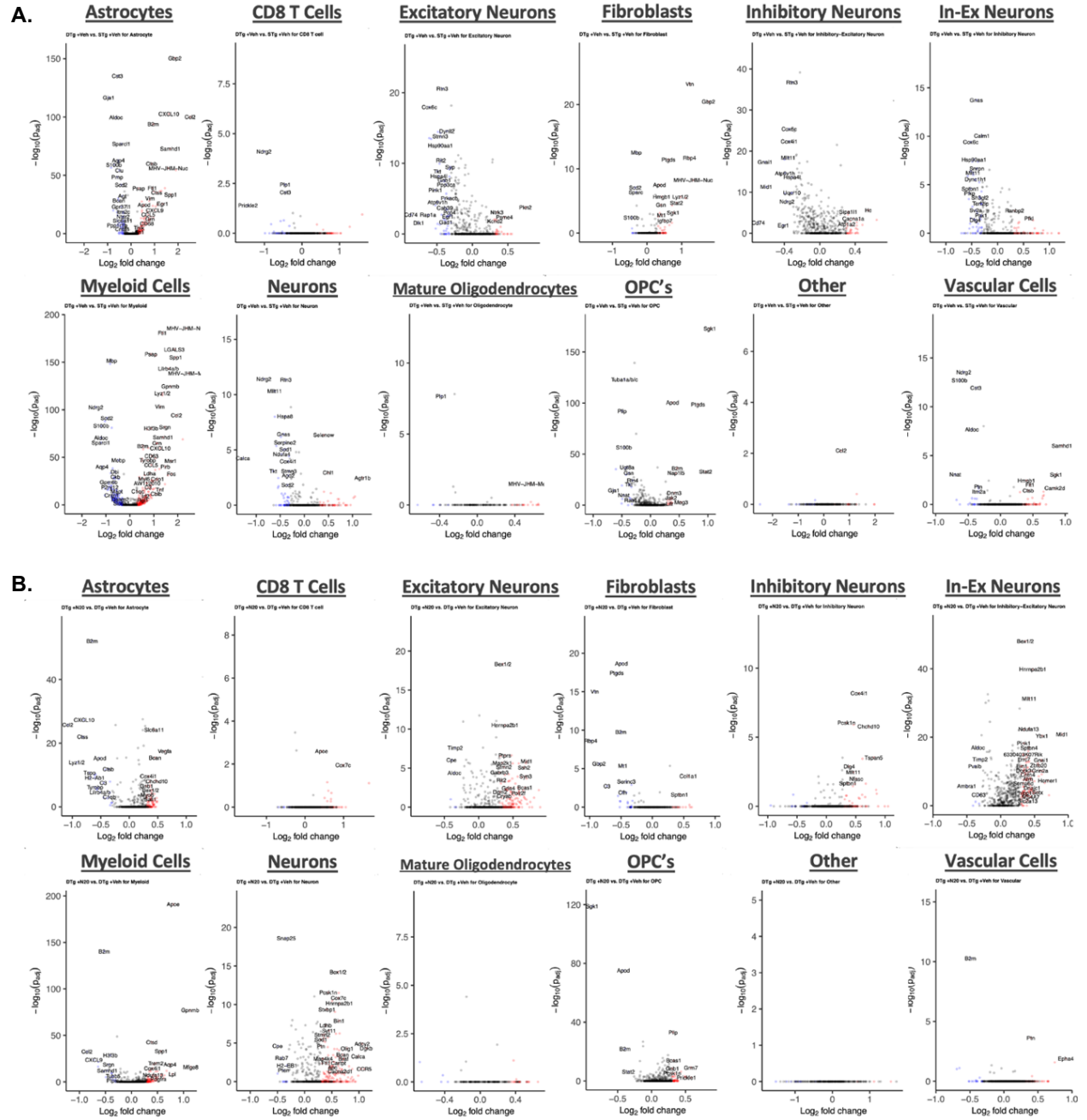


Figure 5: DGE analysis of spatial transcriptomic imaging. (A) Differential gene expression (DGE) analysis volcano plots of broad cluster cell types depicting upregulated and downregulated genes for DTg+Veh spinal cords as compared to STg+Veh. **(B)** DGE volcano plots of broad cluster cell types depicting upregulated and downregulated genes for DTg+N20 spinal cords as compared to DTg+Veh.

DISCUSSION

Various reports indicate neutrophils are dynamically involved in the development of MS, in both relapsing-remitting MS (RRMS) as well as in primary-progressive MS (PPMS). Neutrophils and neutrophil chemokines are increased in the circulation and cerebrospinal fluid of RRMS patients during relapses and neutrophil counts are found in increased numbers in the choroid plexus of PPMS patients (11,12,41–43). The paucity of these cells in studies of postmortem MS lesions coupled with their brief lifespan has deterred investigation of their potential role in disease (44). Previous research in our lab has indicated that tetracycline-inducible continuous expression of CXCL1, a neutrophil chemokine, within the CNS of JHMV infected mice elicits sustained infiltration of neutrophils that correlates with increased demyelinating pathology (28,30). Here, transcriptional analysis, flow cytometry, pharmacological inhibition, and spatial transcriptomics were used to determine mechanisms via which neutrophil-potentiated demyelination may be enacted in this model.

Previous scRNAseq analysis conducted in this model yielded transcriptional differences between DTg and STg neutrophils that prompted further analysis of immune activation pathways. Differential upregulation of transcripts such as ASPRV1, CD63, MMP9, S100a8, and S100a9 signaled potential activation of pathways related to neutrophil EFs in DTg mice as compared to controls. GSEA assessment of neutrophil transcriptional profiles between DTg and STg mice yielded a role for neutrophil degranulation pathways in the development of DTg pathology, as well as overall upregulation of pathways related to neutrophil activation of immune response and neutrophil-mediated cytotoxicity, both of which entail substantive transcript overlap with neutrophil degranulation pathways (45,46). Many of these pathways have been similarly reflected in clinical studies of neutrophils from MS patients, where they have been shown to also upregulate degranulation pathways as well as intracellular immune signaling cascades, antigen presentation,

and overall neutrophil cytotoxicity (47). Interestingly, neutrophil degranulation pathways have also been reported to be upregulated in clinical patients of both, cases of autoimmune encephalitis and cases of infectious encephalitis, providing evidence for the active role neutrophils can play in eliciting CNS immune activation and tissue damage (48).

Evaluation of surface markers via flow cytometry for degranulation markers CD63 and CD11b, in DTg mice as compared to controls, confirmed an increased number of spinal cord degranulating neutrophils and further supported a role for active degranulation in the spinal cords of DTg mice. Neutrophil primary granules store the cell's most potent antimicrobial and cytotoxic molecules, containing enzymes like neutrophil elastase, cathepsin G, and MPO, which has the capability of potentiating oxidative molecules like ROS (49,50). Dysregulated exocytosis of these granules can in turn result in local tissue damage as well as in amplification of inflammation (24,51). Small molecule degranulation inhibitor N20 was used here to provide functional insight into the contribution of neutrophil granule exocytosis to white matter injury. N20 targets the Rab27a–JFC1 interaction required for docking and releasing primary granules and a subset of JFC1-associated tertiary granules, each containing the surface markers CD63 and CD11b, respectively (33). Consistent with its mechanism of action, N20 treatment reduced clinical disease severity and spinal cord demyelination without substantially altering neutrophil infiltration, overall neutrophil numbers, or surface marker expression of either CD63 or CD11b. These results potentially suggest that once neutrophils have trafficked into the CNS, limiting their ability to degranulate—rather than preventing their recruitment—is sufficient to attenuate tissue damage. Importantly, N20 did not broadly suppress neutrophil function in the periphery, as circulating MMP-9 and MPO levels were comparable across groups. This supports a model in which the primary effects of N20 occur at the level of granule exocytosis within inflamed tissue rather than

through systemic immunosuppression. Simultaneously, it is important to underscore that the pharmacodynamics of N20 remain uncharacterized. While mass spectrometric analysis confirmed the presence of N20 in both the CNS and periphery, the measured concentrations of the drug hovered near the limit of detection. As tissues were collected ~24h after the last N20 injection, and neutrophils have an approximate 24h lifespan, it is possible that N20 concentrations at the time of sacrifice were suboptimal, and therefore reestablished physiological neutrophil degranulation levels may have impacted neutrophil signature at the time of collection. Consequently, it may be that expression of degranulation markers measured at the time of collection may not be representative of neutrophil activation during the period of N20 treatment.

Since no immediate reduction in neutrophil infiltration, degranulation markers, or granule content release was yielded by these analyses, we hypothesized that mechanistic insight on N20 reduction of DTg pathology may instead be reflected in transcriptional reprogramming of infiltrating immune cells rather than in changes in their abundance or frequency. To analyze changes between conditions we utilized the CosMx spatial transcriptomics platform which allowed us to visualize infiltrating neutrophils in situ and accompanying infiltrating immune cells such as macrophages and T cells, as well as resident cells like reactive astrocytes, disease associated microglia and infected oligodendrocytes within the context of demyelinating lesions. Analysis of cell proportion per cluster by condition determined that the myeloid cluster demonstrated the greatest amount of differential proportions between DTg and STg conditions, where the cluster was comprised by ~30% from myeloid cells in the DTg+Veh condition and ~40% from myeloid cells in the DTg+N20 condition, for a total of about 70% of myeloid cells derived from DTg mice. This difference in proportional makeup suggested, as expected, a potential role for myeloid cells in mediating DTg pathology. A closer look at subclusters within the myeloid category identified

virus-associated myeloid cells, neutrophils, DAM's, and APC's as the main cell types comprising this cluster. Of these, the neutrophil and DAM subclusters showed the largest disparity in proportionality between the DTg+Veh and DTg+N20 conditions, highlighting these cells as potential players in the N20-mediated amelioration of pathology seen in DTg+N20 mice. Surprisingly, both neutrophils and DAM's displayed about a 20% increase in presence, rather than decrease, in the spinal cords of DTg+N20 mice. This was unexpected, as we had hypothesized that N20 treatment would reduce neutrophil degranulation, consequently dampening the inflammatory cascade and lowering the chemotactic recruitment of neutrophils and macrophages. Interestingly, differential gene expression (DGE) analysis of the broad myeloid cluster in DTg+N20 mice as compared to DTg+Veh controls demonstrated overall downregulation of inflammatory genes like *B2m*, *CCL2*, *CXCL9*, *Srgn*, *Samhd1*, and *Ptprc* while upregulating DAM genes such as *Apoe*, *Gpnmb*, *Ctsd*, *Spp1*, *Trem2*, *Lpl*, and *Mfge8*, which have all been associated with context-dependent anti-inflammatory and tissue-protecting roles. With downregulation of *CCL2* and *CXCL9* leading to decreased monocyte, macrophage, and T cell recruitment, decreases in *B2M* transcription, an MHC I component, eliciting less immune infiltration, and downregulation of *Ptprc* (CD45) resulting in overall decreased immune activation, this would all seem to point to a general decrease in stimulation of the inflammatory milieu. Additionally, the downregulation of *Srgn* is particularly notable as it is one of the proteoglycans responsible for stabilizing proteases like neutrophil elastase within neutrophil tertiary granules, and its production is associated with active degranulation (52,53).

Overall, these transcriptional changes signal to a decrease in pro-inflammatory signaling and suggest a shift towards an anti-inflammatory and potentially tissue-restoring environment. In further support of this is the simultaneous coordinated upregulation of the DAM signature, which

is defined as upregulation of a core set of genes, including *Apoe*, *Gpnmb*, *Ctsd*, *Spp1*, *Trem2*, and *Lpl*, associated with anti-inflammatory activity in certain contexts.

Importantly, the increased DAM presence in DTg+N20 spinal cords, rather than being a paradoxical finding, is consistent with a growing body of literature establishing DAMs as a protective, phagocytic myeloid state. Originally defined in Alzheimer's disease models by Keren-Shaul et al. (2017), the DAM signature—characterized by coordinated upregulation of *Trem2*, *Apoe*, *Lpl*, *Ctsd*, *Spp1*, and *Gpnmb*—has been associated with enhanced phagocytic clearance of pathological debris and neuroprotection across multiple neurological contexts (54,55). *Trem2*, *Apoe*, and *Lpl* are central regulators of lipid processing in microglia and macrophages, are critical for the clearance of myelin debris, and have been associated with critical tissue repair, reduction of pro-inflammatory cytokines, and remyelination processes post-injury (56–59). Efficient lipid handling is particularly important in demyelinating contexts, since the accumulation of cholesterol-rich debris can stimulate inflammation and exacerbate tissue damage (60). Upregulation of *Ctsd*, a lysosomal protease, further suggests enhanced lysosomal degradation capacity, consistent with active myelin phagocytosis (61,62). Additionally, *Gpnmb* and *Spp1* have been linked to reparative or remodeling myeloid states active during tissue post-injury recovery and remyelination (63,64). Thus, treatment of DTg mice with N20 seems to promote an overall decrease in stimulation of inflammation which in turn allows for an overall shift towards activation of anti-inflammatory pathways and initiation of tissue repair programs.

Taken together, these data support a potential key role for neutrophil degranulation in amplifying white matter injury in a model of sustained neutrophil infiltration during chronic JHNV-induced demyelination. Rather than serving solely as transient innate responders, under chronic stimulation, as in RRMS and PMS conditions, neutrophils can accumulate in increased

numbers and maintain a chronic transcriptional program that favors granule mobilization and release, thereby creating a microenvironment conducive to ongoing tissue damage. Pharmacologic inhibition of granule exocytosis with N20 disrupts these processes and significantly reduces disease burden in this pre-clinical model of chronic neutrophil activation, highlighting neutrophil degranulation as a modifiable contributor to CNS pathology in disorders like MS. The current findings provide strong evidence that targeting neutrophil granule exocytosis represents a promising approach to limiting immune-mediated demyelination while preserving core antimicrobial functions.

METHODS & MATERIALS

Mice and viral infection: pBI-CXCL1-rtTA double transgenic mice (C57BL/6, H-2^b background) were generated and bred as previously described [17-18]. Doxycycline (50 mg/kg) administration via daily intraperitoneal (i.p.) injection for a period of 12 days to either DTg or STg mice results in increased CXCL1 production within the CNS of DTg, but not STg, mice as measured by RT-PCR, enzyme-linked immunosorbent assay (ELISA), immunohistochemical staining, and neutrophil accumulation in the CNS through flow cytometry and immunohistochemical staining (28). DTg and STg mice (male and female, 6-10 weeks of age) were injected intracranially (i.c.) in the right brain hemisphere with 2300 plaque-forming units (PFU) of JHMV strain suspended in 30μL of sterile Hanks balanced sterile solution (HBSS), and animals were euthanized at defined times post-infection (p.i.). To determine viral titers within brains, experimental animals were sacrificed at day 14 p.i. and transcardially perfused with 20mL of HBSS, and brains were isolated, homogenized, and plaque assays were performed on DBT astrocytoma cell line as described previously (28). As a control, uninfected STg and DTg mice were treated with Doxycycline (50mg/kg) via i.p. injection for 12 days and animals were sacrificed two days later to assess neutrophil accumulation within the spinal cords by flow analysis and the presence of demyelination via Luxol Fast Blue (LFB) and Hematoxylin/Eosin staining. Clinical disease severity was assessed using a 4-point scoring scale as previously described (28). All animal studies were reviewed and approved by both the University of California, Irvine -Institutional Animal Care and Use Committee (IACUC).

Nexinhib20 Compounding and Administration: Nexinhib20 (N20) was acquired from Dr. Sergio Catz (Scripps Research Institute) at a concentration of 100mM and diluted in 100% dimethyl sulfoxide (DMSO). Subsequently, N20 was diluted 1:40 in 1x PBS at standard room

temperature each day of N20 administration. 200ml (15mg/kg) of diluted N20 were administered i.p. per mouse contralaterally to the site of daily doxycycline administration. On the days of N20 administration 4-6hrs were allowed to pass after doxycycline administration prior to administration of N20. Vehicle consisted of 100% DMSO dissolved in 1X PBS at a concentration equivalent to treatment of 2.5% DMSO.

Cell isolation and flow cytometry: Flow cytometry was performed to identify inflammatory leukocytes infiltrating into the CNS using established protocols (28,30). Single cell suspensions were generated from isolated tissue samples using Miltenyi Neural Tissue Dissociation Kit(P) (Cat#130-092-628) and Miltenyi gentleMACS OctoDissociator with Heaters. Isolated cells were incubated with anti-CD16/32 Fc block (BD Biosciences, San Jose, CA) at a 1:200 dilution. Subsequently, cells were stained with fluorescently tagged anti-mouse IgG antibodies for the following cell surface markers: CD63, CD45, Ly6g, Ly6c, and CD11b. A detailed list of antibodies used is provided in Table 1. Samples were analyzed using either an Agilent Technologies NovoCyte2060R or an Amnis ImageStream Mark II Imaging Flow Cytometer (Millipore-Sigma) and analyzed with FlowJo (Tree Star Inc.).

Histology (Regular & CosMx): Experimental mice were euthanized according to IACUC guidelines at defined timepoints p.i. and transcardially perfused with 20mL of HBSS. Spinal columns were removed, fixed overnight in 4% paraformaldehyde at 4°C, effaced to excise spinal cords after approximately 24h of fixation, and cryoprotected by storage in 30% sucrose in 1x PBS for a minimum of 4 days. Spinal cords were cut coronally using a straight blade and separated into eight 1.5mm sections beginning at the first thoracic vertebrae. Sections were then embedded in O.C.T. (VWR, Radnor, PA, USA). 10 or 12 µM thick coronal sections were cut via cryostat and either processed for CosMx staining, immunohistochemical staining, or staining with Luxol fast

blue (LFB) and hematoxylin/eosin (H&E). Between 4-8 sections per mouse comprised an n=1 for each type of analysis. In brief, for scoring and calculation of spinal cord demyelination: areas of total white matter versus areas of demyelinated white matter were determined using Image J Software area analysis and demyelination was then calculated as a percentage of total demyelination from spinal cord sections analyzed (30).

CosMx Analysis: CosMx analysis was performed on tissues acquired and processed as described in the Histology section. Tissues were sliced onto Leica BOND Plus Slides (Cat#S21.2113). Slides were then processed and stained as according to the CosMx Fixed Frozen RNA Assay Protocol using a 2-slide kit with the CosMx Mouse Neuroscience 1K RNA Panel with Custom Immune Marker Panel (curated by the Lane Lab; custom gene list available on Table 2). Staining was followed by slide imaging using the CosMx Spatial Molecular Imager, with imaging protocol lasting approximately ~4.5 days. The resulting spatial transcriptomics data was then acquired via AtoMx Control Center and analyzed using R packages.

Single cell RNA sequencing (scRNASeq): 10x scRNASeq was performed as previously described (30). Immune cells were isolated as described above from the spinal cord (4 mice pooled per condition) at day 12 p.i and stained with DAPI and APC conjugated anti-CD45 for 20 minutes on ice in 1X PBS containing 0.5% bovine serum albumin (BSA). Live CD45⁺ cells were enriched using a BD FACSAria flow sorter and washed once with 0.04% BSA. Samples were then processed for single cell RNA sequencing via the 10X Genomics platform performed at the Huntsman Cancer Institute High Throughput Genomics Shared Resource Core Facility at the University of Utah Health Science Center. RNA sequencing was performed via Illumina NovaSeq 6000 next generation sequencer. Sequencing data was processed using the 10X Genomics CellRanger pipeline and analyzed using the Seurat R package. Gene expression signatures defining

cell clusters were analyzed from double-tg and single-tg controls at day 12 p.i in spinal cord. Cells from each aggregated sample dataset were clustered into corresponding immune cell populations by a shared nearest neighbor modularity optimization-based clustering algorithm using the Seurat package. The resulting clusters were defined using an immune-cell scoring algorithm that compares the gene signatures of each cluster in the experimental dataset with the microarray data available in the Immunological Genome Project Database (30). Expression levels and distribution of population-specific immune cell markers were then analyzed to further refine the identified clusters and expose any subpopulations that should be separated as independent clusters. Once the clusters were established and identified, plots were generated using Seurat, ggpubr and fgsea R packages.

Mass Spectrometry: Experimental animals were sacrificed at 14DPI, with blood collected for analysis via intracardiac puncture and mice subsequently transcardially perfused with 20mL of HBSS. Brains were harvested, divided into left and right hemispheres, frozen fresh on dry ice and stored at -80°C for eventual mass spectrometry analysis. Blood samples were collected in heparinized tubes, stored on ice for approximately 1 hour and centrifuged at 4,000g for 15min at 4°C to separate plasma which was then stored at -80°C for further processing. For plasma sample preparation plasma samples (~300ul per sample) was evaporated to dryness at 10°C in Speedvac and brain samples were dried down overnight in temperature controlled Speedvac at 10 °C. Samples were resuspended in 50:50 MeOH:H₂O with a final volume of 100ul, sonicated for 10 minutes, centrifuged at 16000g for 10 minutes at 4°C to pellet insolubles, then transferred to an autosampler vial for analysis. The samples were run on an Agilent Ultivo Triple quadrupole Mass Spectrometer. The column used was a Waters XSelect CSH 2.5um 2.1 x 150mm C18. The mobile phase was composed of A= Water/0.1% formic acid, B=ACN/0.1% formic acid. The flowrate was

set to 350 μ L/min and the sample injection volume was 5 μ L. The gradient was T0 = 95/5; T1 = 95/5; T4 5/95; T7 = off. There was a 3min re-equilibration step. Operating in positive ion mode, the source conditions were as follows: Dry gas temp = 230; Dry Gas flow = 9L/min; Sheath Gas temp = 200; Sheath gas flow = 12L/min; Neb pressure = 30psi; Capillary voltage = 3500V; and Nozzle voltage = 2000V. Data was processed using Agilent Quantitative Analysis software. Calibration standards were prepared by dilution of stocks. The internal standard was added at 100nM to each calibration standard.

ELISA: To determine tissue protein level, tissue was homogenized in RIPA buffer, centrifuged, and supernatant aliquoted. Protein assays were run using MMP-9, MPO DuoSet sandwich ELISA kit (R&D Systems, Minneapolis, MN) following manufacturer specifications

Statistical analysis: For flow cytometry analysis unpaired Student's t test, one-way ANOVA, or two-way ANOVA was used to determine significance and a p value of < 0.05 was considered statistically significant. GraphPad Prism was used to perform statistical analyses. Data for each experiment is presented as mean+standard error of mean (SEM). Wilcoxon test was used for analyzing gene expression in scRNASeq clusters and the resulting p values were corrected for multiple comparisons by Holm-Sidak method and a p value of < 0.05 was considered statistically significant.

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SUPPLEMENTARY MATERIALS

Table 1. Antibodies used throughout flow cytometric experiments.

Table 2. CosMx custom Lane lab 50-gene immune-based panel.

Table 3. Mass spectrometric analysis of N20 concentration in blood plasma samples

Table 4. Mass spectrometric analysis of N20 concentration in brain tissue samples

Table 1

Flow Antibody	Clone	Cat #	Company
CD63	NVG-2	143904	BioLegend
CD62L	MEL-14	104435	BioLegend
CD45	30-F110	17-0451-82	eBioscience/ThermoFisher
CD8a	RPA-T8	301049	BioLegend
LY6G	1A8	551460	BD Biosciences
CXCR4	2B11	565019	BD Biosciences
CD11b	M1/70	101212	BioLegend
CD4	RM4-5	17-0042-82	Invitrogen

Table 2

	Gene		Gene
1	ASPRV1	26	FOXP3
2	AW112010	27	GATA3
3	CCL25	28	GZMB
4	CCL3	29	HP
5	CCL5	30	IFITM1
6	CCR2	31	IL2RA(CD25)
7	CCR5	32	IL4
8	CD163	33	ISG20
9	CD19	34	ITGB2
10	CD4	35	LCN2
11	CD62L	36	LGALS3
12	CD63	37	MHV-JHM-Mem
13	CD68	38	MHV-JHM-Nuc
14	CD69	39	MHV-JHM-Spike
15	CD79A	40	MS4A6D
16	CD8A	41	MS4A7
17	CHIL3	42	PDCD1
18	CXCL1	43	PRF1
19	CXCL10	44	RETNLG
20	CXCL9	45	S100a8
21	CXCR2	46	S100a9
22	CXCR3	47	SARSCoV2-N
23	CXCR4	48	SARSCoV2-S
24	CXCR5	49	SLAMF7
25	F13A1	50	TCF7

Table 3

Sample	Final Volume	Final Concentration
3	100ul	~1nM
5	100ul	~1nM
8	100ul	~1nM
14	100ul	~1nM

Table 4

Sample	Tissue weight	Final Volume	Final Concentration
1	17 mg	100 uL	<1nM
3	14 mg	100 uL	<1nM
5	15 mg	100 uL	<1nM
6	15 mg	100 uL	<1nM
8	14 mg	100 uL	<1nM
10	15 mg	100 uL	<1nM
14	14 mg	100 uL	<1nM

CHAPTER 4

INFECTION OF 5XFAD MICE WITH A MOUSE-ADAPTED SARS-COV-2 DOES NOT ALTER ALZHEIMER'S DISEASE NEUROPATHOLOGY YET INDUCES WIDE-SPREAD CHANGES IN GENE EXPRESSION ACROSS DIVERSE CELL TYPES

Alzheimer's & Dementia, 2026

Infection of 5xFAD mice with a mouse-adapted SARS-CoV-2 does not alter Alzheimer's disease neuropathology yet induces wide-spread changes in gene expression across diverse cell types

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ABSTRACT

Introduction: The COVID-19 pandemic underscored the impact of SARS-CoV-2 infection on worsening the severity of Alzheimer's disease (AD) neuropathology and disease progression.

Methods: Aged 5xFAD and wildtype (WT) mice were infected with a mouse-adapted SARS-CoV-2 (MA10) and extensive characterization of molecular and cellular changes within the brains was performed.

Results: MA10 infection induced acute viral pneumonia. Viral RNA was undetectable within the brains of infected mice and there was no evidence of glial activation or neuroinflammation. MA10 infection did not affect Ab plaque volume or numbers in 5xFAD mice compared to uninfected mice. Spatial transcriptomics revealed altered expression of genes associated with homeostatic function in neurons, glia, and vascular endothelial cells.

Discussion: Collectively, these findings demonstrate that while MA10 infection did not affect AD neuropathology yet there were numerous down-stream effects on gene expression associated with resident CNS cell functions that may impact neurologic disease.

Keywords: *Alzheimer's disease neuropathology, mouse-adapted SARS-CoV-2, neuroinflammation, viral infection, spatial transcriptomics.*

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of age-related dementia, impairing memory and cognitive function. Pathology includes the accumulation of amyloid plaques and neurofibrillary tangles, accompanied with neuronal loss and neuroinflammation.¹ AD is a multifactorial disorder influenced by several risk factors including age², genetics³, and environmental factors⁴. Some studies suggest viral infections may accelerate disease onset or exacerbate existing pathology and clinical symptoms associated with neurodegenerative diseases, including AD⁵ and other studies have implicated microbial pathogens in AD pathogenesis^{6,7}, highlighting the potential contribution of infection to AD and related dementias.

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and rapidly developed into a global pandemic in 2020, affecting hundreds of millions of people worldwide. Infection presents with mild-to-moderate respiratory symptoms⁸, however severe disease can result in acute respiratory distress syndrome (ARDS) and respiratory failure⁹. Beyond respiratory problems, neurological symptoms and complications have been reported including headache, dizziness, and olfactory and gustatory dysfunction¹⁰. Severe COVID-19 has also been associated with ischemic stroke, cerebrovascular disease, and seizures¹¹.

Risk factors contributing to COVID-19 severity and mortality include advanced age¹² and pre-existing dementia¹³, and among patients with dementia, AD was the most prevalent cognitive impairment in those who died of COVID-19¹⁴. Additionally, COVID-19 appears to impact brain structure and function, and some studies suggest that COVID-19 may induce or accelerate neurodegenerative processes, including those related to dementia and AD¹⁵. Furthermore, coronaviruses such as SARS-CoV-1¹⁶ and more recently SARS-CoV-2¹⁷ possess proteins with

amyloidogenic properties that may promote amyloid aggregation. In addition, infection may alter resident CNS cell function that can also contribute to alterations in neurologic dysfunction.

Neuroinflammation is a hallmark of many neurodegenerative diseases and is influenced by reactive microglia and astrocytes that lose their homeostatic functions, resulting in the release of proinflammatory cytokines that disrupt the blood–brain barrier (BBB), impair synaptic function, and promote neuronal death¹⁸. In the context of COVID-19, glial cells and neurons have been identified as targets of SARS-CoV-2 infection in patients and animal models^{19,20}, although the virus is considered to have limited neuroinvasive capacity. Post-mortem analyses of COVID-19 patient brains have reported gliosis, axonal damage, and BBB disruption^{21,22}. Similar findings have been observed in animal models, where infection with SARS-CoV-2 or the mouse-adapted SARS-CoV-2 MA10 strain leads to BBB disruption, gliosis, and the development of neuronal proteinopathies, even in the absence of direct CNS infection^{23,24}. Cases where direct viral infiltration in the brain was observed also noted similar results along with increased expression of AD risk genes²⁵. Together, these findings suggest that COVID-19–associated neuroinflammation may contribute to hippocampal injury and neuronal and synaptic dysfunction.

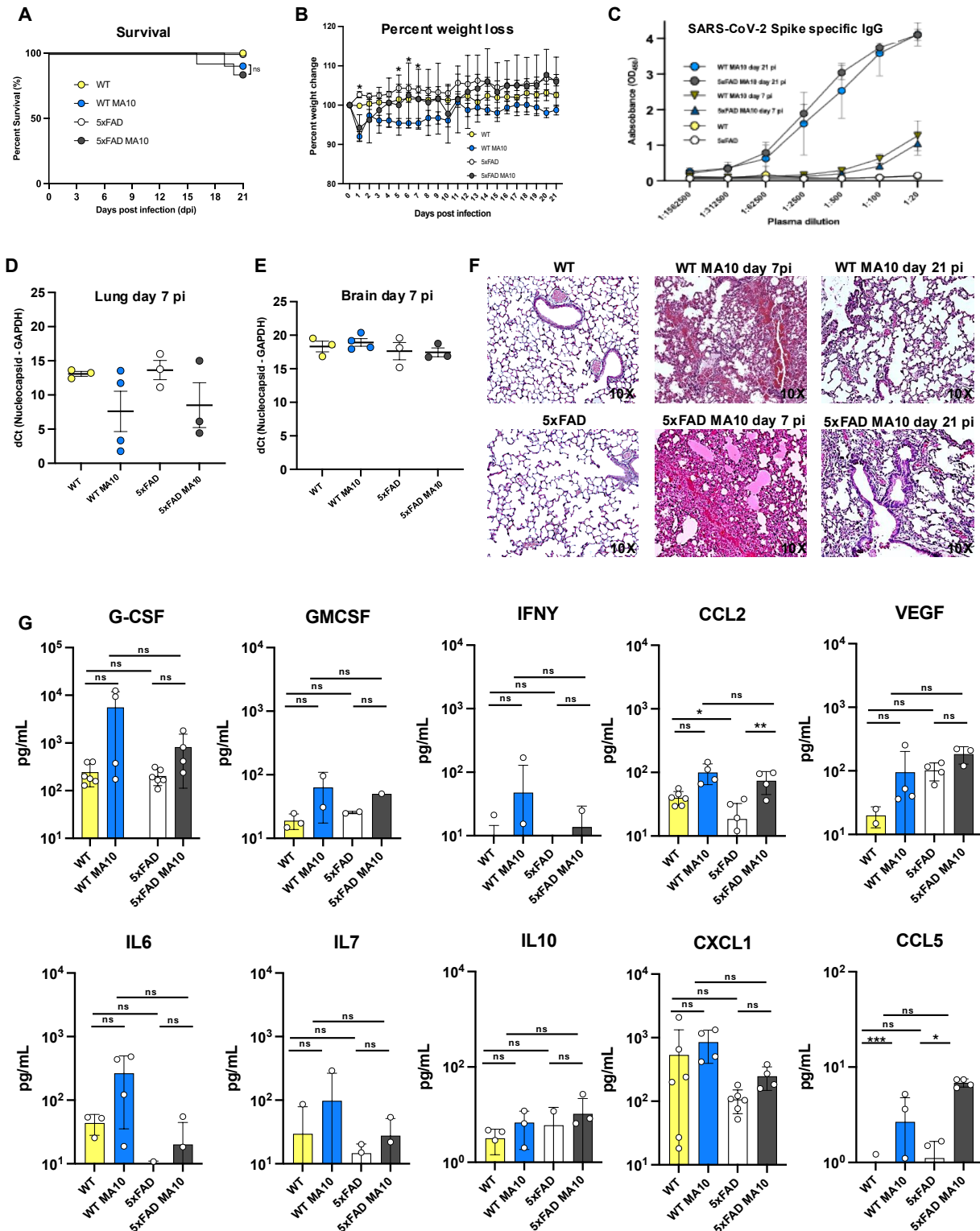
In this study, we infected aged C57BL/6 wild-type (WT) mice and 5xFAD mice with mouse-adapted SARS-CoV-2 (SARS-CoV-2 MA10)²⁶ to evaluate infection-associated neurological changes and determine whether SARS-CoV-2 infection influences AD-related neuropathology. The 5xFAD model is a widely used transgenic model of AD that overexpresses mutant human APP and PS1, resulting in early and progressive amyloid plaque deposition, neuroinflammation, synaptic dysfunction, and cognitive decline²⁷. Utilizing bulk RNA sequencing and spatial transcriptomic analyses, we examined infection-induced changes in neuronal, glial, and vascular pathways to better understand how SARS-CoV-2 infection may impact brain function in

aging and AD-relevant contexts and found that despite the absence of CNS infection alterations in neuronal, glial, and vascular pathways occurred , while amyloid beta ($A\beta$) plaque burden in 5xFAD mice remained unchanged.

RESULTS

MA10 infection of WT and 5xFAD mice. C57BL/6 (WT) and 5xFAD mice (10-14 months of age) were intranasally (i.n.) infected with 5×10^3 pfu of MA10. Animals were sacrificed at days 7 and 21 (p.i.) and lungs and brains harvested to assess tissue pathology, presence of viral RNA, and effects on AD-associated neuropathology. By 4 months of age, 5xFAD mice develop A β plaques in the brain which increase in number and size over time, plateauing between 10-12 months of age.^{27,29} By day 21 p.i., there was no significant difference in mortality, with ~20% of infected 5xFAD mice and ~15% of infected WT mice dying in response to infection (**Figure 1A**). Daily weighing of mice showed MA10 infected WT mice exhibited significant weight loss compared to their uninfected controls only at 1 and 5-7 p.i., while MA10 infected 5xFAD mice did not demonstrate significant weight loss in comparison to their uninfected controls (**Figure 1B**). Spike-specific IgG antibodies were detected at days 7 and 21 p.i. in both WT and 5xFAD mice confirming infection with MA10 compared to SHAM-infected mice (**Figure 1C**). Viral RNA was examined via qPCR in lungs and brains of infected mice at 7 days p.i. Low levels of viral RNA

FIGURE 1. MA10 infection elicits mild disease in infected mice, and genotype does not affect mortality. (A) Percent survival of uninfected WT and 5xFAD compared to MA10 infected WT and C57BL/6 mice. (B) Average body weight changes each day p.i normalized to the body weight on the day of infection. “#” indicating significance ($p < 0.05$) between MA10 infected WT and uninfected WT. (C) Neutralizing IgG anti-spike antibodies were present in the plasma of MA10-infected mice at days 7 and 21 p.i. as assessed by ELISA. RT-qPCR was performed on (D) lung and (E) brain homogenates at day 7 p.i. to quantify MA10 nucleocapsid (N) gene expression. The data are presented as dCt values, calculated by subtracting the Ct of the housekeeping gene GAPDH from the Ct of the viral N gene. Lower dCt values indicate higher viral RNA abundance. Each dot represents an individual mouse; bars represent the mean $\pm$ SEM. (F) Representative H&E-stained lung images at 10 $\times$ magnification from uninfected and MA10-infected WT and 5xFAD mice at both 7 and 21 days p.i., are shown. Pathological features associated with infection included airway edema, vascular congestion, and intra-alveolar hemorrhage, peri-bronchiolar lymphocytic cuffing, and interstitial vascular congestion and lymphocytic infiltrates. (G) Cytokine concentrations in plasma were measured using a multiplex bead-based immunoassay on the Luminex platform. Data for individual cytokines are shown with each dot representing an individual mouse and bars indicating the mean $\pm$ SEM. * $p < 0.05$ between MA10 infected WT and uninfected WT.



were present at day 7 p.i. within the lungs (**Figure 1D**). No viral RNA was detected in the brains at 7 days p.i. (**Figure 1E**) consistent with previous reports indicating MA10 does not readily infect

the CNS.²³ There were no significant differences between uninfected and infected cohorts, and any Ct values yielded from infected cohorts in qPCR were assumed to be background, as they were like those of uninfected/control mice. Hematoxylin and eosin (H&E) staining of lungs of infected WT and 5xFAD mice at day 7 p.i. demonstrated both alveolar and interstitial lesions, with alveolar hemorrhage and edema, interstitial congestion, and lymphocytic infiltrates within the lungs and by day 21 p.i. there was still evidence of inflammation in both MA10-infected WT and 5xFAD mice although this was reduced in comparison to day 7 p.i. (**Figure 1F**). Examination of proinflammatory cytokines and chemokines within the blood of MA10-infected WT and 5xFAD mice at day 7 p.i. showed a trend toward increased levels of proinflammatory cytokines in infected mice compared to their controls, however, the differences did not reach statistical significance for all markers. MA10 infection did result in a significant increase in CCL2 and CCL5 in both WT and 5xFAD mice compared to uninfected controls (**Figure 1G**).

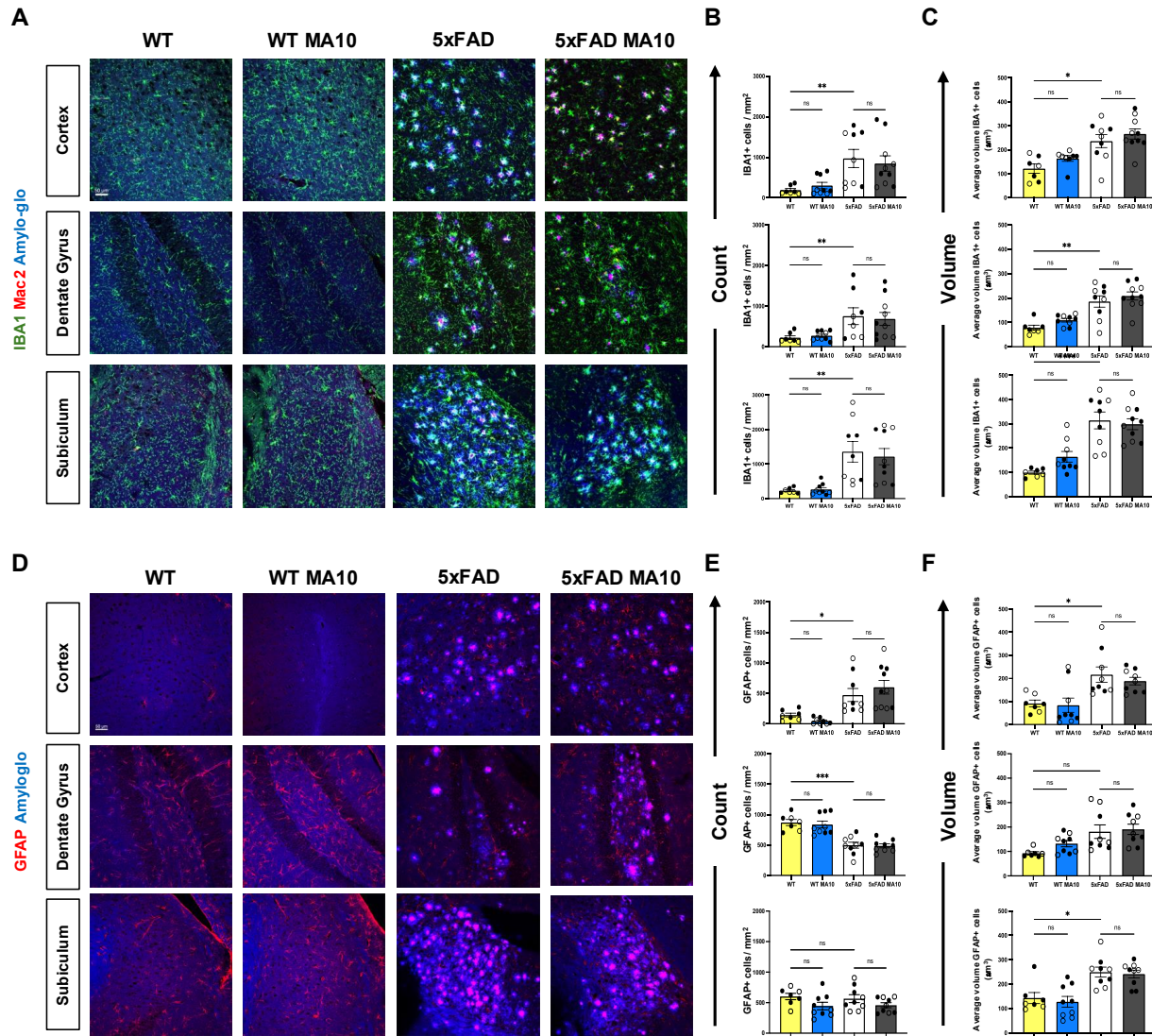
Microglia and astrocytes in MA10-infected mice do not exhibit changes in numbers nor volume. Brains from MA10-infected and control mice were immunofluorescently labeled for microglia (IBA1) and astrocytes (GFAP) to determine if there was an increase in glial activation in response to MA10 infection at day 21 p.i. We elected to examine the cortex (CRTX), dentate gyrus (DG), and subiculum (SUB) as previous studies of 5xFAD mice indicate changes in AD associated pathology in these regions.²⁹ As a metric of cell activation, we measured either GFAP+ or IBA1 cell numbers and volume in control and MA10-infected WT and 5xFAD mice. Representative images of IBA1+ microglia and Mac2+ monocytes along with Amyloglo counterstain for A β plaques (5xFAD mice) were acquired within the CRTX, DG, and SUB from uninfected and MA10-infected WT and 5xFAD mice are shown (**Figure 2A**). Quantification of microglia numbers (**Figure 2B**), and volume (**Figure 2C**) does not indicate significant changes in

MA10-infected WT or 5xFAD mice compared to their uninfected controls. There was an increase in microglia number and volume in both uninfected and infected 5xFAD mice compared to uninfected and infected WT mice in all brain regions examined (**Figure 2C**).

