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Trends in neuroimmunology: Cross-talk between brain-resident and peripheral immune cells in both health and disease

Edited by
Estela Maris Muñoz, Rajnikant Mishra and Veronica Martinez Cerdeño

Coordinated by
Janina Edith Borgonovo, Shashank Kumar Maurya and Suryanarayan Biswal

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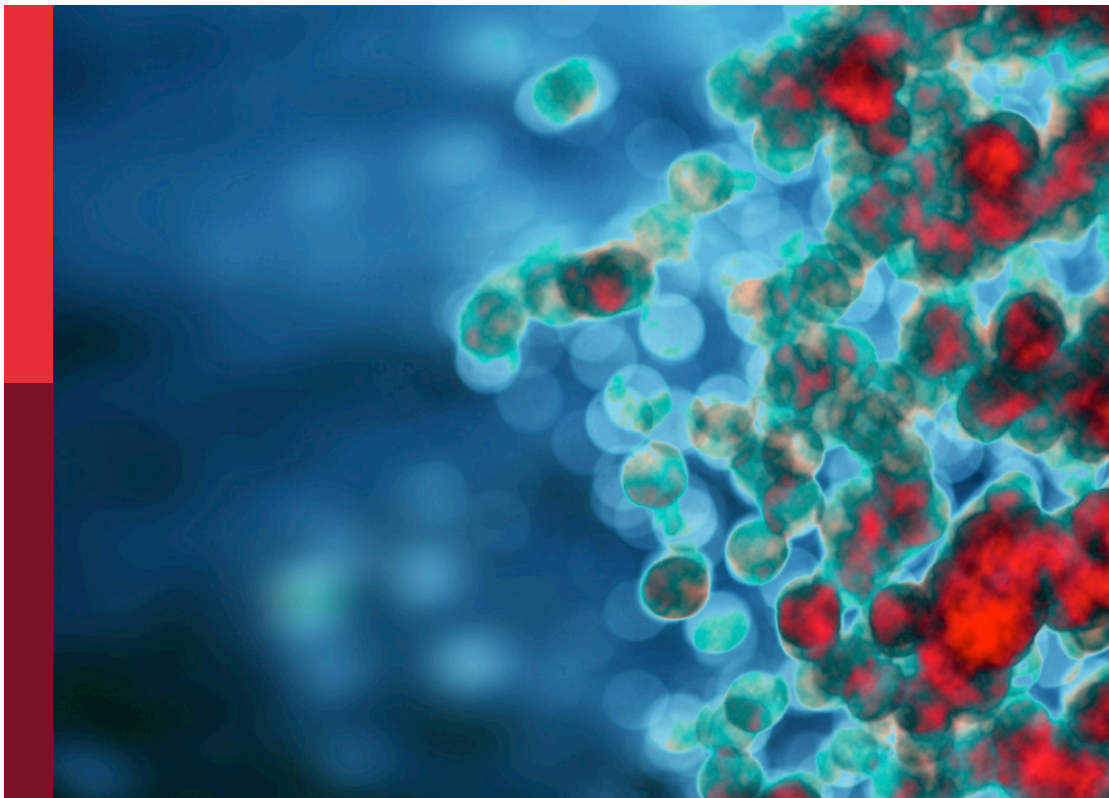

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Trends in neuroimmunology: Cross-talk between brain-resident and peripheral immune cells in both health and disease

Topic editors

Estela Maris Muñoz — Institute of Histology and Embryology of Mendoza
Dr. Mario H. Burgos (IHEM)-UNCuyo-CONICET, Argentina
Rajnikant Mishra — Banaras Hindu University, India
Veronica Martinez Cerdeño — University of California, Davis, United States

Topic coordinators

Janina Edith Borgonovo — University of Chile, Chile
Shashank Kumar Maurya — University of Delhi, India
Suryanarayan Biswal — Central University of Punjab, India

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EDITED AND REVIEWED BY
Hans-Peter Hartung,
Heinrich Heine University, Germany

*CORRESPONDENCE
Shashank K. Maurya
smaurya1@zoology.du.ac.in;
maurya.shashankkumar@gmail.com
Rajnikant Mishra
rmishraa@bhu.ac.in
Estela M. Muñoz
munoz.estela@fcm.uncu.edu.ar;
emunoz@mendoza-conicet.gob.ar

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Editorial: Trends in neuroimmunology: cross-talk between brain-resident and peripheral immune cells in both health and disease

Shashank K. Maurya[1]*, Janina E. Borgonovo[2], Suryanarayan Biswal[3], Verónica Martínez-Cerdeño[4], Rajnikant Mishra[5]* and Estela M. Muñoz[6]*

[1]Biochemistry and Molecular Biology Laboratory, Department of Zoology, Faculty of Science, University of Delhi, Delhi, India, [2]Integrative Biology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile, [3]Department of Human Genetics and Molecular Medicine, Central University of Punjab, Bathinda, India, [4]Department of Pathology and Laboratory Medicine, Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children of Northern California, and MIND Institute at the UC Davis Medical Center, University of California, Davis School of Medicine, Sacramento, CA, United States, [5]Biochemistry and Molecular Biology Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, India, [6]Institute of Histology and Embryology of Mendoza (IHEM), National University of Cuyo (UNCuyo), National Scientific and Technical Research Council (CONICET), Mendoza, Argentina

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Editorial on the Research Topic

Trends in neuroimmunology: cross-talk between brain-resident and peripheral immune cells in both health and disease

The functional anatomy of organisms is maintained by the coordination of different systems, which often rely on interactions between specialized cells and between macromolecules. The immune system works with the circulatory and lymphatic systems to protect most of the organs. However, some organs are considered immune privileged due to the presence of highly selective and regulated barriers, such as the blood-brain barrier (BBB) within the brain (1). The BBB controls periphery-brain molecule exchange and prevents immune effector cells from entering the homeostatic brain. BBB-associated elements, such as endothelial cells, pericytes, astrocytes, and microglia, potentially can function as antigen-presenting cells (APC). Pathological scenarios that induce dysfunction of the BBB and its associated cells may lead to the infiltration of lymphocytes, crossing over from the blood to the brain. Similarly, traumas can also enable B and T lymphocytes to pass bidirectionally between the central nervous system (CNS) and the periphery, via the meningeal lymphatic vessels, which drain into the cervical lymph nodes. Research in animals and humans has revealed that B and T cells are involved in the progression of neurological diseases (NDs). It has been shown that under certain conditions, T cells establish themselves and become resident in the brain, from where they can exert either beneficial or detrimental effects on brain function. Amazing efforts have been made to further comprehend interactions between brain-specific cells and peripheral immune cells,

especially their roles and impact on the onset, progression, and eventual resolution of diverse brain pathologies (2–4). The Research Topic discussed herein represents an effort of Frontiers Media S.A. and the authors of this Editorial to develop another special volume related to the healthy and diseased brain (5–8). This Research Topic, which is available for the scientific community and the public, focuses on understanding the complexity of central immune cells and peripheral immune cells, and their cross-talk mechanisms in diverse CNS pathologies. Eleven peer-reviewed manuscripts including four original articles, six reviews, and one systematic review, encompass this special volume. Seventy-five authors from research laboratories located in six countries: Australia, China, Germany, Japan, United Kingdom, and United States took part in this initiative.

Among the interesting contributions, an *in vitro* study on primary murine glia by Li et al. showed differential substrate-dependent and time-dependent phagocytic behavior and phenotypic plasticity among M0-like (unstimulated), M1-like (pro-inflammatory) and M2-like (anti-inflammatory) microglia subtypes. Although the application of M1/M2 terminology in the microglia field has been dismissed, the coexistence of pro-inflammatory and anti-inflammatory microglial states has been documented, including in circumventricular organs (9–12). Li et al. differentiated cultured glial cells into M1-like and M2-like microglia subtypes by treating them either with granulocyte colony stimulating factor and interferon-gamma (GM-CSF/IFNγ), or with macrophage colony-stimulating factor and interleukin-4 (M-CSF/IL-4), respectively. No supplements were added to obtain M0-like microglia. Phagocytosis assays using *E. coli*-rhodamine particles or IgG-FITC beads revealed different preferences and dynamics for the substrates among the microglia subtypes. M1-like microglial cells engulfed more bacteria particles than beads after 3 hours. The opposite behavior was observed with the anti-inflammatory subtype, where M0-like microglia internalized both substrates equally. The authors reported further differences among the three differentiated microglial phenotypes during incubation with both substrates for 16 hours. M2-like microglia showed discontinuous phagocytosis after 8 hours, while M0-like and M1-like microglial cells continuously internalized substrates with different profiles. One interesting observation after a prolonged exposure for 5 days to either *E. coli* particles or IgG-opsonized beads, was that M1-like states and M0/2-M1 transitions were both enhanced, indicating phenotypic plasticity like it occurs in neurodegenerative conditions (13, 14). The study by Li et al. complements the existing knowledge about microglia diversity and plasticity (15, 16), and it opens therapeutic avenues to intervene in microglia-mediated inflammation and neurodegeneration.

Neumaier et al. reviewed current knowledge and therapeutic potential of midkine (MDK), which is a neurotrophic growth factor with dual functions in the healthy and diseased CNS and periphery (17, 18). Due to its multi-functionality, MDK has been involved in the progression or suppression of numerous CNS-related pathologies including autoimmune disorders, such as multiple sclerosis (MS), brain tumors, acute injuries, and other conditions that imply neuroinflammation and neurodegeneration. In the CNS, MDK is spatio-temporally expressed by oligodendrocytes, astrocytes, and neuronal lineages, and maybe also by microglia in response to inflammatory stimuli. In the periphery, hematopoietic and non-hematopoietic cells can produce MDK. This regulator acts through multimolecular receptor complexes, with protein tyrosine phosphatase ζ (PTPζ) as one of the most established components. In addition, multiple signaling pathways are involved depending on the cellular context, thereby facilitating MDK's multifaceted functions. Interestingly, the authors discussed the role of MDK as a mediator of the neuro-immune cell-to-cell cross-talk in CNS inflammatory scenarios that involve a dysfunctional or leaky BBB. These conditions facilitate the infiltration of MDK-expressing immune cells from the periphery. The recruitment of peripheral immune actors such as macrophages and T cells, and the impact of MDK-signaling events on CNS-resident cells are also addressed within the context of neoplastic diseases (19). The findings included in this review support the importance of MDK as a mediator of tumorigenesis and inflammatory disorders, irrespective of the tissue and cell type, and they emphasize the need for further research to better understand its mechanisms and biomarker potential in neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (PD and AD, respectively).