MAC2 is a unique marker for peripheral monocyte-derived macrophages that allows discrimination between these cells and microglia.³⁰ Immunostaining and quantification do not indicate changes in MAC2-positive cell number or volume within the cortex, dentate gyrus, or subiculum as a result of MA10 infection in either WT or 5xFAD mice (**Supplemental Figure 1**). There were increased numbers ($p < 0.0001$) of MAC2-positive cells in the brains of uninfected 5xFAD mice compared to uninfected WT mice, reflective of the neuroinflammation occurring within the brains of the 5xFAD mice as a result of ongoing AD neuropathology and A β plaque accumulation (**Supplemental Figure 1**). Although MAC2 can be expressed by some activated microglial subsets, our spatial transcriptomic data did not identify a unique MAC2-high myeloid population distinct from the resident clusters. Moreover, other studies have demonstrated distinct macrophage populations that were separate from resident microglia and our findings support these

FIGURE 2. Effect of MA10 infection on glial activation in WT and 5xFAD mouse brains.

Brains from uninfected and MA10-infected WT and 5xFAD mice at day 21 p.i. were immunostained for IBA1 and MAC2 to assess changes in microglia and infiltrating monocytes/macrophages, respectively and GFAP to detect astrocytes. Dense-core A β plaques were stained via Amylo-Glo. (A) Representative images (20X magnification) from brains isolated from uninfected and infected WT and 5xFAD mice showing the cortex (CRTX), dentate gyrus (DG), and subiculum (SUB) stained for microglia (IBA1), macrophages (MAC2), and Amylo-Glo. (B) Average IBA1+ cells per mm² in CRTX (upper graph), DG (middle graph) and SUB (bottom graph) from experimental mice. (C) IBA1 average volume per μm^3 in CRTX (upper graph), DG (middle graph) and SUB (bottom graph) from experimental mice. (D) Representative images from brains isolated from uninfected and infected WT and 5xFAD showing GFAP expression and A β plaques. Average GFAP+ cells per mm² (E) and average volume per μm^3 (F) in the CRTX (upper graphs), DG (middle graphs) and SUB (bottom graphs). Immunohistological data were analyzed using two-way ANOVA. Tukey's post-hoc test was employed to examine biologically relevant interactions. Female and male mice are indicated by open or closed circles respectively. Data are represented as MEAN $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Scale bar in (A) = 50 μm .

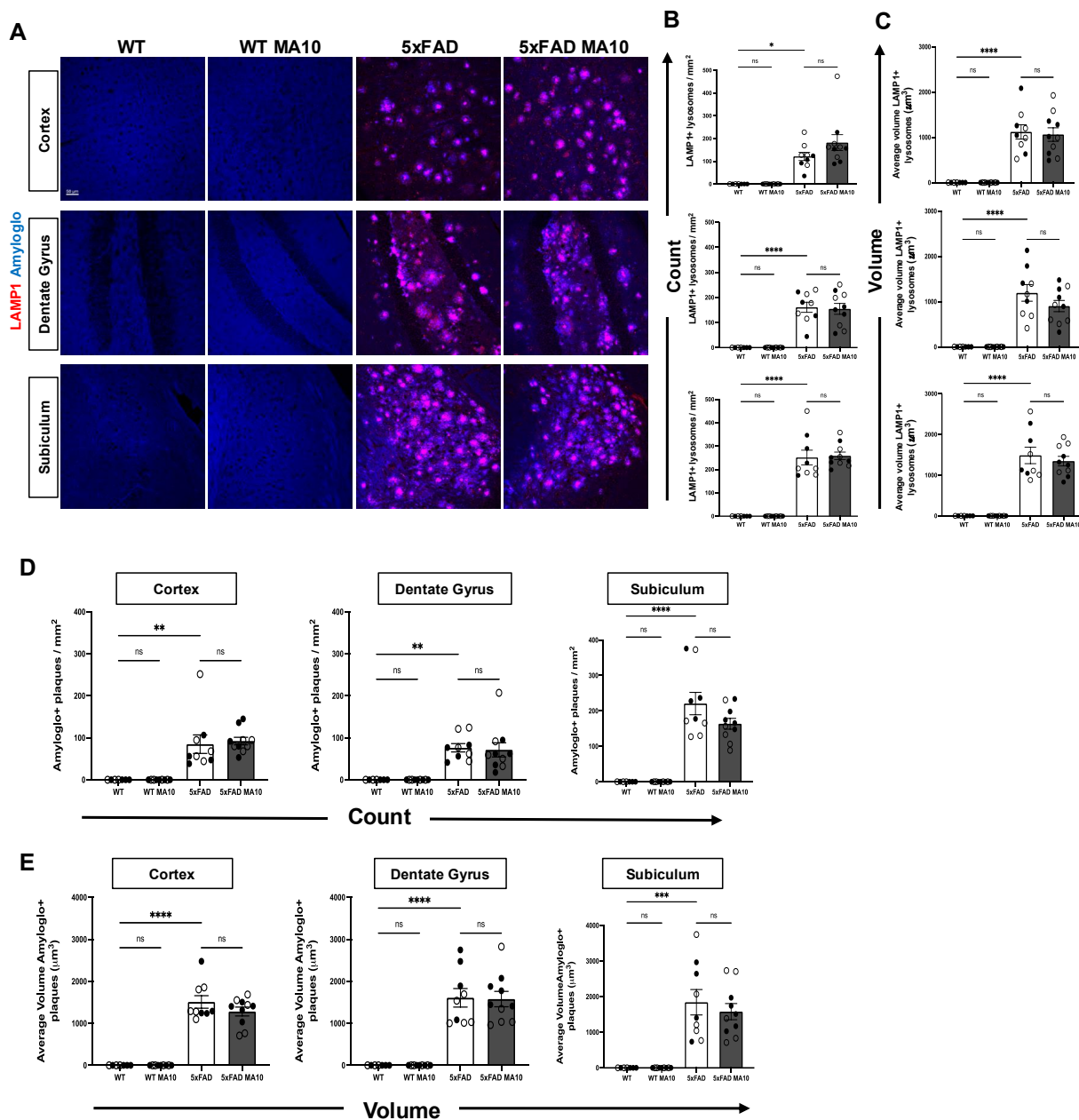


observations³¹. Immunofluorescent staining of GFAP+ astrocytes in uninfected and MA10-infected WT and 5xFAD mice along with Amyl-glo staining of A β plaques in control and MA10-infected 5xFAD mice within the CTX revealed no differences in GFAP expression (**Figure 2D**). Quantification of both astrocyte numbers and volume revealed no differences between control and infected WT and 5xFAD mice, indicating that infection did not result in increased astrocyte activation at 21 days p.i. (**Figures 2E-F**). There were increased numbers of GFAP+ cells within the cortex of uninfected 5xFAD mice compared to uninfected WT mice while volume of GFAP+ cells was increased in both the cortex and subiculum in uninfected 5xFAD mice compared to

uninfected WT mice (**Figure 2F**). GFAP numbers were reduced in the dentate gyrus of uninfected 5xFAD mice compared to uninfected WT mice (**Figure 2F**). Overall, these findings indicate that MA10 infection of either WT or 5xFAD does not result in sustained activation of either microglia or astrocytes by 21 days p.i.

Dystrophic neurite and A β plaque burden in MA10-infected mice. Dense core A β plaques, a key feature of AD pathology, are surrounded by dystrophic neurites, which are abnormal swollen neuritic processes that can be detected by immunostaining for lysosome-associated membrane protein 1 (LAMP1)²⁹. Representative images of LAMP1+ lysosomes with Amylo-glo-positive A β plaques were acquired for the cortex, dentate gyrus, and subiculum (**Figure 3A**). Dystrophic neurites and A β plaque pathology are only present in 5xFAD, and infection does not affect or induce these pathologic features in WT mice (**Figure 3A**). Immunostaining and quantification do not indicate changes in count or volume of dystrophic neurites in response to MA10 infection of either WT or 5xFAD mice compared to uninfected controls in any of the brain regions examined in either WT or 5xFAD mice (**Figures 3B-C**). There were no differences in either A β plaques count or volume in MA10 infected 5xFAD mice compared to uninfected 5xFAD mice (**Figure 3D-E**). Synaptic puncta were next quantified via co-localization of the presynaptic

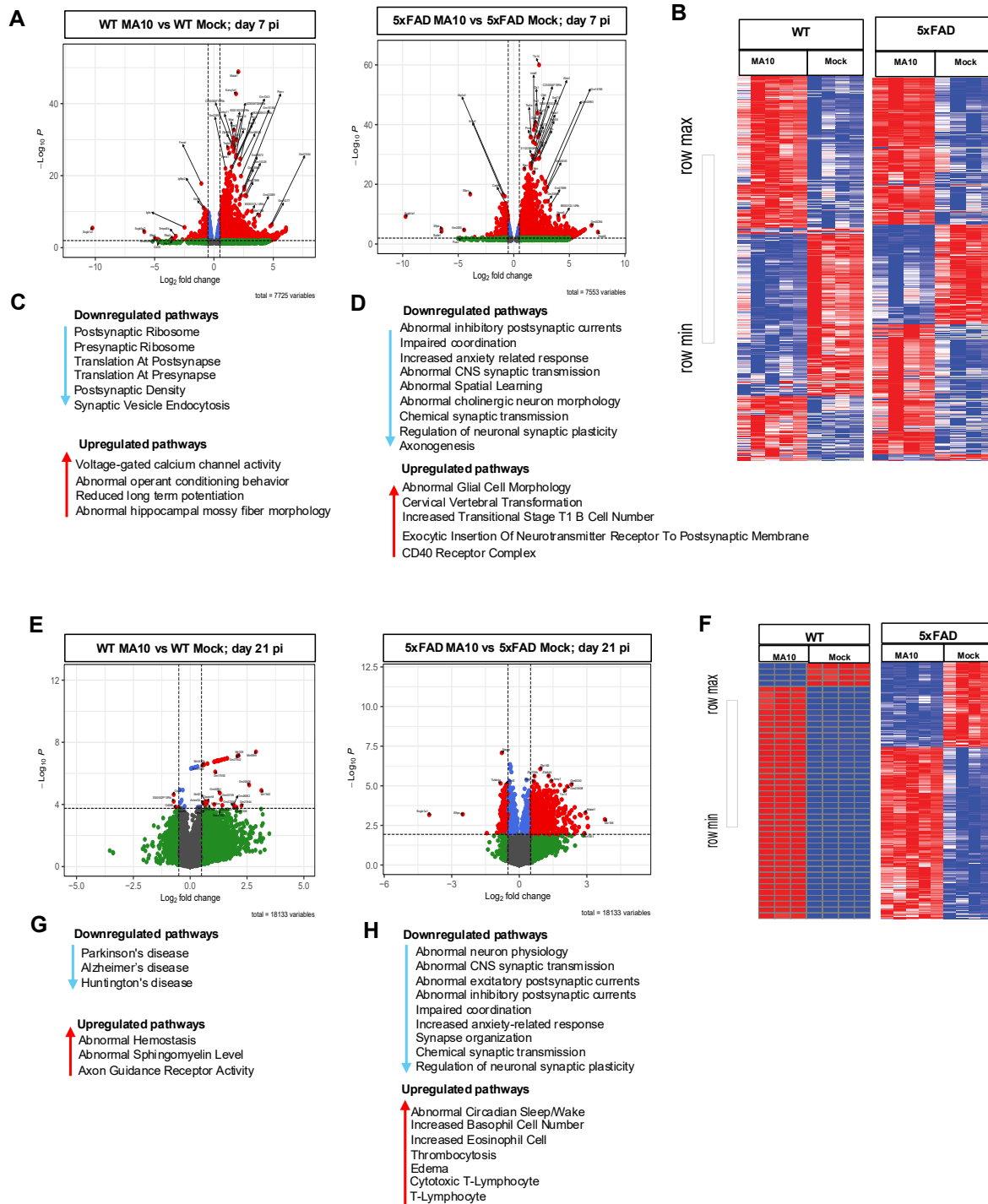
FIGURE 3. MA10 infection does not affect dystrophic neurites or A β plaques in 5xFAD mice. Brains from uninfected and MA10-infected mice at 21 days post-infection were immunostained with LAMP1 to visualize dystrophic neurites and with Amylo-Glo to detect dense-core A β plaques. **(A)** Representative images of LAMP1 and Amylo-Glo staining in the CRTX, DG, and SUB of uninfected and infected WT and 5xFAD mice. **(B)** Quantification of LAMP+ lysosomes density **(B)** and volume **(C)** shows no differences in MA10-infected WT or 5xFAD mice compared to uninfected controls. A β plaque burden was determined through quantifying Amylo-Glo⁺ plaque number as shown in **(D)** and summarizes the average volume of these plaques **(E)**. Immunohistological quantifications were analyzed using two-way ANOVA followed by Tukey's post hoc test. Female and male mice are represented by open and closed circles, respectively. Data are presented as mean $\pm$ SEM; $p \leq 0.05$ (*), ≤ 0.0001 (****). Scale bar in **(A)** = 50 μ m.



marker, synaptophysin, and postsynaptic marker PSD95 to assess effects of MA10 infection on neuronal dysfunction. Representative images of synaptophysin puncta, and PSD95 positive puncta were acquired (**Supplemental Figure 2A**). Immunostaining and quantification did not indicate changes in synapse numbers in the brains of either WT or 5xFAD mice at 21 dpi (**Supplemental Figure 2B**).

Bulk RNA sequencing of brains from MA10-infected mice. Bulk RNA sequencing of differentially expressed genes was performed on the brains of experimental mice at day 7 and 21 p.i. Volcano plots of brain gene expression in MA10 infected and control WT and 5xFAD at days 7 (**Figures 4A**) and 21 p.i. (**Figures 4E**) were plotted. Differentially expressed genes were selected as significant with a $\log_2(\text{Fold Change}) > 0.5$ and a false discovery rate (FDR) < 0.05 . On both days 7 and 21p.i., infected WT and 5xFAD groups show differential expression of genes when compared to control groups. At day 7 p.i, both groups exhibit a strong transcriptional response, with numerous genes significantly upregulated (**Figure 4A**). By day 21p.i, MA10-infected WT mice showed fewer numbers of DEGs compared to MA10-infected 5xFAD mice, suggesting a more persistent molecular response in MA10-infected 5xFAD mice (**Figure 4E**). Heatmaps were created to compare significant DEGs from control and MA10-infected WT and 5xFAD mice. DEGs were selected as significant if $\text{Log}_2\text{Fold Change}$ was greater than 0.5 and false discovery rate/ adjusted p-value as less than 0.05 ($\text{FC} > 0.5$, $\text{FDR} < 0.05$). Genes that met such criteria were then used to plot heatmaps of their expression from the brains of experimental mice at days 7 p.i. (**Figure 4B**) and 21p.i. (**Figure 4F**). GO analysis of MA10-infected WT at day 7 p.i. revealed increased expression of pathways associated with voltage-gated calcium channel activity,

FIGURE 4. SARS2 MA10 infection in brains of WT and 5xFAD mice is associated with differential expression of genes involved in regulation of synaptic activity at both days 7 and 21 p.i. RNA sequencing was performed on brains from MA10 infected and control WT and 5xFAD male mice at 7 days p.i. (**A**) Volcano plot of differentially expressed genes (DEG) displaying fold change of genes ($\log_2$ scale) and P values. (**B**) Heatmap of selected ($\text{FC} = 0.5$, $\text{FDR} < 0.05$) DEGs from MA10- infected WT and 5xFAD and uninfected (mock) controls at day 7 p.i. Enriched pathways of interest examined via Gene Ontology (GO) expressed in (**C**) MA10-infected WT and (**D**) 5xFAD mice showing downregulated and upregulated pathways at day 7 p.i. Volcano plot of differentially expressed genes (DEG) displaying fold change of genes ($\log_2$ scale) and P values in (**E**) MA10-infected WT and 5xFAD mice at day 21 p.i. (**F**) Heatmap of selected ($\text{FC} = 0.5$, $\text{FDR} < 0.05$) DEGs from MA10-infected WT and 5xFAD and uninfected (mock) controls at day 21 p.i. Enriched pathways of interest examined via Gene Ontology (GO) expressed in (**G**) MA10-infected WT and (**H**) 5xFAD mice showing downregulated and upregulated pathways at day 21 p.i.



abnormal operant conditioning, reduced long-term potentiation, and abnormal hippocampal mossy fiber morphology (Figure 4C). In the MA10-infected 5XFAD at day 7 p.i. analysis indicated changes in pathways associated with abnormal postsynaptic currents, synaptic transmission,

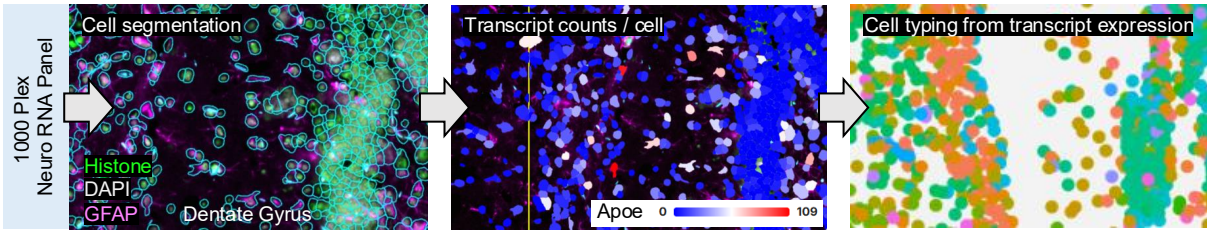
neuron morphology, and spatial learning as well as impaired coordination and increased anxiety-related responses. Additionally, there are changes in pathways associated with synaptic transmission and plasticity as well as axonogenesis (**Figure 4D**). Brains of both the WT and 5xFAD groups did indicate lasting changes in gene expression (**Figure 4E**); however, heatmap analysis indicated that the MA10 infected 5xFAD group had dramatically more lasting differentially expressed genes in comparison to the MA10 infected WT group (**Figure 4F**). Moreover, when examining pathways of interest, MA10-infected WT mice showed only one identified down-regulated gene (*UQCRFS1*) that is associated with AD.³² (**Figure 4G**). Analysis on the 5xFAD group at 21 days p.i. indicated changes of many more pathways, most of which were associated with neuronal and synaptic dysfunction, impaired coordination, and anxiety-related responses (**Figure 4H**). Furthermore, the brains of MA10-infected WT mice indicated changes in gene expression in pathways associated with abnormal sphingomyelin levels and axon guidance receptor activity (**Figure 4G**) while the brains of MA10-infected 5xFAD mice indicated changes in pathways associated with edema and abnormal circadian cycles (**Figure 4H**).

Altered gene expression in resident CNS cells following MA10 infection of WT and 5xFAD mice. WT and 5xFAD MA10-infected and uninfected control brains were sectioned and processed for spatial transcriptomic analysis and analyzed for 1,000 genes using the Nanostring CosMx Spatial Molecular Imager platform (n = 3 per group, 1216 total FOVs, ~100 FOVs per brain section) (**Figure 5A**; examples of cell segmentation in **Supplemental Figure 3**). 787,580 cells were captured with a mean transcript count of ~600 per cell. Unbiased cell clustering identified 46 transcriptionally distinct cell clusters (**Figure 5B and Supplemental Figures 4A and B**), which were manually annotated based on gene expression and location. Projection of clusters in XY space revealed distinct localization to specific anatomical regions (**Figure 5C and**

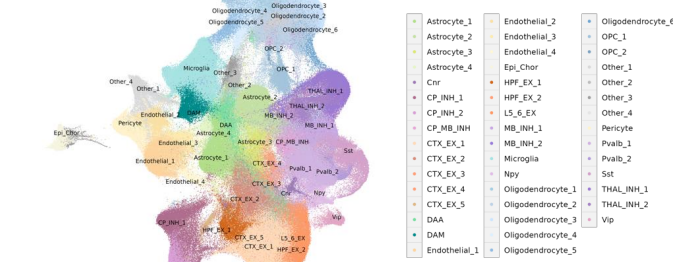
Supplemental 5). Separation of cell clusters by group (WT control, WT MA10 infected, 5xFAD control, 5xFAD MA10 infected) revealed distribution of distinct cell populations within the brains of each experimental group (**Figure 5D**). Clusters from the same broad cellular subtype were then combined into broad cell types (i.e. Astrocyte 1, Astrocyte 2, etc. were assigned to the Astrocyte cell type) for subsequent analyses. The proportion of cells in each major cluster from experimental groups was then plotted per genotype (**Figure 5E**). To broadly assess overall deviation from WT in a given group, DEG scores were calculated for each cellular subtype. To calculate DEG scores for each group, the log₂fold change values were summed for DEGs that were significantly upregulated or downregulated in comparison to WT (i.e., $p_{adj} \leq 0.05$), yielding DEG scores for each subtype. Subtypes were then plotted in XY space colored by DEG score for upregulated and downregulated genes to give a visual impression of the spatial distribution of DEGs and overall degree of deviation from WT control in the three experimental groups (**Figure 5F**). As expected, the 5xFAD MA10 infected brains had the highest upregulated and downregulated DEG scores, indicative of broad deviation from WT gene expression patterns. As disease-associated microglia (DAMs) are considered important in A β pathology in 5xFAD-infected mice,³³ we next plotted the

FIGURE 5. Spatial transcriptomic analysis of the MA10-infected WT and 5XFAD mouse brain. (A) Experimental workflow for targeted 1000-plex single-cell spatial transcriptomics. Fields of view (FOVs) were selected in hippocampal, corpus callosum, upper thalamic, upper caudate, and cortical regions for each of the six sections then imaged with DNA, rRNA, Histone, and GFAP markers for cell segmentation. Transcript counts for each gene were acquired per cell. (B) Uniform Manifold Approximation and Projection (UMAP) of 787,580 cells across 6 brains were captured with a mean transcript count of ~600 per cell. Unbiased cell clustering identified 46 transcriptionally distinct cell clusters which were annotated with a combination of automated and manual approaches with reference to Allen Brain Atlas single-cell RNA-seq cell types, gene expression, and anatomical location in space. (C) 46 clusters plotted in XY space. (D) Separation of clusters by group (WT control, MA10-infected WT, 5xFAD control, MA10-infected 5xFAD). (E) Proportional distributions across experimental groups. Bar plots display the relative abundance of each cluster in each group. (F) Downregulated DEG scores in XY space in comparison to WT control (left panel), upregulated DEGs (right panel). (G) DAM cell counts across groups (left panel), distribution of DAMs plotted in XY space in uninfected and MA10 infected 5xFAD.

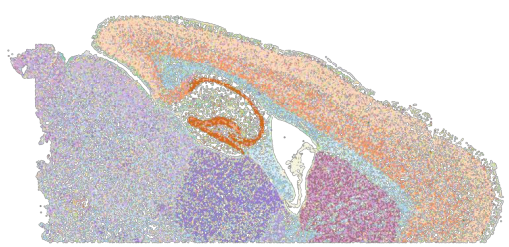
A) Workflow for spatial transcriptomics



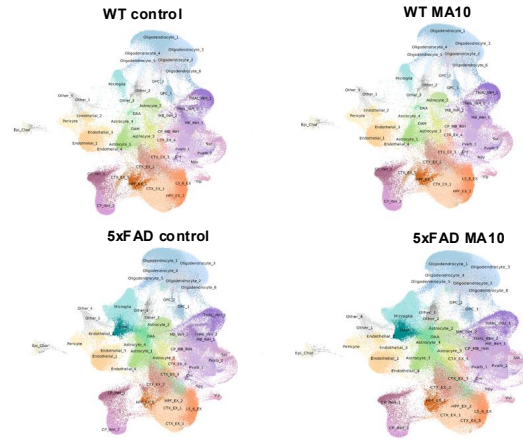
B) UMAP



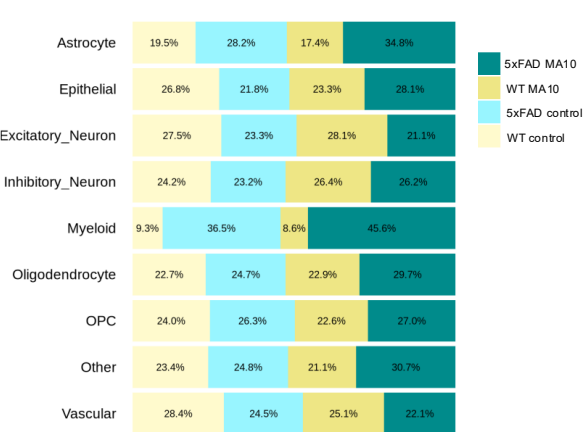
C) Clusters in XY space



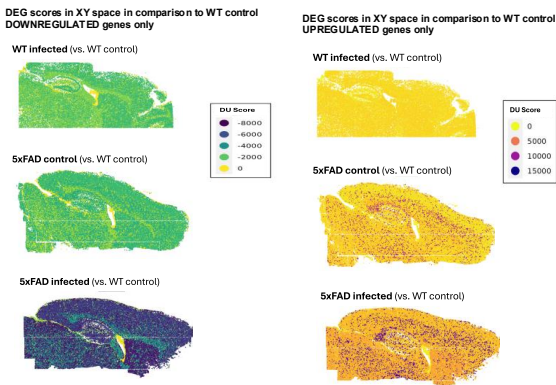
D) UMAP split by group



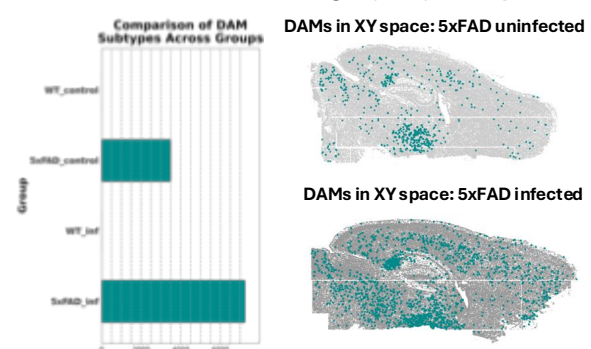
E) Broad cell type cell proportions



F) DEG scores in XY space



G) Disease Associated Microglia (DAM) in XY space



DAM cluster in XY space revealing that DAMs are distributed in both uninfected and MA10-infected 5xFAD mice in plaque-laden areas i.e., the subiculum of the hippocampus, throughout the cortex (**Figure 5G**).

MA10 infection alters gene expression in glial cells and neurons. Differential gene expression analysis revealed that MA10 infection led to the appearance of differentially expressed genes (DEGs) within broad cell types in both WT and 5xFAD mice, including microglia, astrocytes, oligodendrocytes, inhibitory neurons and vascular endothelial cells (**Figures 6A-J**). Many DEGs were identified between infected and uninfected control brains with infection in both 5xFAD and WT mice. In both mice, microglia demonstrated loss of numerous homeostatic signature genes ³⁴ (i.e. *Hexb*, *Csf1r*, *P2ry12*, *Cx3cr1*) (**Figures 6A and B**). Astrocytes indicated a downregulation of *Slc1a2*, *Gjal1*, *Plpp3*, *Atp1a2*, *Ptprz1*, genes identified to be involved in astrocytic functions that support neuronal communication and homeostasis³⁵⁻³⁷ (**Figures 6C and D**). Furthermore, oligodendrocytes showed decreased expression of genes related to myelination, lipid metabolism, and neuroprotection (*Malat1*, *Mag*, *Ugt8a*, and *Plp* ³⁸⁻⁴⁰ as well as an increased expression in genes related to cellular stress responses (*Sgk1*) ⁴¹ (**Figures 6E and F**). Other cell types such as inhibitory neurons exhibited a downregulation of genes associated with neurotropic support (*Ndr4*, *Sst*, and *Npy*)⁴²⁻⁴⁴ (**Figures 6G and H**), while vascular endothelial cells indicated consistent changes across genotypes in both the downregulation of genes associated with blood brain barrier (BBB) integrity (*Bsg*, *Cldn5*, and *Pecam1*)^{45,46} as well as the upregulation of genes associated with other vascular adaptations (*Apod*, and *Acta2*)^{47,48} (**Figures 6I and J**). WT brains demonstrated a greater number of significant DEGs and/or a greater average difference than

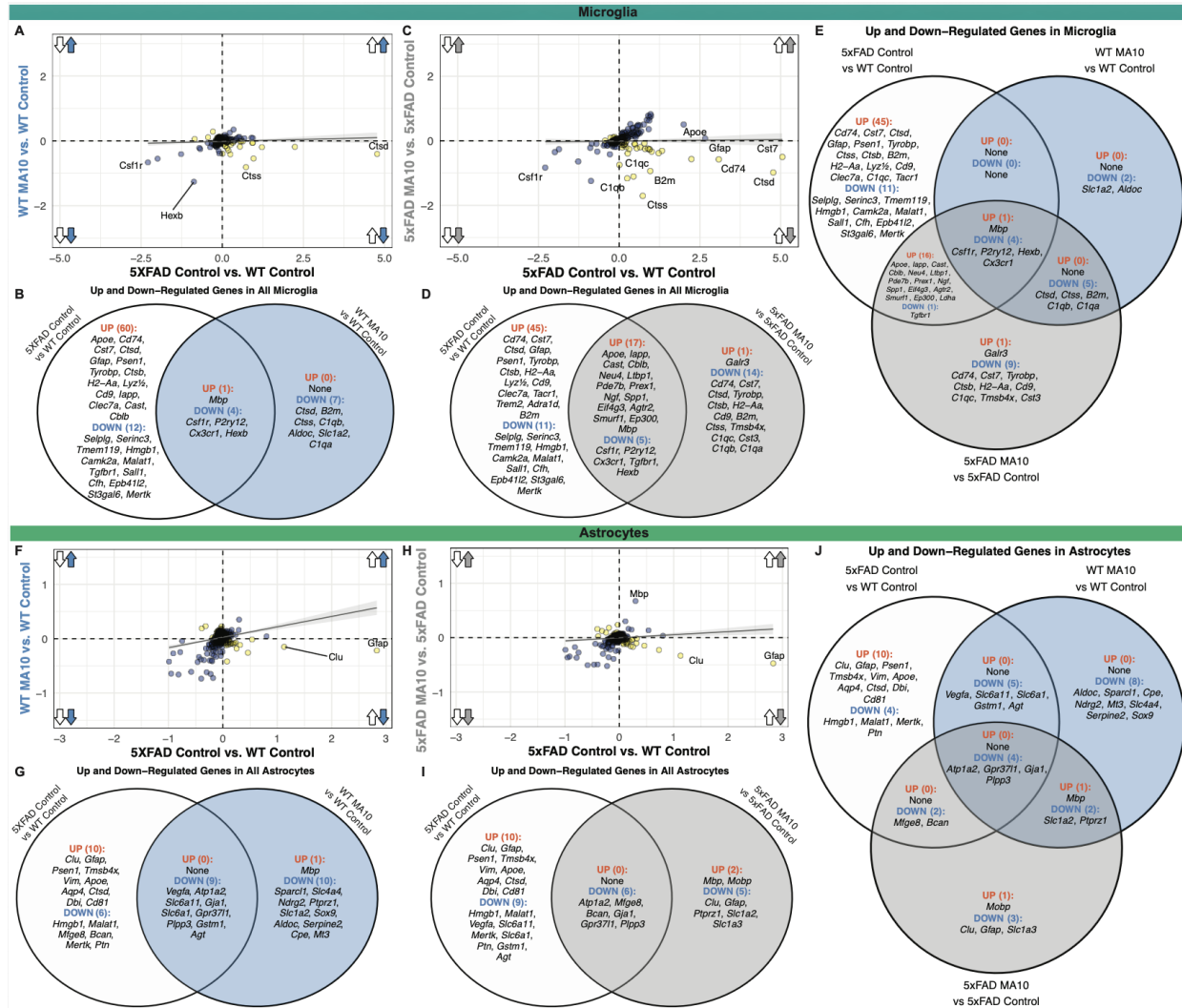
FIGURE 6. SARS2 MA10 infection leads to changes in gene expression within resident CNS cells, as revealed by spatial transcriptomic analysis of WT and 5xFAD mouse brains. Volcano plots of DEGs between each of the following broad cell type: (A) microglia, (C) astrocyte (E) oligodendrocyte, (G) inhibitory neuron and (I) vascular of both MA10-infected WT vs WT control (left panel) and MA10-infected 5xFAD MA10 vs 5xFAD control (right panel). (B), (D), (F), (H) and (J) represent corresponding dot plots showing the expression of individual genes across experimental groups (WT control, 5xFAD control, WT MA10, and 5xFAD MA10). Dot size represents the percentage of cells expressing the gene, while color indicates the average scaled expression level.

showed loss of homeostatic microglial markers after infection in comparison to their respective uninfected controls, the average difference ($\log_2$ fold change) and significance (p_{adj}) in 5xFAD microglia were higher than WT. In addition to conservation of the majority of downregulated genes from WTs, 5xFAD microglia also had a number of upregulated genes that were not present in WTs, including *Cblb*, *Cast*, *Neu4*, *Iapp*, *ApoE*, *Ltbp1*, *Eif4g3*, *Ngf*, and *Smurf1*, among others (**Figure 6B**), indicating a further loss of microglial homeostatic functions such as survival, communication, migration and phagocytic capacity to an even more reactive state suggesting that 5xFAD microglia are more affected by MA10 infection than WT microglia at the transcriptomic level. A complete comparison of differentially expressed genes in MA10-infected WT and 5xFAD mice compared to uninfected controls is provided in **Supplemental Figure 6** and **Supplemental Figure 7**, respectively.

Comparing effects of A β pathology and MA10 infection on cell-type specific differential gene expression. We have demonstrated that MA10 infection in the periphery shifts the transcriptional state of microglia towards a greater loss of homeostatic markers. To investigate whether these dysregulated responses in microglia are a consequence of peripheral MA10 infection, the presence of A β pathology, or unique to the presence of both inflammatory stimuli, we compared the average differences in gene expression within all microglia in our spatial transcriptomic dataset. We plotted all DEGs found in comparing control 5xFAD vs. control WT samples (i.e., response to A β -associated pathology) and those found comparing MA10 infected WT vs. control WT (i.e., response to MA10 infection) using the average difference of each gene among the two comparisons (**Figure 7A**). In response to either A β pathology or MA10 infection, microglia shared only four significantly down-regulated DEGs (*Csf1r*, *P2ry12*, *Cx3cr1*, *Hexb*), all of which are signature microglial homeostatic markers (**Figure 7B**). Meanwhile, there were

several genes that were uniquely differentially expressed in one inflammatory stimulus over the other. Specific to MA10 infection, microglia demonstrated seven significantly down-regulated genes (*Ctsd*, *B2m*, *Ctss*, *C1qb*, *Aldoc*, *Slc1a2*, and *C1qa*). In contrast, microglia demonstrated 60 significantly up-regulated genes (*Apoe*, *Cd74*, *Cst7*, *Ctsd*, *Ctsb*, *Cd9*, *Clec7a* etc.) and 12 significantly down-regulated genes (*Selpg*, *Serinc3*, *Tmem119*, *Hmgbl*, *Camk2a*, *Malat1*, *Tgfbr1*,

FIGURE 7. Microglia and astrocytes have differential responses to the presence of amyloid pathology, peripheral MA10 infection, or both concurrently. (A) Scatterplot of the average difference of all microglia for all significant genes ($p_{adj} < 0.05$) between 5xFAD control vs. WT control (x-axis, only amyloid pathology) and WT MA10 vs. control WT (y-axis, only MA10 infection) comparisons. Arrows indicate direction of dysregulation for each comparison. Directly correlated genes (blue) occur in the same direction for both comparisons (i.e., both up-regulated or both down-regulated), while inversely correlated genes (orange) occur in opposite directions for each comparison. Linear regression line demonstrates the relationship between the two comparisons. (B) Venn diagram depicting the significant up- and down-regulated genes unique to the 5xFAD Control vs. WT Control (left, white) and WT MA10 vs. WT Control (right, blue), while demonstrating up- and down-regulated genes commonly shared across the two comparisons (middle). (C) Scatterplot of the average difference of all microglia for all significant genes between 5xFAD Control vs. WT Control, now with 5xFAD MA10 vs. 5xFAD Control (y-axis, both amyloid and MA10 infection). Red text demonstrates genes up-regulated in the same direction in the first scatterplot, but down-regulated in the MA10 5xFAD vs. Control 5xFAD comparison. (D) Venn diagram depicting the significant up- and down-regulated genes unique to 5xFAD Control vs. WT Control and 5xFAD MA10 vs. 5xFAD Control (right, purple), while demonstrating up- and down-regulated genes commonly shared between the two comparisons. (E) Three-way Venn diagram of all significantly up- and down-regulated genes within microglia between the three comparisons: 5xFAD Control vs. WT Control (white), WT MA10 vs. WT Control (blue), and 5xFAD MA10 vs. 5xFAD Control (grey). Genes are considered significantly correlated if the log2 Fold Change magnitude is greater than 0.3. (F) Scatterplot of the average difference of all astrocytes for all significant genes between 5xFAD Control vs. WT Control and WT MA10 vs. WT Control. (G) Venn diagram depicting the significant up- and down-regulated genes in astrocytes unique to 5xFAD Control vs. WT Control (left, white) and WT MA10 vs. WT Control (right, blue), while demonstrating shared dysregulated genes between the two comparisons. (H) Scatterplot of the average difference of all astrocytes for all significant genes between 5xFAD Control vs. WT Control. (I) Venn diagram depicting the significant up- and down-regulated genes in astrocytes unique to 5xFAD Control vs. WT Control (left, white) and 5xFAD MA10 vs. 5xFAD Control (right, grey), while demonstrating shared dysregulated genes between the two comparisons. (J) Three-way Venn diagram of all significantly up- and down-regulated genes within astrocytes between the three comparisons: 5xFAD Control vs. WT Control (white), WT MA10 vs. WT Control (blue), and 5xFAD MA10 vs. 5xFAD Control (grey).



etc.) that were unique to responses to A β pathology (**Figure 7B**). Therefore, while the microglia activation response to peripheral MA10 infection and A β pathology down-regulated similar homeostatic markers, this comparison highlights the nuanced differences in related groups of genes when considering the magnitude of their inflammatory response.

To investigate the effect of the concurrence of both inflammatory stimuli (e.g., amyloid pathology and MA10 infection) on the gene expression within microglial cells, we plotted all DEGs between Control 5xFAD vs. Control WT (only amyloid pathology) and MA10 5xFAD vs. Control 5xFAD (both amyloid pathology and MA10 infection) (**Figure 7C**). Many differential

gene expression changes were shared between the two conditions, with several common genes up-regulated or down-regulated. For example, 17 genes were significantly up-regulated within myeloid cells (*Apoe*, *Iapp*, *Cast*, *Cblb*, *Neu4*, *Spp1*, *Ltbpl*, *Prex1*, etc.) in the presence of both inflammatory stimuli together, while 5 genes were significantly down-regulated in both (*Csf1r*, *P2ry12*, *Cx3cr1*, *Tgfb1*, *Hexb*) (**Figure 7D**).

To determine which set of DEGs in microglial cells were unique to the interaction of both A β pathology and MA10 infection, we compared significant DEGs (adjusted p-value < 0.05) from all microglial cells across the three comparisons for the three conditions: MA10 WT vs. Control WT (only peripheral MA10 infection), Control 5xFAD vs. Control WT (only A β pathology), and MA10 5xFAD vs. Control 5xFAD (both peripheral MA10 infection and A β pathology). As previously demonstrated, this analysis also demonstrated four homeostatic genes (*Csf1r*, *P2ry12*, *Hexb*, *Cx3cr1*) were significantly down-regulated in the context of all three conditions. Among DEGs that were uniquely down-regulated only in the context of both amyloid pathology and MA10 infection together were *Cd74*, *Cst7*, *Tyrobp*, *Ctsb*, *H2-Aa*, *Cd9*, *Clqc*, *Tmsb4x*, and *Cst3*. Meanwhile, these DEGs were actually significantly up-regulated in the presence of A β pathology or peripheral MA10 infection, independently (**Figure 7E**). To determine the effects on other cell types within the CNS, we performed a similar analysis with astrocytes (**Figure 7F-J**), vascular cells, oligodendrocytes, and inhibitory neurons (**Supplemental Figure 8**).

Astrocytes showed substantial overlap in transcriptional suppression across conditions. Nine down-regulated DEGs were commonly downregulated in response to either A β pathology or MA10 infection (*Vegfa*, *Atpla2*, *Slc6a11*, *Gjal*, *Slc6a11*, *Gpr37l1*, *Plpp3*, *Gstm1*, *Agt*) (**Figure 7G**). MA10 infection induced ten additional down-regulated DEGs (*Sparcl1*, *Slc4a4*, *Ndr2*, *Ptprz1*, *Slc1a2*, *Sox9*, *Aldoc*, *Serpine2*, *Cpe*, *Mt3*) and one upregulated DEG (*Mbp*), while A β

pathology alone resulted in six down-regulated (*Hmgb1*, *Malat1*, *Mfge8*, *Bcan*, *Mertk*, *Ptn*) and ten up-regulated DEGs (*Clu*, *Gfap*, *Psen1*, *Tmsb4x*, *Vim*, *Apoe*, *Aqp4*, *Ctsd*, *Dbi*, *Cd81*). Six genes were commonly down-regulated with both stimuli (*Atp1a2*, *Mfge8*, *Bcan*, *Gja1*, *Gpr371l*, *Plpp3*) (**Figure 7I**), and four genes (*Atp1a2*, *Gpr371l*, *Gja1*, *Plpp3*) were consistently downregulated across all three conditions (**Figure 7J**).

Vascular cells shared six down-regulated DEGs in response to either A β pathology or MA10 infection, (*Rgs5*, *Slc1a2*, *Flt1*, *Sparcl1*, *Bsg*, *Pecam1*) (**Supplemental Figure 8B**). MA10 infection resulted in six additional down-regulated (*Cpe*, *B2m*, *Pltp*, *Sparc*, *Cldn5*, *Vtn*) and four up-regulated DEGs (*Slc2a1*, *Vim*, *Apod*, *Acta2*), whereas A β pathology alone produced one down-regulated (*Slc2a1*) and three upregulated DEGs (*Gfap*, *Psen1*, *Clu*) (**Supplemental Figure 8B**). Three genes (*Flt1*, *Bsg*, *Pecam1*) were consistently downregulated under both stimuli and across all three conditions (**Supplemental Figure 8D-E**).

Oligodendrocytes showed no shared DEGs between A β pathology and MA10 infection (**Supplemental Figure 8G**). MA10 infection induced six down-regulated (*Pllp*, *Malat1*, *Myrf*, *Mag*, *Ptgds*, *Ugt8a*) and two upregulated DEGs (*Sgk1*, *Mbp*), while A β pathology resulted in one down-regulated (*Hmgb1*) and ten up-regulated DEGs (*Apoe*, *B2m*, *Clu*, *Cstd*, *Gfap*, *Psen1*, *Pllp*, *Cst7*, *Cd9*, *Plp1*) (**Supplemental Figure 8G**). No shared DEGs were detected under combined or all three conditions (**Supplemental Figure 8J**).

Inhibitory neurons shared three down-regulated DEGs in response to either A β pathology or MA10 infection, (*Atp2a2*, *Hspa8*, *Sst*) (**Supplemental Figure 8L**). MA10 infection induced seven additional down-regulated (*Hsp90aa1*, *Tcf7l2*, *Gnas*, *Sparcl1*, *Ywhag*, *Cpe*, *Sparc*) and four upregulated DEGs (*Ppp1r1b*, *Penk*, *Arpp21*, *Pcp4*), whereas A β pathology resulted in five down-regulated (*Cplx1*, *Syp*, *Meg3*, *Snhg11*, *Penk*) and one upregulated DEG (*Psen1*) (**Supplemental**

Figure 8L). With both stimuli, two DEGs were commonly downregulated (*Penk*, *Sst*) (**Supplemental Figure 8N**) and only *Sst* remained downregulated across all three conditions (**Supplemental Figure 8O**).

DISCUSSION

Recent studies have reported that infection with either SARS-CoV-2 or mouse-adapted SARS-CoV-2 (MA10) has led to the presence of gliosis and other CNS changes in animal models in the absence of virus in the brain, arguing that peripheral inflammatory responses to viral infection can impact CNS resident cell response^{23–25}. While COVID-19 most commonly presents with respiratory symptoms, neurological symptoms have been extensively documented, and can emerge during acute infection and persist beyond viral clearance⁴⁹. The full extent of neuropathological changes in the CNS that results from SARS-COV-2 infection is still not fully understood. Given the association between COVID-19, neuroinflammation, and neurodegenerative processes, further characterization of CNS alterations following infection and their potential contribution to neurodegeneration is needed.

To address these questions, we infected aged (10–14 month old) C57BL/6 wild-type (WT) and 5xFAD mice with mouse-adapted SARS-CoV-2 (MA10) to assess neuropathological changes following chronic infection and examine infection-induced alterations in brain gene expression. We utilized aged 5xFAD mice to evaluate the effects of SARS-CoV-2 infection on a brain with already established amyloid pathology. This approach was intended to model the clinical scenario of infection in elderly patients with pre-existing dementia. It remains possible that infection at a younger age, prior to the onset of significant A β deposition, might more profoundly influence the trajectory or magnitude of subsequent pathology—a question that warrants further investigation.

As both microglia and astrocytes can contribute to neuroinflammatory and neurodegenerative processes¹⁸, changes in microglia and astrocyte number and volume were quantified. In contrast to prior reports suggesting infection-induced microgliosis²⁴, we observed no significant changes in microglial number or volume in the brains of infected WT or 5xFAD

mice compared to uninfected controls. Similarly, plasma levels of interleukin-1 β (IL-1 β), a cytokine previously implicated in neuroinflammation, plasticity and memory deficits⁵⁰, were unchanged following infection.

Additionally, we detected no increase in MAC2+ monocytes or macrophages within the brains of MA10-infected WT or 5xFAD mice at day 21 p.i. Astrocyte number and volume were also unchanged at day 21 p.i., consistent with reports showing minimal astrocytic changes at later time points following infection⁵¹, and despite transient increases in GFAP expression reported during the acute infection²³. Uninfected 5xFAD mice exhibited increased astrocyte number and volume in the cortex relative to WT mice, consistent with astrocyte activation in response to progressive A β plaque accumulation²⁹. The absence of overt microglial or astrocytic activation following MA10 infection may reflect the timing of analysis, the age of the mice, the use of a mouse-adapted viral strain, or the infectious dose employed.

While our histological analysis did not reveal overt changes in glial numbers or volume, we acknowledge the limitations of IHC in fully capturing complex activation phenotypes. Future studies utilizing flow cytometry would be beneficial to provide a more robust, quantitative assessment of protein-level changes in microglial homeostatic markers. Furthermore, while MAC2 staining suggested minimal peripheral myeloid cell infiltration, more comprehensive profiling is needed to rule out the involvement of other peripheral immune populations, such as T cells, which could subtly influence the CNS transcriptomic environment.

Both MA10-infected WT and 5xFAD mice exhibited changes in gene expression that were associated with neuronal dysfunction. RNASeq analysis indicates an upregulation in genes associated with reduced long-term potentiation (LTP) in WT mice, and downregulation genes associated with synaptic function and plasticity in 5xFAD mice. Given the established role of LTP

as a cellular correlate of learning and memory and its impairment in memory disorders⁵², these transcriptional changes likely contribute to cognitive deficits reported in both clinical and experimental studies. Notably, 5xFAD mice exhibited a greater number of differentially expressed genes compared to WT mice at day 21 p.i., likely reflecting heightened vulnerability associated with pre-existing A β pathology.

Although RNASeq analyses suggested neuronal and synaptic dysfunction, MA10 infection did not induce or exacerbate overt neuropathological hallmarks of AD. Specifically, we observed no changes in dystrophic neurites, A β plaque burden in 5xFAD mice, or neuronal loss, or synapse loss in either genotype. These findings were somewhat unexpected given reports that coronavirus proteins, including the envelope (E) protein, possess amyloidogenic properties¹⁶ and that MA10 infection can influence other AD-related pathologies such as phosphorylated tau^{24,25}. Together, these data indicate that, at the viral dose employed, MA10 infection does not drive overt neurodegeneration or exacerbate classical AD pathology.

Despite the absence of overt neuropathological changes, widespread transcriptomic dysregulation revealed subtle but potentially consequential molecular alterations across multiple CNS-resident cell types. Significant changes in homeostatic gene expression were observed in microglia, astrocytes, oligodendrocytes, inhibitory neurons, and vascular endothelial cells, suggesting early signatures of neuroinflammation and homeostatic disruption that may precede longer-term neurodegenerative processes.

In both WT and 5xFAD mice, microglia exhibited downregulation of established homeostatic genes, including *Hexb*, *Csflr*, *P2ryl2*, and *Cx3cr1*, genes typically suppressed during microglial activation or transition to a disease-associated microglia (DAM) phenotype³⁴. Downregulation of these genes suggests a shift away from a homeostatic microglial state,

consistent with observations in viral encephalitis and neurodegenerative disorders such as AD⁵³. Notably, in MA10-infected 5xFAD mice only, microglia showed upregulation of genes involved in immune regulation (*Cblb*)⁵⁴, protease inhibition (*Cast*)⁵⁵, lysosomal degradation (*Neu4*)⁵⁶, and inflammatory signaling and metabolic dysfunction (*Iapp*)⁵⁷. Increased expression of *ApoE*, a defining feature of DAMs, supports the emergence of a reactive microglial phenotype⁵⁸.

Astrocytes showed downregulation of key genes involved in neurotransmitter regulation (*Slc1a2*), gap junctional communication (*Gjal*), phospholipid metabolism (*Plpp3*), ion homeostasis (*Atp1a2*), and neurodevelopment (*Ptprz1*). Reduced expression of *Slc1a2*, encoding the astrocytic glutamate transporter EAAT2/GLT-1, is associated with excitotoxicity and neuronal injury in aging and neurodegenerative disease⁵⁹. Downregulation of *Atp1a2* may impair potassium clearance and neuronal excitability⁶⁰, while reduced *Gjal* (connexin43) expression disrupts astrocyte communication and has been associated with neuroinflammation⁶¹. *Plpp3* downregulation disrupts lipid-mediated signaling, impairing astrocyte–neuron communication, leading to deficits in neurotransmission⁶². *Ptprz1* has established supporting roles enabling neurogenesis and plasticity⁶³. Together, these changes indicate compromised astrocytic support functions that may contribute to neuronal dysfunction without causing overt degeneration.

In addition to astrocytes and microglia, oligodendrocytes, inhibitory interneurons, and vascular endothelial cells also displayed pronounced transcriptional changes. Oligodendrocytes showed downregulation of myelination-associated genes, including *Mag*⁶⁴, *Ugt8a*⁶⁵, and *Plp*⁶⁶, as well as *Malat1*, which has neuroprotective functions⁶⁷. Upregulation of *Sgk1* has been associated with metabolic stress in the brain, neurodegeneration and cognitive impairment and potential mediator of tau phosphorylation⁶⁸. These changes suggest oligodendrocyte dysfunction or early

demyelinating processes, potentially contributing to not only neurodegenerative processes but also white matter abnormalities reported in post-COVID imaging studies⁶⁹.

Inhibitory interneurons showed reduced expression of *Ndr4*, *Sst*, and *Npy*. *Sst* (somatostatin) and *Npy* (neuropeptide Y) which are critical neuropeptides involved in regulating neuronal excitability and synaptic transmission⁴². Their downregulation may indicate impaired inhibitory control, potentially contributing to disrupted cortical circuitry and excitatory-inhibitory imbalance. Similarly, the reduction in *Ndr4*, a gene involved in synaptic vesicle trafficking and neuronal survival⁴³. Overall findings suggest the presence of early synaptic vulnerability in the post-infectious brain.

Endothelial cells displayed downregulation of *Bsg*, *Cldn5* and *Pecam1*, all of which are vital for blood-brain barrier (BBB) integrity⁷⁰⁻⁷². *Cldn5* encodes a tight junction protein critical for BBB permeability, and its downregulation may reflect early BBB disruption, a finding consistent with neuropathological reports in COVID-19 patients²². Conversely, upregulation of *Apod* and *Acta2* may indicate vascular remodeling suggesting vascular changes in the brain not related to BBB disruption and dysfunction but associated with remodeling of affected vessels, in a maladaptive manner, due to the stress caused by an initial insult such as inflammation and infection^{73,74}.

Results from this study contribute to growing information providing insight into mechanisms by which SARS-CoV-2 infection contributes to neurologic deficits. Our results have shown there are dramatic changes in genes impacting homeostatic gene functions in microglia, astrocytes, oligodendroglia as well as neurons and vascular endothelial cells. These subtle, yet potentially important, changes in cell function can occur in the absence of viral infection of the CNS and impact neurologic functions including behavior and memory. Furthermore, MA10

infection did not impact the severity of AD-associated neuropathology in the 5xFAD model, although there were numerous changes in glial and neuron gene expression within the brains. We believe that one mechanism by which this may occur is in response to the peripheral inflammation that occurs in response to MA10 infection of the lungs leading to increased pro-inflammatory cytokines/chemokines that can access the CNS and alter resident cell function. Collectively, these findings underscore the need for further research to elucidate the molecular mechanisms underlying COVID-19-related CNS changes and their long-term impact on neurodegenerative processes.

CONCLUSION

Ongoing studies have demonstrated that SARS2 infection impacts the severity of clinical disease in elderly patients with established dementia. This study was undertaken to better understand the effects of SARS2 infection on impacting the severity of AD pathology using experimental infection of aged 5xFAD and WT mice with MA10, a mouse-adapted SARS2. While MA10 infection of 5xFAD mice did not result in either increased neuroinflammation nor a reduction in A β plaque size/volume, there was a dramatic effect on expression of genes encoding homeostatic function in glia, neurons, and vascular endothelial cells in both 5xFAD and WT mice. These findings argue that in the absence of viral infection of the brain, peripheral MA10 infection and the ensuing inflammatory response results in subtle yet sustained gene expression within the brain that has implications for long-term neurologic deficits.

MATERIALS AND METHODS

Viral infection of mice: Male and female 10-14 month old C57BL/6 WT and 5xFAD were used for these studies. Tg (APPSwFILon, PSEN1*M146L*L286V) 6799Vas/ Mmjax RRID:MMRRC034848-JAX, was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at The Jackson Laboratory, an NIH-funded strain repository, and was donated to the MMRRC by Robert Vassar, Ph.D., Northwestern University. Mouse-adapted SARS-Related Coronavirus 2 MA10 Variant (in isolate USA-WA1/2020 backbone), Infectious Clone (ic2019-nCoV MA10) in Calu-3 Cells, NR-55329 developed by Baric and colleagues²⁸ was used for infection of experimental mice. Age-matched (10-14 months) 5xFAD and WT mice were intranasally inoculated with 5×10^3 pfu of SARS-CoV-2 MA10 in 50 μ l of 1x PBS or sham inoculated. Infected and uninfected mice were monitored daily for signs of clinical illness.

Histology and immunohistochemical staining: Mice were euthanized at defined times post-infection (p.i.) and brain and lungs were collected. Half brains were fixed in 4% PFA for 24 hours at 4 °C, then transferred into 30% sucrose in 1 X PBS solution for cryoprotection for 48 hours at 4 °C, embedded in O.C.T and 15 μ m sagittal sections were cut via cryostat (Thermo 95 664 OEC70 Micron HM525). Brain sections were desiccated overnight, and slides were rinsed with 1X TBS to remove residual O.C.T. Samples were blocked with 5% normal goat serum and 0.1% Triton-X in TBS, followed by overnight at 4 °C incubation with appropriate primary antibodies. On the second day, slides were treated with appropriate secondary antibodies following TBS rinsing. Amylo-Glo staining (TR-300-AG; Biosensis) was performed according to the manufacturer's instructions. A complete list of antibodies and dilutions is provided in **Table 1**. Lungs were fixed and processed in the same manner as described above and stained with hematoxylin/eosin (H&E).

Imaging and quantitative analysis: High-resolution fluorescent images were obtained at 20X magnification using a Leica TCS SPE-II confocal microscope and LAS-X software. With confocal imaging, one field of view (FOV) per brain region was captured per mouse. Three brain regions per mouse were imaged: cortex (CRTX), dentate gyrus (DG) and subiculum (SUB). Cell counts and plaque burden were quantified using the spots module in Imaris. Volumetric measurements were automatically acquired using the surfaces module with confocal images of each brain region. Super-Resolution Lattice Structure Illumination Microscopy (Lattice-SIM) was performed using an Elyra 7 microscope system. The same three distinct anatomical regions (CRTX, DG, and SUB) per mouse were imaged at 40X magnification. Images were processed using ZEN SIM2 on the ZEN software before being analyzed on Imaris. For quantification of both pre- and post-synaptic puncta (synaptophysin and PSD95), the Imaris spots module was used to count the number of puncta for each z-stack. Z-stacks were 5-8 μm and the number of co-localized spots was normalized to z-stack volume. Overlapping puncta were defined as spots that were $< 0.2 \mu\text{m}$ apart.

RNA extraction and PCR: Mouse tissue was added to TRIzol and homogenized with the Bead Ruptor 12 (Omni International) and 1.4mm ceramic beads (Omni- International, 19-627). RNA was extracted via RNeasy minikit (Qiagen, 74106) using the "Purification of Total RNA, Including Small RNAs, from Animal Tissues" protocol, with Buffer RWT substituted by Buffer RW1. Library preparation, RNA sequencing, and read mapping analysis were performed by Novogene Co. Gene expression values were normalized into log₂ FPKM (fragments per kilobase of transcript per million mapped reads). Heatmaps were created using Morpheus (Morpheus, <https://software.broadinstitute.org/morpheus>). Volcano plots were created using custom code in R. Differentially expressed genes were selected as significant with a log₂(Fold Change) > 0.5 and a

false discovery rate (FDR) <0.05. From the comparisons, lists of genes of interest were chosen to plot a heatmap of their expression and a GO term enrichment analysis using enrichR (<https://amp.pharm.mssm.edu/Enrichr/>).

cDNA synthesis and gene analysis: cDNA was made following the “First Strand cDNA Synthesis” protocol by New England Biolabs, using AMV Reverse Transcriptase (New England Biolabs, M0277L), Random Hexamers (Invitrogen, N8080127), RNase inhibitor (New England Biolabs M0314L) and AMV Buffer (New England Biolabs B0277A). qPCRs were performed using the QIAquant 96 2 plex and iTaq Universal SYBER Green Supermix (BioRad, 1725120). Standard protocol for iTaq Universal SYBER GREEN Supermix was followed. Reactions were 10µl, and the machine was set to run for 1 cycle (95°C for 3 min), followed by 40 cycles (95°C for 15 s, then 58°C for 30 s). Sequences for mouse GAPDH, forward primer: AACTTTGGCATTGTGGAAGG, reverse primer: GGATGCAGGGATGATGTTCT; SARS-CoV-2 Nucleocapsid primers were purchased from Integrated DNA Technologies, forward (IDT, 10006821) and reverse (IDT, 10006822).

Enzyme-linked immunosorbent assay: SARS-CoV-2 spike protein-specific antibodies in plasma of infected mice were measured by ELISA. 96-well plates were coated with 100 ng per well of Recombinant SARS-CoV (2019-nCoV) Spike S1+S2 ECD-His Recombinant Protein # 40589-V08B1 from Sinobiological and incubated overnight at 4°C. Plates were then washed three times with 1X PBS and blocked with 3% BSA in 1X PBS for 1 hour at room temperature. Following blocking, serial dilutions of mouse plasma (100 µl/well) were added and incubated for 2h at RT. Bound antibodies were detected using HRP-conjugated goat anti-Mouse IgG (H+L) Catalog # 31430 from Invitrogen and developed with TMB substrate (ThermoFisher, REF #

34028). The reaction then stopped with Stop reagent for TMB substrate catalog number S514-100ML from Millipore. Plates were read with the ELISA reader at 450nm.

Cytokine profiling using Luminex platform: One aliquot of frozen plasma samples from mice were thawed to perform cytokine profiling using the Luminex Intelliflex platform. We first performed a titration assay, 3 samples from each cohort were used and diluted at 1:25, 1:50, and 1:100. Then, these three dilutions and a neat control were screened using the MILLIPLEX® MAP mouse kit “Milliplex Mouse Cytokine/Chemokine MAGNETIC BEAD Premixed 32 Plex Kit.”, which was the same kit intended for general assay. After measuring analytes and comparing concentrations, neat samples were chosen as the best representatives of concentration values in the samples measured. Hence, plasma samples were used in a neat state (no dilution) in the general assay and 10µl of each sample were assessed in a 384-well plate for cytokine profiling. The Milliplex Mouse Cytokine/Chemokine MAGNETIC BEAD Premixed 32 Plex Kit (G-CSF, EOTAXIN, GMCSF, IFNY, IL1a, IL1b, IL2, IL4, IL3, IL5, IL6, IL7, IL9, IL10, IL12P40, IL12P70, LIF, IL13, LIX, IL15, IL17, IP10, KC, MCP1, MIP1A, MIP1B, MCWF, MIP2, MIG, RANTES, VEGF, TNFA) was used to measure plasma cytokine and chemokines. To adjust the manufacturer’s protocol to our 384-well plate format, all other reagents, including antibodies, magnetic beads, and detection reagents, were used at 10 µL. Outcomes from wells with <50 beads for each analyte were excluded from analysis. Plates were read using a xMAP INTELLIFLEX® System (Luminex). Quality control was performed on cytokine quantification data using the Belysa® Immunoassay Curve Fitting Software (Millipore). This consisted of evaluating standard curves for all 32 analytes and quality control samples (provided in each kit as QC1 and QC2) measured in the experiment. The interpolated concentrations of the plasma samples were exported in an excel file format for statistical analysis. Graph Pad Prism v. 10.5 was used for analysis and

image generation. For statistical analysis a two-way ANOVA was fit to model the log expression of each cytokine. Significant differences were evaluated by group using the overall F test followed by pairwise comparisons under the Tukey's Honest Significant Differences procedure.

Spatial transcriptomic analysis: Formalin-fixed brain hemispheres were embedded in optimal cutting temperature (OCT) compound (Sakura Fintek, 4583) and stored frozen at -80°C. Sagittal sections were cut at 10um the day before starting the protocol on a Leica cryostat (LeicaBiosystems, CM1950). Six brain sections were mounted onto VWR Superfrost Plus slides (Avantor, 48311–703), allowed to dry at room temperature for 15 minutes to promote tissue adherence, and stored overnight at -80°C. Tissue was processed in accordance with the Nanostring CosMx fresh-frozen slide preparation manual for RNA (NanoString University). On day one of the slide preparation protocol, slides were removed from -80°C and baked at 60°C for one hour. Slides were then processed for CosMx: three 5-minute washes in 1X PBS, one 2-minute wash in 4% sodium dodecyl sulfate (Thermo Fisher Scientific, AM9822), : three 5-minute washes in 1X PBS, one 5-minute wash in 50% ethanol, one 5-minute wash in 70% ethanol, and two 5-minute washes in 100% ethanol before allowing slides to air dry at room temperature for 10 minutes. A pressure cooker was used to maintain slides in 100°C for 15 min in preheated 1X CosMx Target Retrieval Solution (Nanostring) for antigen retrieval. Slides were then cooled in DEPC-treated water (Thermo Fisher Scientific, AM9922) for fifteen seconds, transferred to 100% ethanol for three minutes, and then isolated at room temperature for 30 minutes to air dry. For effective tissue permeabilization, slides were transferred to a digestion buffer (3 µg/mL Proteinase K in 1X PBS; Nanostring) to incubate, then washed twice for five minutes in 1X PBS. Fiducials for imaging were diluted to 0.00015% in 2X SSC-T and left for five minutes on slides to incubate. Slides were shielded from light following fiducial application. A 10% neutral buffered formalin solution (NBF;

CAT#15740) was applied for one minute to post-fix tissues. Slides were then washed for five minutes with NBF Stop Buffer (0.1M Tris-Glycine Buffer, CAT#15740) twice and washed for five minutes with 1x PBS. An NHS-Acetate (100 mM; CAT#26777) solution was then administered to the slides and incubated for fifteen minutes at room temperature. Slides were then washed twice for five minutes with 2X SSC and incubated in modified 1000-plex Mouse Neuroscience panel (Nanostring) with the addition of an rRNA segmentation marker for in situ hybridization in a 37°C hybridization oven overnight for 16–18 hours. On day 2 of slide preparation, slides were placed in preheated stringent wash solution (50% deionized formamide [CAT#AM9342]) in 2X saline-sodium citrate [SSC; CAT#AM9763]) at 37°C twice for 25 minutes each. Slides were then washed in 2X SSC twice for two minutes each. Slides were then incubated with DAPI nuclear stain for fifteen minutes, washed for five minutes with 1X PBS, incubated with histone and GFAP cell segmentation markers for one hour, and washed 3 times for five minutes each in 1X PBS. Adhesive flow cells (Nanostring) were placed in each slide to create a fluidic chamber for spatial imaging. Slides were loaded into the CosMx instrument and automatically processed. On each slide, approximately 600 total fields of view (FOVs; ~100 FOVs per brain section) were chosen that captured hippocampal, corpus callosum, upper thalamic, upper caudate, and cortical regions for each of the six sections. Imaging progressed for approximately seven days and data were uploaded automatically to the Nanostring AtoMx online platform. Pre-processed data was exported from AtoMx as a Seurat object for analysis. Spatial transcriptomics datasets were processed with R 4.3.1 software as previously described. Principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) analysis were performed to reduce dataset dimensionality and visualize clustering. Data-driven clustering at 1.0 resolution yielded 46 clusters. Clusters were manually annotated based on transcript expression of known

marker genes and location in XY space. The detection of oligodendrocyte-associated transcripts e.g. *Mbp* in glial clusters can result in “doublets” and reflect the technical limits of spatial segmentation in highly myelinated regions, a common observation in high-resolution spatial platforms. Cell proportion plots were generated by first visualizing the number of cells in each cell type, then scaling relative to 1) normalized percentages per group, calculated by dividing the number of cells in each cell type-group pair by the total number of cells, and 2) dividing by the sum of the proportions across the cell type to account for differences in sample sizes. MAST was used on scaled expression data to perform differential gene expression analysis per cell type between groups to compute the average difference, defined as the difference in log-scaled average expression between the 2 groups for each non-specific cell type. The absolute $\log_2$ fold change values of all genes with statistically significant gene (i.e., $\text{padj} < 0.05$) differential expression patterns between two groups were summed to calculate the DEG scores between group pairs for each subcluster. ggplot2 3.4.4174 was used to generate data visualizations.

Statistical analysis: GraphPad Prism was used to perform statistical analysis on immunohistochemical data. Data were analyzed using two-way ANOVA, and Tukey’s post-hoc tests used to evaluate biologically relevant interactions. Each data set was examined for sex-differences, and if none were found, both male and female data were combined; p-value of <0.05 was considered statistically significant. Data for each experiment is presented as mean $\pm$ SEM.

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CONTRIBUTIONS

S.F., KN.G. and T.E.L. designed the study; R.S.B. provided mouse-adapted SARS-CoV-2 and assisted in experimental design; S.F., L.Z., G.M.O., Y.C., C.P., and K.F. performed experiments and analyzed data; G.P.S., L.Z. and D.A.N. performed and analyzed cytokine/Luminex data; R.A.E. performed histologic analysis of lungs; S.F. and D.I.J. performed bulk RNA sequencing analysis; K.I.T performed spatial transcriptomics; K.I.T., L.Z., D.I.J., and L.L analyzed spatial transcriptomic data. S.F., L.Z. D.I.J. and T.E.L. wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

DATA AVAILABILITY STATEMENT

Protocols, data and results will be available via the AD Knowledge Portal.

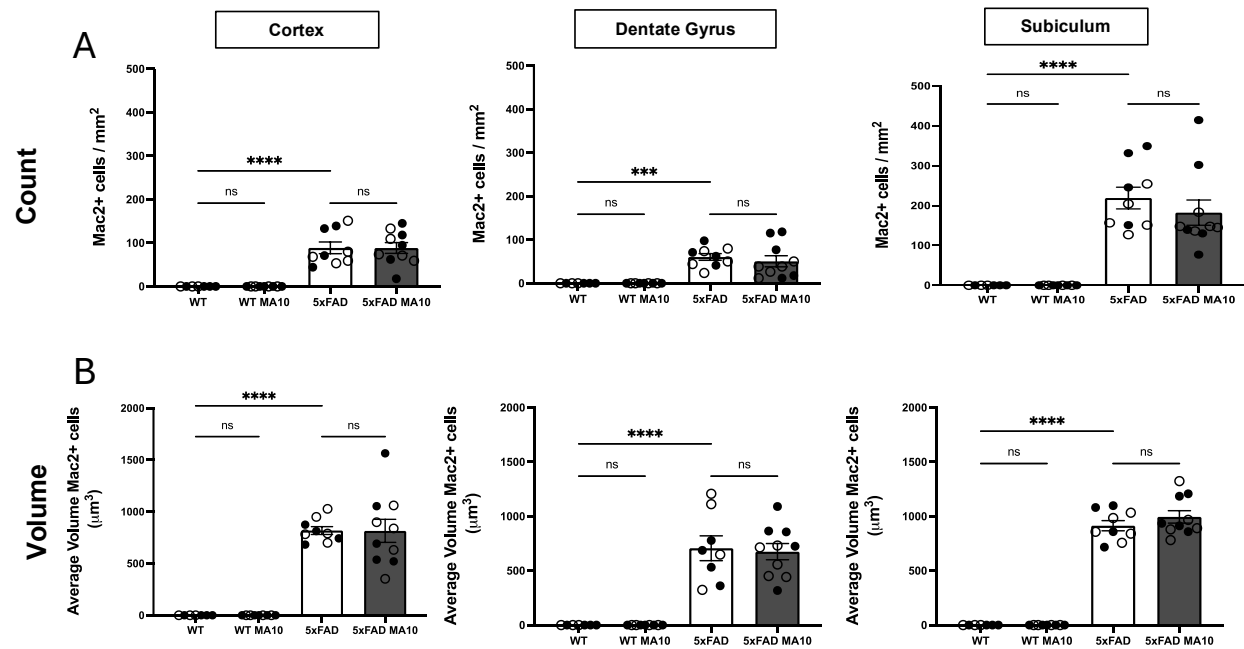
CONSENT STATEMENT

No human subjects were used for the present study. Therefore, consent was not necessary.

SUPPLEMENTARY MATERIALS

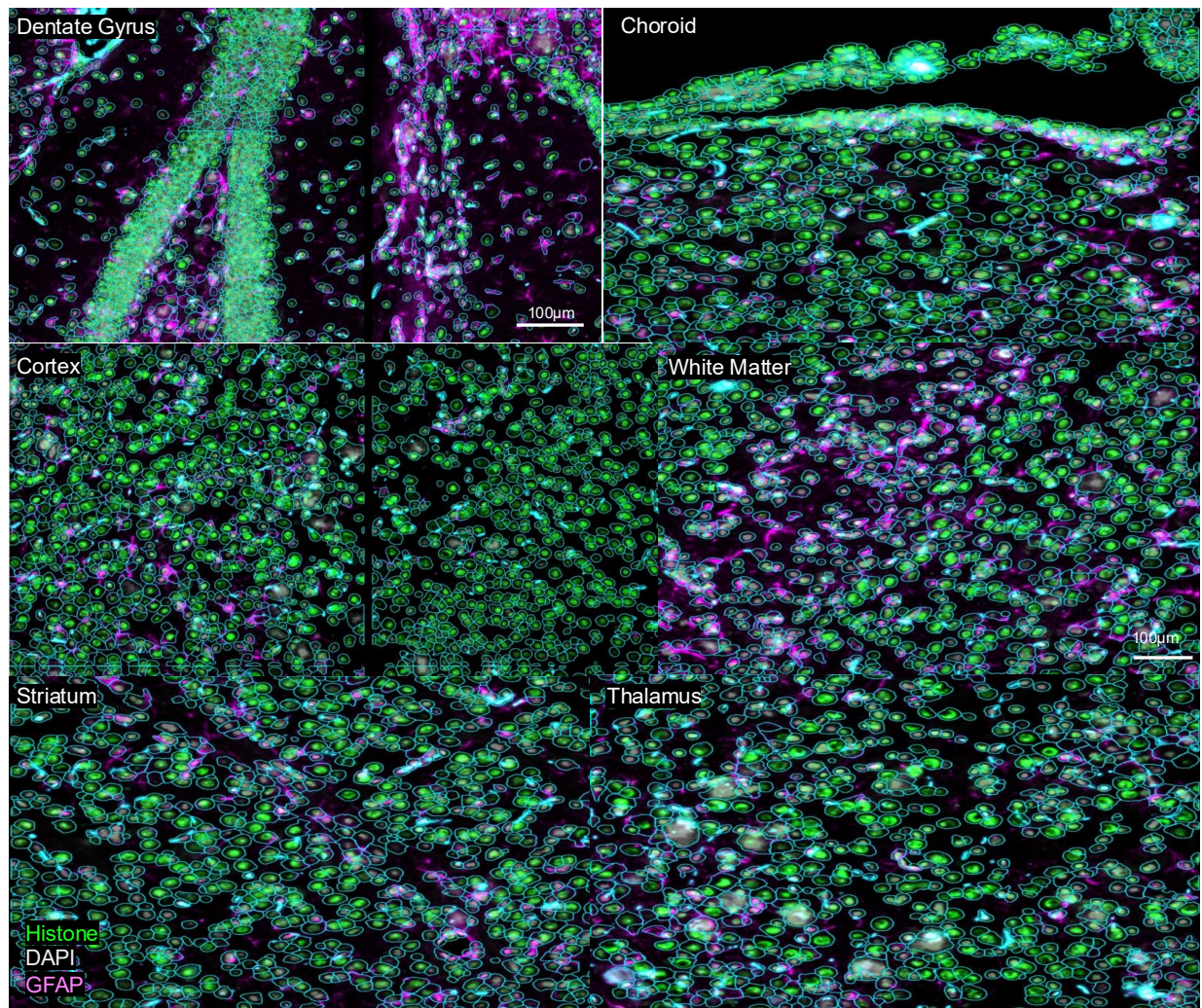
TABLE 1: Primary, secondary antibodies, and associated information

Primary antibody	Source	Catalog #	Dilution	Secondary antibody	Source	Catalog #	Dilution
IBA1	Wako	019-19741	1:500	Goat anti-rabbit IgG, AlexaFluor 488	ThermoFisher	A11008	1:200
GFAP	Abcam	ab134436	1:1000	Goat anti-chicken IgY Alexa Fluor 594	Abcam	ab150172	1:400
Mac2	Cedarlane	CL8942AP	1:500	Goat anti-rat IgG Alexa Fluor 594	Abcam	ab150160	1:200
PSD95	Abcam	ab18258	1:500	Goat anti-rabbit IgG, AlexaFluor 488	ThermoFisher	A11008	1:200
Synaptophysin	Sigma-Aldrich	S5768	1:1000	Goat anti-mouse IgG Alexa Fluor 555	ThermoFisher	A21424	1:200
NeuN	Abcam	ab104225	1:100	Goat anti-rabbit IgG, AlexaFluor 488	ThermoFisher	A11008	1:350
LAMP1	Abcam	ab25245	1:200	Goat anti-rat IgG Alexa Fluor 594	Abcam	ab150160	1:200



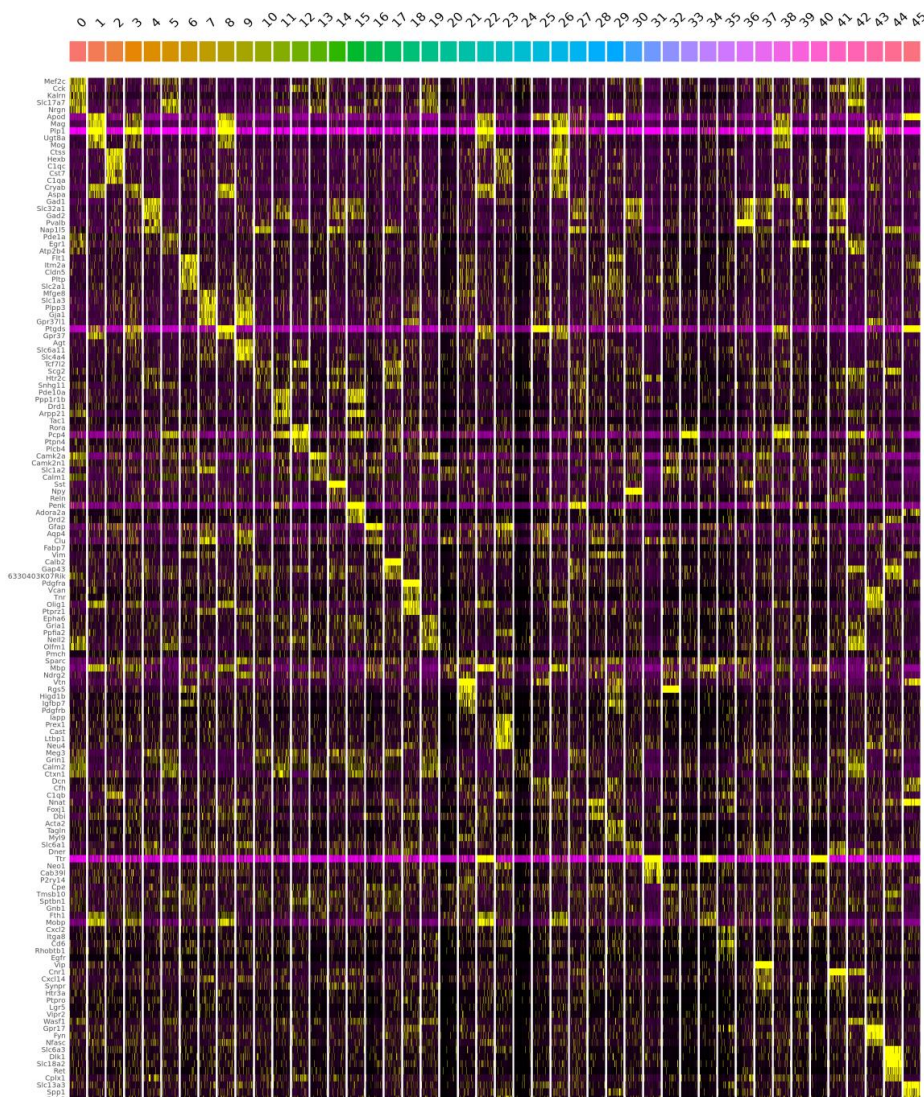
SUPPLEMENTAL FIGURE 1. (A) Quantification of Mac2⁺ cells and their volumes (B) in the cortex, dentate gyrus, and subiculum of MA10-infected WT and 5xFAD mice at day 21 p.i. Immunohistological data were analyzed using two-way ANOVA. Tukey's post-hoc test was employed to examine biologically relevant interactions. Female and male mice are indicated by open or closed circles respectively. Data are represented as mean $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

Cell segmentation examples

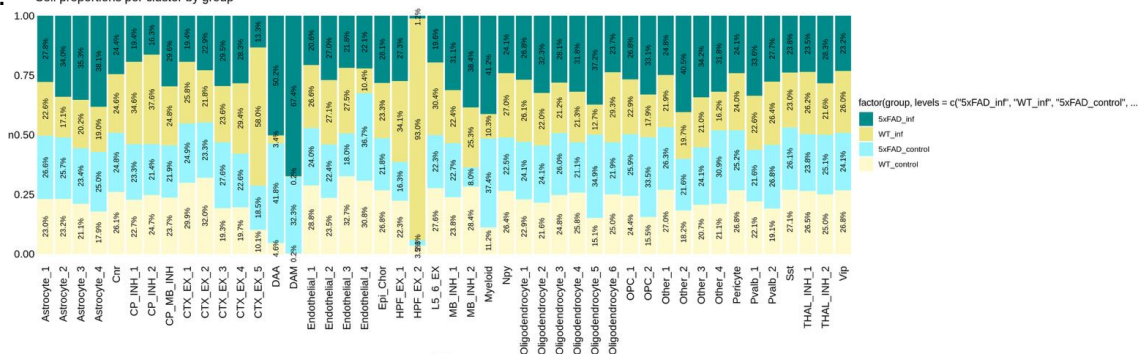


SUPPLEMENTAL FIGURE 3. Representative images demonstrating cell segmentation in dentate gyrus, choroid plexus, cortex, white matter, striatum and thalamus. Cells were imaged with rRNA (not shown), histone, DAPI, and GFAP markers and segmented automatically.