Neuroinflammation and neurodegeneration are associated with traumatic spinal cord injuries (SCI), which are highly debilitating pathologies. SCI progresses through various phases: acute (up to 3 days post-injury; dpi), subacute (3–14 dpi), and chronic (more than 14 dpi) stages. Yao et al. investigated differential gene expression profiles and pathways in macrophages and microglia across these SCI phases to pinpoint potential therapeutic targets for SCI. The authors applied bioinformatic analysis to the existing scRNA-seq dataset GSE159638 (total 30,958 cells; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159638), which was generated in a mouse model of thoracic contusion SCI (20). Then, they validated the results in a mouse model of cervical SC hemi-contusion injury [wild type and APOE$^{-/-}$ mice; (21)]. They identified apolipoprotein E (APOE) as a central gene of interest in both macrophages and microglia during the subacute and chronic phases of SCI. These cells exhibited high activity, suggesting a crucial role in regulating SCI-associated inflammation. On the other hand, APOE has been linked to pathways related to debris and dead cell clearance (phagocytosis), lipid metabolism, and lysosomal function (22–24). Subsequent experiments demonstrated that APOE knockout (KO) in mice exacerbated neurological deficits, increased neuroinflammation, and worsened white matter loss after SCI at the cervical level. Following SCI, ultrastructural analysis of the KO mice revealed myelin uptake and accumulation of lipid droplets, lysosomes, and needle-like cholesterol crystals in macrophages and microglia. APOE is vital for cholesterol homeostasis within the CNS (25). Together, these results make APOE and its associates promising therapeutic targets for reducing neuroinflammation and for enhancing recovery after SCI.

Zhang et al. contributed to this Research Topic with a comprehensive review of the impact of inflammation and the involvement of infiltrated regulatory T cells (Treg cells) on neuropathic pain (NP) following spinal cord injury (SCI), as well as the potential of cellular therapeutic interventions in SCI-related conditions. The authors discussed the mechanisms behind

inflammation and NP after SCI, which include a plethora of cells and mediators. Glial cells, including astrocytes and microglia, and infiltrated immune cells, such as monocytes, macrophages, B cells, and T cells, are involved in these scenarios. These cells, when activated, release inflammatory mediators including chemokines (e.g., CXCL1 and CXCL2) and cytokines (e.g., TNF-α, IL-1β, and IL-6) (26). These molecules affect neurons through multiple signaling pathways, leading to neurotransmitter and ion channel imbalance, increased neuronal excitability, decreased neuronal inhibition, and boosted pain transmission (27). In particular, the authors reviewed the mechanisms by which astrocytes, microglia, and immunosuppressive Treg cells intervene in the pathogenesis of SCI and the subsequent NP. In fact, the inflammatory response following SCI has been tightly linked to a reduction in the number of Treg cells (28). Finally, this review provides a framework for thinking about strategies and challenges (i.e., cell purity, stability, and functionality) in the application of Treg cell therapy in SCI patients who suffer from neuropathic pain.

Chronic inflammation has been associated with different neurological disorders (NDs). Cumulative evidence showed that the recruitment of peripheral immune cells into the CNS is a common characteristic in various NDs (29–32). Among these neuroinflammatory cells, T helper (Th) 17 lymphocytes play an active role in the pathogenesis of CNS-related diseases. The biology of this $CD4^+$ Th cell subtype in NDs is addressed in this volume by Shi et al. Th17 cells and their cytokines (e.g., IL-17A, IL-23, IL-21, IL-6, and IFN-γ) contribute to the disruption of the BBB, promote the infiltration of other immune cells into the CNS, excessively activate microglia, and can cause direct cytotoxic damage to neurons (33). The authors described Th17 lymphocytes, including the signaling pathways that induce their differentiation. They also introduced ND-linked environmental factors that may induce the pathogenic potential of Th17 cells, such as peripheral inflammation, enhanced oxidative stress, and changes in the microbiota or diet that affect the gut-brain axis. Shi et al. further discussed the possible immunopathological mechanisms of Th17 cells in AD, PD, MS, amyotrophic lateral sclerosis (ALS), and major depressive disorder (MDD). Finally, therapeutic strategies targeting Th17 lymphocytes, their associated cytokines, and Th17-related molecular mechanisms to treat neurodegenerative diseases, are also addressed.

Grotemeyer et al. elaborated a detailed summary of the interconnected innate and adaptive immune responses in the context of Parkinson's disease (PD), which is a ND characterized by neuroinflammation and dopaminergic neurodegeneration (34, 35). Interestingly, they propose a mechanism for how neuroinflammation is triggered in PD. They hypothesized that pathological forms of alpha-synuclein (pαSYN), the key protein in PD, might act as a damage-associated molecular pattern (DAMP) to induce and maintain a pro-inflammatory shift of the immune system, via pattern recognition receptor (PRR)-mediated processes (36). Central (e.g., microglia) and peripheral (e.g., T and B cells) immune cells and their mechanisms in the pathophysiology of PD in humans and animal models (e.g., MPTP, 6-OHDA, viral vector, and preformed fibrils), are discussed. Circulating and infiltrated $CD4^+$ and $CD8^+$ T cells are among the immune effector cells in PD, and their roles are both beneficial and detrimental. The authors also summarized current clinical trials on anti-inflammatory therapy in PD, focusing on the regulation of glucose metabolism, intestinal microbiota, and oxidative stress. Then, they discussed different signaling pathways associated with inflammation and neurodegeneration, such as the pentose phosphate pathway (PPP) (30) and the renin-angiotensin [-aldosterone] system (RA[A]S) (37), to use them as potential therapeutic targets. Finally, the authors suggested that dopaminergic neurodegeneration could be halted by administrating neuroprotective/anti-inflammatory agents early in the course of PD, before severe symptoms have developed.

The involvement of innate and adaptative immune cells in the pathophysiology of multiple sclerosis (MS), and their regulation by physical exercise, are addressed in this Research Topic by Zong et al. The pathogenesis of this neuroinflammatory and autoimmune disease is driven by the dysfunctional activity of immune cells, including those recruited from the periphery into the CNS (38). Aberrant immune responses damage oligodendrocytes and thus, cause severe demyelination, impaired remyelination, axonal degeneration, and altered neurotransmission (39, 40). This results in a spectrum of motor and non-motor symptoms. The disease has no cure and pharmacotherapy is considered the primary treatment. However, drugs have low efficacy, several side effects, and high costs. Alternative MS-modifying interventions, such as physical exercise, have gained attention as a new therapy to alleviate patients' symptoms (41, 42). In summary, the authors present morphological, cellular, and molecular evidence from animal models (e.g., EAE and toxin and/or virus-induced demyelination models) and human studies of how this type of adjunctive intervention regulates innate and adaptive immune cells, reducing peripheral immune cell infiltration, and eventually leading to a reduction of the autoimmune responses and their concomitant negative effects in the CNS. The authors focused this review specifically on T cells (e.g., $CD8^+$ and $CD4^+$ cells, including Th17 and Treg cells), B cells, dendritic cells, neutrophils, microglia/macrophages, and astrocytes. Zong et al. also raised a critical view towards the need to conduct more studies in humans, stratifying patients by gender, disease stage, and type, duration, intensity, and cycle of exercise, to better understand the potential of the physical therapy in treating MS.

Beyond the immunological roles, immune cells can participate in other physiological responses that are essential to maintain the homeostasis of the organisms (43, 44). One instance of this is the interplay between enteric C1q-producing macrophages and the enteric nervous system to regulate neuronal and smooth muscle cell functions and thus, gastrointestinal motility and homeostasis (45). It was previously reported that bidirectional signaling between muscularis macrophages and enteric neurons is necessary to ensure gut peristalsis in healthy mice (46). Macrophage-derived bone morphogenetic protein 2 (BMP2) and neuronal colony stimulatory factor 1 (CSF1) are involved in this cross-talk mechanism. In this context, Yip et al. contributed to this Research Topic with an original article in which they studied the participation of CD163 intestinal macrophages and inhibitory interneurons of the myenteric plexus in the regulation of colonic

motility. They used a conditional KO *Cx3cr1* (chemokine receptor)-*Dtr* (diphtheria toxin receptor) rat model to transiently deplete resident macrophages in combination with the nitric oxide synthase (NOS) inhibitor NOLA (Nω-nitro-L-arginine), and *ex vivo* video imaging (47, 48). The authors showed that the resident intestinal macrophages are crucial in regulating colonic motility in the absence of the inhibitory neuronal input driven by NO. Whereas, under control conditions, these macrophages might not be relevant. They also showed that these immune cells are important in maintaining healthy intestinal structure. The authors highlighted CD163-positive intestinal macrophages as a potential therapeutic target for gastrointestinal disorders in which inhibitory neuronal input is impaired, such as gastroparesis and achalasia (49, 50). However, Yip et al. pointed out the need for further research to dissect the cell subtypes and to investigate the mechanisms of these functions.

Viengkhou and Hofer reviewed the dual pivotal roles of type I interferons (IFN-Is) in regulating cellular and molecular homeostasis within the CNS, as well as inflammation and immunity associated with diverse NDs, from chronic infections and autoimmune conditions to trauma, aging, and neurodegeneration. Some of these conditions are known as interferonopathies. The authors initially discussed mechanisms by which levels of IFN-Is are altered, especially those mediated by innate immune sensors (e.g., cyclic GMP-AMP synthase/STING signaling pathway), by genetic alterations (e.g., trisomy 21 and mutations in *USP18* or *ISG15*), and by therapeutic interventions for diseases like chronic viral infections, MS, and certain cancers (51, 52). They further presented the canonical and non-canonical IFN-I signaling pathways that imply binding to cell surface receptors and activation of distinct response phases, including an early widespread protein phosphorylation stage and changes in the expression of several IFN-regulated genes (IRGs) (53). The classic path involves the interferon-stimulated gene factor 3 (ISGF3) complex (54), which consists of the transcription factors STAT1 (signal transducer and activator of transcription 1), STAT2, and interferon regulatory factor 9 (IRF9). Then, Viengkhou and Hofer focused the review on the specific responses to IFN-Is mounted by each cell type in the CNS, especially those mediated by neurons, glial cells, and BBB-associated cells. Understanding the diversity in cell responses has been facilitated by single-cell technologies. Moreover, it has been accepted that a diverse spectrum of cellular response states coexists within the diseased CNS, instead of a single prevalent response. Neurons respond to limit the impact of viral infections, but they can suffer neurotoxic effects from increased IFN-I signaling, including fewer dendrites, impaired neurogenesis, and altered neurotransmission (55). Although basal IFN-I signaling in astrocytes is crucial for brain health (56), its contribution to IFN-I neurotoxicity seems yet unclear. A small IFN-I-hyperresponsive microglia subset was identified by single-cell sequencing, which has been associated with age-dependent cognitive decline and synaptic stripping (57, 58). Due to that chronic inflammation has been related to NDs and that IFN-I therapy has been shown to have adverse effects, the authors finished their review discussing the implications and mechanisms of IFN-Is in cerebral interferonopathies, such as Aicardi-Goutières Syndrome (AGS) and chronic viral encephalopathies, as well as in aging, and in diseases with abnormal protein aggregation, including AD and PD (59–61). The authors pointed out that understanding the complexity of IFN-I responses in the CNS is critical for developing targeted therapies for neurological disorders that occur with IFN-I dysregulation. These therapies should consider factors such as cell type, signaling duration, and disease context.