A. Top 5 marker genes by cluster

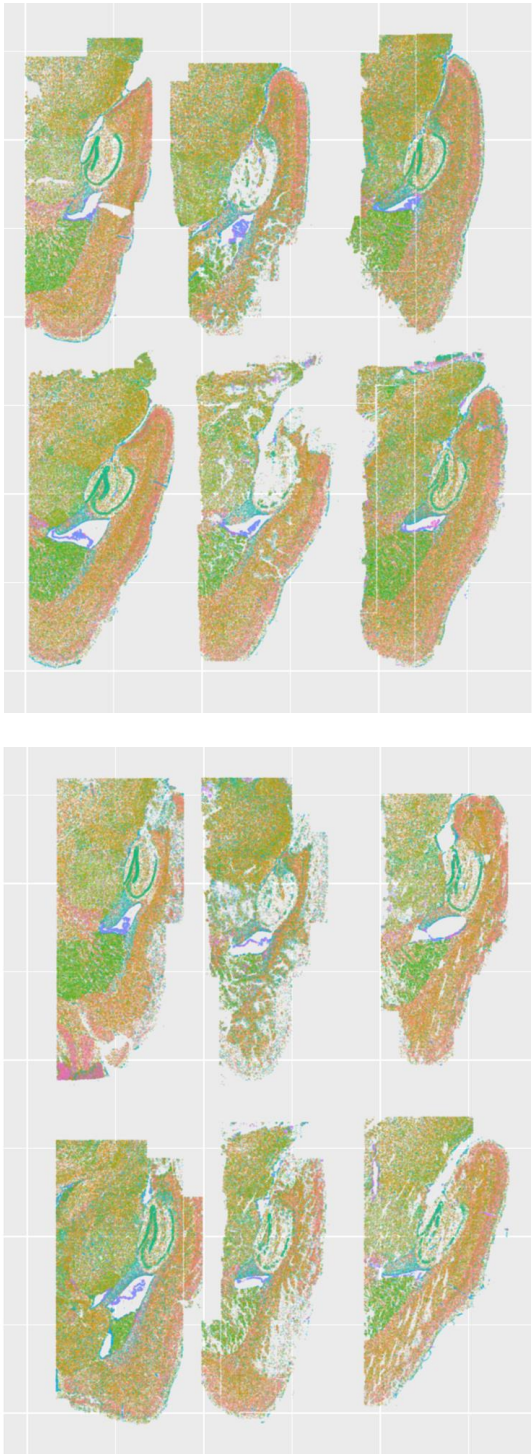


B. Cell proportions per cluster by group



SUPPLEMENTAL FIGURE 4. (A) Heatmap of top 5 marker genes for all spatial transcriptomics subclusters. **(B)** Proportional distribution of Seurat clusters across experimental groups. Bar plots display relative abundance of each cluster in each group.

Cell clusters in XY space, all brains

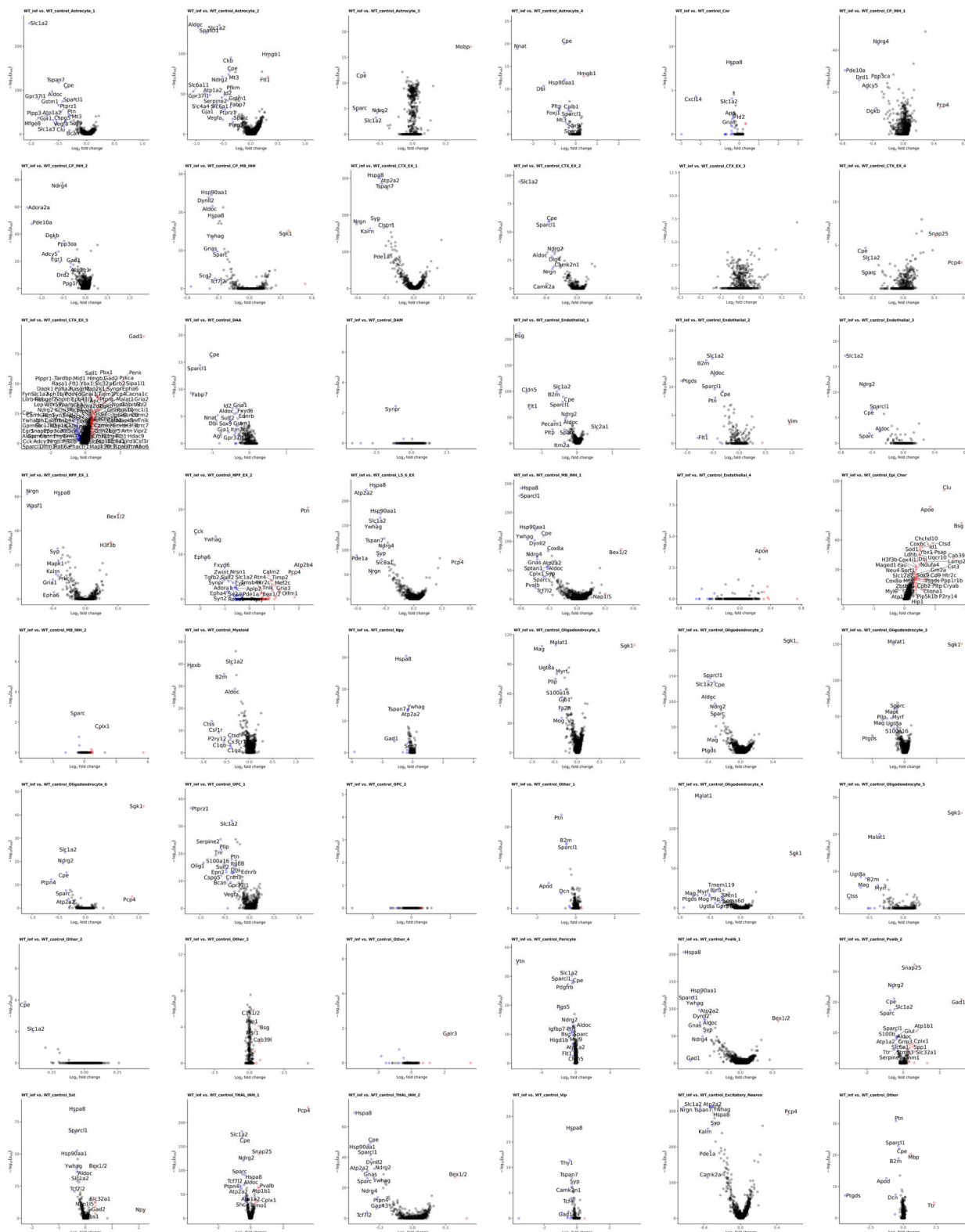


Cluster	Cell type	Sub-type
0	Excitatory Neuron	CTX EX 1
1	Oligodendrocyte	Oligodendrocyte 1
2	Myeloid	Myeloid
3	Oligodendrocyte	Oligodendrocyte 2
4	Inhibitory Neuron	Pvalb 1
5	Excitatory Neuron	L5 6 EX
6	Vascular	Endothelial 1
7	Astrocyte	Astrocyte 1
8	Oligodendrocyte	Oligodendrocyte 3
9	Astrocyte	Astrocyte 2
10	Inhibitory Neuron	MB INH 1
11	Inhibitory Neuron	CP INH 1
12	Inhibitory Neuron	THAL INH 1
13	Excitatory Neuron	CTX EX 2
14	Inhibitory Neuron	Sst
15	Inhibitory Neuron	CP INH 2
16	Astrocyte	DAA
17	Inhibitory Neuron	THAL INH 2
18	OPC	OPC 1
19	Excitatory Neuron	HPF EX 1
20	Astrocyte	Astrocyte 3
21	Vascular	Pericyte
22	Oligodendrocyte	Oligodendrocyte 4
23	Myeloid	DAM
24	Excitatory Neuron	CTX EX 3
25	Other	Other 1
26	Oligodendrocyte	Oligodendrocyte 5
27	Inhibitory Neuron	CP MB INH
28	Astrocyte	Astrocyte 4
29	Vascular	Endothelial 2
30	Inhibitory Neuron	Npy
31	Epithelial	Epi Chor
32	Vascular	Endothelial 3
33	Excitatory Neuron	CTX EX 4
34	Other	Other 2
35	Vascular	Endothelial 4
36	Inhibitory Neuron	Pvalb 2
37	Inhibitory Neuron	Vip
38	Oligodendrocyte	Oligodendrocyte 6
39	Excitatory Neuron	CTX EX 5
40	Other	Other 3
41	Inhibitory Neuron	Cnr
42	Excitatory Neuron	HPF EX 2
43	OPC	OPC 2
44	Inhibitory Neuron	MB INH 2
45	Other	Other 4



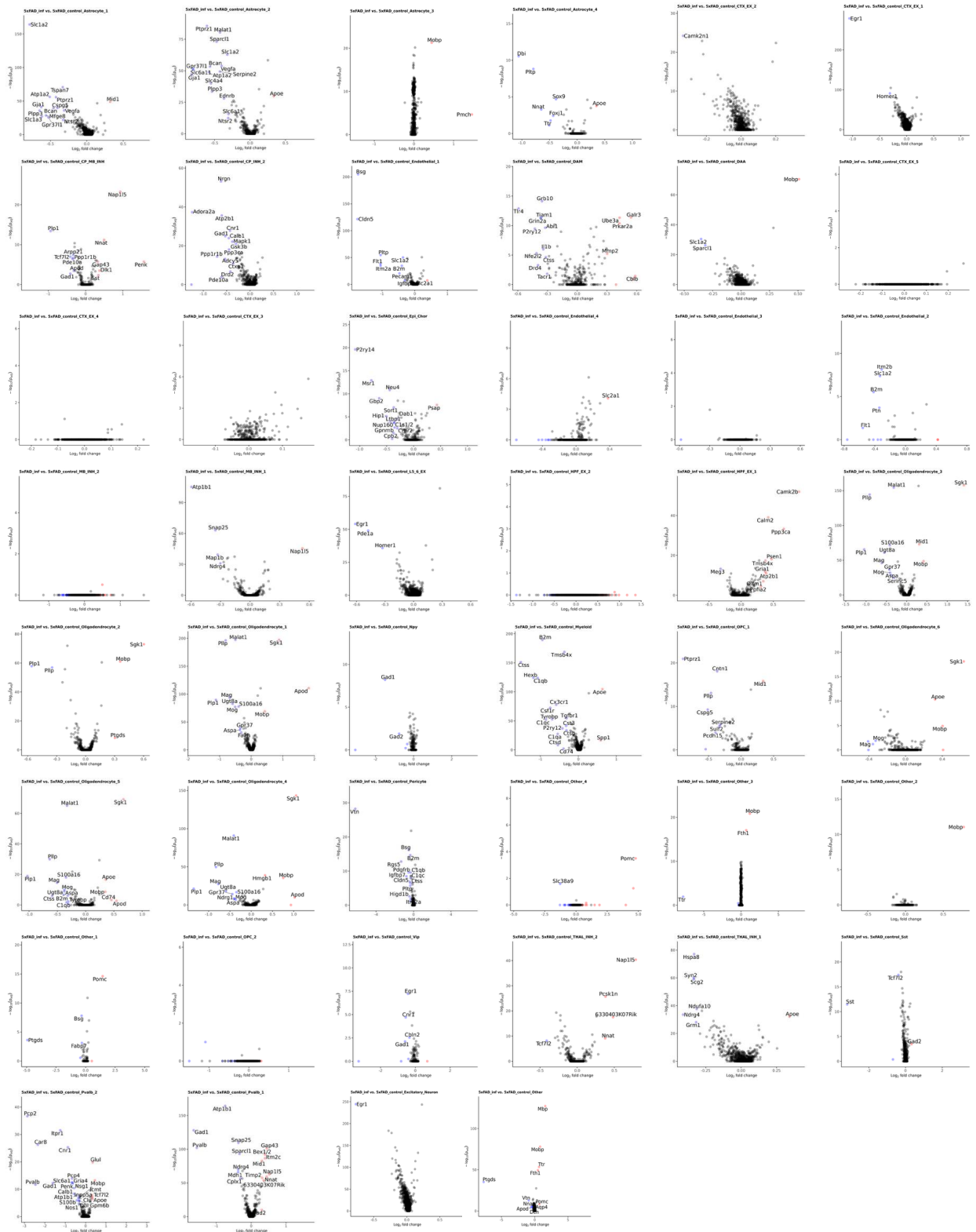
SUPPLEMENTAL FIGURE 5. 46 clusters plotted in XY space in all 6 brains from WT (n=3) and 5xFAD (n=3) mice, both infected and uninfected, at day 21 p.i. (n=3/group).

Infected WT vs. con WT



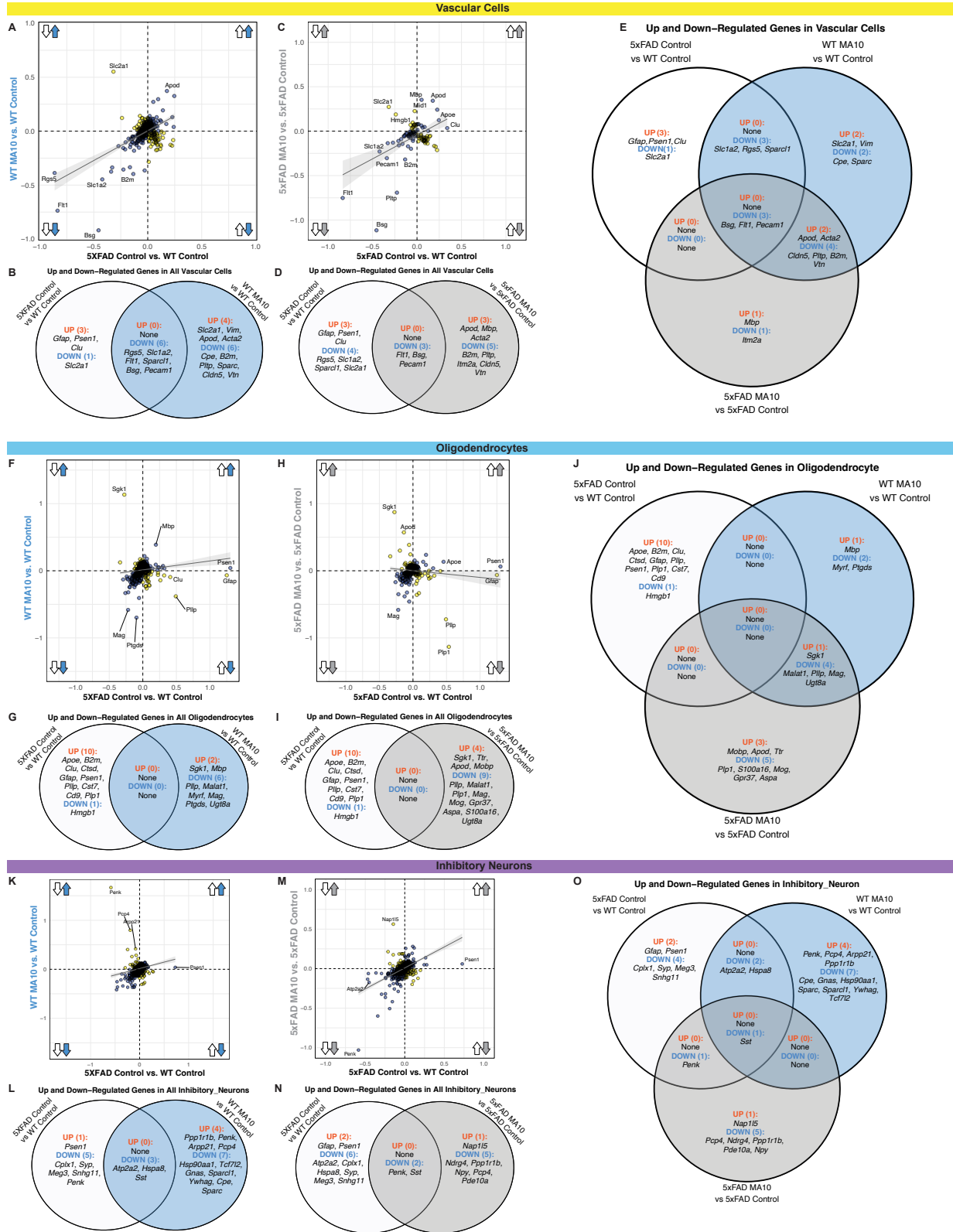
SUPPLEMENTAL FIGURE 6. Volcano plots for all spatial transcriptomics subcluster in MA10 infected WT vs Control WT.

Infected 5xFAD vs. con 5xFAD



SUPPLEMENTAL FIGURE 7. Volcano plots for all spatial transcriptomics subcluster in MA10 infected 5xFAD vs Control 5xFAD.

SUPPLEMENTAL FIGURE 8. Investigating gene dysregulation within vascular cells, oligodendrocytes and inhibitory neurons the presence of amyloid pathology, peripheral MA10 infection, or both concurrently. (A) Scatterplot of the average difference of all vascular cells for all significant genes between 5xFAD Control vs. WT Control and WT MA10 vs. WT Control. (B) Venn diagram depicting the significant up- and down-regulated genes in vascular cells unique to 5xFAD Control vs. WT Control (left, white) and WT MA10 vs. WT Control (right, blue), while demonstrating shared dysregulated genes between the two comparisons. (C) Scatterplot of the average difference of all vascular cells for all significant genes between 5xFAD Control vs. WT Control. (D) Venn diagram depicting the significant up- and down-regulated genes in vascular cells unique to 5xFAD Control vs. WT Control (left, white) and 5xFAD MA10 vs. 5xFAD Control (right, grey), while demonstrating shared dysregulated genes between the two comparisons. (E) Three-way Venn diagram of all significantly up- and down-regulated genes within vascular cells between the three comparisons: 5xFAD Control vs. WT Control (white), WT MA10 vs. WT Control (blue), and 5xFAD MA10 vs. 5xFAD Control (grey). (F) Scatterplot of the average difference of all oligodendrocytes for all 50 significant genes ($p_{adj} < 0.05$) between 5xFAD Control vs. WT Control (x-axis, only amyloid pathology) and WT MA10 vs. Control WT (y-axis, only MA10 infection) comparisons. Arrows indicate direction of dysregulation for each comparison. Directly correlated genes (blue) occur in the same direction for both comparisons (i.e., both up-regulated or both down-regulated), while inversely correlated genes (orange) occur in opposite directions for each comparison. Linear regression line demonstrates the relationship between the two comparisons. (G) Venn diagram depicting the significant up- and down-regulated genes unique to the 5xFAD Control vs. WT Control (left, white) and WT MA10 vs. WT Control (right, blue), while demonstrating up- and down-regulated genes commonly shared across the two comparisons (middle). (H) Scatterplot of the average difference of all oligodendrocytes for all significant genes between 5xFAD Control vs. WT Control, now with 5xFAD MA10 vs. 5xFAD Control (y-axis, both amyloid and MA10 infection). Red text demonstrates genes up-regulated in the same direction in the first scatterplot, but down-regulated in the MA10 5xFAD vs. Control 5xFAD comparison. (I) Venn diagram depicting the significant up- and down-regulated genes unique to 5xFAD Control vs. WT Control and 5xFAD MA10 vs. 5xFAD Control (right, purple), while demonstrating up- and down-regulated genes commonly shared between the two comparisons. (J) Three-way Venn diagram of all significantly up- and down-regulated genes within oligodendrocytes between the three comparisons: 5xFAD Control vs. WT Control (white), WT MA10 vs. WT Control (blue), and 5xFAD MA10 vs. 5xFAD Control (grey). Genes are considered significantly correlated if the log₂ Fold Change magnitude is greater than 0.3. (K) Scatterplot of the average difference of all inhibitory neurons for all significant genes between 5xFAD Control vs. WT Control and WT MA10 vs. WT Control. (L) Venn diagram depicting the significant up- and down-regulated genes in inhibitory neurons unique to 5xFAD Control vs. WT Control (left, white) and WT MA10 vs. WT Control (right, blue), while demonstrating shared dysregulated genes between the two comparisons. (M) Scatterplot of the average difference of all inhibitory neurons for all significant genes between 5xFAD Control vs. WT Control. (N) Venn diagram depicting the significant up- and down-regulated genes in inhibitory neurons unique to 5xFAD Control vs. WT Control (left, white) and 5xFAD MA10 vs. 5xFAD Control (right, grey), while demonstrating shared dysregulated genes between the two comparisons. (O) Three-way Venn diagram of all significantly up- and down-regulated genes within inhibitory neurons between the three comparisons: 5xFAD Control vs. WT Control (white), WT MA10 vs. WT Control (blue), and 5xFAD MA10 vs. 5xFAD Control (grey).



CHAPTER 5

ACUTE VIRAL ENCEPHALITIS IMPACTS DENSE-CORE AMYLOID PLAQUE PATHOLOGY AND DYSREGULATES MYELOID RESPONSES TO AMYLOID PLAQUES

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Acute viral encephalitis impacts dense-core amyloid plaque pathology and dysregulates myeloid responses to amyloid plaques

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ABSTRACT

Background: Recent epidemiological datasets have associated viral encephalitis exposure (i.e., viral-induced neuroinflammation) with increased risk of Alzheimer's disease (AD) and dementia, highlighting the need to uncover how it may impact AD neuropathology.

Methods: Aged 5xFAD and wildtype (WT) mice were infected with JHMV, a neurotropic strain of murine coronavirus to comprehensively determine how coronavirus-induced encephalitis may induce molecular and cellular changes that impact A β neuropathology.

Results: JHMV-induced encephalitis at 12 days post-infection resulted in minimal changes to overall A β protein, despite increased CD4⁺ and CD8⁺ T cell infiltration and *Lgals3*/MAC2-expressing macrophages surrounding more compact A β plaques in the brain. Spatial transcriptomic imaging and pathway analysis of differentially expressed genes (DEGs) within myeloid cells demonstrate down-regulated disease-associated (DAM) pathways involving A β clearance, response to lipids, and macrophage activation within infected 5xFAD brains.

Conclusions: JHMV encephalitis induces dysregulated gene expression and myeloid cell responses to A β plaque burden in 5xFAD mouse brains.

Keywords: *Viral encephalitis, coronavirus infection, Alzheimer's disease, myeloid cells, spatial transcriptomic imaging, amyloid-beta neuropathology, 5xFAD*

INTRODUCTION

Alzheimer's Disease (AD) is the most common cause of dementia impacting almost 7 million people in the United States with cases projected to increase over 13 million by 2050^{1,2}. As a progressive neurodegenerative disease, AD-associated neuropathology include widespread aggregation of neuritic beta-amyloid (A β) plaques and neurofibrillary tau tangles (NFTs), which are proposed to occur consecutively or work synergistically towards neuroinflammation, neuronal loss, and eventual cognitive decline^{5,6}. Genome-wide association studies (GWAS) have been a critical tool to determine polymorphic variants within alleles that increase genetic risk and identify physiological processes contributing to AD pathogenesis in late-onset AD⁸⁸. Numerous large GWAS have repeatedly identified AD risk genes associated with immunity and specifically expressed in myeloid cells, implicating neuroimmune processes and neuroinflammation as a major factor of AD etiology. Myeloid cells within the central nervous system (CNS) are primarily comprised of two distinct populations: CNS-resident microglia and monocyte-derived macrophages. Many studies have demonstrated a close association between A β plaques and microglia, which are considered the principal immune cell within the CNS. Once activated, microglia undergo a morphological and functional change into rounder, amoeboid shapes with short, thick processes that extend out to enable migration towards the inflammatory stimulus, cytokine secretion, and phagocytosis^{127,129}.

Microbial infection has been broadly explored as a mechanism through which both genetic and environmental contributions interact together to influence AD risk and neuropathology. Pathogenic microbes that invade the CNS can cause significant neuroinflammation through CNS-resident immune cells and infiltration of peripheral immune cells such as monocyte/macrophages and T cells. To illustrate the association of viral infection with several neurodegenerative diseases,

Levine et al.¹²⁵ mined medical record data from both the Finnish biobank (FinnGen) and UK Biobank. Their findings demonstrated several viral pathogens tightly associated with different neurological diseases, while the strongest association was between AD and viral encephalitis¹²⁵. Viral encephalitis is defined as viral-mediated inflammation of the brain parenchyma. While the blood-brain-barrier (BBB) prevents most viral pathogens from neuroinvasion, neurotropic viruses can circumvent these barriers to infect and replicate within the CNS¹¹². The resulting inflammation via indirect neuroimmune mechanisms may induce acute and chronic neurologic damage with potential consequences in the onset or severity of neurodegenerative disease^{113,167,168}. Therefore, it is unsurprising that majority of identified viral pathogens associated with neurodegenerative disease and dementia are neurotropic (i.e., herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein-Barr Virus, and flaviviruses) and systemic infections leading to viral encephalitis (i.e., human immunodeficiency virus (HIV) and severe acute respiratory syndrome-Coronavirus 2 (SARS-CoV-2)^{186,229,230}. While the field's current understanding of the contributions of these viruses and their resulting inflammation on AD remains nuanced and complex, ongoing investigations are required to precisely identify direct and indirect viral mechanisms and inflammatory pathways impacting AD neuropathology.

The neurotropic John Howard Mueller (JHM) strain of murine hepatitis virus (JHMV, a member of the *Betacoronaviridae* genus) has previously been utilized to model molecular and cellular mechanisms of host defense and disease in response to viral encephalitis within the CNS^{231,232}. Intracranial inoculation of susceptible mice with JHMV results in an acute encephalomyelitis characterized by wide-spread replication of virus in glia with relative sparing of neurons. The resulting innate immune responses include expression of type I interferon, which limit the spread of virus early following infection. Ultimately, virus-specific CD4⁺ and CD8⁺ T

cells are attracted into the CNS in response to T cell chemoattractant chemokines and control viral replication via expression of anti-viral cytokines including IFN- γ and cytolytic activity^{233,234}. However, sterile immunity is not achieved and virus will persist within white matter tracts, where immune-mediated demyelination will occur due to chronic infiltration of macrophages and T cells^{235,236}. Based upon evidence that neuroinflammation impacts AD-associated neuropathology and that viral encephalitis is now recognized as a major risk factor associated with AD, this study utilizing JHMV infection of aged C57BL/6 and 5xFAD mice to determine the molecular and cellular mechanisms by which viral encephalitis impacts existing AD pathology and neuroinflammation.

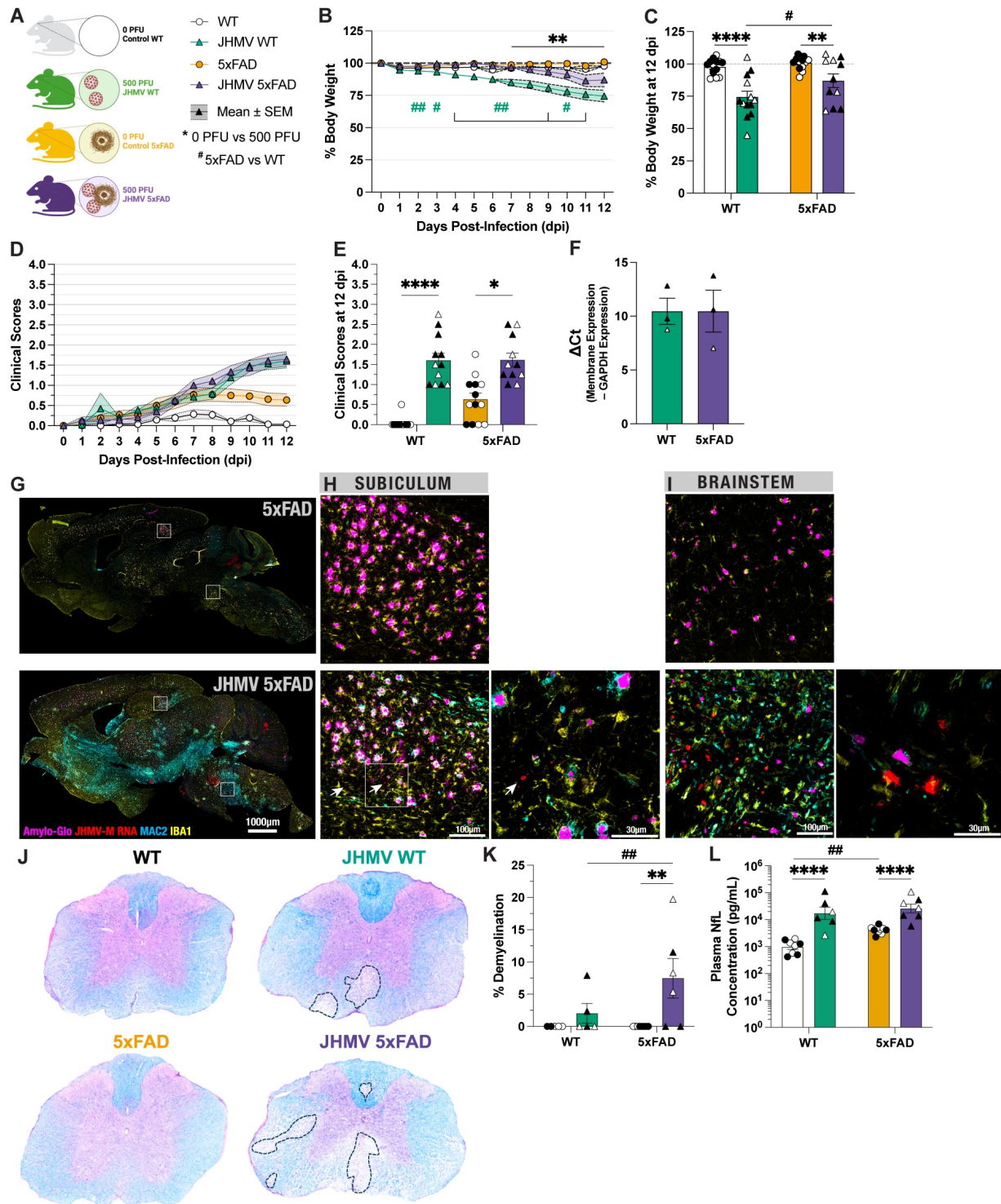
RESULTS

5xFAD mice exhibit similar weight loss, mild clinical disease, and control of viral replication within the CNS following intracranial JHMV infection: To determine whether familial AD mutations and its ongoing neuroinflammation impacted morbidity following JHMV infection of the CNS, we utilized 10-month-old 5xFAD and wildtype (WT) C57BL/6 mice to measure viral replication and onset of neurological disease during acute JHMV-mediated encephalitis at 12 days post-infection (p.i) (**Figure 1A**). By 12 days p.i., both JHMV-infected WT mice and JHMV-infected 5xFAD mice had significant reductions in body weight compared to uninfected controls (**Figures 1B&C**, $p<0.0001$ and $p<0.01$, respectively). When comparing the infected groups together, JHMV-infected wildtype mice had greater body weight loss compared to JHMV-infected 5xFAD mice ($p<0.05$). When considering clinical disease severity assessed by hindlimb paralysis, both JHMV-infected WT and JHMV-infected 5xFAD mice displayed similar progression of motor impairment and clinical disease by 12 days p.i. (**Figure 1D**). By 12 days p.i., both JHMV-infected WT and JHMV-infected 5xFAD mice had significant progressive motor impairments compared to their uninfected controls (**Figure 1E**, $p<0.0001$ and $p<0.05$, respectively). Using qPCR to quantify levels of viral RNA in infected brains by 12 days p.i., no significant differences were observed between JHMV-infected WT and JHMV-infected 5xFAD, suggesting similar control over viral replication at 12 days p.i. (**Figure 1F**). This is corroborated in younger 6-month-old WT and 5xFAD mice, which also displayed similar levels of viral RNA via qPCR by 10-14 days p.i. (**Supplementary Figure 1A**).

JHMV viral RNA are present across similar brain regions in the CNS of infected WT and 5xFAD mice: Despite effective viral clearance, sterile immunity is not achieved and viral antigen and RNA will continue to persist within white matter of the CNS. Using antibodies against

JHMV nucleocapsid protein, histological staining revealed presence of viral antigen within white matter regions (e.g., brainstem and peduncles) in addition to the subiculum hippocampal region within JHMV-infected 5xFAD brains at 12 days p.i. (**Supplementary Figure 1B-C**). Notably, viral antigen is not observed in somatosensory cortical regions. In addition to viral antigen, *in situ* hybridization (ISH) via RNAscope also reveals the widespread active replication of viral RNA throughout the wildtype brain at 7 days p.i. within specific regions where viral antigen is present, specifically the subiculum and brainstem (**Supplementary Figure 1D-F**). This is also corroborated in JHMV-infected 5xFAD brains, which reveal a lingering presence of JHMV RNA in the subiculum region, but more so in the brainstem (**Figure 1G-I**). Despite similar viral control efficacy, JHMV-infected 5xFAD mice exhibited greater immune-mediated demyelination in Luxol-Fast Blue (LFB)-stained spinal cord sections at 12 days p.i. compared to JHMV-infected

FIGURE 1. JHMV infection increases clinical disease severity and spinal cord demyelination by 12 days p.i. **A)** Schematic of each experimental group. **B)** Line chart depicting changes in initial body weights across each day post-infection for uninfected WT (white), JHMV-infected WT (green), uninfected 5xFAD controls (yellow), and JHMV-infected 5xFAD mice (purple). **C)** At 12 dpi, percent changes in body weight were compared across each experimental group. **D)** Line chart depicting the progression of clinical disease severity and hindlimb paralysis across each day post-infection. **E)** At 12 dpi, clinical disease scores were compared across each experimental group. **F)** qPCR with extracted RNA from brain homogenates demonstrate similar transcript levels of viral membrane protein RNA between JHMV-infected WT and 5xFAD brains at 12 dpi. **G)** Representative whole-brain scanned images of sagittal brain sections from uninfected and JHMV-infected 5xFAD brains co-stained with Amylo-Glo, MAC2, and IBA1 in addition to *in situ hybridization* targeting JHMV RNA. Representative 20X and 63X confocal images of subiculum (**H**) and brainstem regions (**I**) in brain sections of uninfected and JHMV-infected 5xFAD mice. Fluorescent signals of viral RNA are indicated with white arrows. **J)** Representative images of H&E/Luxol Fast Blue-stained spinal cord sections across each experimental group. Dashed lines demarcate areas of demyelination. **K)** The percentage of demyelination was compared across each experimental group. **L)** Plasma concentration of NfL protein in blood plasma was compared across each experimental group. n=7-10 per group. Data is presented as mean $\pm$ SEM. Unpaired t-tests was used when appropriate and two-way ANOVA followed-by Holm-Sidak post-hoc tests were performed to examine biological relevant interactions. Clinical disease scores were compared using Kruskal-Wallis tests followed by Dunn's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Males are represented with closed symbols and females are represented with open symbols.

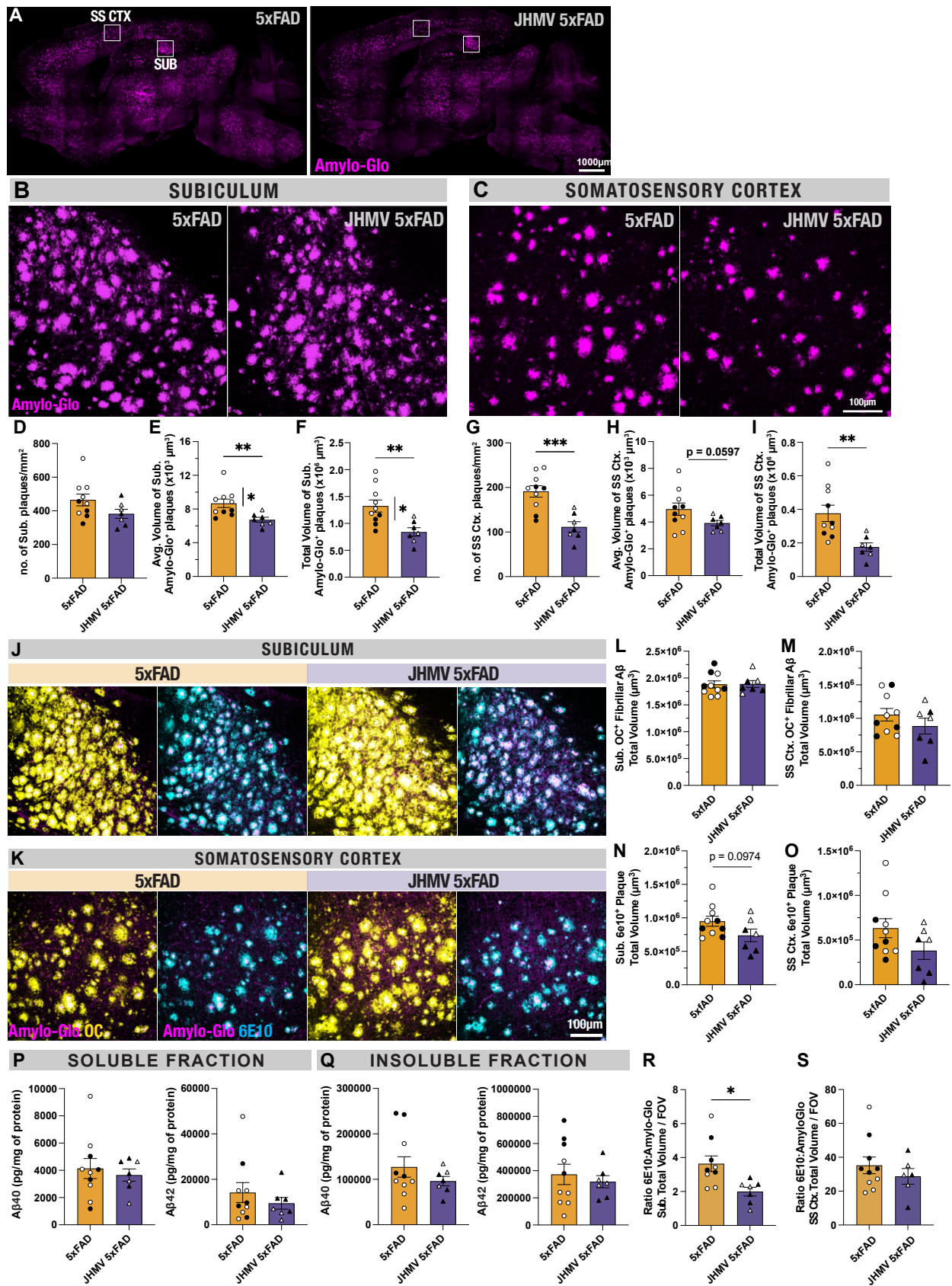


WT mice (**Figure 1J-K**, $p < 0.01$). To assess levels of neuronal damage resulting from JHMV infection, we measured concentration of plasma neurofilament light chain (NfL). In line with previous studies, we found significant increases in plasma NfL protein concentrations in 5xFAD

mice compared to WT mice (**Figure 1L**). Additionally, we found that JHNV infection further increased plasma NfL concentration in JHNV-infected WT and 5xFAD compared to their respective, uninfected controls (**Figure 1L**).

Dense-core A β plaque burden is reduced in JHNV-infected 5xFAD mice in subiculum and somatosensory cortical regions: The significance of viral RNA present in the subiculum compared to the somatosensory cortex may suggest that areas of viral RNA persistence may influence A β plaque deposition in 5xFAD mice^{131,237}. In JHNV-infected 5xFAD brains, Amylo-Glo staining for dense-core A β plaques revealed significant reductions in A β plaque volume in the subiculum (**Figure 2A-F**, $p < 0.01$). However, in the somatosensory cortex where viral RNA was not observed, JHNV infection significantly reduced the number of dense-core A β plaques with only a trending reduction in the average volume of Amylo-Glo⁺ dense-core plaques (**Figure 2G-I**).