Sun et al. conducted a Mendelian randomization (MR) study to explore the causal relationship between immune cell surface antigens and post-stroke functional outcomes, and to identify novel biomarkers and therapeutic targets for ischemic stroke. The authors employed Genome-Wide Association Studies (GWAS) summary statistics for a two-sample MR analysis, followed by several alternative methods and sensitive approaches. They sourced genetic variants linked to immune cell surface antigens (measured by median fluorescence intensities, MFIs) from the publicly available GWAS catalog (62); outcome data from the Genetics of Ischemic Stroke Functional Outcome (GISCOME) network (63, 64), and statistics about the risk of ischemic stroke from the MEGASTROKE consortium (65). The cohorts were primarily of European ancestry, aged 18 and above. A total of 389 MFIs with surface antigens were included in seven panels (maturation stages of T cell, Treg cell, TBNK, DC, B cell, monocyte, and myeloid cell, respectively). The authors identified genetic variants including single nucleotide polymorphisms (SNPs) associated with MFIs of immune cell surface markers, as measured from samples of peripheral blood. They meticulously selected SNPs that were strongly linked to markers and less likely influenced by non-genetic factors like lifestyle, and they treated them as instrumental variables (IVs) for the MR analysis (66, 67). After a comprehensive analysis, Sun et al. identified 13 suggestive immune cell surface antigens that appear to be associated with post-stroke outcomes. Notably, elevated levels of CD20 on switched memory B cells and of PDL-1 on monocytes appeared to be linked to worse stroke outcomes and severity. In contrast, surface antigen CD25 on $CD39^{+}$ resting Treg cells was found to be associated with favorable post-stroke functional outcomes, possibly due to enhanced Treg cell survival supported by IL2 affinity (68). CD39 was highlighted for its immunosuppressive role, which may be crucial for long-term immune balance after stroke (69). The authors discussed limitations of their analysis including those related to the nature of the sourced data. Overall, this study uncovers potential novel biomarkers and therapeutic strategies targeting immune cell surface antigens to enhance post-stroke recovery, and it warrants further exploration and validation across diverse populations and stroke subtypes.

Considering that research in Treg cells in NDs continues to be a topic of interest (70, 71), Gao et al. contributed to this Research Topic with a bibliometric analysis of the field, spanning from 1991 to 2023, and including 2,739 documents between articles and review articles from the Web of Science Core Collection. The authors used Tableau Public, VOSviewer, and CiteSpace software to perform the

study. The research course was categorized into three phases: 1991–2003 (early stage), 2004–2019 (rapid expansion period), and 2020–2023 (fluctuating yet productive phase). A total of 85 countries/regions investigating Treg cells in NDs were identified with the United States, China, and Germany leading in document output. Collaboration among countries/regions was widespread, again with the United States cooperating most (with 57 countries/regions). Notably, Harvard Medical School showed exceptional productivity, citations, link strength, and centrality, reflecting its prolific research and collaborations. Studies examining Treg cells in NDs were published in 859 journals. Among them, the top 11 journals contributed 618 documents, with Frontiers in Immunology, Journal of Immunology, and Journal of Neuroinflammation as the most prominent publishers. The associations of high-frequency keywords, such as "multiple sclerosis", "inflammation", and "regulatory T cells", were found to change throughout the research evolution. Initially, they appeared linked with neuroprotection, neuroimmunology, and immunoregulation (2014), and currently, they shifted towards ischemic stroke, gut microbiota, and the gut-brain axis. Gao et al. identified the top 10 most-cited documents, with three emphasizing the roles of cytokines in autoimmune neurological diseases (72–74), and others examining gut microbiota impact on immune responses and the influence of tumor microenvironment in tumorigenesis (75). Although the United States has led in document output and citations, China emerged as a significant contributor, rising to the forefront in 2022. The study conducted by Gao et al. acknowledges limitations such as language barriers and publication bias, but it emphasizes the need for continual updates to reflect ongoing scientific avenues. This review provides valuable insights for shaping future research directions and therapeutic strategies in this dynamic field.

Overall, the original research and review articles on this Research Topic illustrate the complexity behind the participation of immune cells in the healthy and diseased central nervous system. We expect this Research Topic will encourage researchers to continue their efforts to further investigate immunity and the brain, with the ultimate hope of finding not only new knowledge but also potential clinical interventions to prevent or ameliorate the devastating consequences of neurological diseases.

Author contributions

SM: Writing – review & editing, Writing – original draft, Conceptualization. JB: Writing – review & editing, Writing – original draft. SB: Writing – review & editing, Writing – original draft. VM: Writing – review & editing. RM: Writing – review & editing. EM: Writing – review & editing, Writing – original draft, Conceptualization.

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Conflict of interest

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REVIEW
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Neuroinflammation in Parkinson's Disease – Putative Pathomechanisms and Targets for Disease-Modification

Alexander Grotemeyer[1], *Rhonda Leah McFleder*[1], *Jingjing Wu*[1], *Jörg Wischhusen*[2] *and Chi Wang Ip*[1]*

[1] Department of Neurology, University Hospital Würzburg, Würzburg, Germany, [2] Section for Experimental Tumor Immunology, Department of Obstetrics and Gynecology, University Hospital of Würzburg, Würzburg, Germany

Parkinson's disease (PD) is a progressive and debilitating chronic disease that affects more than six million people worldwide, with rising prevalence. The hallmarks of PD are motor deficits, the spreading of pathological α-synuclein clusters in the central nervous system, and neuroinflammatory processes. PD is treated symptomatically, as no causally-acting drug or procedure has been successfully established for clinical use. Various pathways contributing to dopaminergic neuron loss in PD have been investigated and described to interact with the innate and adaptive immune system. We discuss the possible contribution of interconnected pathways related to the immune response, focusing on the pathophysiology and neurodegeneration of PD. In addition, we provide an overview of clinical trials targeting neuroinflammation in PD.