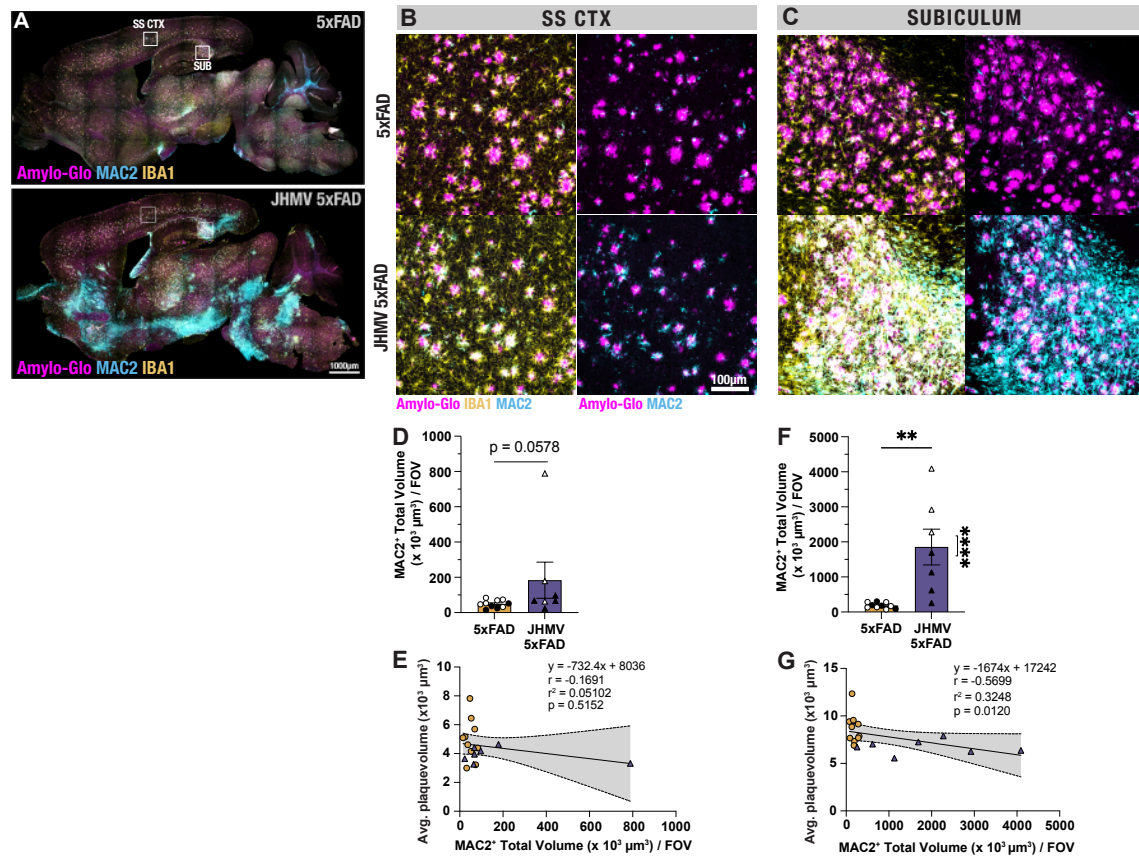
FIGURE 2. JHNV infection reduces dense-core amyloid plaque pathology, but not overall A β burden. A β plaque burden was assessed using Amylo-Glo staining for dense-core plaques in 30 μ m sagittal brain sections. **A**) Representative whole-brain images of 12dpi JHNV-infected and control 5xFAD mouse brains stained with Amylo-Glo. Representative 20X confocal images of subiculum (**B**) and somatosensory cortex (**C**) depicting Amylo-Glo⁺, dense-core A β plaques. Quantification of plaque densities (**D, G**), individual plaque volumes (**E, H**), and average plaque volumes (**F, I**) with the field-of-view (FOV) of JHNV-infected and control 5xFAD brain sections. Representative confocal images of brain sections costained with Amylo-Glo, conformation-specific OC antibody against amyloid fibrils, and 6E10 antibody against A β ₁₋₁₆ residues in subiculum (**J**) and somatosensory cortex (**K**) of uninfected and JHNV-infected 5xFAD mice. Quantification of OC fibrillar A β volumes around dense-core plaques in subiculum (**L**) and somatosensory cortex (**M**). Quantification of 6E10⁺ diffuse plaque volumes in subiculum (**N**) and somatosensory cortex (**O**). Protein concentration of A β 40 and A β 42 within detergent-soluble fractions (**P**) or insoluble fractions (**Q**) of frozen cortex homogenates. (**R**) Calculated ratio of 6E10⁺ diffuse A β total volume to Amylo-Glo⁺ dense-core plaque total volume in the subiculum. (**S**) Calculated ratio of 6E10⁺ diffuse A β total volume to Amylo-Glo⁺ dense-core plaque total volume in the somatosensory cortex. $n = 7-10$ per group. Data is presented as mean $\pm$ SEM. Unpaired t-tests were used to examine statistically significant differences between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Males are represented with closed symbols and females are represented with open symbols



To assess the role of JHNV infection on plaque composition, we utilized conformation-specific OC antibody against fibrillar A β and 6E10 antibody against A β ₁₋₁₆ peptides to visualize diffuse plaque structures associated with increased neurotoxicity¹³⁰. Interestingly, staining for OC⁺ fibrillar A β surrounding the denser plaques revealed no changes in either 5xFAD subiculum or somatosensory cortex regions following JHNV-infection (**Figure 2J, L-M**). Staining for A β ₁₋₁₆ residues with 6E10 demonstrated similar results, with only trending reductions in 6E10⁺ diffuse plaque volume within the subiculum following JHNV infection (**Figure 2K, N-O**). This is further corroborated by biochemical analysis demonstrating no differences in soluble and insoluble A β 40 and A β 42 protein concentration in cortex homogenates of JHNV-infected and control 5xFAD brains via MULTI-ARRAY assay (Mesoscale Discovery) (**Figure 2P-K**). Interestingly, we observed a reduced ratio of diffuse 6E10⁺ plaques to dense-core Amylo-Glo⁺ plaques in subiculum regions of JHNV-infected 5xFAD mice compared to uninfected controls (**Figure 2R-S**), indicating an increased compaction of diffuse A β peptides into plaques following infection^{130,238}. Overall, these results demonstrate JHNV infection and its resulting encephalitis may distinctly impact dense-core plaques as opposed to overall A β burden in the 5xFAD mouse brain.

MAC2⁺ macrophages infiltrate into the CNS following JHNV infection and strongly correlate with brain regions demonstrating reduced A β plaque burden: We have previously demonstrated that inflammatory *Lgals3*/MAC2-expressing monocyte/macrophages migrate to areas of viral replication in response to chemokine expression using a combination of single-cell RNA sequencing and histology¹⁵⁰. Immunostaining *Lgals3*/MAC2 in JHNV-infected 5xFAD brains demonstrated high infiltration of peripheral monocyte/macrophages in the same brain regions where JHNV viral antigen is present, namely the subiculum, peduncles, and brainstem (**Figure 3A**). Representative confocal images of AD-relevant brain regions like the somatosensory

cortex and subiculum also illustrated localized interactions between MAC2⁺ and A β plaques (Figure 3B-C). Moreover, quantifying the activation state MAC2⁺ cells via assessment of cell volume within AD-relevant regions revealed increased volumes of MAC2⁺ cells within the



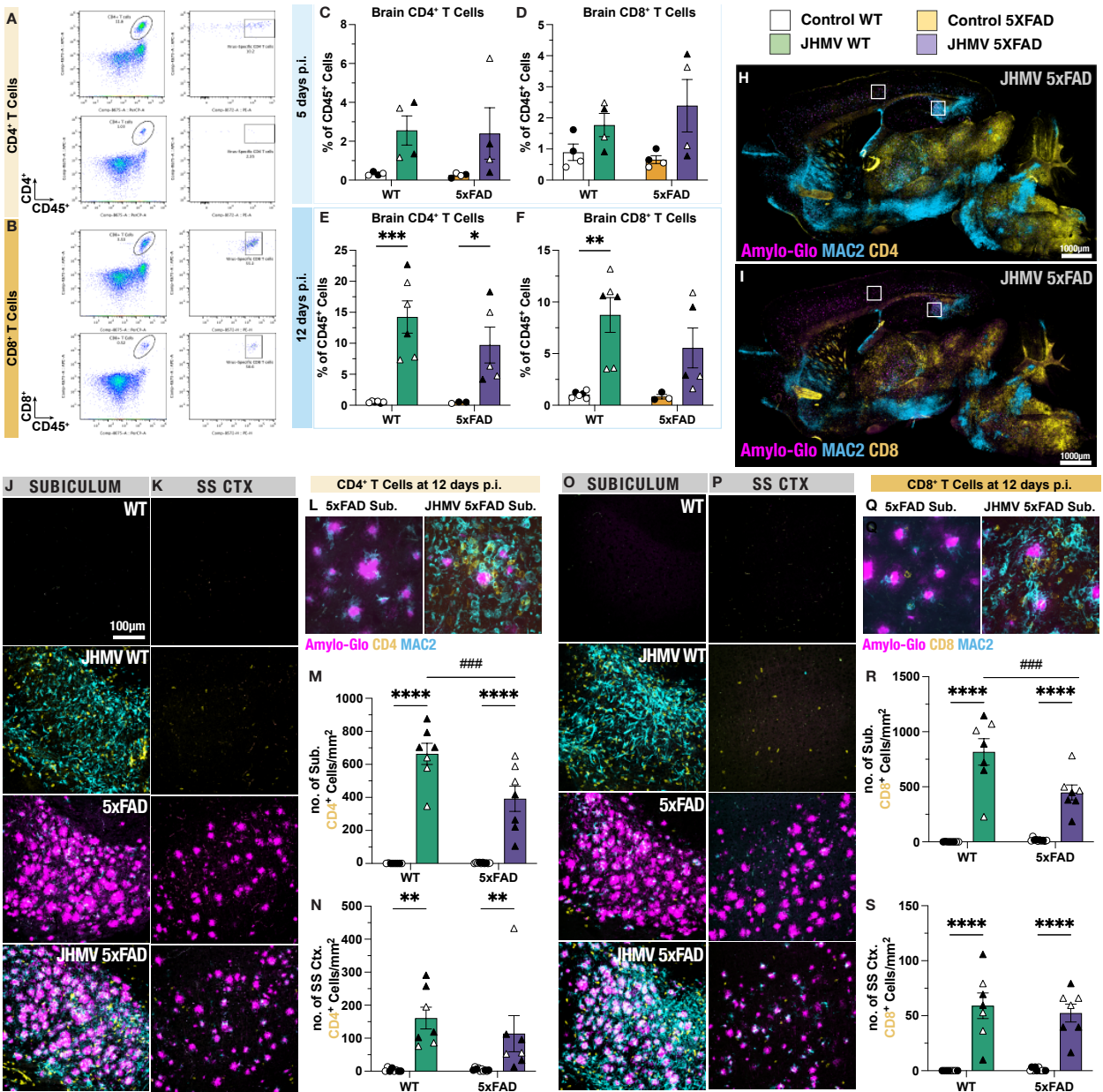
somatosensory cortex ($p<0.0578$) and a significant increase ($p<0.01$) in volume of these cells in the subiculum (**Figures 3D&F**). Moreover, there was a significant ($p<0.05$) correlation between enriched MAC2⁺ infiltration within the subiculum and associated with reduced A β plaque volumes (**Figure 3G**, $r^2=0.3248$, $p=0.0120$). These findings suggest that inflammatory monocyte/macrophages that are recruited into the CNS in response to JHNV replication during acute disease contribute to reduced morphology of dense-core A β plaques.

CD4⁺ and CD8⁺ T cells infiltrate into the CNS following JHNV infection and tightly associate with MAC2⁺ interactions with A β plaques: To address the potential involvement of CNS inflammatory CD4⁺ and CD8⁺ T cells during acute JHNV infection in 5xFAD mice, flow cytometry was performed to quantify the frequency of CD45⁺ CD4⁺ and CD45⁺ CD8⁺ T cells at 5 and 12 days p.i. (**Figure 4A-B**). By 5 days p.i., there was moderate infiltration of both CD4⁺ and CD8⁺ T cells in both JHNV-infected WT and 5xFAD brains, although no significance was detected (**Figure 4C-D**). However, both JHNV-infected WT and 5xFAD brains exhibited significant levels of infiltrating CD4⁺ T cells at 12 days following JHNV infection compared to uninfected controls (**Figure 4E**, $p<0.001$ and $p<0.05$, respectively). Interestingly, JHNV-infected

FIGURE 4. T cells infiltrate into the brains of JHNV-infected WT and 5xFAD mice at 12 days p.i. Gating strategy to select CD45⁺ CD4⁺ T cells (A) and CD45⁺ CD8⁺ T cells (B) following cell isolation, staining, and flow cytometry. Flow cytometric analysis of CD4⁺ T cell frequency (C) and CD8⁺ T cell frequency (D) from brain isolates at 5 dpi. Flow cytometric analysis of CD4⁺ T cell frequency (E) and CD8⁺ T cell frequency (F) from brain isolates at 12 dpi. Infiltration of CD4⁺ and CD8⁺ T cells into brain regions were assessed with brain sections co-stained with Amylo-Glo, MAC2, and either CD4 (H) or CD8 (I) antibody. Representative 20X confocal images of subiculum (J) and somatosensory cortex (K) depicting the presence of CD4⁺ T cells within brain sections from each experimental group. (L) Representative confocal images of control 5xFAD and JHNV 5xFAD subiculum. Quantification of CD4⁺ densities per FOV in subiculum (M) and somatosensory cortex (N) and within each experimental group. Representative 20X confocal images of subiculum (O) and somatosensory cortex (P) depicting the presence of CD8⁺ T cells within brain sections from each experimental group. (Q) Representative confocal images of control 5xFAD and JHNV 5xFAD subiculum. Quantification of CD8⁺ densities per FOV in subiculum (R) and somatosensory cortex (S) within each experimental group. $n=4-10$ per group. Data is presented as mean $\pm$ SEM. Two-way ANOVA followed by Holm-Sidak post-hoc tests was performed to examine biological relevant interactions. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Males are represented with closed symbols and females are represented with open symbols.

5xFAD brains showed a trending increase in infiltrating CD8⁺ T cells despite JHNV-infected WT mice exhibiting a greater significant infiltration of CD8⁺ T cells compared to uninfected WT controls (**Figure 4F**, $p < 0.01$).

To visualize the infiltration of T cells into the brain parenchyma, we utilized fluorescent immunostaining of CD4⁺ and CD8⁺ T cells in tissue sections for confocal microscopy to also determine T cell populations in regions where reduced A β pathology was observed following viral



encephalitis (**Figures 4H-I**). In parallel with the obtained flow cytometric data, JHNV-infection mediated the infiltration of effector T cells, resulting in significant increases in the density of CD4⁺ T cells in both subiculum and somatosensory cortex regions of infected WT and 5xFAD brains at 12 days p.i. (**Figure 4J-N**). Furthermore, immunostaining for CD8⁺ T cells also reveals increased infiltration of CD8⁺ T cells in subiculum and somatosensory regions within infected WT and 5xFAD brains (**Figure 4O-S**). Interestingly, the infiltration of CD4⁺ and CD8⁺ T cells was significantly diminished in JHNV-infected 5xFAD mice compared to JHNV-infected WT mice (**Figure 4M&R**).

Bulk RNA sequencing reveals upregulation of genes associated to host immunity and viral-induced neuroinflammation in JHNV-infected mice: To investigate transcriptional changes within 5xFAD brains following viral encephalitis, we isolated RNA from frozen hemi-brain samples from each experimental group at days 7 and 14 p.i. and performed bulk sequencing analysis for dysregulated or differentially expressed genes (DEGs) for pathway analysis by gene ontology at each timepoint. Volcano plots were plotted to compare the average difference in gene expression (shown as Log₂ fold change) in comparison with its respective control group. Genes were determined to be significantly dysregulated if the magnitude of average difference from baseline was greater than 0.5 with a false discovery rate/adjusted p-value less than 0.05 (i.e. |Log₂FC| > 0.5, FDR > 0.05). Significant DEGs were then plotted on volcano plots and heatmaps to illustrate its level of differential expression. JHNV-induced encephalitis at both day 7 and 14 p.i. resulted in significantly down-regulated DEGs in JHNV-infected 5xFAD brains include *Sqle*, *Msmo1*, *Hmgcs1*, and *Gm9946*. Meanwhile, significantly DEGs in JHNV-infected 5xFAD brains such as *Lyz2*, *Cd68*, *Gpnmb*, *Ctss*, *Ctsb*, *Ctsd*, *Lgals3*, *Il7r*, *Ly6c2*, and *Cxcl9* were significantly up-regulated at day 7 p.i. (**Supplementary Figure 2A&J**). Heatmap plots of the JHNV 5xFAD

vs. Control 5xFAD comparison depict the magnitude of significant DEGs resulting from viral encephalitis (**Supplementary Figure 2B&K**). Using Gene Ontology (GO), we conducted pathway analysis for down-regulated pathways enriched in DEGs impacted by viral encephalitis in the 5xFAD brain and observed enriched pathways associated with cholesterol and steroid biosynthesis, neuronal transmission, axonogenesis, and lipid and lipoprotein metabolism. As expected, viral encephalitis in 5xFAD brains also up-regulated pathways involved in immunity, such as the IL-2 signaling, cytokine signaling, T cell regulation of apoptosis, neutrophil activation, and lysosome functioning (**Supplementary Figure 2C&L**). By day 14 p.i., several of these DEGs and enriched pathways impacted by viral encephalitis continue to be up-regulated, in addition to *Spp1*, *Ccl5*, and *B2m* (**Supplementary Figure 2J-L**).

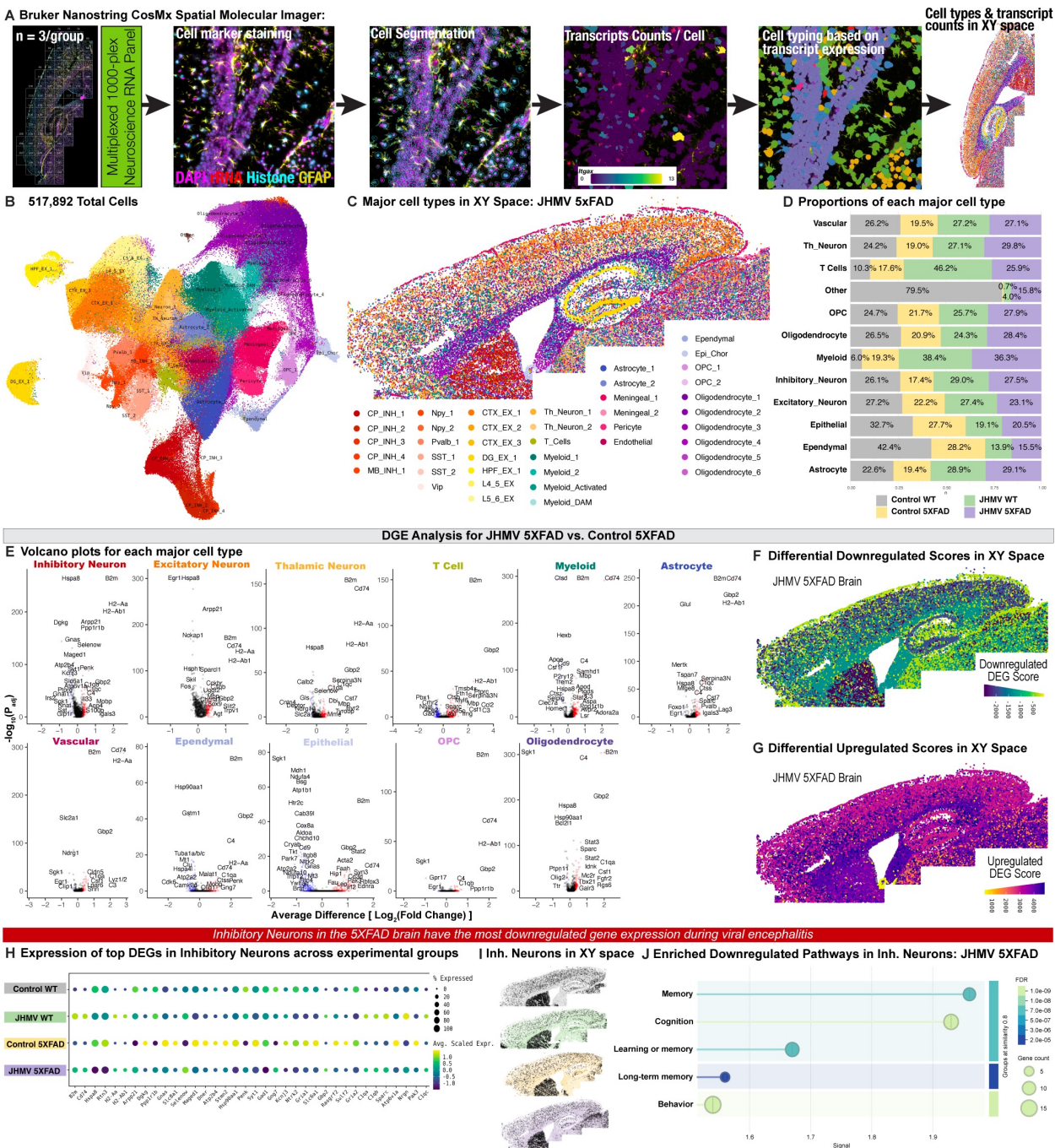
To determine whether these changes were specifically associated with JHMV-induced encephalitis, we have also performed bulk sequencing and DEG analysis on hemibrains from non-transgenic wildtype mice infected with JHMV at 7 and 14 days p.i. (**Supplementary Figures 2D-F & 2M-O**). Indeed, many significant DEGs and enriched pathways were also shared in JHMV-infected wildtype mouse brains at 7 (**Supplementary Figures 2D-F**) and 14 days p.i. (**Supplementary Figures 3M-O**). Comparing gene expression differences between JHMV 5xFAD vs. JHMV wildtype mouse brains minimal differences in genes associated with immunity at day 7, yet several significant genes appeared up-regulated in JHMV-infected 5xFAD brains such as *Plin4*, *Hif3a*, *Chil3*, *Cdc25c*, *Fam107a*, *Gm29650*, and *Kdm5d*. Gene ontology analysis of significant DEGs in JHMV 5xFAD vs. JHMV wildtype brains reveal enriched down-regulated pathways involved in BDNF signaling, while enriched up-regulated pathways include hemostasis, platelet activation, IL-6 regulation, and blood-brain-barrier transport (**Supplementary Figures 2G-I & 2P-R**).

Utilizing spatial transcriptomics to explore the impact of acute viral encephalitis on

CNS cell types: While these bulk sequencing methods have identified key biological pathways impacted by viral encephalitis in the 5xFAD brain, we lack the spatial and single-cell resolution to determine which key cell types within the CNS are mediating changes associated with A β pathology. To determine the impact of acute viral encephalitis following JHMV infection on cells within the mouse brain we investigated transcriptional changes to myeloid cells and other CNS cell types using spatial transcriptomic imaging. This approach facilitates gene expression analysis with single-cell resolution of CNS cell types, previously limited by technique-induced expression changes associated with single-cell and nucleus RNA-seq²³⁹. Furthermore, this technique retains gene expression of cells in distinct spatial regions of the brain, enabling *in situ* quantification and profiling of cell populations within a brain slice. We performed this experiment utilizing a high-plex *in situ* analysis platform (Bruker Nanostring CosMx Spatial Molecular Imager) with a 950-plex RNA mouse neuroscience panel and a 50-plex Custom Add-On RNA panel (Bruker

FIGURE 5. Spatial transcriptomic analysis of JHMV-infected and control WT and 5xFAD mice. **A)** Experimental workflow for targeted 1000-plex single cell spatial transcriptomic imaging of 12 sagittal mouse brain sections (n=3/group). Fields of view (FOVs) was selected in dentate gyrus to represent the cell marker staining, segmentation, transcript counts, and cell typing based on transcript expression. DAPI, rRNA, Histone, and GFAP were used as cell markers. **B)** Uniform Manifold Approximation and Projection (UMAP) of 517,892 cells across all brain samples were captured with a mean transcript count of 841 transcripts per cell. Unbiased cell clustering at 1.0 resolution identified 42 clusters, which were manually annotated with a combination of automated and manual approaches with reference to Allen Brain Atlas single-cell RNA-seq cell types, gene expression, and anatomic location in XY space. **C)** 42 annotated clusters plotted in XY space with a representative JHMV-infected 5xFAD brain. **D)** Proportions of each major CNS cell type grouped by experimental group. **E)** Volcano plots of DEGs within each major CNS cell type across JHMV-infected 5xFAD vs. control 5xFAD. **F)** Differential downregulation (DD) and **G)** differential upregulation (DU) scores for JHMV-infected 5xFAD vs. control 5xFAD in each cluster plotted in XY space using a representative JHMV-infected 5xFAD brain. **H)** Pseudo-bulk expression of top DEGs in inhibitory neurons between JHMV-infected 5xFAD vs. control 5xFAD, grouped by experimental group. **I)** Inhibitory neurons in XY space in representative brain sections from each experimental group. **J)** Pathways enriched via Gene Ontology (GO) of downregulated DEGs in JHMV-infected 5xFAD vs. control.

Nanostring)²³⁹. Three sagittal hemibrain slices per group were processed capturing the cortex and hippocampus regions, resulting in a dataset comprising of 517,892 cells from 12 brain sections. The CosMx SMI platform performed cell segmentation using DAPI, histone, ribosomal RNA, and GFAP staining (Figure 5A, Supplementary Figure 3A) and transcript counts per cell for each of



the 1000 gene targets per cell were calculated with an average of 841 transcripts per cell for 517,892 cells (**Figure 5B, Supplementary Figures 3B&C**). Cell clustering was obtained using a community detection approach on a k-nearest neighbor graph, followed by dimensionality reduction via Uniform Manifold Approximation and Projection (UMAP). Clusters were manually annotated based on top gene expression, expression of canonical CNS cell markers, and its spatial XY location in the brain, identifying 42 annotated clusters comprised of 11 clusters of inhibitory neurons, 7 clusters of excitatory neurons, 2 clusters of thalamic neurons, 2 clusters of astrocytes, 4 clusters of endothelial and vascular-related cell types, 4 clusters of myeloid cells, 2 clusters of oligodendrocyte precursor cells (OPCs), 6 clusters of oligodendrocytes, 1 cluster of T cells, 1 cluster of ependymal cells, 1 cluster of epithelial cells, and 1 cluster designated “other” (**Figure 5B&C, Supplementary Figures 3D&E**). Each of the 42 clusters were condensed into major clusters comprised of similar cell types (e.g., Astrocyte_1 and Astrocyte_2 were combined into a broader “Astrocyte” cluster) (**Supplementary Figure 3F**). The proportions of each major cell type cluster were then plotted per experimental group to illustrate any changes associated with JHMV-induced encephalitis, A β pathology, or both (**Figure 5D, Supplementary Figure 3F**). Since cell clustering relies purely on gene expression levels and not spatial location, we verified the correct assignment of cell populations in neuroanatomical locations by assessing cell type proportions across each experimental group and by plotting cells in XY space (**Supplementary Figures 4A&B**). For each cluster, we also determined cell counts across each cluster of cell types within the CNS (**Supplementary Figures 4C&D**). Clusters with fewer than 500 cells (i.e., “Other”) were removed from downstream analysis. In concordance with histology and flow cytometry, we observed increased proportion of cells in myeloid and T cell clusters following JHMV encephalitis (**Supplementary Figures 4C&D**).

To validate the impact of acute viral encephalitis on the transcriptional state of various cell types within the brain, we next conducted differential gene expression (DGE) analysis on JHMOV-infected WT and control, uninfected WT. Volcano plots depict several changes in gene expression within each CNS subtype and major cell type in the brains of these two groups (**Supplementary Figure 5 & 6A**). To visualize the spatial distribution of all cell types with changes in gene expression, we generated a DEG score representing the magnitude of fold change between each comparison of two experimental groups. This was calculated using the sum of products between two variables: the average difference in gene expression between two groups and the negative log₁₀ p-value for each gene²³⁹. The calculated value provides each cluster with a value plotted in XY space, depicting the DEG score of all differentially down-regulated (DD score) or up-regulated genes (DU score) (**Supplementary Figures 6B-C**). Notably, the myeloid cell cluster demonstrated the greatest DD score within JHMOV-infected WT brains, indicating the greatest down-regulation of genes in this cell population. Visualizing the location of myeloid cells in XY space on a JHMOV-infected WT brain demonstrates that several myeloid cells exhibiting the most down-regulation are found throughout cortical regions and the subiculum of the hippocampus, as well as the fornix (**Supplementary Figure 6B**). Pathway analysis of down-regulated DEGs within myeloid cells illustrate impacts on neuron projection morphogenesis, behavior, regulation of tissue and bone remodeling, and modulation of synaptic transmission (**Supplementary Figure 6D**). Furthermore, myeloid cell populations significantly upregulate expression of several DEGs involved in gliogenesis, lymphocyte immunity, immune effector processes, and antigen-presentation consistent with previous single-cell sequencing analyses on JHMOV infection of the CNS (**Supplementary Figure 6E**).

In addition to myeloid cells also exhibiting high DU scores in JHMV WT brains, astrocytes and oligodendrocytes demonstrate the greatest magnitude of up-regulated genes during acute viral encephalitis (**Supplementary Figure 6A**). Indeed, visualizing DU scores of each CNS cell in XY space illustrates the highest scores along white matter areas such as the corpus callosum and fornix, but also distributed throughout the cortex and hippocampal formation (**Supplementary Figure 6C**). Pathway analysis using gene ontology (GO) of significantly up-regulated genes within astrocytes and oligodendrocytes reveal biological processes associated with immune responses, immune effector processes, gliogenesis, and synaptic pruning during acute viral encephalitis (**Supplementary Figures 6F&G**).

Spatial transcriptomic imaging reveals a shift in disease-associated myeloid cells surrounding A β plaque pathology following acute JHMV-induced encephalitis: To first determine the consequences of plaque deposition on cell types within the CNS, we performed DGE analysis on control 5xFAD and control WT brains. Volcano plots demonstrate the widespread transcriptional changes within each CNS subtype and major cell type impacted by plaque deposition (**Supplementary Figure 7 & 8A**). In calculating the DEG score to compare the magnitude of fold change in DEGs within different CNS cell types, we found that myeloid cells demonstrated the greatest magnitude of down- and up-regulated DEGs compared to other CNS cell types. Displaying the DEG score of each cell in XY space further depicts the distribution of dysregulated gene expression across myeloid cells in plaque-laden areas, particularly the cortex and subiculum of 5xFAD mice (**Supplementary Figure 8B&C**). In total, the presence of amyloid plaque deposition resulted in 190 significantly down-regulated DEGs within myeloid cells (defined as $p_{adj} < 0.05$ and absolute average difference > 0.3) between 5xFAD and WT brains. In line with previous sequencing studies, myeloid cells impacted by the presence of amyloid plaque

pathology exhibited down-regulated homeostatic genes (*P2ry12*, *Tmem119*, *Csf1r*). Additionally, pathway analysis via gene ontology revealed down-regulation in pathways involved in modulation of neurotransmission, cell morphogenesis, cell migration, development of neuron projections, and learning and memory (**Supplementary Figure 8D**). Furthermore, myeloid cells exhibited 77 significantly up-regulated DEGs between 5xFAD and WT brains, several of which are associated with disease-associated microglia (DAM) signatures (*Cst7*, *Itgax*, *Spp1*, *Gpnmb*, *Ctss*, *Ctsz*, *Csf1*, *Lpl*, etc.) (**Supplementary Figure 8A**). Meanwhile, additional up-regulated DEGs within myeloid cells were associated with GO pathways involving regulation of tumor necrosis factor and interleukin-6 production, gliogenesis and glial cell proliferation, and negative regulation of cell activation (**Supplementary Figure 8E**).

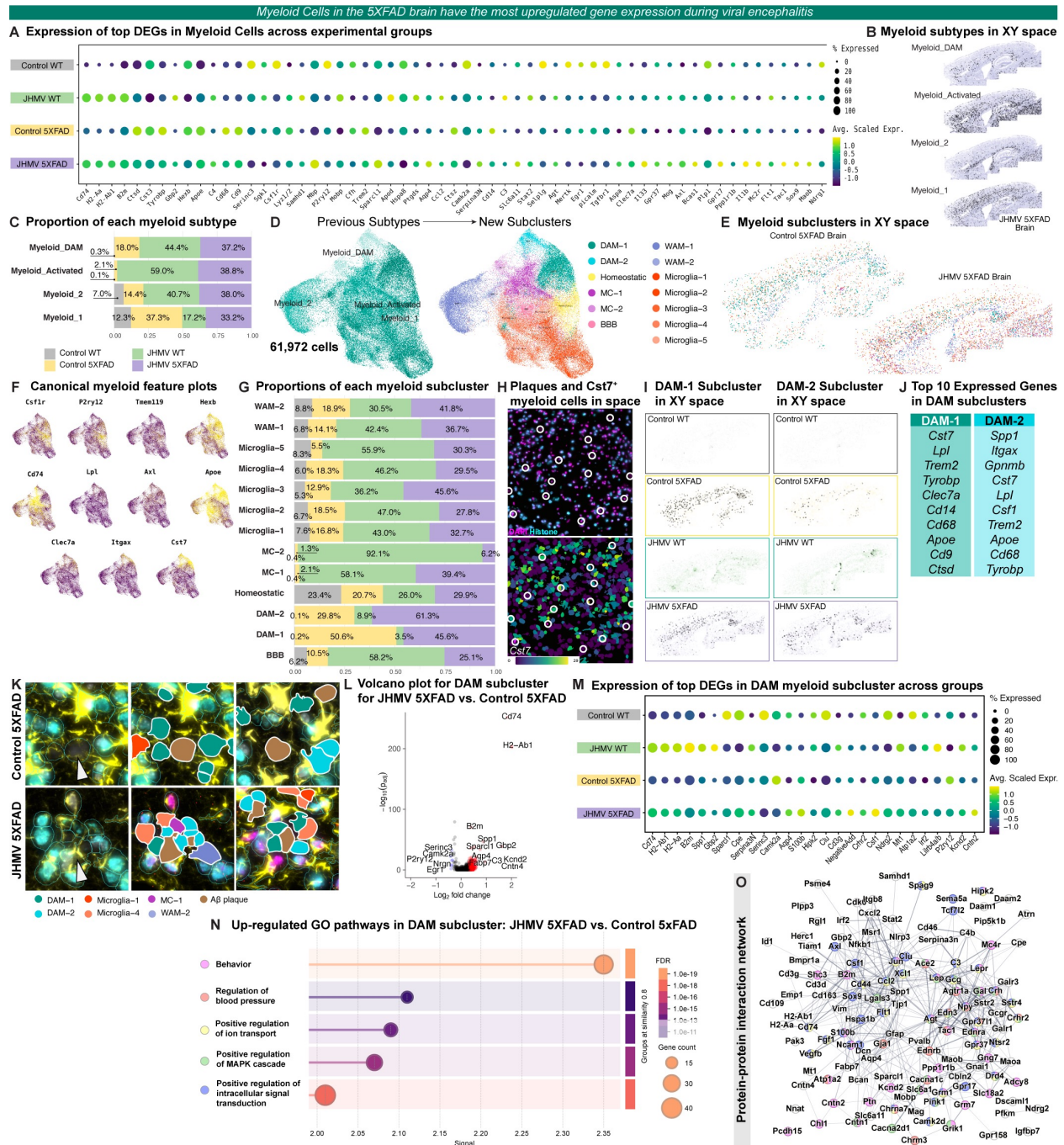
We next performed differential gene expression (DGE) analysis to investigate how the acute viral encephalitis impacted CNS cell types surrounding A β plaque pathology. Non-aggregate DGE analysis of JHNV-infected 5xFAD vs. 5xFAD brains revealed dysregulated gene expression across the 42 cell clusters and major cell types (**Supplementary Figure 9 & Figure 5E**). To visualize the spatial distribution of all cell types with changes in gene expression, we generated a DEG score representing the magnitude of fold change between each comparison of two experimental groups (**Figures 5F&G**). Visualizing down-regulated DEG scores across brain sections demonstrates widespread genetic changes during acute JHNV-induced encephalitis, however inhibitory and excitatory neuron clusters exhibited the greatest DD score. Replotting the inhibitory neuron clusters in XY space validated the correct cell type clustering with spatial specificity. To visualize genetic changes in inhibitory neurons across the different experimental groups, we plotted the DEGs within this cluster as pseudo-bulked expression values and in XY space (**Figure 5H&I**). Within inhibitory neurons in JHNV-infected 5XFAD brains, several genes

were downregulated involved in memory, cognition, and behavior such as *Cck*, *Cnr1*, *Vip*, *Sgkl*, and *Egr1* (**Figure 5J**).

Among all the cell types, myeloid cells expressed the greatest differential upregulation (DU) score between JHMOV-infected 5xFAD mice and uninfected 5xFAD controls, implicating a post-acute encephalitis response that may interact with existing amyloid pathology. We observed an increased proportion of myeloid cells during viral encephalitis in 5xFAD brains, in concordance with increased infiltration following JHMOV infection²⁴⁰ (**Figure 5D**). Using DGE analysis, we found that several genes associated with immune activation and antigen presentation are upregulated in the myeloid cell cluster (*CD74*, *H2-Aa*, *H2-Ab1*; **Figure 5E**). Interestingly, several DAM genes were downregulated within this myeloid population (*CD9*, *Trem2*, *Clec7a*, *Ctsz*, *Dtsd*; **Figure 5E**), as well as several homeostatic microglial genes (*Csf1r*, *P2ry12*, *Hexb*, *Cst3*; **Figure 5E**). To determine the biological pathways enriched in myeloid DEGs, we performed gene ontology (GO) pathway analysis of dysregulated genes in the myeloid cluster. We found that JHMOV-infection induced several up-regulated myeloid DEGs in 5xFAD brains associated with gliogenesis, glial cell development, glial cell differentiation, inflammatory responses, and myelination (**Supplementary Figure 10A**). We also utilized protein network analysis to illustrate the protein-protein interactions of closely associated up-regulated myeloid DEGs (**Supplementary Figure 10B**). Interestingly, GO pathway analysis of down-regulated myeloid DEGs depicted enriched pathways involved in regulation of macrophage migration, host-pathogen processes, ischemia responses, and neuronal death (**Supplementary Figure 10C&D**).

Pseudo-bulk analysis of these top DEGs between JHMOV-infected 5xFAD and 5xFAD brains was performed, demonstrating the downregulation of several DAM genes following acute viral encephalitis in 5xFAD myeloid cells despite DAM upregulation relative to control WT myeloid cells (**Figure 6A**). Cell clustering of all sampled cells produced four subtypes within the myeloid cluster: DAM, Activated, Myeloid 1, and Myeloid 2. Plotting these clusters in XY space reveals that the DAM and Myeloid 1 subtype to be distributed in plaque-laden areas (i.e. the subiculum of the hippocampus and throughout the cortex; **Figure 6B, Supplementary Figures 11A&D**). Meanwhile, the Activated Myeloid cluster appears to be pre-dominantly distributed throughout the brain in regions associated with viral replication, whereas the Myeloid 2 population is primarily localized to white-matter (i.e. the corpus callosum; **Figure 6B, Supplementary Figures 11B&C**). Concordant with our histology and flow cytometry analysis, we found an

FIGURE 6. Sub-clustering analysis of myeloid cells. **A)** Expression of top DEGs in myeloid cells between JHMOV-infected 5xFAD vs. control 5xFAD across each experimental group. **B)** Myeloid cell subtypes in XY space on a representative section of a JHMOV-infected 5xFAD brain. **C)** Proportion of each myeloid cell subtype split by each experimental group. **D)** Myeloid cells were re-clustered at 0.5 resolution yield 13 new myeloid subclusters. UMAP of 61,972 subsetted myeloid cells with original myeloid annotations (left) and new myeloid subcluster annotations (right). **E)** Myeloid cell subclusters in XY space on a representative control 5xFAD and JHMOV-infected 5xFAD brain section. **F)** Feature plots representing expression of several canonical myeloid cell markers on the new UMAP. **G)** Proportion of the number of cells in each myeloid subcluster, grouped by experimental group. **H)** Representative FOV of DAPI⁺ plaques and Histone-expressing cells in the subiculum region of JHMOV-infected 5xFAD mouse (top). Cst7-expressing cells in the same FOV (below). **I)** DAM-1 (left) and DAM-2 (right) subclusters in XY space on representative sections from each experimental group. **J)** List of the top 10 expressed genes in DAM-1 (left) or DAM-2 (right) subclusters. **K)** Representative FOVs of subiculum in JHMOV-infected 5xFAD (top) and control 5xFAD (bottom) with arrows pointing to DAPI-positive amyloid-beta dense core plaques surrounded by myeloid subtypes. **L)** Volcano plot for DAM subcluster in JHMOV-infected 5xFAD vs. control 5xFAD. **M)** Pseudo-bulk sequencing analysis of top DEGs for JHMOV-infected 5xFAD and control 5xFAD within DAM myeloid subcluster. **N)** Pathway analysis by Gene Ontology of enriched DEGs in the DAM myeloid subcluster for JHMOV-infected 5xFAD vs. control 5xFAD comparison. **O)** Protein-protein interactions (PPI) network of all significantly up-regulated DEGs within DAM myeloid subcluster in JHMOV-infected 5xFAD vs control 5xFAD. Nodes are colored according to enriched pathway analysis. Connection line thickness between nodes indicates the degree of confidence in the prediction of the PPI.



increased proportion of cells within the DAM and Activated Myeloid subtype following acute viral encephalitis in both 5xFAD and WT brains (Figure 6C).

To further investigate the myeloid population, we captured all 61,972 myeloid cells across the four experimental groups and re-clustered them into 13 new subclusters (Figure 6D). We

performed manual cell annotation once more using top gene expression and their spatial location in the brain (**Figure 6E, Supplementary Figure 11E**). Among the sub-clustered myeloid cells, we identified two subclusters within the DAM subtype (DAM-1 and DAM-2). Visualization of cellular expression of canonical DAM markers *Lpl*, *Axl*, *Clec7a*, *Itgax*, and *Cst7* corroborates the identification of these DAM subclusters (**Figure 6F**). Cell proportions were calculated for each myeloid sub-cluster across the four experimental groups, revealing a higher proportion of both DAM sub-clusters are represented in 5xFAD brains (**Figure 6G, Supplementary Figure 11F-I**). While the DAM-1 sub-cluster is equally represented in both JHMV-infected and control 5xFAD brains, we observed a greater proportion of cells in the DAM-2 sub-cluster in 5xFAD brains during viral encephalitis (**Figure 6G, Supplementary Figure 9I**). In the cell segmentation imaging, we identified several DAPI-positive “cells” that were only present in 5xFAD brains, anucleate, and lacked expression of histone markers, suggesting these to be DAPI-positive A β plaques surrounded by *Cst7*-expressing myeloid cells (**Figure 6H**).

Plotting these DAM subclusters in XY space demonstrates that both DAM-1 and DAM-2 subclusters are distributed throughout the 5xFAD brain in plaque-laden areas, representing plaque-associated myeloid cells (**Figure 6I**). Furthermore, both DAM-1 and DAM-2 sub-clusters are characterized by high expression of canonical DAM markers (*Cst7*, *Lpl*, *Trem2*, *Apoe*) and phagocytosis (*Tyrobp*, *CD68*), likely attributed to plaque-associated microglia. However, cells within the DAM-1 sub-cluster differs in its high expression of *Clec7a*, *Cd9*, and *Cd14* while the DAM-2 sub-cluster exhibit a high expression of *Itgax*, *Csf1*, *Spp1*, and *Gpnmb* (**Figure 6J**). To determine the association amyloid pathology and cells with each DAM sub-cluster, we examined the proximity of DAM-1 and DAM-2 cells surrounding A β plaques that stained positive DAPI but lacked histone markers (**Figure 6K**). We observed several DAM-1 cells surrounding A β plaques

in control 5xFAD mice, corroborating that DAM-1 likely represent plaque-associated microglia. Meanwhile, we confirmed a greater proportion of DAM-2 cells surrounding A β plaques in JHNV-infected 5xFAD brains. Furthermore, these DAM-2 cells appear to be in direct contact with dense-core A β plaque pathology following encephalitis, unlike DAM-1 cells in control 5xFAD brains (**Figure 6K**). This data appears in line with our histological data, suggesting that cells within the DAM-2 sub-cluster could be responsible for the higher association of inflammatory MAC2⁺ myeloid cells surrounding A β plaques in response to JHNV infection.

To investigate the transcriptional changes that may occur in cells within the DAM sub-cluster, we performed pseudo-bulk analysis of DEGs across all the experimental groups (**Figures 6L&M, Supplementary Figure 12A**). Relative to control 5xFAD brains, cells in the DAM sub-cluster had increased expression of genes associated with immune activation and antigen-presentation (*CD74*, *H2-Ab1*, *H2-Aa*, *B2m*) and IFN- γ -induced signaling (*Gbp2*, *Irf2*) (**Figures 6L&M**). Gene ontology pathway analysis of these up-regulated DEGs in DAMs depict effects on behavior, regulation of blood pressure, ion transport, regulation of MAPK cascade, and regulation of intracellular signal transduction (**Figure 6N**). To visualize the protein-protein interactions and pathways associated with significant DEGs in DAM cells following viral encephalitis, we visualized interacting networks of related proteins expressed by up-regulated DEGs (**Figure 6O**). Furthermore, performing DGE analysis on each DAM sub-cluster reveals a differential impact of JHNV infection on DAM-1 and DAM-2 sub-clusters in the 5xFAD brain (**Supplementary Figures 12B&C**). In the DAM-1 subcluster, pathway analysis illustrates enriched pathways for up-regulated DEGs involved in regulation of MAPK cascade, gliogenesis, blood pressure, and secretion (**Supplementary Figure 12D**). Down-regulated genes within DAM-2 cells enrich GO pathways involved in regulation of macrophage fusion, microglial cell activation, and responses

to lipoprotein particles, while up-regulated genes are associated with antigen presentation via MHC Class II, T cell activation, and regulation of adaptive immunity (**Supplementary Figure 12E**).

Corroborating previous studies detailing the infiltration of monocyte-derived macrophages during JHMOV-induced encephalitis²⁴⁰, we have also observed high proportions of monocyte-derived cells (MC) within JHMOV-infected WT and JHMOV-infected 5xFAD brains in our spatial transcriptomic myeloid dataset (**Figure 6G, Supplementary Figure 12F**). Plotting the MC subclusters in XY space illustrates a significant spread of MC-1 throughout the brains of JHMOV-infected mice, yet MC-2 appears more specific to JHMOV-infected WT mice (**Supplementary Figure 12G**). When comparing transcriptional differences of monocyte-derived cells within the MC subcluster between JHMOV-infected brains vs. Control 5xFAD brains, we find that JHMOV infection results in decreased expression of *Lyz1*, *Mrc1*, *Tyrobp*, *Clec7a*, *Cd44*, and *Ctsd* (**Supplementary Figure 12H&I**). Utilizing pseudo-bulk sequencing analysis, we have also identified dysregulated DEGs among other myeloid cell types: WAM, Homeostatic myeloid cells, BBB-associated myeloid cells, and microglia (**Supplementary Figure 13**).

Comparison of myeloid differential gene expression effects of A β pathology and JHMOV-induced encephalitis: We have demonstrated that acute viral encephalitis through JHMOV infection shifts the transcriptional signature, particularly in disease-associated myeloid cells surrounding A β plaques. To investigate whether dysregulated responses in myeloid cells is a consequence of encephalitis, the presence of A β pathology, or unique to the presence of both inflammatory stimuli, we compared the average differences in gene expression within all myeloid cells in our spatial transcriptomic dataset. We plotted all DEGs between Control 5xFAD vs. Control WT (response to amyloid pathology) and JHMOV WT vs. Control WT (response to viral

encephalitis) using the average difference of each gene among the two comparisons (**Figure 7A**). Many gene expression changes were shared and correlated between the two inflammatory stimuli. In response to either amyloid or JHMOV encephalitis, myeloid cells shared down-regulation of 59 genes with similar homeostatic markers (e.g., *P2ry12*, *Cx3cr1*, *Tmem119*, *Csf1r*, etc.), while sharing up-regulation in 47 genes related to DAM signatures (e.g., *Apoe*, *Clec7a*, *Cst7*, *Ctsb*, *Itgax*, *Axl*, etc.). Notably, several genes were more upregulated in response to one stimulus such as *H2-Aa*, *Cd74*, *H2-Ab1*, *Gpnmb*, *Lgals3*, which demonstrated greater upregulation in response to JHMOV infection (**Figure 7B**). Meanwhile, there were several genes that were uniquely differentially expressed in one inflammatory stimulus over the other. For example, *Cd9*, *Trem2*,

FIGURE 7. Myeloid cells have differential responses to viral encephalitis, amyloid pathology, or both inflammatory stimuli concurrently. **A)** Scatterplot of the average difference of all myeloid cells for all significant genes ($p_{adj} < 0.05$) between Control 5xFAD vs. Control WT (x-axis, only amyloid pathology) and JHMOV WT vs. Control WT (y-axis, only viral encephalitis) comparisons. Arrows indicate direction of dysregulation for each comparison. Directly correlated genes (blue) occur in the same direction for both comparisons (i.e., both up- or both down-regulated), while inversely correlated genes (orange) occur in opposite directions for each comparison. Linear regression line demonstrates the relationship between the two comparisons. **B)** Venn diagram depicting the significant up- and down-regulated genes unique to the Control 5xFAD vs. Control WT (left, yellow) and JHMOV WT vs. Control WT (right, green), while demonstrating up- and down-regulated genes commonly shared across the two comparisons (middle). **C)** Scatterplot of the average difference of all myeloid cells for all significant genes between Control 5xFAD vs. Control WT, now with JHMOV 5xFAD vs. Control 5xFAD (y-axis, both amyloid and viral encephalitis). Red text demonstrates genes up-regulated in the same direction in the first scatterplot, but down-regulated in the JHMOV 5xFAD vs. Control 5xFAD comparison. **D)** Venn diagram depicting the significant up- and down-regulated genes unique to Control 5xFAD vs. Control WT and JHMOV 5xFAD vs. Control 5xFAD (right, purple), while demonstrating up- and down-regulated genes commonly shared between the two comparisons. **E)** Three-way Venn diagram of all significantly up- and down-regulated genes between the three comparisons: amyloid pathology (yellow), viral encephalitis WT (green), both amyloid and viral encephalitis (purple). Genes are considered significantly correlated if the $\log_2$ Fold Change magnitude is greater than 0.3. **F)** Gene ontology pathways enriched by all significantly up-regulated DEGs unique to JHMOV 5xFAD vs. Control 5xFAD. **G)** PPI of the significantly up-regulated DEGs unique to the JHMOV 5xFAD vs. Control 5xFAD. Nodes are colored according to enriched pathway analysis. Connection line thickness between nodes indicates the degree of confidence in the prediction of the PPI.

A Average difference in all myeloid cells

B Up and Down-Regulated Genes in All Myeloid Cells

C Average difference in all myeloid cells

D Up and Down-Regulated Genes in All Myeloid Cells



To determine the effect of the concurrence of both inflammatory stimuli (e.g., amyloid pathology and viral encephalitis) on the gene expression within myeloid cells, we plotted all DEGs between Control 5xFAD vs. Control WT and JHMV 5xFAD vs. Control 5xFAD (**Figure 7C**). Once again, many differential gene expression changes were shared in the context of only amyloid pathology (Control 5xFAD) or *both* amyloid and viral encephalitis. Myeloid cells shared 14 up-regulated genes involving MHC class II presentation, adaptive immunity activation, and other inflammatory mechanisms like *Cd74*, *B2m*, *H2-Aa*, *H2-Ab1*, *Stat3*, *Gbp2* (**Figure 7D**). Additionally, 9 homeostatic microglia genes were similarly downregulated in both conditions, such as *P2ry12* and *Csf1r*.

Notably, to determine which set of DEGs were unique to the interaction of both amyloid pathology and viral encephalitis, we compared DEGs from all myeloid cells across three comparisons: Control 5XFAD vs. Control WT (only amyloid pathology), JHMV WT vs. Control WT (only JHMV infection), or JHMV 5XFAD vs. Control 5XFAD (both amyloid pathology and JHMV infection). We have identified 143 genes that uniquely become differentially expressed under the occurrence of both amyloid pathology and JHMV infection. Several DAM genes are among the 11 uniquely downregulated in the interaction of both inflammatory stimuli in JHMV-infected 5xFAD brains: *Apoe*, *Cd9*, *Clec7a*, *Ctsd*, *Lyz1/2*, *Tyrobp*, *Trem2*, *Ctsz*, and *Cd68*. Notably, these same set of DAM genes were observed to be upregulated in the presence of both amyloid pathology and viral encephalitis, independently (**Figure 7F**). Additionally, 132 genes were observed to be uniquely upregulated in the interaction of both inflammatory stimuli (**Figure 7F**). Pathway analysis of these up-regulated genes in JHMV 5XFAD brains reveal impacts on glial cell development, gliogenesis, adenylate cyclase-modulating G protein-coupled receptor signaling pathways, myelination, and neuroinflammatory responses (**Figure 7G**). Additionally, mapping out

protein-protein interactions of significantly up-regulated DEGs unique to JHNV 5xFAD myeloid cells demonstrate closely linked genes that transcribe proteins associated with interactions or similar cellular mechanisms.

DISCUSSION

With increasing evidence identifying how A β -dependent pathways in combination with innate immune responses influence AD onset in pre-clinical mouse models of AD, microbial infection and its resulting peripheral inflammation has been put forward as a major environmental risk factor for AD onset^{112,167,241–244}. Importantly, viral exposure has recently been associated with increased risk of numerous neurodegenerative diseases, with the strongest association occurring between viral encephalitis and AD¹²⁵. Viral encephalitis is defined as viral-mediated inflammation of the brain parenchyma. While the BBB prevents most viral pathogens from invading from the periphery, neurotropic viruses can circumvent these barriers to infect and replicate within the CNS while the effects of neurovirulent viruses replicating in the periphery can indirectly induce neurological effects¹¹². The resulting encephalitis can therefore lead to neurologic damage and potential consequences to the onset or severity of neurodegenerative disease^{113,167,168}. There is a critical need to understand post-infectious neuroimmune mechanisms and how they may interact with A β plaque deposition and modulate the onset and severity of AD pathology.

This study was performed to better elucidate the interaction between viral-induced inflammation and amyloid pathology associated with Alzheimer's disease using experimental infection of aged 5xFAD and WT mice with JHMV, a neurotropic murine coronavirus. We demonstrate that acute JHMV infection in 5xFAD mice results in similar inflammatory responses in parallel to significant reductions in A β plaque sizes despite no significant changes to overall A β plaque load. we observed compaction of dense-core A β plaques and reduced numbers associated with MAC2⁺ macrophages within subiculum and somatosensory cortex regions, respectively. Within regions where viral RNA/antigen persists, increased recruitment of inflammatory monocyte/macrophages and T cells significantly correlate with more compact A β plaques.

Furthermore, we utilized spatial transcriptomic imaging to investigate the transcriptional impact of viral encephalitis with single-cell resolution and determine altered gene expression within specific cell types surrounding A β pathology in the 5xFAD brain. While several cell types were dysregulated following viral encephalitis, we show that myeloid cells demonstrate a suppressed DAM response in the brain of JHMV-infected 5xFAD mice, particularly those surrounding amyloid pathology. These findings reveal that viral encephalitis and its resulting inflammatory mechanisms impacts the expression of key genes within myeloid cells responding to A β plaque pathology, which has implications to the role of viral encephalitis as a risk factor for Alzheimer's disease.

During neurodegenerative diseases like AD, microglia transition from homeostatic states into activated inflammatory states, which correlate with cognitive decline and neurodegeneration^{136,137}. Single-cell transcriptomic and proteomic studies have revealed activated microglia further assume functionally distinct microglial cell populations in during different diseased states within the CNS. In AD, the prominent transcriptional signature within microglia are disease-associated microglia (DAMs), microglia neurodegenerative phenotype (MGnD), and activated response microglia (ARMs)^{127,137}. The functional role of many upregulated DAM genes are also implicated in phagocytosis, chemotaxis, damage-mediated cytokine release, while downregulated genes are typical of microglial homeostasis¹³⁷. During early, asymptomatic stages, increased microglia activation into DAM states can promote A β clearance and confer neuroprotection, which can shift to a neurodegenerative, proinflammatory state reminiscent of the MGnD phenotype as disease progresses further¹³⁸.

Other myeloid cells contributing to neuroinflammation are non-parenchymal macrophages, which undergo development from bone marrow (BM)-derived monocytes and represent

functionally distinct cell populations from microglia under steady-state conditions^{143–145}. However, studies have reported that inflammatory and diseased conditions (e.g., AD) result in robust infiltration of peripheral-derived monocytes into the brain, which differentiate into macrophages upon immune activation with similar transcriptional and phenotype changes as microglia^{146–150}. In post-mortem AD tissue, circulating monocyte/macrophages have been identified, infiltrating into brain parenchyma and localizing amyloid plaques¹⁴⁸. In pre-clinical AD models, infiltration of peripheral BM-derived monocyte/macrophages have also been found closely associating with A β plaques^{150–152}. Notably, these BM-derived macrophages appear to be more efficient than resident microglia in phagocytosis and clearance of A β within the brain parenchyma, while protecting synapses from loss^{152–154}. The ability of BM-derived macrophages to reduce A β is further illustrated in selective ablation studies resulting in exacerbated cortical and hippocampal A β pathology. Conversely, disease stage may impact the phagocytic capacity of peripheral myeloid cells in clearing A β during the late-stage disease¹⁶⁰. Further supporting the importance of timing monocyte and macrophage infiltration, glatiramer acetate used to recruit immunomodulatory BMDMs only modestly reduced A β burden in 5xFAD mice during the development stage of pathology and increased A β levels during later stages of neuropathology¹⁶¹. These results provide additional evidence of the importance of myeloid cells in regulating neuroinflammation and shaping AD pathology, specifically highlighting the nuanced disease-modifying role of peripheral BMDMs recruited into the CNS. These results provide additional evidence of the importance of myeloid cells like monocyte/macrophages in regulating neuroinflammation and shaping AD pathology^{158,159}.

This study builds upon an ongoing question in the field about the role of infectious microbes, its result inflammation, and its effects on the progression and severity of AD

pathologies. Previously, our lab has reported on the impact of JHNV encephalitis on the 3xTg-AD transgenic mouse model, which uniquely combines the autosomal dominant Swedish mutation in human *APP* to reproduce amyloid pathology and two human *MAPT* mutations associated with frontotemporal dementia (FTD) to reproduce tauopathies²⁴⁵. While JHNV infection did not impact A β pathology in 3xTg-AD brains, the resulting encephalitis did appear to worsen tau pathology²⁴⁵. Other viral models have also been explored as potential risk factors for AD. Post-mortem analysis has identified colocalization of HSV-1 viral DNA with A β plaques in AD brains¹⁷³. This is also supported with a study reporting that HSV-1 infection led to seeding of surface glycoproteins within A β oligomers and accelerated A β deposition in the 5xFAD mouse model of familial AD, suggesting A β playing a potentially protective role in anti-viral immunity¹⁷⁴. However, recent studies have also complicated this prevailing theory in which unique post-mortem AD brain tissue with concurrent HSV encephalitis revealed HSV-infected cells localized close to A β plaques neurofibrillary tangles, but not directly associated with worsening AD pathology¹⁷⁵. Additionally, 5xFAD nor combined LOAD mutations (e.g., humanized A β , *APOE4*, and *TREM2*^{R47H} transgenes) in mice did not significantly protect against HSV-1 exposure nor initiate A β aggregation following HSV-1 infection, respectively^{176,177}. Interestingly, the researchers did observe limited viral invasion in regions with high A β plaque densities attributed with phagocytic activity of reactive microglia¹⁷⁶. Additionally, HSV-1 infection of LOAD mice resulted in extensive infiltration of peripheral leukocytes and phagocytic activity of myeloid cells¹⁷⁷. Rather than a direct cause, HSV-1 infection may be a modulating risk factor for AD with a combination of both environmental and genetic factors contributing to neurological damage.

While this study reports a dampened DAM transcriptional response to amyloid pathologies following encephalitis, there are several limitations that warrant future investigations. Here, we

utilize single-cell spatial transcriptomic imaging as a powerful method of retaining both spatial and transcriptional information within unique cell types. However, the pre-selected 1000-plex mouse neuroscience probe list limits the depth of exploration and unbiased investigation compared to whole genome approaches offered by traditional single-cell and single-nucleus techniques. Specifically, the available 1000-plex probe list offers probes against key gene transcripts involved in major biologically-relevant pathways, yet the limited selection of probes for inflammation and immunity pathways yield surface-level analyses on immune cell types. Interestingly, DGE analysis of each CNS cell type in the spatial transcriptomic dataset illustrates up-regulation of antigen presentation genes *H2-Aa* and *H2-Ab1*, even among cells that are not typically associated with antigen presentation. These are likely due to segmentation overlaps with antigen-presenting cells such as microglia and CNS-associated macrophages, highlighting some limitations in the cell segmentation process for identifying captured cells within the tissue sample. Furthermore, it will be necessary to carefully consider and interpret the impact on pathologies observed in the 5xFAD transgenic model, which lacks AD-related tauopathies. While we have previously investigated the impact of viral encephalitis on a mouse model with both amyloidosis and tauopathy, its reliance on transgenes to overexpress rare autosomal-dominant mutations limits the clinical relevance of this approach. With the advent of better humanized mouse models that have the potential of recapitulating amyloidosis and tauopathy together, future investigations investigate both the acute and post-acute impact of viral encephalitis on these mice may offer more relevant and comprehensive understanding of its interactions with AD pathologies and its severity. Committing to defining these post-infectious neuroimmune mechanisms and their interaction with AD pathogenesis holds promise, with potential to offer both treatments for current patients and

preventative interventions to improve public health outcomes and reducing AD risk in an aging population.

MATERIALS & METHODS

2.1. Mice: Male and female C57BL/6 WT and 5xFAD at 6- and 10-months of age were used for these studies. Tg (APPSwFILon, PSEN1*M146L*L286V) 6799Vas/ Mmjax RRID:MMRRC034848-JAX, was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at The Jackson Laboratory, an NIH-funded strain repository, and was donated to the MMRRC by Robert Vassar, Ph.D., Northwestern University and have been previously described and characterized in detail[22,23]. In brief, the 5xFAD mouse expresses five familial AD genes (APP Swedish, Florida, and London; PSEN1 M146L+L286V) and recapitulate progressive amyloid pathology as early as 3-4 months of age, leading to chronic neuroinflammation, synaptic loss, and dystrophic neurites. All studies were conducted following the NIH, American Physiological Society, and UCI Animal Care Guidelines. Any necessary veterinary care was provided by vivarium technicians, who check on mice daily for any changes in the health or well-being of the mice. Consistent with the recommendation of the Panel of Euthanasia of the AVMA, mice with continued discomfort and distress will be euthanized by inhalant overdose of CO₂, followed by secondary euthanasia via cervical dislocation. All animal experiments involving mice were approved by UC Irvine Institutional Animal Care and Use Committee and conducted in compliance with all relevant ethical regulations for animal testing and research. All experiments involving mice also comply with the Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines.

2.2. Viral infection: All infections were performed on mice under deep anesthesia through intraperitoneal (i.p.) injections of a mixture containing 85 mg/kg ketamine and 10 mg/kg xylazine. Subsequently, mice were infected intracranially (i.c.) with 500 plaque-forming units (PFU) of JHMV in 30µL of sterile Hanks balanced sterile solution (HBSS); control mice receive i.c.

injections only containing HBSS. Infected and control mice were weighed and monitored daily using a well-accepted scoring criteria to screen clinical disease symptoms such as significant weight loss, inactivity, lack of grooming, and other indicators of distress or discomfort[24,25].

2.3. RNA Extraction: Brains were isolated at defined times 12 days post-infection (p.i.) was also homogenized with RNA extraction, cDNA synthesis, and qPCR for comparison of JHMV Membrane (M) mRNA levels performed as previously described[25]. Briefly, mouse brain tissue was added to TRIzol and homogenized using the Bead Ruptor 12 (Omni International) and 1.4mm ceramic beads (Omni International, 19-627). RNA was extracted via RNAeasy Minikit (Qiagen, 74106) using the “Purification of Total RNA, Including Small RNAs, from Animal Tissues” protocol from the manufacturer and Buffer RW1 to substitute Buffer RWT.

2.4. cDNA synthesis and qPCR: cDNA was made from extracted RNA using previously described methods[26]. Briefly, cDNA was synthesized using the “First Strand cDNA Synthesis” protocol by New England Biolabs, using AMV Reverse Transcriptase (New England Biolabs, M0277L), Random Hexamers (Invitrogen, N8080127), RNase Inhibitor (New England Biolabs M0314L) and AMV Buffer (New England Biolabs B0277A). Primer sequences used were GAPDH (forward: AACTTTGGCATTGTGGAAGG; reverse: GGATGCAGGGATGATGTTCT), JHMV Matrix glycoprotein (forward: TCAACCCCGAAACAAACAACC; reverse: GGCTGTTAGTGTATGG TAATCCTCA). qPCR was performed using the Bio-Rad iQ5 and iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA). Reactions were performed using 10µL and the machine was set to run using the following parameters: 1 cycle (95°C for 3 minutes) followed by 40 cycles (95°C for 10 seconds, then 55°C for 30 seconds). Ct values for each sample were normalized to an internal control (GAPDH), yielding dCt values where lower dCt values indicated higher mRNA levels present

while higher dCt values represented lower mRNA levels as more cycles of amplification was required to detect a signal across threshold.

2.5. Cell Isolation and Flow Cytometry: Flow cytometry was performed to identify inflammatory cells entering the CNS using established protocols[25,27]. In brief, single cell suspensions were generated from tissue samples by grinding with frosted microscope slides. Immune cells were enriched via a 2-step Percoll cushion (90% and 63%), and cells were collected at the interface of the two Percoll layers. Before staining with fluorescent antibodies, isolated cells were incubated with anti-CD16/32 Fc block (BD Biosciences, San Jose, CA) at a 1:100 dilution. Immuno-phenotyping of cells was performed using commercially available antibodies specific for the following cell surface markers: CD4 (Invitrogen, 11-0042-82), CD8a (Invitrogen, 17-0081-82), CD11b (Abcam, ab24874) and CD45 (BioLegend, 103114; 103130). Cells were simultaneously incubated with LIVE/DEAD Aqua Dead Cell Stain (Invitrogen, L34966). The following flow cytometric gating strategies were employed for inflammatory cells isolated from the CNS: macrophages/myeloid cells (CD45^{hi} CD11b⁺) and microglia (CD45^{lo} CD11b⁺); FITC-conjugated rat anti-mouse CD4 and a PE-conjugated tetramer specific for the CD4 immunodominant epitope present within the JHMV matrix (M) glycoprotein, spanning amino acids 133-147 (M133-147 tetramer), to determine total and virus-specific CD4⁺ cells (37, 38) ; APC-conjugated rat anti-mouse CD8a and a PE- conjugated tetramer specific for the CD8 immunodominant epitope present in the spike (S) glycoprotein, spanning amino acids 510-518 (S510-518), to identify total and virus-specific CD8⁺ cells (37, 38). Data were collected using a Novocyte flow cytometer and analyzed with FlowJo software (Tree Star Inc.).

2.6. Histopathology: 6-month and 10-month-old 5xFAD hemizygous and wildtype non-transgenic mice were euthanized by inhalant overdose of Isoflurane at 7, 12, or 14-days post-

infection before opening the thoracic cavity as a secondary method of euthanasia. Before transcardial perfusion with 1X phosphate buffered saline (PBS), blood plasma was collected from mice via cardiac puncture. In all experiments, brains were dissected, and the hemispheres were divided along the midline. One hemisphere of each brain was fixed in 4% paraformaldehyde (PFA) in PBS for 24 hours at 4°C for immunohistochemical and spatial single-cell transcriptomics. The other hemisphere was either fresh-frozen in dry ice for sample preparation to perform biochemical and protein analysis or placed in an enzyme digestion mix for cell isolation and staining to perform Flow cytometry. Additionally, spinal columns were isolated and stored in 4% PFA for 24-36 hours prior to analyzing spinal cord histopathology using Luxol Fast Blue staining protocols.

2.7. Immunofluorescence Staining: After 24 hours, PFA-fixed brain hemispheres were transferred into 30% sucrose in 1X PBS for cryoprotection stored in 4°C for 48 - X hours before they were embedded in optimum cutting temperature (OCT) compound (VWR, Radnor, PA, USA) and stored in -80°C. Sagittal brain sections were cut at 30µm using a cryostat (Thermo 95 664 OEC70 Micron HM525) and collected while free-floating in an anti-freeze solution of 30% ethylene glycol and 30% glycerol in 1X PBS kept in -20°C. Selected brain sections were chosen for immunohistological analyses between 1.10mm and 1.95 mm lateral to Bregma). One representative brain section from each mouse within the identical experimental cohort (i.e., same age, sex, genotype, and experimental condition) were subjected to simultaneous staining in the same container as described[22,28,29]. Briefly, the selected free-floating brain sections underwent several washes, at room temperature unless otherwise stated as follows: three 1X PBS washes for 5 minutes. For Amylo-Glo staining, free-floating brain sections were washed in 70% ethanol for 5 minutes followed by rinsing with deionized water for 2 minutes before immersed in Amylo-Glo RTD Amyloid Plaque Staining Reagent (1:100 dilution in 0.9% saline solution; TR-200-AG;

Biosensis, Thebarton, South Australia) for 10 minutes per manufacturer's instructions. Post-incubation, the free-floating sections were rinsed with 0.9% saline solution for 5 minutes before a brief wash in deionized water for 15 seconds. After Amylo-Glo staining, the brain sections continued onto standardized indirect immunohistochemical procedures as previously described[22,28,29]. Briefly, sections were immersed in blocking serum solution (5% normal goat serum with 0.2% Triton-X in 1X PBS) for 1 hour before an overnight incubation at 4°C in primary antibodies diluted in blocking serum solution. A complete list of antibodies with their respective dilutions is provided in Table 1. The following day, the brain sections were incubated with secondary antibodies for 1 hour in the dark after three washes of 1X PBS. Before mounting the brain sections onto microscope slides for imaging, the stained brain sections were once more rinsed in three washes of 1X PBS

2.8. Confocal Microscopy and Imaris Quantitative Analysis: Brain sections were imaged with a Zeiss Axio Scan Z1 Slidescanner using a 10X 0.45 NA Plan-Apo objective. High-resolution confocal fluorescence images were also obtained using a 20X 0.75 NA objective on a Leica TVS SPE-II confocal microscope. Two brain regions per mouse were selected for imaging: subiculum (SUB) and somatosensory cortex (SS CTX), unless otherwise stated and one field of view (FOV) per brain region was obtained for image analysis using Bitplane Imaris Software for quantification. Cell counting and volumetric measurements were acquired using the Spots and Surfaces modules through batch analysis of each imaged brain region.

2.9. Protein Extraction, Biochemical Analysis via Meso Scale Discovery (MSD) Assays: Sample preparation and quantification of A β followed established protocols[28,29]. Hippocampal and cortical regions of each mouse brain hemisphere were micro-dissected and flash-frozen. Samples were pulverized using a Bessman Tissue Pulverizer. For A β biochemical analysis,

pulverized hippocampal tissue was homogenized in 150 μ L of Tissue Protein Extraction Reagent (TPER; Life Technologies, Grand Island, NY). Cortical tissue was homogenized in 1000 μ L/150mg of TPER. The formulation of TPER contains 25mM bicine and 150 mM sodium chloride (pH 7.6) to effectively solubilize proteins within brain tissue post-homogenization. Protease (Roche) and phosphatase inhibitors (Sigma-Aldrich) were added to the homogenized samples and centrifuged at 100,000 g for 1 hour at 4°C to generate TPER-soluble fractions. To further create formic acid fractions, pellets from TPER-soluble fractions were homogenized in 70% formic acid: either 75 μ L or half of the added TPER volume for hippocampus or cortical tissue, respectively. Samples were once again centrifuged at 100,000 x g for 1 hour at 4°C. Proteins in this insoluble fraction were normalized to the respective brain region weight, while protein in the TPER-soluble fractions was normalized to the protein concentration determined via Bradford Protein Assay. Formic acid neutralization buffer (1 M TRIS base, 0.5 M Na₂HPO₄, 10% NaN₃) was used to adjust pH before running ELISA assays. Quantitative biochemical analysis of human A β soluble and insoluble fraction levels were obtained using the V-PLEX A β Peptide Panel 1 (6E10) (K15200G-1; Meso Scale Discovery, Rockville, MD). Quantitative biochemical analysis of neurofilament-light chain (NfL) in plasma samples was performed using the R-Plex Human Neurofilament L Assay (K1517XR-2; Mesoscale Discovery).

2.10. Luxol Fast Blue (LFB) Staining: Preparation for spinal cord histology was performed using previously described protocols[24,26]. In brief, PFA-fixed spinal columns were dissected to carefully extract the spinal cord from thoracic vertebrae 6-10 and cryoprotected in 30% sucrose for three days at 4°C. Spinal cords were then cut in 1mm transverse blocks and subsequently embedded in optimum cutting temperature (OCT) compound (VWR, Radnor, PA, USA) and frozen at -80°C. Spinal cord tissue was then coronally cryosectioned with a thickness of 8

micrometers (μm) and mounted on slides for luxol fast blue (LFB) staining using established protocols. Sections were stained with hematoxylin and eosin (H&E) in combination with LFB. Between 5–8 sections per mouse spinal cord were imaged using brightfield microscopy for analysis of demyelination. Areas of total white matter and demyelinated white matter were obtained using FIJI ImageJ Software, while demyelination was scored as a percentage of total white matter from the analyzed spinal cord sections as previously described.

2.11. Bulk RNA Sequencing: RNA was extracted from 6-month-old 5xFAD mouse brains infected with 500 PFU JHMV at 7- and 14-days p.i. as described above. Library preparation, RNA sequencing, and read mapping analysis were performed by Novogene Co. Gene expression values were normalized into Log_2 FPKM (fragments per kilobase of transcript per million mapped reads). Heatmaps were created using Morpheus (Morpheus, <https://software.broadinstitute.org/morpheus>). Volcano plots were created using custom code in R. Differentially expressed genes (DEGs) were filtered as significant with the magnitude of Log_2 (Fold change) greater than 0.5 ($|\text{Log}_2\text{FC}| > 0.05$) and a false discovery rate (FDR) < 0.05 . For each experimentally relevant comparison, gene ontology (GO) term enrichment analysis was performed on significant DEGs using enrichR (<https://amp.pharm.mssm.edu/Enrichr/>).

2.12. Spatial Transcriptomic Tissue Preparation: PFA-fixed brain hemispheres were embedded in OCT and stored frozen at -80°C . 24 hours prior to processing tissue for CosMx Spatial Molecular Imaging (SMI), sagittal brain sections were cut at $10\mu\text{m}$ and mounted on VWR Superfrost Plus microscope slides (Avantor, 48311-703). In each slide, six sagittal sections equally representing each experimental group were mounted and allowed to dry at room temperature for 15 minutes to promote tissue adherence and stored overnight at -80°C with desiccant. The following day, the brain tissue slides were processed (a total of 12 brain sections) in accordance with the Bruker

Nanostring CosMx specifications. Unless otherwise noted, the following steps were performed at room temperature under a fume hood in a cleaned, RNase free area. Slides were dried in an oven for 60 minutes at 60°C before briefly incubated in pre-cooled 10% neutral-buffered formalin for 15 minutes at 4°C and rinsed in three 1X PBS washes afterwards. The slides were placed back into the oven at 60°C for 45-60-minutes to further improve tissue adherence. After three washes in 1X PBS for 5 minutes, the slides were incubated in 4% sodium dodecyl sulfate (SDS) for 2 minutes, then washed in three washes of 1X PBS before dehydrating the tissue in subsequent 50%, 70% and 100% ethanol washes for 5 minutes each. After allowing the slides to air dry flat for 10-30 minutes, the slides were placed in a pre-heated container of 1X CosMx Target Retrieval Solution (Nanostring) at 100°C for 5 minutes, maintained by a steamer set to 100°C. After the antigen retrieval step, the slides were cooled in DEPC-treated water (ThermoFisher Scientific, AM9922) for 15 seconds and 100% ethanol for three minutes before 30 minutes of air drying. To permeabilize tissue, the sections were incubated in a digestion buffer (3 µg/mL Proteinase K in 1X PBS; Nanostring) for 30 minutes before a rinse in NBF Stop Buffer (0.1M Tris-Glycine Buffer, Cat# 15740) for 5 minutes, then rinsed in three 1X PBS washes for five minutes each. Meanwhile, a fiducial solution (Nanostring) was prepared to a 0.0015% dilution in 2X SSC-T and applied on tissue for 5 minutes. From this point, the slides were shielded from light. After two 1X PBS washes, slides were post-fixed in 10% NBF for five minutes and washed in two NBF Stop Buffer washes for five minutes each. Afterwards, the slides were left to incubate in an NHS-acetate (100mM; Cat#26777) solution for 15 minutes before being rinsed in 2X saline-sodium citrate (SSC, Cat#AM9763) for 5 minutes. Finally, the tissue was left to incubate with probe mix containing segmentation markers for ribosomal RNA (rRNA), CosMx Mouse Neuroscience Panel, 1000-plex, RNA with a custom RNA Add-On set, and RNase inhibitor in Buffer R (Nanostring).

The slides were placed inside a staining tray and left inside oven set to 37°C for hybridization over 16-18 hours overnight at which point, the hybridization probe mix was removed from the slides, and these were placed in two pre-heated stringent washes (50% deionized formamide, CAT #AM9342) in 2X SSC at 37°C for 25 minutes each. After two washes in 2X SSC for two minutes, the tissue was incubated in DAPI nuclear stain for 15 minutes and then washed in 1X PBS for 5 minutes. Meanwhile, a segmentation marker mix for GFAP and histones was prepared and left to incubate on the tissue for 60 minutes. After slides were rinsed in three 1X PBS washes for 5 minutes, the tissue was once again incubated in NHS-Acetate before finally being rinsed in two 2X SSC washes for 5 minutes. Before loading into the CosMx Spatial Molecular Imaging instrument, adhesive flow cells (Nanostring) were placed in each slide to create a fluidic chamber for imaging according to manufacturer's instructions. Once loaded and processed in the machine according to the manufacturer's instructions for a 2-slide run, approximately 850 fields of view (FOVs, about 75 FOVs per brain section) was chosen to capture the following brain regions: cortical regions, corpus callosum, hippocampus, upper thalamus, and upper caudate striatal regions for each of the 12 brain sections. Imaging continued for approximately 6 days and the raw data was collected onto the Nanostring AtoMx online platform.

2.13. Spatial Transcriptomic Analysis: For spatial transcriptomic analysis, raw datasets were exported from Nanostring AtoMx as a Seurat object and processed with R 4.3.1 software as previously described[29,30]. Briefly, principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) analysis was performed to reduce dataset dimensionality and visualize cell clustering at 1.0 resolution to yield 42 clusters. Clusters were manually annotated based on top transcript expression markers of known marker genes and its location in XY space. Furthermore, cell count and proportion plots were generated by plotting the number of cells in

each cell type and scaling values relative to 1) normalized percentages per experimental group by obtaining the ratio of cell counts in each cell type-group pair by the total number of cells and 2) dividing by the sum of the proportions across the cell type to account for differences in sample sizes. MAST was used on scaled expression data to enable differential gene expression analysis per cell type between group comparisons to compute the average difference as defined by the difference in log-scaled average expression across the compared groups for each cell type. DEG scores were calculated by take the sum of the absolute $\log_2$ fold change values of all transcripts with statistically significant ($p_{\text{adj}} < 0.05$) differential gene expression patterns between two group comparisons for each cluster. To generate data visualization, we utilized ggplot2 3.4.4174.

2.14. Statistics: Sample sizes were calculated using power analyses based on pilot data (G*power parameters: power 0.95, Type I Error Rate 5%, Effect Size: 1.56) and on historical experience (i.e., mice lost due to acute infection or aging), while being powered to detect relevant sex differences. Animal groups are randomized at weaning, and age-matched control cohorts will be generated alongside their respective experimental groups to maximize rigor and reproducibility. Data was analyzed using two-tailed unpaired t-tests for comparison between two groups or ANOVA (one- or two-way with mixed effects) for comparisons of up to four groups with appropriate post-hoc tests (Holm-Šidák's multiple comparisons) when necessary. For non-parametric data, Kruskal-Wallis tests were used to compare group differences followed by Dunn's multiple comparisons test. Statistical significance is defined as $p < 0.05$ and statistical trends at $p < 0.1$. Read mapping analysis and statistics for bulk RNA sequencing data were performed by Novogene Co. Statistics for transcriptomic data (CosMx Spatial Molecular Imager) will be calculated using standard R packages, with a False Discovery Rate (FDR) < 0.05 .

2.15. Availability of data and materials

Protocols, data, and results generated during the current study will be available via the AD Knowledge Portal (<https://adknowledgeportal.synapse.org/>). The AD Knowledge Portal is a platform for accessing data, analyses, and tools generated by the Accelerating Medicines Partnership (AMP-AD) Target Discovery Program and other National Institute on Aging (NIA)-supported programs to enable open-science practices and accelerate translational learning. The data, analyses, and tools are shared early in the research cycle without a publication embargo on secondary use. Data is available for general research use according to the following requirements for data access and data attribution (<https://adknowledgeportal.org/DataAccess/Instructions>)

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Not applicable

CONFLICTS

The authors declare that they have no competing interests.

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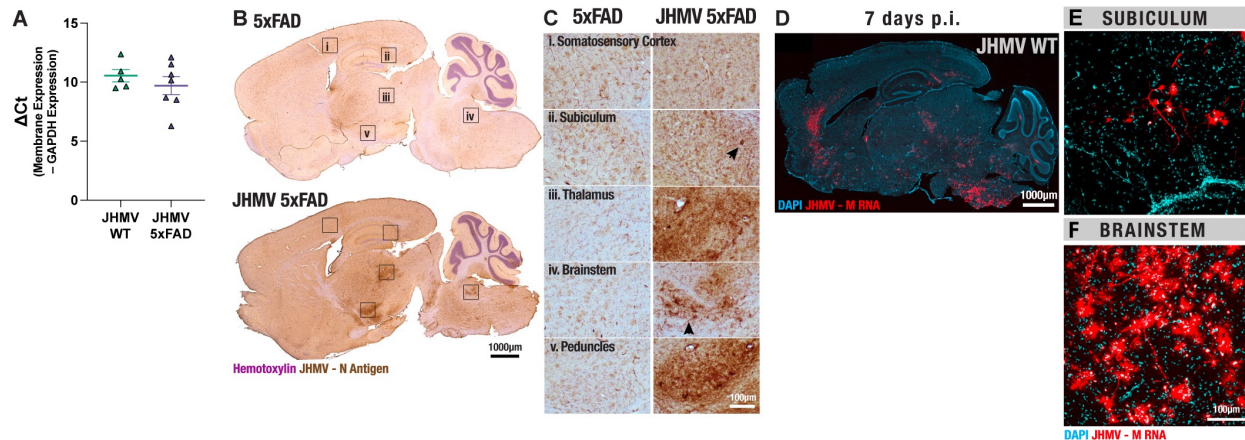
AUTHOR CONTRIBUTION STATEMENT

DIJ, KNG, and TEL designed the study; SF, LL, KF, JM, VS, and RJ assisted DIJ in performing experiments; DIJ collected and analyzed experimental data; DIJ performed bulk RNA sequencing analysis; DIJ performed spatial transcriptomics; DIJ analyzed spatial transcriptomic data; KIT and NEK provided technical advice and scripts for spatial transcriptomic analysis. DIJ wrote the main manuscript text and prepared figures. All authors reviewed the manuscript.