Keywords: Parkinson's disease, neuroinflammation, T cells, microglia, neurodegeneration, animal models, inflammatory cascades

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Luke Michael Healy, McGill University, Canada

***Correspondence:**
Chi Wang Ip
Ip_C@ukw.de

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1 INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD) (1–4). Motor symptoms such as bradykinesia, rigidity, resting tremor, and postural instability (5, 6) are hallmarks of PD and essential for staging. PD patients also exhibit a wide variety of non-motor symptoms, ranging from anosmia, rapid eye movement (REM) sleep disorders, and constipation to severe psychiatric symptoms such as dementia (6). Some of these non-motor symptoms are observed long before motor counterparts occur and are therefore essential to early PD diagnosis (7). From the defined histological description, a staging model arose in which progression of PD symptoms can be matched with intracellular deposits of pathological α-synuclein aggregates (pαSYN) or Lewy pathology (LP) of the corresponding brain areas (8–11). However, LP is also observed in the brains of older patients with no PD symptoms during their lifetime, indicating that LP is not specific as a standalone for PD diagnosis (10, 12). Moreover, it remains unclear why pαSYN occurs, why pαSYN spreads from cell to cell (10, 13–16), and how cell death is mediated by pαSYN (17). Since α-synuclein (αSYN) has been described as a critical mediator of inflammation and immune responses, and is released from inflamed neurons, αSYN may trigger a self-amplifying proinflammatory response (18). Intriguingly, neuroinflammation has emerged as a key aspect in PD (19–22) – but the extent to which neuroinflammation contributes to the development and maintenance of PD is controversial. Hence, knowledge transfer from neuroinflammatory

diseases, where the concept of immune system-induced neuronal cell damage is established, could help to elucidate the still-obscure role of the immune system in PD.

Immunomodulatory therapies are established as effective treatments in 'classical' inflammatory neurological diseases, such as multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD), or myasthenia gravis (MG) (23–27). Although the pathophysiology differs regarding the antigen in NMOSD and MG, common therapeutic strategies are implemented (28). In both entities, antibodies are pathophysiologically relevant and inhibition of the complement system has proven beneficial (29, 30). This supports the idea that similar inflammatory processes can be addressed across different neuroinflammatory diseases.

Various concepts have been tested as a causal treatment for PD, including vaccination against αSYN (31, 32). However, none have yet proven successful and traditional treatment of PD is still symptomatic and largely based on dopaminergic medication (33–35). To determine the relevance of neuroinflammation in PD pathogenesis, large-scale clinical observational studies are essential to generate appropriate hypotheses, which can be subsequently tested in animal models. In our review, we discuss the evidence for neuroinflammation in PD and potential future targets. We further illustrate immunological pathways linked to neuroinflammation and dopaminergic neurodegeneration.

2 PATHOMECHANISMS OF NEUROINFLAMMATION IN PD PATIENTS

The pathophysiologic role of the immune system in PD is still enigmatic. Neuroinflammation was proposed by James Parkinson himself in the initial description of PD (1). There is also early literature discussing PD as an 'autoimmune disease' (36). Yet interest in the topic was only rekindled in 1988, when microglia activation was described in brain autopsies of PD and AD patients (21). Since then, evidence has accumulated that neuroinflammation is likely to play a critical role in PD (36–38). Significant elevation of inflammatory cytokines in the blood and cerebrospinal fluid (CSF) of PD patients has now been confirmed in a meta-analysis over 25 clinical studies (39). Increased expression of NLRP3 and caspase-1 genes in peripheral blood mononuclear cells (PBMC) and elevated protein levels of NLRP3, caspase-1, and IL-1B in the blood plasma were found to correlate with PD severity (40), further supporting the perception of PD as a chronic systemic inflammatory disease. Evidence suggests that both innate and adaptive immune responses are involved in PD progression (41–44).

2.1 Innate Immune Response

Microglia are permanently in contact with astrocytes, neurons, and endothelial cells, constantly monitoring surrounding tissue for an ongoing infection or trauma *via* thin processes (45). In response to injury or infection, resting microglia undergo morphological alterations, change their transcriptional activity, and present antigens *via* the major histocompatibility complexes (MHC)-I and -II (46–48). Unlike MHC-I, which is constitutively expressed on all nucleated cells and platelets (49–52), MHC-II is specifically expressed on antigen-presenting cells such as microglia, astrocytes, monocytes, macrophages, dendritic cells, and granulocytes, and can be induced upon activation.

As brain-resident innate immune cells, microglia are typically the first responders to altered homeostasis within the CNS. Microglia activation increases the amount of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFKB) and NLR family pyrin domain-containing 3 (NLRP3), with subsequent upregulation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and cytokines such as interleukin-1 beta (IL-1B) and tumor necrosis factor alpha (TNF) (53, 54). Phagocytic activity and MHC-II expression are also enhanced (55, 56). An increase in activated microglia has been observed in both PD patients and healthy older people, indicating that various triggers might induce a proinflammatory shift with age (21, 57). Compared to age-matched controls, PD patients exhibited significantly elevated levels of proinflammatory cytokines such as IL-1B, IL-6, and TNF in CSF and blood (39, 58). Furthermore, ([^{11}C](*R*)-PK11195)-based positron emission tomography (PET) has revealed a higher density of activated microglia in the midbrain and putamen of early PD patients (59, 60), which correlated with decreased activity of dopamine transporter ligands ([^{11}C] CFT). Similar results were described in patients suffering from a REM sleep behavior disorder (60, 61). While the presence of chronic inflammatory processes in PD is now widely accepted, the underlying reason for neuroinflammation is still unclear. Due to its function as a damage-associated molecular pattern (DAMP) (53, 62, 63), pαSYN might trigger and maintain a proinflammatory shift of the immune system. In addition, DAMPs may be released from dying or damaged cells. Well-known DAMPs triggering an innate immune response upon interaction with pattern recognition receptors (PRRs) are IL-1α or mitochondrial reactive oxygen species (mROS), which activate NLRP3. Consecutive NLRP3 activation leads to increased IL-1B synthesis as a trigger for further innate immune responses (64). Initial microglia activation in PD (21, 65) may therefore result from PRR-mediated responses to DAMPs.

In addition to DAMPs, pathogen-associated molecular patterns such as viral RNA or the bacterial cell wall component lipopolysaccharide (LPS) may also maintain neuroinflammation. While a correlation between viral infection and PD has not strictly been established, preceding infections might still modulate the risk for developing PD (66). For example, there is evidence for toxin-related PD, which implies that toxins released by infectious agents might also be responsible for some cases of 'sporadic' PD. Other toxins such as the pesticide rotenone have neurotoxic and neuroinflammatory effects that may kill dopaminergic cells and thereby cause PD in people exposed to higher concentrations (67).

Possible links between PD and infection have become even more topical with the still-ongoing worldwide COVID-19 pandemic. While there is very little consolidated knowledge regarding possible interactions between the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and PD, there are some interesting observations on pathogenic coronaviruses and neuroinflammation or, more specifically,

PD. Antibodies against coronavirus antigens have been found in the CSF of PD patients (68). In addition, intranasal infection of hACE2 transgenic mice with SARS-CoV-1 caused severe CNS infections that spread from the olfactory bulb to the basal ganglia and midbrain within approximately four days (69). Considering that COVID-19 patients often experience anosmia, SARS-CoV-2 is also likely to reach the CNS *via* the olfactory bulb. Disconcertingly, *in vitro* assays have shown that the nucleocapsid(N)-protein of SARS-CoV-2 accelerates pαSyn-aggregation by a factor of 10 (70). Thus, it is concerning that COVID-19-related inflammation may trigger or accelerate PD in patients at risk. Yet to date, there are only a few case studies describing the development of PD after SARS-CoV-2 infection (71, 72). Whether infection with SARS-CoV-2 impacts the development of PD remains to be explored.

2.2 Adaptive Immune Response in PD: T Cells Friends and Foes

Neuroinflammation in PD is not confined to the innate immune system; it also involves adaptive immune responses. Several findings indicate that various T cell subpopulations may contribute to PD pathophysiology (73).

Neuroinflammation within the substantia nigra (SN) is well-described in PD. Significant infiltration of both $CD4^+$ and $CD8^+$ T cells into the SN of PD patients has been described (42), particularly elevated levels of $CD8^+$ T cells (74, 75). It is possible that $CD8^+$ T cells have an important role in early PD, even before pαSYN can be detected in the SN, as relevant infiltration into the SN has been observed in very early PD that subsides with disease progression (74). Altered counts of $CD4^+$ T cell populations in the blood of PD patients have also been reported, but remain controversial. Many studies describe a decrease in the overall $CD4^+$ T cell population in PD patients (44, 76–78). However, both elevated (76) and reduced T_{reg} cell counts (79) were found in the blood of PD patients, although the studies used a relatively non-specific marker ($CD4^+CD25^+$) to identify T_{reg} cells. Increased numbers of $CD4^+FoxP3^+$ T_{reg} have been observed in the blood of PD (aged 46-80 years) and AD (62-82 years) patients and healthy older people (51-87 years) compared to healthy young controls (23-40 years) (80). An increase in T_{reg} cell activity with age was also seen, which was significantly more pronounced in PD and AD patients compared to young controls (80). In contrast, decreased levels of $CD4^+CD25^{high}CD127^{low}$ T_{reg} cells (44, 81) and reduced suppressive activity of T_{reg} cells has also been found in PD patients (77). Furthermore, peripherally-reduced T_{reg} cells and increased Th1 cell counts were found to correlate with the severity of motor dysfunction (assessed by Unified Parkinson's Disease Rating Scale III [UPDRS III]) (44, 81). In addition, decreased Th2 cell numbers and a relative increase in Th1 cells were seen in PD patients (44). Despite these discrepancies, most studies highlight an increased peripheral immune response in PD, with a reduced amount of T_{reg} cells ($CD4^+CD25^{high}CD127^{low}$) (44, 81) (**Table 1**).

These population and activity shifts of T cells, along with an increase in HLA-DR positive antigen-presenting microglia in PD patients (82), support the idea that $CD4^+$ T cells might contribute to neurodegeneration in PD. Studies on the role of Th17 cells in PD have generally confirmed this concept. Addition of IL-17 to autologous cocultures between T cells and pluripotent stem cells (iPSC)-derived mid brain neurons (MBN) from PD patients also induced NFKB-dependent cell death. iPSC-derived MBN from PD patients further showed higher expression of IL-17 receptors, together with enhanced susceptibility towards death induction by recombinant IL-17 or autologous Th17 cells (73). Interestingly, Th17 cells are increased in PD patients, which is consistent with the results of previous studies (81, 83, 84). However, one study in PD patients reported a decrease in the Th17 cell count, along with unchanged levels of IL-17 (44). Based on these studies, Th17 cells and IL-17 are likely to favor the progression of PD, but their distinct role remains unresolved (85, 86). Consequently, a comparison of different analytical approaches might be needed to rule out possible methodological issues.