SUPPLEMENTARY MATERIALS

Table 1: List of primary and secondary antibodies used in this study

Primary Antibody	Source	Catalog Number	Dilution	Secondary Antibody	Source	Catalog Number	Dilution
IBA1	Wako	09-19741	1:2000	Goat anti-rabbit IgG, Alexa Fluor 488	ThermoFisher	A11008	1:200
MAC2	Cedarlane	CL8942AP	1:500	Goat anti-rat IgG Alexa Fluor 594	ThermoFisher	ab150160	1:200
6e10 A β 1-16	BioLegend	8030001	1:2000	Goat anti-mouse, Alexa Fluor 555	ThermoFisher	A21424	1:200
OC	Sigma Aldrich	2286	1:1000	Goat anti-rabbit IgG, Alexa Fluor 488	ThermoFisher	A11008	1:200
CD4	Abcam	ab183685	1:500	Goat anti-rabbit IgG, Alexa Fluor 488	ThermoFisher	A11008	1:200
CD8	Abcam	ab217344	1:500	Goat anti-rabbit IgG, Alexa Fluor 488	ThermoFisher	A11008	1:200



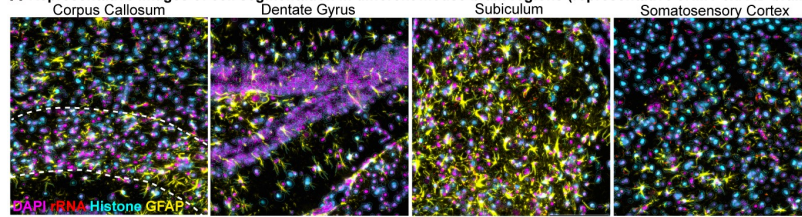
Supplementary Figure 1. **A)** qPCR with extracted RNA from brain homogenates demonstrate similar transcript levels of viral membrane protein RNA between JHMV-infected WT and 5xFAD brains at 6-months of age at 10-14 dpi. **B)** Representative whole-brain scanned image of sagittal brain sections stained with H&E/DAB targeting JHMV nucleocapsid protein. **C)** Representative 10X brightfield images of different brain regions with or without presence of JHMV nucleocapsid protein (indicated with black arrowheads). **D)** Representative whole-brain scanned image of a sagittal brain section from a JHMV-infected WT mouse at 7 dpi depicting *fluorescent in situ hybridization* targeting JHMV viral RNA encoding membrane protein. Representative 20X confocal images of subiculum (**E**) and brainstem (**F**) visualizing the presence of JHMV RNA at 7dpi in an infected WT brain. $n=7-10$ per group $n=4-10$ per group. Data is presented as mean $\pm$ SEM. Unpaired t-tests was used to examine statistically significant differences between groups. Males are represented with closed symbols and females are represented with open symbols.

Supplementary Figure 2. Bulk RNA sequencing was performed on extracted RNA from brain homogenates of 6-month-old WT and 5xFAD mice inoculated with either JHNV or vehicle controls at 7 or 14 days p.i. **A)** Volcano plot displaying fold change of DEGs ($\log_2$ scale) at 7 days p.i. compared between JHNV 5xFAD vs. Control 5xFAD groups. **B)** Heatmap analysis depicts DEGs compared between JHNV 5xFAD vs. Control 5xFAD at 7 days p.i. Downregulated genes ($\log_2\text{FoldChange} < 0.5$) are in blue, while upregulated genes ($\log_2\text{FoldChange} > 0.5$) are in orange. **C)** Gene ontology (GO) pathway analysis depicts enriched pathways of down-regulated DEGs (blue) or up-regulated pathways (orange) at 7 days p.i. **D, G)** Volcano plot displaying fold change of DEGs ($\log_2$ scale) at 7 days p.i. compared across respective groups (JHNV WT vs. Control WT and JHNV 5xFAD vs. JHNV WT, respectively). **E, H)** Heatmap analysis depicts DEGs compared across respective groups at 7 days p.i. Downregulated genes ($\log_2\text{FoldChange} < 0.5$) are in blue, while upregulated genes ($\log_2\text{FoldChange} > 0.5$) are in orange. **F, I)** Enriched pathways of either downregulated (in blue) or upregulated (in orange) DEGs at 7 days p.i. through gene ontology pathway analysis across each respective group comparison. **J)** Volcano plot displaying fold change of DEGs ($\log_2$ scale) at 14 days p.i. compared between JHNV 5xFAD vs. Control 5xFAD groups. **K)** Heatmap analysis depicts DEGs compared between JHNV 5xFAD vs. Control 5xFAD at 14 days p.i. Downregulated genes ($\log_2\text{FoldChange} < 0.5$) are in blue, while upregulated genes ($\log_2\text{FoldChange} > 0.5$) are in orange. **L)** Gene ontology (GO) pathway analysis depicts enriched pathways of down-regulated DEGs (blue) or up-regulated pathways (orange) at 14 days p.i. **M, P)** Volcano plot displaying fold change of DEGs ($\log_2$ scale) at 14 days p.i. compared across respective groups (JHNV WT vs. Control WT and JHNV 5xFAD vs. JHNV WT, respectively). **N, Q)** Heatmap analysis depicts DEGs compared across respective groups at 14 days p.i. Downregulated genes ($\log_2\text{FoldChange} < 0.5$) are in blue, while upregulated genes ($\log_2\text{FoldChange} > 0.5$) are in orange. **O, R)** Enriched pathways of either downregulated (in blue) or upregulated (in orange) DEGs at 14 days p.i. through gene ontology pathway analysis across each respective group comparison.



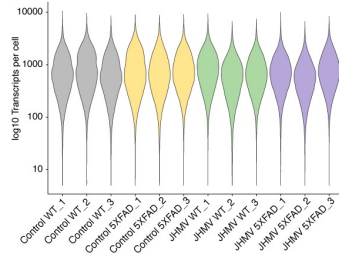
Supplementary Figure 3. **A)** Representative images of cell segmentation within corpus callosum (dashed area), dentate gyrus, subiculum, and somatosensory cortex of a JHNV 5xFAD brain, respectively. **B)** Volcano plot depicting the total number of transcripts per cell across each sample. **C)** Volcano plot depicting the number of unique gene transcripts identified per cell across each sample. **D)** Heatmap depicting the top 5 expressed transcripts within each major CNS cell type to aid in the manual annotation of each cluster. **E)** Expression of canonical markers of common CNS cell types and their location in the UMAP. Included markers for neurons (*Snap25*, *Syt1*), excitatory neurons (*Rbfox3*, *Camk2a*, *Slc17a7*), inhibitory neurons (*Calb2*, *Sst*, *Pvalb*, *Gad1*, *Gad2*), other neurons (*Rasgrf2*, *Reln*), OPCs (*Olig1*, *Pdgfra*), oligodendrocytes (*Olig2*, *Plp1*, *Mag*, *Mbp*, *Mobp*, *Mog*), microglia (*Csf1r*, *Cd74*, *P2ry12*, *Tmem119*), disease-associated microglia (*Lpl*, *Axl*, *Apoe*, *Clec7a*, *Itgax*, *Cst7*), astrocytes (*Gjal1*, *Aqp4*, *Aldh1l1*, *S100b*, *Gfap*, *Slc1a2*), vascular cells (*Rgs5*), endothelial cells (*Flt1*, *Kdr*), and smooth muscle cells (*Pdgfrb*). **F)** UMAP of each major CNS cell type split by experimental group.

A Representative images of cell segmentation in different mouse brain regions (representative JHNV 5xFAD brain)

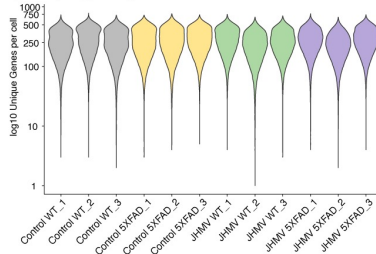


Control WT JHNV WT
Control 5xFAD JHNV 5xFAD

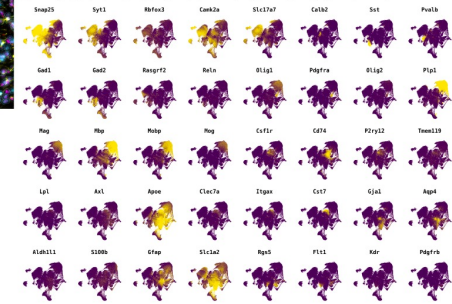
B Total transcripts per cell



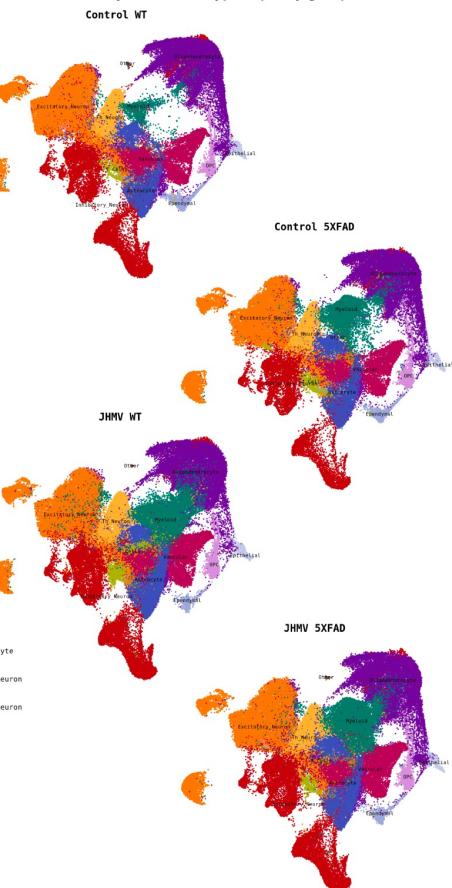
C Unique genes per cell



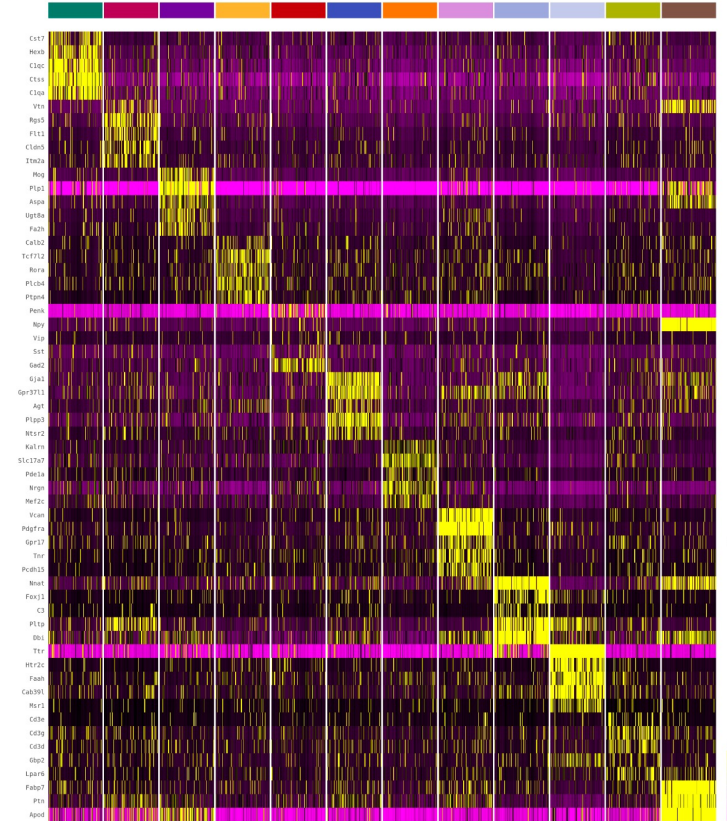
E Expression of canonical CNS cell markers within UMAP



F UMAP of major CNS Cell Types split by group

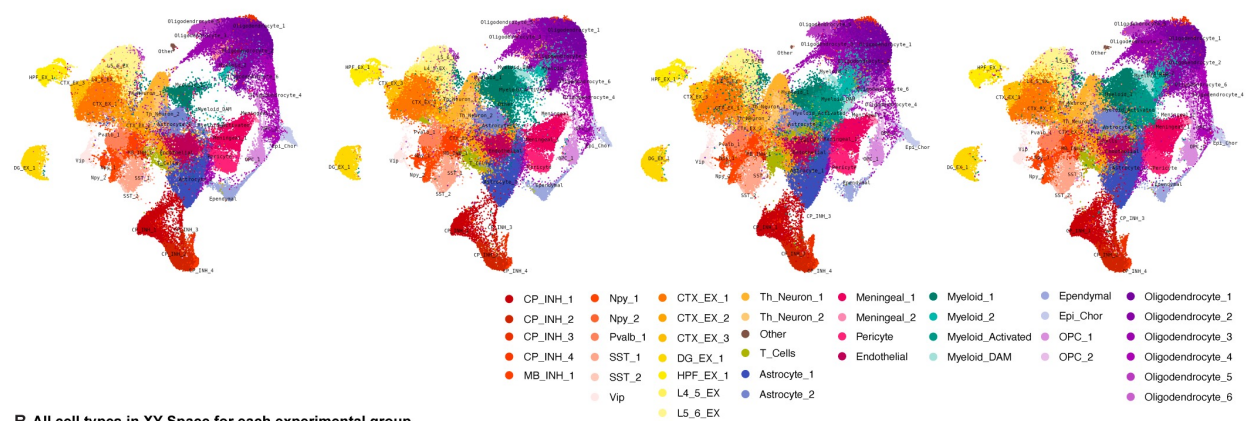


D Top 5 Marker Genes per major CNS Cell Type

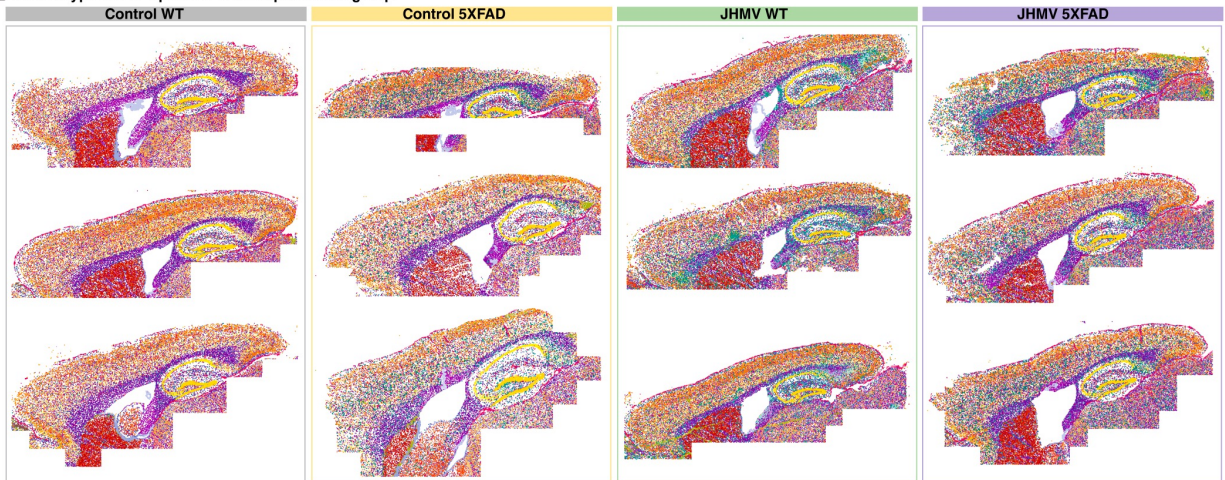


Supplementary Figure 4. **A)** UMAP of each subtype within major CNS cell types, split by experimental group. **B)** All CNS subtypes overlaid in XY space within each brain sample in the spatial transcriptomic analysis. **C)** Plot depicting the number of cells associated with each major CNS cell type, split by experimental group. **D)** Plot depicting the number of cells within each CNS subtype, split by experimental group. Abbreviations include CP INH: caudate putamen inhibitory neurons, CTX EX: cortex excitatory neurons, DG EX: dentate gyrus excitatory neurons, Epi Chor: epithelial cells in choroid plexus, HPF EX: hippocampal formation excitatory neurons, L4 5 EX: layer 4/5 excitatory neurons, L5 6 EX: layer 5/6 excitatory neurons, MB INH: midbrain inhibitory neurons, Myeloid DAM: disease-associated myeloid cells, Npy: neuropeptide Y-expressing inhibitory neurons, OPC: oligodendrocyte precursor cells, Pvalb: parvalbumin-expressing inhibitory neurons, SST: somatostatin-expressing inhibitory neurons, Th Neuron: thalamus neurons, Vip: vasoactive intestinal polypeptide-expressing neurons.

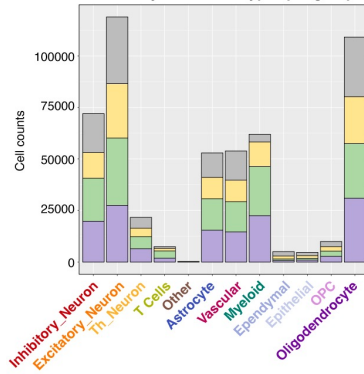
A UMAP split by experimental group
Control WT



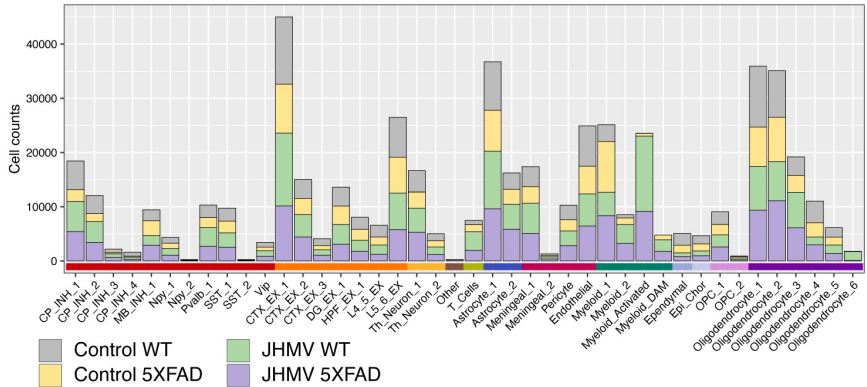
B All cell types in XY Space for each experimental group



C Cell counts of major CNS Cell Types per group

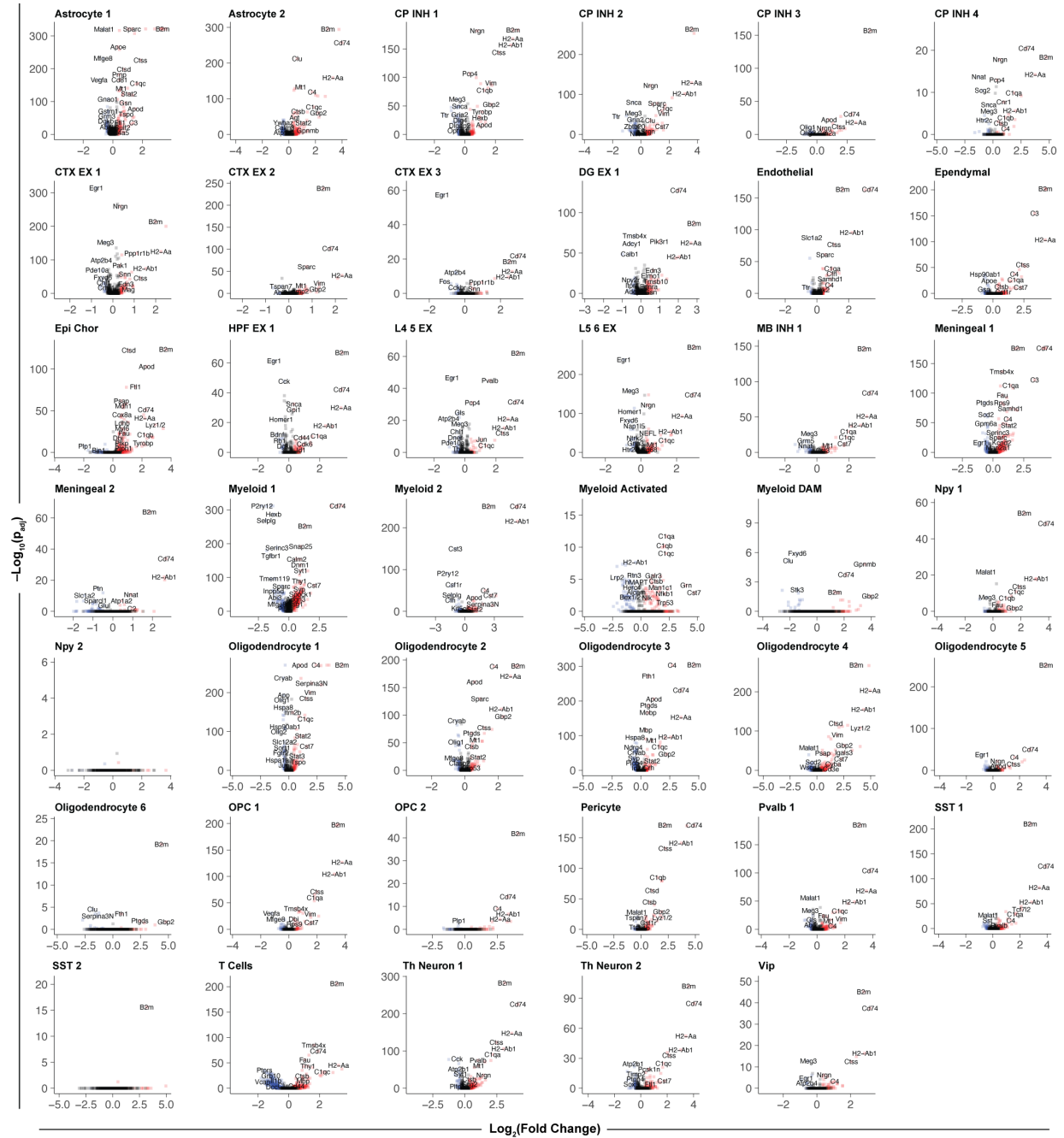


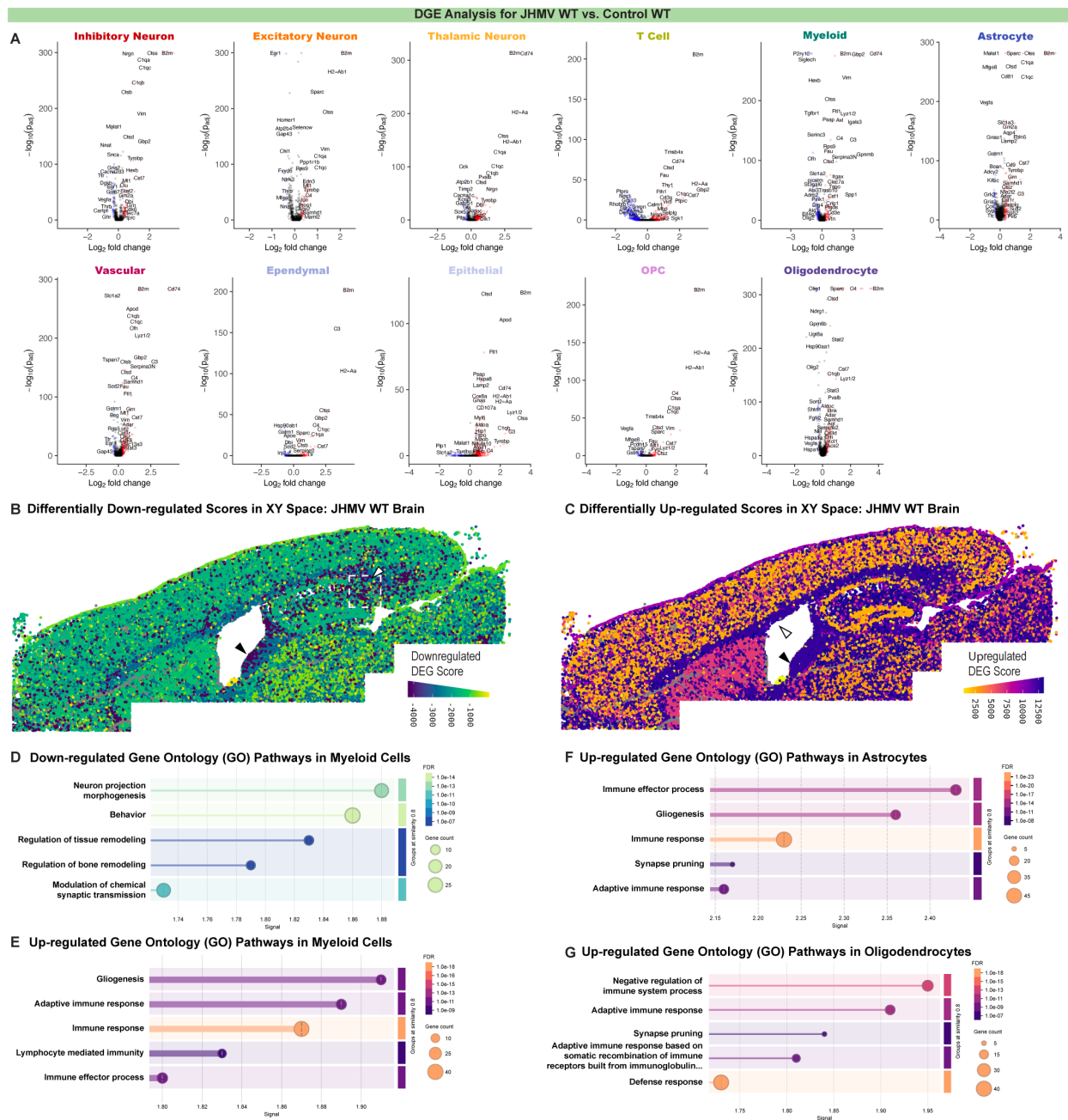
D Cell counts of all cell subtypes per experimental group



Supplementary Figure 5. DGE Analysis of CNS subtypes for JHNV WT vs. Control WT at 12 days p.i. Volcano plots of DEGs within each CNS subtype type across JHNV-infected WT vs. control WT comparison at 12 days p.i. Abbreviations include: CP INH, caudate putamen inhibitory neurons; CTX EX, cortex excitatory neurons; DG EX, dentate gyrus excitatory neurons; Epi Chor, epithelial cells in choroid plexus; HPF EX, hippocampal formation excitatory neurons; L4 5 EX, layer 4/5 excitatory neurons; L5 6 EX, layer 5/6 excitatory neurons; MB INH, midbrain inhibitory neurons; Myeloid DAM, disease-associated myeloid cells; Npy, neuropeptide Y-expressing inhibitory neurons; OPC, oligodendrocyte precursor cells; Pvalb, parvalbumin-expressing inhibitory neurons; SST, somatostatin-expressing inhibitory neurons; Th Neuron, thalamus neurons; Vip, vasoactive intestinal polypeptide-expressing neurons.

DGE Analysis for JHMV WT vs. Control WT: All CNS Subtypes

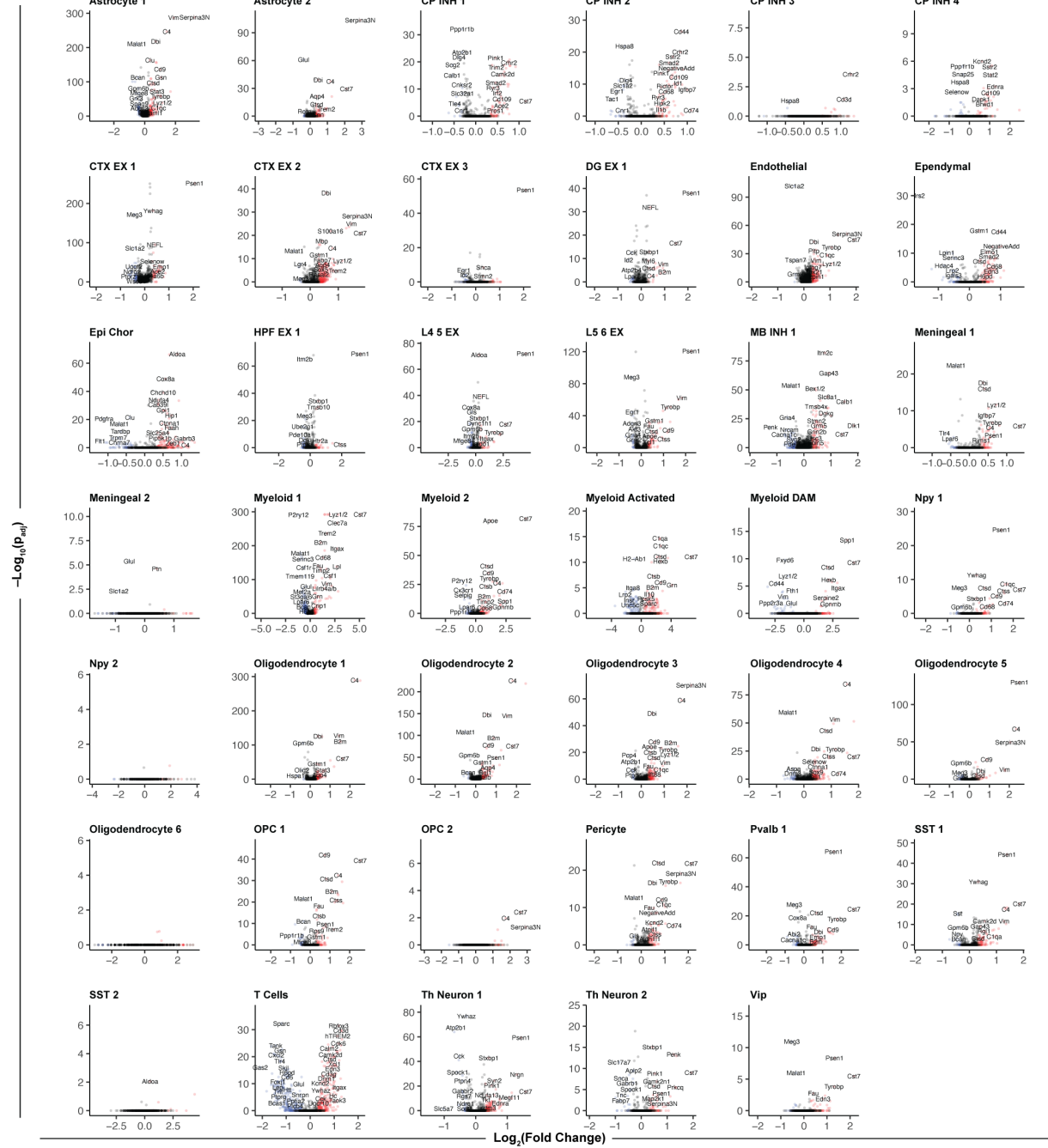


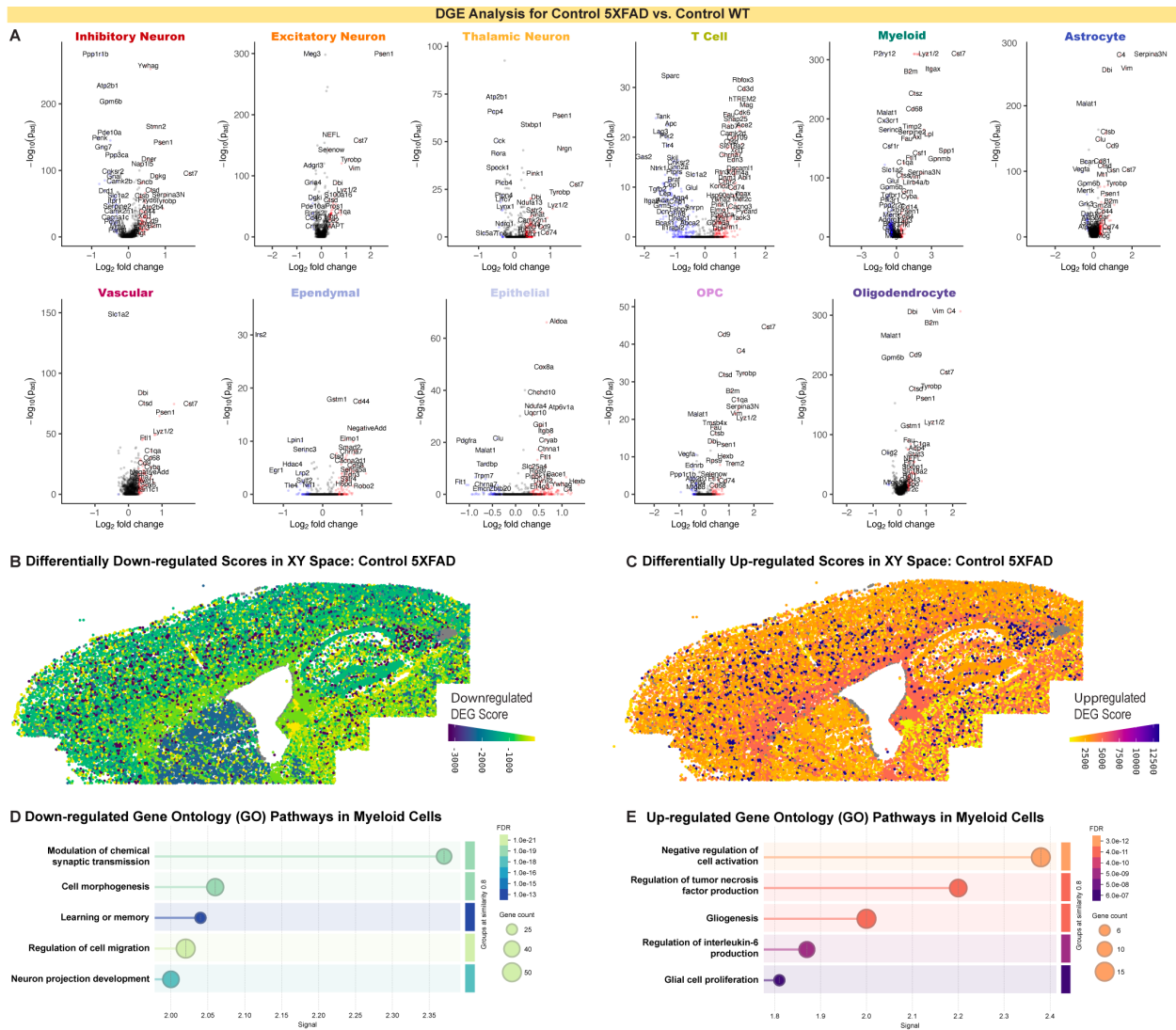


Supplementary Figure 6. DGE Analysis of each cell type for JHMV WT vs. Control WT comparison. **A)** Volcano plots of DEGs within each major CNS cell type across JHMV-infected 5xFAD vs. control 5xFAD. **B)** Differential downregulation (DD) and **C)** differential upregulation (DU) scores for JHMV-infected 5xFAD vs. control 5xFAD in each cluster plotted in XY space using a representative JHMV-infected 5xFAD brain. Arrowheads indicate major white matter tracts enriched in dysregulated cell types, specifically the fornix (black) and corpus callosum (white). **D)** GO Pathways enriched in down-regulated DEGs in myeloid cells. GO Pathways enriched in up-regulated DEGs in myeloid cells (**E**), astrocytes (**F**), and oligodendrocytes (**G**).

Supplementary Figure 7. DGE Analysis of CNS subtypes for Control 5xFAD vs. Control WT at 12 days p.i. Volcano plots of DEGs within each CNS subtype type across Control 5xFAD vs. control WT comparison at 12 days p. i. Abbreviations include: CP INH, caudate putamen inhibitory neurons; CTX EX, cortex excitatory neurons; DG EX, dentate gyrus excitatory neurons; Epi Chor, epithelial cells in choroid plexus; HPF EX, hippocampal formation excitatory neurons; L4 5 EX, layer 4/5 excitatory neurons; L5 6 EX, layer 5/6 excitatory neurons; MB INH, midbrain inhibitory neurons; Myeloid DAM, disease-associated myeloid cells; Npy, neuropeptide Y-expressing inhibitory neurons; OPC, oligodendrocyte precursor cells; Pvalb, parvalbumin-expressing inhibitory neurons; SST, somatostatin-expressing inhibitory neurons; Th Neuron, thalamus neurons; Vip, vasoactive intestinal polypeptide-expressing neurons.

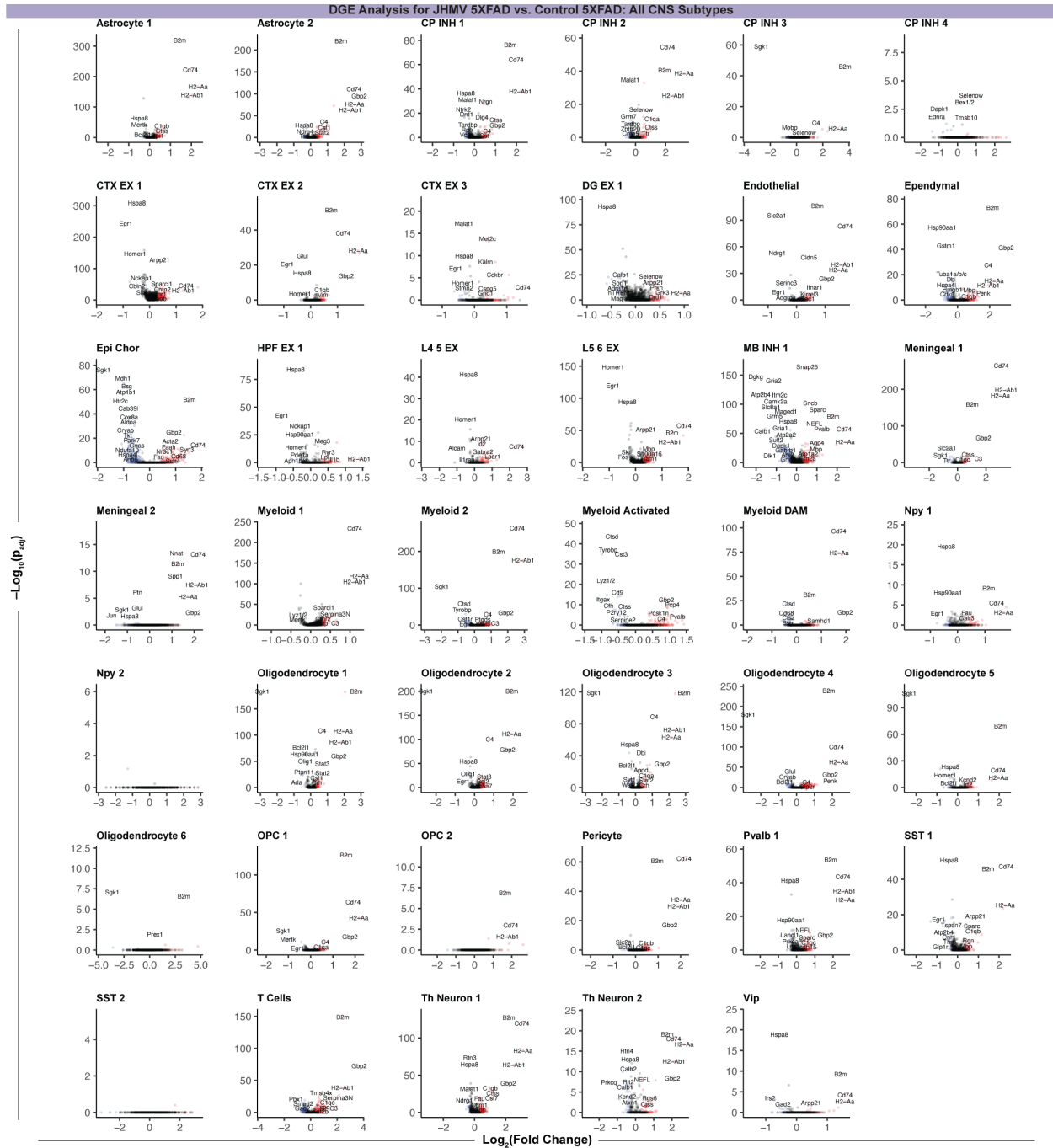
DGE Analysis for Control 5XFAD vs. Control WT: All CNS Subtypes

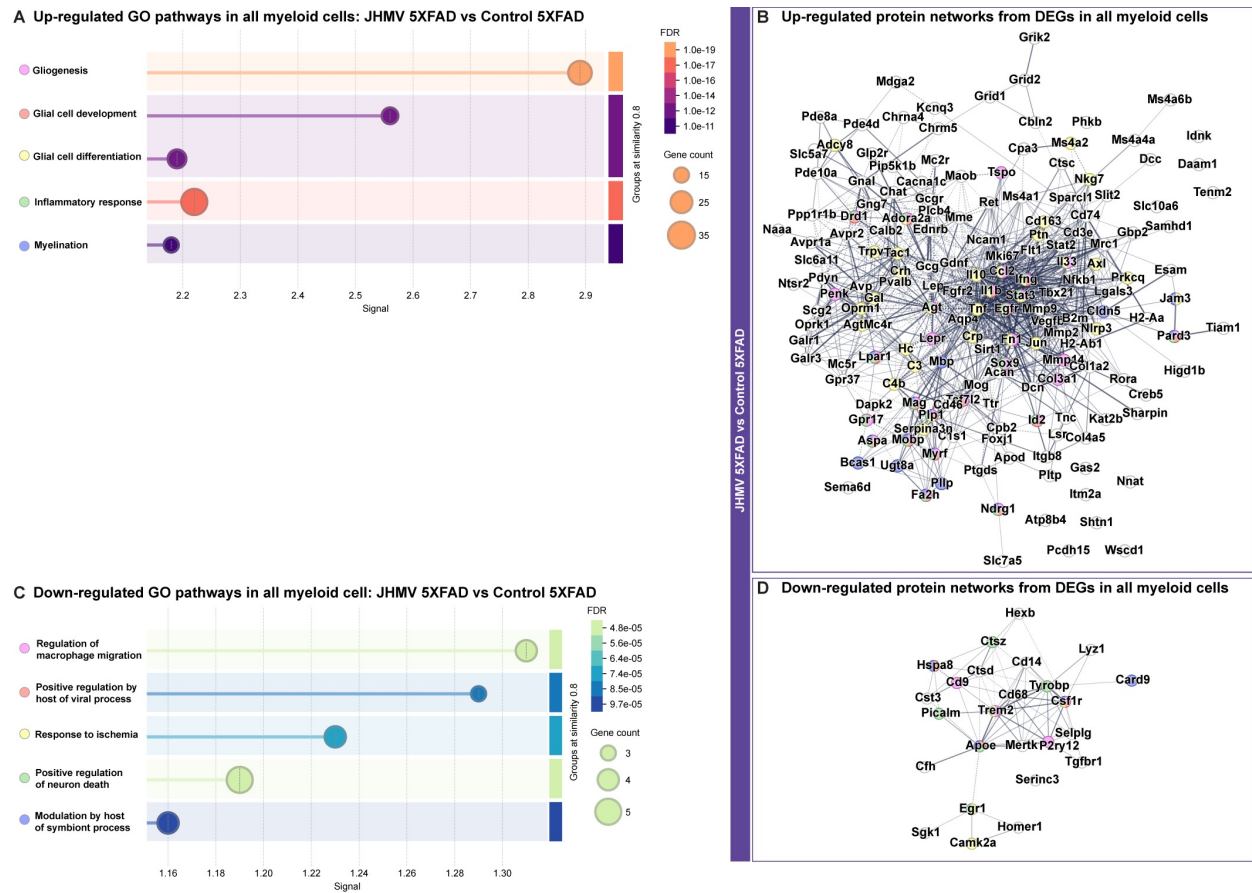




Supplementary Figure 8. DGE Analysis of each cell type for Control 5xFAD vs. Control WT comparison. **A)** Volcano plots of DEGs within each major CNS cell type across JHMY-infected 5xFAD vs. control 5xFAD. **B)** Differential downregulation (DD) and **C)** differential upregulation (DU) scores for JHMY-infected 5xFAD vs. control 5xFAD in each cluster plotted in XY space using a representative JHMY-infected 5xFAD brain. **D)** GO Pathways enriched in down-regulated DEGs in myeloid cells. **E)** GO Pathways enriched in up-regulated DEGs in myeloid cells.

Supplementary Figure 9. DGE Analysis of CNS subtypes for JHNV 5xFAD vs. Control 5xFAD at 12 days p.i. Volcano plots of DEGs within each CNS subtype type across JHNV-infected 5xFAD vs. control 5xFAD comparison at 12 days p.i. Abbreviations include: CP INH, caudate putamen inhibitory neurons; CTX EX, cortex excitatory neurons; DG EX, dentate gyrus excitatory neurons; Epi Chor, epithelial cells in choroid plexus; HPF EX, hippocampal formation excitatory neurons; L4 5 EX, layer 4/5 excitatory neurons; L5 6 EX, layer 5/6 excitatory neurons; MB INH, midbrain inhibitory neurons; Myeloid DAM, disease-associated myeloid cells; Npy, neuropeptide Y-expressing inhibitory neurons; OPC, oligodendrocyte precursor cells; Pvalb, parvalbumin-expressing inhibitory neurons; SST, somatostatin-expressing inhibitory neurons; Th Neuron, thalamus neurons; Vip, vasoactive intestinal polypeptide-expressing neurons.

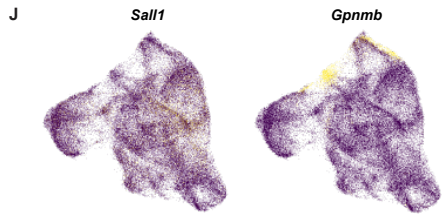
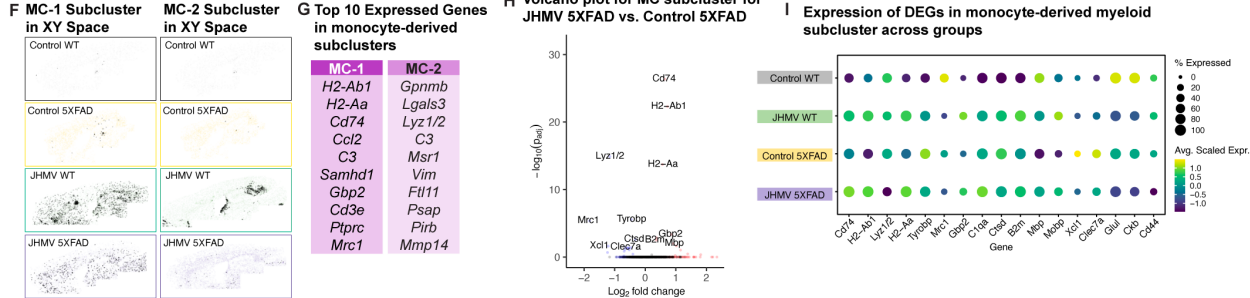
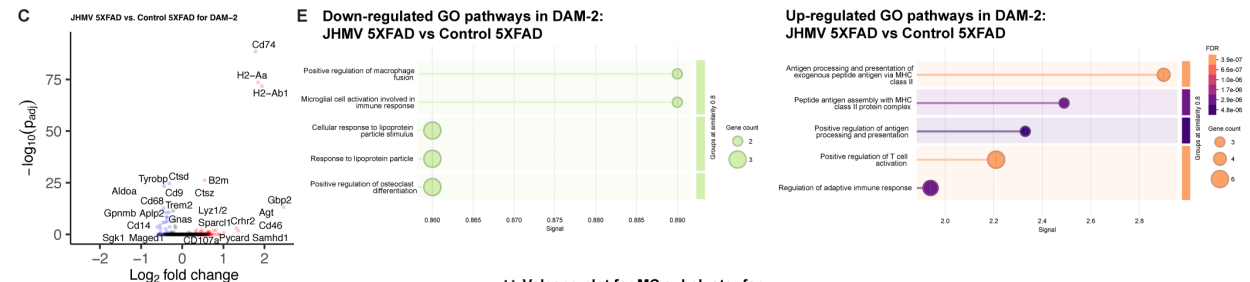
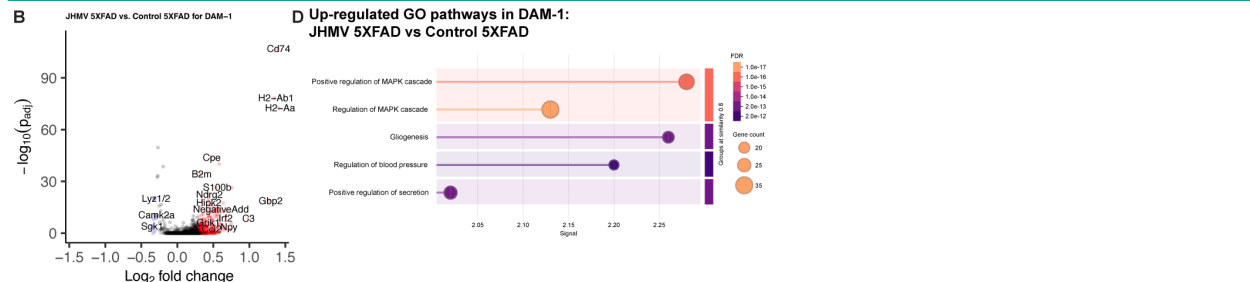
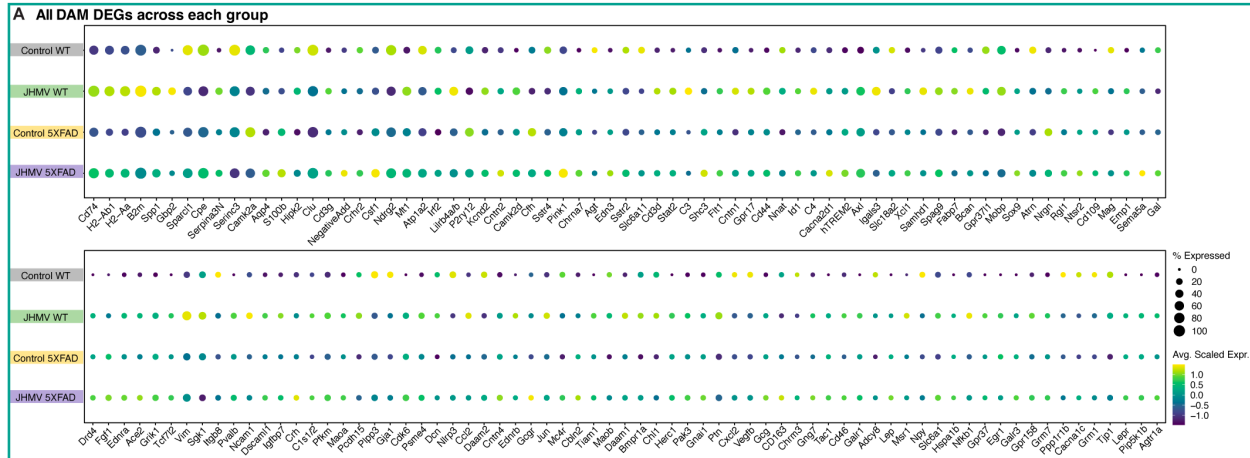


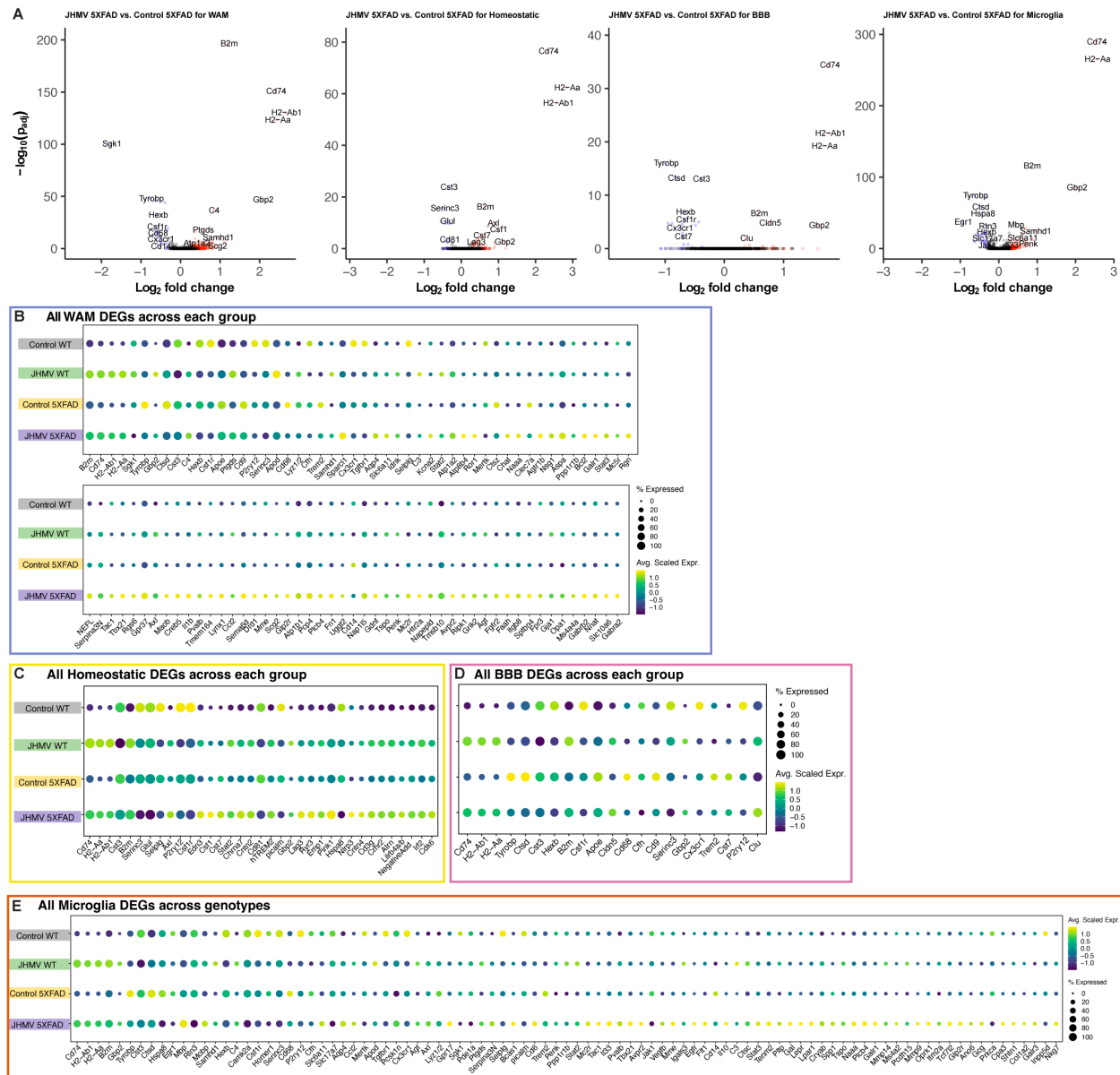


Supplementary Figure 10. A) Pathway analysis by gene ontology for pathways enriched by significantly up-regulated DEGs within myeloid cells for JHMV 5xFAD vs. Control 5xFAD comparison. **B)** Protein-protein interactions (PPI) network of all significantly up-regulated DEGs with myeloid cells for the JHMV 5xFAD vs. Control 5xFAD comparison. **C)** Pathway analysis by gene ontology for pathways enriched by down-regulated DEGs within myeloid cells for JHMV 5xFAD vs. Control 5xFAD comparison. **D)** PPI network of all significantly down-regulated DEGs with myeloid cells for the JHMV 5xFAD vs. Control 5xFAD comparison. Nodes are colored according to its respective enriched pathway analysis. The thickness of each line connection between nodes indicates the degree of confidence in the prediction of the PPI network.

Supplementary Figure 11. Spatial transcriptomic analysis of the myeloid cluster. **A)** Cluster 5 location (highlighted in red) in the original UMAP annotated as Myeloid_1 based on the top 10 expressed genes and its distribution in XY space across all samples. **B)** Cluster 7 location in the original UMAP annotated as Myeloid_Activated based on the top 10 expressed genes and its distribution in XY space across all the samples. **C)** Cluster 22 location in the original UMAP annotated as Myeloid_2 based on the top 10 expressed genes and its distribution in XY space across all the samples. **D)** Cluster 29 location in the original UMAP annotated as Myeloid_DAM based on the top 10 expressed genes and its distribution in XY space across all the samples. **E)** Heatmap of the top 5 genes expressed in each myeloid subcluster. **F)** UMAP of myeloid subclusters split between each experimental group. **G)** Proportions of each myeloid cell type within each experimental group. **H)** Cell counts of each myeloid cell type within each experimental group. **I)** Plot depicting the cell counts of each myeloid subcluster split by experimental group. Abbreviations include: DAM, disease-associated myeloid cells; MC, monocyte-derived cells; BBB, blood-brain-barrier-associated myeloid cells; WAM, white matter-associated myeloid cells.

Supplementary Figure 12. Pseudo-bulk DGE analysis for Myeloid Subclusters DAM and Monocyte-derived cell. **A)** Pseudo-bulk sequencing analysis across all experimental groups depicting expression of DEGs within DAMs identified from the JHMOV 5xFAD vs. Control 5xFAD comparison. **B-C)** Volcano plot depicting DEGs within DAM-1 (B) or DAM-2 (C) myeloid subcluster for the JHMOV 5xFAD vs. Control 5xFAD comparison. **D)** Gene ontology (GO) pathway analysis of up-regulated DEGs in DAM-1 myeloid subcluster in the JHMOV 5xFAD vs. Control 5xFAD comparison. **E)** GO pathway analysis for down-regulated (left) and up-regulated (right) DEGs in the DAM-2 myeloid subcluster in the JHMOV 5xFAD vs. Control 5xFAD comparison. **F)** Monocyte-derived cells-1 (MC-1) and MC-2 subclusters in XY space on representative sections from each experimental group. **G)** List of the top 10 expressed genes in monocyte-derived cell subcluster MC-1 (left) and MC-2 (right). **H)** Volcano plot depicting DEGs in monocyte-derived subcluster for JHMOV 5xFAD vs. Control 5xFAD comparison. **I)** Pseudo-bulk sequencing analysis across all experimental groups depicting expression of DEGs from the JHMOV 5xFAD vs. Control 5xFAD comparison. **J)** Feature plots of *Sal11* and *Gpnmb* expression and their location in the Myeloid Subcluster UMAP.





Supplementary Figure 13. A) Volcano plots displaying fold change of DEGs ($\log_2$ scale) within WAM, homeostatic myeloid cells, BBB, and Microglia subclusters from the JHMY 5xFAD vs. Control 5xFAD comparisons. **B)** Pseudo-bulk sequencing analysis across all experimental groups depicting expression of DEGs within white-matter associated myeloid cells (WAMs) identified from the JHMY 5xFAD vs. Control 5xFAD comparison. **C)** Pseudo-bulk sequencing analysis across all experimental groups depicting expression of DEGs within homeostatic myeloid cells identified from the JHMY 5xFAD vs. Control 5xFAD comparison. **D)** Pseudo-bulk sequencing analysis across all experimental groups depicting expression of DEGs within myeloid cells associated with the blood-brain-barrier (BBB) identified from the JHMY 5xFAD vs. Control 5xFAD comparison. **E)** Pseudo-bulk sequencing analysis across all experimental groups depicting expression of DEGs within microglia identified from the JHMY 5xFAD vs. Control 5xFAD comparison.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1 SUMMARY

Ablation of microglia following infection of the central nervous system with a neurotropic murine coronavirus infection leads to increased demyelination and impaired remyelination

Intracranial inoculation of susceptible C57BL/6 mice with the neuroadapted JHM strain of mouse hepatitis virus (JHMV, a member of the *Coronaviridae* family) results in an acute encephalomyelitis characterized by wide-spread replication of virus in glia with relative sparing of neurons¹. Innate immune responses characterized by the expression of type I interferon is critical with regards to controlling viral replication². Virus-specific CD4⁺ and CD8⁺ T cells are attracted into the CNS in response to T cell chemoattractant chemokines³⁻⁶ and control viral replication via expression of anti-viral cytokines including IFN- γ and cytolytic activity^{7, 8}. Although T cells are efficient in controlling viral replication, sterile immunity is not achieved and virus persists primarily in spinal cord white matter tracts^{1, 9-11}. Persistent viral infection within glial cells leads to expression of chemokine gene expression resulting in chronic inflammation comprised primarily of inflammatory monocyte/macrophages and T cells that contribute to immune-mediated demyelination¹²⁻²². While the contributions of inflammatory CD4⁺ and CD8⁺ T cells and monocyte/ macrophage in contributing to host defense and demyelination have been extensively studied, the contributions of glial cells in these responses during persistent infection remain to be better characterized.

Microglia serve various functions under neuroinflammatory conditions resulting from either infection, injury, or onset of disease by influencing antigen presentation, which either amplifies or restricts disease progression via secretion of regulatory factors that, in turn control the activation state of inflammatory immune cells^{23, 24}. Pharmacologic depletion of microglia via administration of PLX5622, a small molecule antagonist for colony stimulating factor 1 receptor

(CSF1R), prior to JHMV infection results in increased morbidity/mortality associated with impaired control of viral replication, muted anti-viral CD4⁺ T cell responses associated with reduced expression of MHC class II expression on inflammatory monocyte/macrophages²⁵⁻²⁷. Microglia depletion in JHMV-infected mice results in a significant increase in the severity of demyelination accompanied by a reduction in remyelination, indicating that microglia augment both host defense as well as restrict myelin damage and influence repair^{26, 28}. Indeed, microglia have been shown to influence remyelination in response to CNS damage and may serve as therapeutic targets to promote remyelination^{29, 30}. Studies in Chapter 2 aimed to further elucidate microglial contributions to immune-mediated demyelination and pathways promoting remyelination following persistent JHMV infection by timing microglial ablation to 14 days post-infection (p.i.).

Feeding C57BL/6 mice with chow containing the CSF1R antagonist molecule PLX5622 or control chow at 14 days following JHMV infection resulted in a significant reduction of microglia within the brain, while inflammatory monocyte-derived macrophages infiltrating from the periphery remained intact. Importantly, there were no differences in survival or severity of clinical disease associated with JHMV infection, nor were there differences in control of viral replication in brains and spinal cords of control or PLX5622-treated mice. Furthermore, timed ablation of microglia at 14 days p.i. did not affect infiltration of T cell into the CNS, including total or virus-specific CD4⁺ and CD8⁺ T cells. These findings demonstrate that microglia play a minimal role in controlling viral replication in persistently infected mice.

To determine whether microglia contribute to pathways associated with host defense, demyelination, and remyelination, we performed bulk RNA sequencing analysis on extracted RNA from spinal cords of JHMV-infected mice fed either PLX5622 chow or control chow. Compared

to infected spinal cords with intact microglia, we observed several differentially expressed genes within microglia-ablated spinal cords that were significantly down-regulated that are associated with immune cell activation, cytokine production, and cytokine-mediated signaling pathways. We also compared the extent of demyelination in spinal cords of JHMV-infected mice with intact or ablated microglia. Through histology and electron microscopy (EM), we observed that PLX5622-treated mice with ablated microglia had significantly higher demyelination and axonal damage at 28 days p.i., despite similar levels of viral RNA burden to control mice. Furthermore, bulk expression analysis of genes associated with remyelination (e.g., *Lpl* and *Cst7*) and MHC class I and II demonstrate significantly lower levels of transcripts in PLX5622-treated mice. These transcriptional changes were corroborated at the protein level using immunofluorescent staining of MHCII⁺ cells in spinal cords of control and PL5622-treated mice. Despite not impacting viral replication within the spinal cord, these findings support microglial contributions in remyelination and promoting myelin repair during the chronic stage of disease.

These findings speak to the nuanced contributions of microglia during different stages of viral infection and immune-mediated demyelination, where they appear to restrict continuing white matter damage and promote remyelination. Interestingly, despite observing greater demyelination and axonal damage in spinal cords with PLX5622-ablated microglia, the mice did not exhibit increased clinical disease. Mechanisms potentially contributing to this include lack of an overwhelming increase in CNS viral titers in PLX5622-treated mice compared to control mice and this would reduce the activation state of resident glia and infiltrating immune cells thereby potentially limiting axonal damage/function. In addition, blocking CSF1R signaling may alter responses in other inflammatory cells e.g. monocyte/macrophages leading to preservation of

axonal function. Collectively, these findings illustrate the complexity of evaluating CSF1R signaling on disease progression and repair in models of viral-induced neurologic disease.

Finally, these findings contribute to the broader view that microglia alter the microenvironment through secreted growth factors and cytokines. Our previous studies also show that PLX5622-mediated ablation of microglia prior to JHMV infection led to increased spinal cord demyelination and a reduction in remyelination. These changes correlated with reduced expression of transcripts encoding molecules associated with remyelination including Cystatin F (*Cst7*) and Lipoprotein lipase (*Lpl*) by inflammatory macrophages as determined by single cell RNA sequencing²⁶. Bulk RNA sequencing indicated that both *Cst7* and *Lpl* transcripts were reduced within the spinal cords of JHMV-infected mice when treatment with PLX5622 commenced at day 14 p.i., further supporting an important role for these molecules in promoting myelin repair. Ongoing studies will continue to define the molecular mechanisms by which microglia tailor the microenvironment leading to altered disease status, which may lead to discovery of novel therapeutics that can be used for treatment of human demyelinating diseases.

Small molecule inhibitor Nexinhib20 reduces the severity of demyelination in a viral-model of multiple sclerosis

Early following JHMV infection, both infected and uninfected resident cells of the CNS secrete pro-inflammatory cytokines and chemokines that drive infiltration by peripheral innate immune cells³¹. Neutrophils infiltrate as early as 1 day post-infection (DPI) and contribute critically to JHMV pathogenesis by secreting matrix metalloproteases (MMP), such as MMP-9, to help permeabilize the blood-brain barrier (BBB)³²⁻³⁴. In turn, this enables subsequent infiltration by monocytes, macrophages, CD4⁺ and CD8⁺ virus-specific T cells that aid in control of viral replication³⁵⁻³⁷. Although viral titers in the brain are reduced below level of detection (as

determined by plaque assay), viral antigen persists in spinal cord white matter tracts and results in demyelination mediated by chronic infiltration of activated T cells and macrophages³¹. While cells of the adaptive immune response have been extensively studied as important drivers of autoimmune disorders, such as MS, accumulating evidence suggests that innate immune cells, like neutrophils, also play a critical and underappreciated role in disease pathogenesis. Neutrophils have been recognized as active players in the pathophysiology of various autoimmune disorders, including rheumatoid arthritis, psoriasis, and neuromyelitis optica³⁸⁻⁴¹. In MS, elevated neutrophil chemokines and neutrophil counts in serum and cerebrospinal fluid, together with post-mortem evidence of neutrophil accumulation in the choroid plexus, have suggested a potential contribution of neutrophils to MS pathogenesis⁴²⁻⁴⁶. However, the mechanisms underlying their involvement remain to be further examined.

Using a mouse model developed in our laboratory in which tetracycline induces expression of CXCL1 from glial fibrillary acidic protein (GFAP)-positive astrocytes (C57BL/6^{pBI-CXCL1-rtTA}, referred to as “DTg”) we demonstrated that sustained CXCL1 expression derived from astrocytes results in sustained neutrophil infiltration that correlates with increased clinical disease severity and demyelination in both the JHMV model of demyelination⁴⁸ as well as experimental autoimmune encephalomyelitis (EAE)⁴⁹, the prototypic pre-clinical model of autoimmune-mediated demyelination. Conversely, blocking sustained neutrophil infiltration in both models ameliorated these effects, arguing that neutrophils contribute to immune-mediated demyelination. Neutrophils isolated from spinal cords of JHMV-infected DTg mice exhibited increased⁴⁸⁻⁴⁹ expression of transcripts *ASPRV1*, *CD63*, *MMP9*, *S100a8*, and *S100a9*, all of which have been associated with activation of neutrophil extravasation, chemotaxis, and degranulation⁵⁰. To better understand mechanisms by which increased neutrophil infiltration and degranulation amplifies

white matter damage we administered Nexinhib20 (N20), a novel small molecule inhibitor that blocks neutrophil granule exocytosis, to JHMOV-infected DTg and STg mice.

STg and DTg mice were administered N20 or vehicle beginning at 5 days p.i., and were then assessed for changes in weight and presentation of clinical symptoms until 14 days p.i. Although DTg mice treated with N20 demonstrated minimal changes to body weight loss, we observed significant reduction in clinical disease severity by 11 days p.i. and decreased demyelination in spinal cords compared to DTg vehicle controls. Interestingly, N20 did not result in changes to neutrophil infiltration and degranulation within spinal cords of JHMOV-infected DTg mice. Therefore, we turned to investigate whether N20 induced transcriptional changes within infiltrating immune cells.

Utilizing spatial transcriptomic imaging, we identified an increased count of myeloid cells in JHMOV-infected DTg mice and observe that N20 administration increases the presence of neutrophils and disease-associated microglia within spinal cord tissues. In evaluating dysregulated gene transcription within these cell types, we performed differential gene expression analysis on myeloid cells within N20-treated DTg compared to vehicle DTg controls, which illustrated down-regulated expression of genes associated with inflammation (e.g., *B2m*, *Ccl2*, *Cxcl9*, *Srgn*, *Samhd1*, and *Ptprc*). Meanwhile, myeloid cells up-regulated expression of genes associated with disease-associated microglial signatures (e.g., *Apoe*, *Gpnmb*, *Ctsd*, *Spp1*, *Trem2*, and *Lpl*) involved in anti-inflammatory and tissue protective pathways. Importantly, the increased DAM presence in N20-treated DTg spinal cords, rather than being a paradoxical finding, is consistent with a growing body of literature establishing DAMs as a protective, phagocytic myeloid state. Thus, treatment of DTg mice with N20 seems to promote an overall decrease in stimulation of inflammation which

in turn allows for an overall shift towards activation of anti-inflammatory pathways and initiation of tissue repair programs.

Taken together, these data support a potential key role for neutrophil degranulation in amplifying white matter injury in a model of sustained neutrophil infiltration during chronic JHMV-induced demyelination. Rather than serving solely as transient innate responders, under chronic stimulation, as in RRMS and PMS conditions, neutrophils can accumulate in increased numbers and maintain a chronic transcriptional program that favors granule mobilization and release, thereby creating a microenvironment conducive to ongoing tissue damage. Pharmacologic inhibition of granule exocytosis with N20 disrupts these processes and significantly reduces disease burden in this pre-clinical model of chronic neutrophil activation, highlighting neutrophil degranulation as a modifiable contributor to CNS pathology in disorders like MS. The current findings provide strong evidence that targeting neutrophil granule exocytosis represents a promising approach to limiting immune-mediated demyelination while preserving core antimicrobial functions.

Infection of 5xFAD mice with a mouse-adapted SARS-CoV-2 does not alter Alzheimer's disease neuropathology yet induces wide-spread changes in gene expression across diverse cell types

Alzheimer's disease (AD) is the most common cause of age-related dementia, impairing memory and cognitive function. Pathology includes the accumulation of amyloid plaques and neurofibrillary tangles, accompanied with neuronal loss and neuroinflammation⁵¹. AD is a multifactorial disorder influenced by several risk factors including age⁵², genetics⁵³, and environmental factors⁵⁴. Some studies suggest viral infections may accelerate disease onset or exacerbate existing pathology and clinical symptoms associated with neurodegenerative diseases,

including AD⁵⁵ and other studies have implicated microbial pathogens in AD pathogenesis^{56,57}, highlighting the potential contribution of infection to AD and related dementias.

Recent studies have reported that infection with either SARS-CoV-2 or mouse-adapted SARS-CoV-2 (MA10) has led to the presence of gliosis and other CNS changes in animal models in the absence of virus in the brain, arguing that peripheral inflammatory responses to viral infection can impact CNS resident cell response⁵⁸⁻⁶⁰. While COVID-19 most commonly presents with respiratory symptoms, neurological symptoms have been extensively documented, and can emerge during acute infection and persist beyond viral clearance⁶¹. The full extent of neuropathological changes in the CNS that results from SARS-COV-2 infection is still not fully understood. Given the association between COVID-19, neuroinflammation, and neurodegenerative processes, further characterization of CNS alterations following infection and their potential contribution to neurodegeneration is needed.

To address these questions, we infected aged (10–14 month old) C57BL/6 wild-type (WT) and 5xFAD mice with mouse-adapted SARS-CoV-2 (MA10) to assess neuropathological changes following chronic infection and examine infection-induced alterations in brain gene expression. We utilized aged 5xFAD mice to evaluate the effects of SARS-CoV-2 infection on a brain with already established amyloid pathology. This approach was intended to model the clinical scenario of infection in elderly patients with pre-existing dementia. It remains possible that infection at a younger age, prior to the onset of significant A β deposition, might more profoundly influence the trajectory or magnitude of subsequent pathology—a question that warrants further investigation.

As expected, the accumulation of amyloid neuropathology induced increased astrocyte activation and microgliosis indicated through GFAP and IBA1 expression within 5xFAD brains, respectively. However, we observed no significant changes in gliosis within the brains of MA10-

infected WT and 5xFAD mice compared to their respective uninfected controls. While bulk sequencing revealed dysregulated pathways associated with neuronal and synaptic function, MA10 did not significantly alter levels of A β plaque burden, dystrophic neurites, neuronal loss, and synapse loss in the 5xFAD brain.

Furthermore, biochemical assays measuring inflammation-associated cytokines present in the plasma such as interleukin-1 β (IL-1 β) remained unchanged following infection. Similarly, immunostaining for *Lgals3*/MAC2 revealed minimal infiltration of peripheral monocyte/macrophages into the brains of MA10-infected mice. However, histological analysis is limited in its capacity to capture phenotypes illustrating the neuroimmune environment in the brain following MA10 infection. Fortunately, the downgrading of MA10 biosafety classification from Biosafety Level 3 to Biosafety Level 2 Plus will now enable more comprehensive profiling of peripheral immune responses impacting the brain through Flow cytometry and fluid biomarkers of neuroinflammation and neurodegeneration at the protein level.

Despite the absence of overt neuropathological changes associated with Alzheimer's disease and neurodegeneration, spatial transcriptomic imaging revealed dysregulated gene expression across multiple CNS-resident cell types that appeared subtle yet potentially carry consequential molecular alterations to key biological pathways. Significant changes in homeostatic gene expression were observed in microglia, astrocytes, oligodendrocytes, inhibitory neurons, and vascular endothelial cells, suggesting early signatures of neuroinflammation and homeostatic disruption that may precede longer-term neurodegenerative processes. One potential mechanism addressing these impacted transcriptional signatures may involve the role of peripheral inflammation responding to replicating MA10 virus in lung tissue, inducing the production of proinflammatory cytokines/chemokines that circulate through the vasculature and entering the

CNS where it might affect glial homeostasis. Importantly, these findings illustrate the far-reaching consequences of peripheral MA10 infection and its resulting inflammatory responses that permeate into the brain and induce subtle dysregulation of homeostatic genes across CNS cell types. Future investigations are well warranted to manipulate molecular mechanisms underlying these MA10-associated neurological effects to better prepare for the potential emergence of downstream COVID-19 consequences on cognitive function and neurodegeneration.

Acute viral encephalitis impacts dense-core amyloid plaque pathology and dysregulates myeloid responses to amyloid plaques

Microbial infection has been broadly explored as a mechanism through which both genetic and environmental contributions interact together to influence AD risk and neuropathology. Pathogenic microbes that invade the CNS can cause significant neuroinflammation through CNS-resident immune cells and infiltration of peripheral immune cells such as monocyte/macrophages and T cells. To illustrate the association of viral infection with several neurodegenerative diseases, Levine et al.⁵⁵ mined medical record data from both the Finnish biobank (FinnGen) and UK Biobank. Their findings demonstrated several viral pathogens tightly associated with different neurological diseases, while the strongest association was between AD and viral encephalitis⁵⁵. Viral encephalitis is defined as viral-mediated inflammation of the brain parenchyma. While the blood-brain-barrier (BBB) prevents most viral pathogens from neuroinvasion, neurotropic viruses can circumvent these barriers to infect and replicate within the CNS⁶². The resulting inflammation via indirect neuroimmune mechanisms may induce acute and chronic neurologic damage with potential consequences in the onset or severity of neurodegenerative disease⁶³⁻⁶⁵.

This study was performed to better elucidate the interaction between viral-induced inflammation and amyloid pathology associated with Alzheimer's disease using experimental

infection of aged 5xFAD and WT mice with JHMV, a neurotropic murine coronavirus. We demonstrate that acute JHMV infection in 5xFAD mice results in similar inflammatory responses in parallel to significant reductions in A β plaque sizes despite no significant changes to overall A β plaque load. we observed compaction of dense-core A β plaques and reduced numbers associated with MAC2⁺ macrophages within subiculum and somatosensory cortex regions, respectively. Within regions where viral RNA/antigen persists, increased recruitment of inflammatory monocyte/macrophages and T cells significantly correlate with more compact A β plaques. Furthermore, we utilized spatial transcriptomic imaging to investigate the transcriptional impact of viral encephalitis with single-cell resolution and determine altered gene expression within specific cell types surrounding A β pathology in the 5xFAD brain. While several cell types were dysregulated following viral encephalitis, we show that myeloid cells demonstrate a suppressed DAM response in the brain of JHMV-infected 5xFAD mice, particularly those surrounding amyloid pathology. These findings reveal that viral encephalitis and its resulting inflammatory mechanisms impacts the expression of key genes within myeloid cells responding to A β plaque pathology, which has implications to the role of viral encephalitis as a risk factor for AD.

This study builds upon an ongoing question in the field about the role of infectious microbes, its result inflammation, and its effects on the progression and severity of AD pathologies. Previously, our lab has reported on the impact of JHMV encephalitis on the 3xTg-AD transgenic mouse model, which uniquely combines the autosomal dominant Swedish mutation in human *APP* to reproduce amyloid pathology and two human *MAPT* mutations associated with frontotemporal dementia (FTD) to reproduce tauopathies⁶⁶. While JHMV infection did not impact A β pathology in 3xTg-AD brains, the resulting encephalitis did appear to worsen tau pathology⁶⁶. to overexpress rare autosomal-dominant mutations limits the clinical relevance of this approach.

With the advent of better humanized mouse models that have the potential of recapitulating amyloidosis and tauopathy together, future investigations investigate both the acute and post-acute impact of viral encephalitis on these mice may offer more relevant and comprehensive understanding of its interactions with AD pathologies and its severity. Committing to defining these post-infectious neuroimmune mechanisms and their interaction with AD pathogenesis holds promise, with potential to offer both treatments for current patients and preventative interventions to improve public health outcomes and reducing AD risk in an aging population.

6.2 FUTURE DIRECTIONS

Our overall findings demonstrate that viral encephalitis is associated with changes to dense-core plaque pathology, while inducing significant changes gene expression across multiple cell types in the brain. For example, myeloid cells like microglia maintain important roles in compacting and clearance of fibrillar A β proteins. JHNV encephalitis down-regulates key genes associated with DAM transcriptional signatures, which are involved in these vital responses to A β pathology. Furthermore, we have shown that even systemic infection of MA10 SARS-CoV-2 in the periphery is sufficient to induce detectable and long-lasting transcriptional changes in the brain associated with neuronal dysfunction and several homeostatic microglial functions such as survival, communication, migration and phagocytic capacity. However, the specific molecular and cellular mechanisms contributing to these changes in myeloid gene expression following viral encephalitis remain unclear and warrant further investigation and mechanistic studies to determine its lasting effect on AD neuropathology.

Future experiments are ongoing to investigate the role of viral encephalitis on hyperphosphorylated tau accumulation tied to AD-associated neurodegeneration and cognitive decline. Building on previous work investigating the impact of JHNV infection on the 3xTg-AD

mouse model, preliminary studies using the PS19 tauopathy mouse model have identified lasting impacts to the progression of tau pathology and will be investigating the role of T cells in mediating these effects. Additionally, it will be necessary to investigate how viral encephalitis impacts the progression of both amyloid and tau pathology through JHMV infection during earlier stages of disease before significant neuropathology appears. Meanwhile, several experiments are expected to address the impact of systemic SARS-CoV-2 infection on key cellular functions within the CNS through mechanistic studies that probe molecular changes leading to neuronal and microglial dysfunction. Fortunately, the advent of new technologies such as spatial transcriptomic and proteomic imaging enables a high throughput method of identifying dysregulated pathways with cellular and spatial resolution at the gene and protein level.

Beyond viral contributions and interactions to the progression of AD neuropathology, other infectious agents have recently demonstrated notable findings that associate overall anti-microbial inflammation responses with AD. For example, Gaire et al. recently identified higher burden of *Chlamydia pneumoniae* bacterial inclusions within the retina and brains of AD patients, which increased with APOE ϵ 4, disease stage, and cognitive deficits⁶⁷. Furthermore, proteomics from these tissue also revealed upregulation of bacterial-infection and NLRP3-inflammasome pathways, which they reproduced in *C. pneumoniae*-infected APP^{SWE}/PS1 ^{Δ E9} mice and found induction of A β , neuroinflammation, neurotoxicity, and worsened cognition⁶⁸. Conversely, parasitic infections of *Toxoplasma gondii* have also been implicated in reducing A β plaque pathology associated with increased monocyte and microglial phagocytosis⁶⁹⁻⁷⁰. Recently, Yanes et al. demonstrated increased expression of DAM genes such as *Aif1*, *Axl*, and *Clec7a* and identified A β plaques surrounded by T cells, patrolling Ly6C^{lo} monocytes, and inflammatory Ly6C^{hi} monocytes recruited from skull bone marrow⁷⁰. Therefore, future investigations may focus

on treatments broadly targeting anti-inflammation pathways as an effective strategy to mediating the progression of AD neuropathology.

As ongoing research is performed identifying how infectious viral agents may contribute or interact with AD pathology, an increasing number of studies have demonstrated potentially protective effects of anti-viral strategies on the progression of AD neuropathologies. For example, patients with HSV-1 who used antiherpetic therapies were less likely to develop AD than those who did not use antiherpetics⁷¹. Notably, data from US medical surveys demonstrated a reduced risk for dementia and cognitive impairments following vaccination against HZ⁷². This relationship between HZ vaccination and reduced dementia incidence was also observed in a population in Wales and South Korea^{73,74}. Even repeated vaccination against influenza seemed to confer a slight reduction to the risk of developing dementia^{73,74}. Unfortunately, while these observation studies demonstrate an association between dementia risk and anti-viral treatments and vaccination, it may not completely mitigate the onset of dementia or neurodegenerative diseases like AD. Future investigations exploring the relationships between anti-viral immunity, AD pathogenesis, and dementia symptoms will be necessary to consider utilizing these treatments as means to prevent or mitigate subsequent neurodegeneration exacerbated by microbial infection.

Broadly, this work contributes to a growing body of exposome research investigating key interactions between genetic risk and environmental contributions to neurodegenerative conditions like Alzheimer's disease⁷⁵. These environmental contributions include exogenous factors such as diet, microbial infection, and exposures to heavy metals and chemical, while behavior factors include substance use, sleep behaviors, and stress⁷⁶. Recently, a massive study leveraging machine learning, brain imaging (fMRI), and electrophysiological techniques (M/EEG) across 18,701 participants in 34 countries identified that exposome burden accounted for 3.3 to 9.1-fold higher

risk of accelerated aging⁷⁷. The advent of more sophisticated methods in analyzing these massive and complex datasets at the systems level will further clarify gene-environmental interactions that confer AD risk across the lifespan not only at the individual level, but also across widespread populations⁷⁸.

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