In addition to flow cytometric analyses, gene expression analyses and genome-wide association studies that investigate alterations in immune response pathways are playing an

TABLE 1 | Overview of human and animal studies on T cells with special regard to T_{reg} cells.

Publication	*derived from*	*origin*	*Major finding (referring to PD)*	*T_{reg} identification (total amount)*
(42), *2009*	brain tissue	human, mouse	sign. infiltration of $CD4^+$ and $CD8^+$ T cells in the SN	-
(74), *2020*	brain tissue	human	sign. infiltration of $CD8^+$ T cells in early stages of PD	-
(75), *2022*	brain tissue, spleen	human (brain), mouse (brain, spleen, lymph nodes)	sign. infiltration of $CD8^+$ T cells in the SN of humans and of $CD4^+$ and $CD8^+$ T cells in the SN of AAV1/2-A53T-αSyn mice	-
(76), *2001*	mesenteric lymph nodes	rat	no changes (6-OHDA); increase of $CD4^+CD25^+$ T cells (MPTP)	$CD4^+CD25^+$
(44), *2018*	blood	human	decrease of $CD4^+$ T cells, Decrease of Th2, Th17 and T_{reg} cells	$CD4^+CD25^{high}CD127^{low}$
(76), 2001	blood	human	total $CD4^+$ T cells decreased, $CD4^+CD25^+$ T cells increased	$CD4^+CD25^+$
(77), *2012*	blood	human	total $CD4^+$ T cells decreased. suppressive activity of T_{reg} impaired	$CD4^+CD25^{high}CD127^{low}$
(79), *2005*	blood	human	decrease of T_{reg}	$CD4^+CD25^+$
(80), *2007*	blood	human	no change of $CD4^+CD25^+$ T cells but increase in $CD4^+FoxP3^+$ T cells	$CD4^+CD25^{high}$ $CD4^+FoxP3^+$
(81), *2015*	blood	human	decrease of $CD4^+$, T_{reg} T cells Increase of Th1, Th2, Th17 T cells	$CD4^+CD25^{high}CD127^{low}$

increasing role in PD research. Expression quantitative trait loci (eQTLs) are obtained by analyzing the genome and transcriptome of patients with correlating alleles and the expression level of transcripts. Based on $CD4^+$ T cells and monocytes from a multi-ethnic cohort of 461 healthy individuals, susceptibility alleles for MS, rheumatoid arthritis, type 1 diabetes, and PD were found to be overrepresented in $CD4^+$ T cells, while disease- and trait-associated cis-eQTLs associated with AD and PD were primarily overrepresented in monocytes (87). Further evidence for the relevance of the immune system in PD pathogenesis comes from the association of PD with an immune haplotype, including the DRB5*01 and DRB1*15:01 alleles, and from a non-coding polymorphism in the region that might increase MHC-II expression (88). Finally, a very recent study stratified PD patients by their T cell responsiveness to α-SYN as a proxy for an ongoing inflammatory autoimmune response (89). Gene expression analysis in peripheral memory T cells then revealed a clear PD-specific gene signature with transcriptomic markers for inflammation, oxidative stress, phosphorylation, autophagy of mitochondria, cholesterol metabolism, and chemokine signaling *via* CX3CR1, CCR5, and CCR1.

2.3 Adaptive Immune Response in PD: B Cells and Humoral Immunity

While T cells are the more prevalent adaptive immune cells in CSF (90), B and T cell immunity are closely intertwined (91). Although immunohistochemical analyses have found B cell-rich tertiary lymphoid structures in most brains from patients with MS, but not in the two PD patients investigated (92), there is ample evidence for the involvement of B cells, antibodies, and humoral immune effector mechanisms in PD (93–95). In a postmortem study of 13 patients with idiopathic PD and three with genetic PD (compared with 12 controls), all PD patients showed IgG (mostly IgG1) but no IgM binding to dopaminergic neurons, while Lewy bodies were strongly immunolabelled with IgG (94). Nearby activated microglia expressed the high-affinity activating IgG receptor FcγRI, which implies a significant role for antibody-dependent cell-mediated cytotoxicity. The inhibitory IgG receptor FcγRII was absent in all cases. Subsequent studies on B cell-related adaptive immune responses in PD support the involvement of B cells and humoral immune effector mechanisms (96–98). Remarkably, some patient sera also contained antibodies capable of neutralizing pαSYN 'seeding' (forming of αSYN aggregates) *in vitro* (99). Elevated levels of serum antibodies against αSYN have also been reported in a comparison of sera from PD patients with AD patients and controls (100). Of note, B cell numbers were found to be reduced in PD patients (96, 101), along with an increase in IgA levels (102) that correlated with non-motor symptoms (96). Moreover, cytokine expression of B cells was altered in PD (96). Cytokine-dependent interactions between B cells and Th1 and Th17 cells (103) imply that B cells may also modulate proinflammatory Th17 cells in PD patients (73). In addition, there are relevant interactions between the humoral immune response and the complement system, as described in a comprehensive review (104).

Given the paucity of studies focusing on the distinct function of B cells in PD or other neurodegenerative diseases (103, 105), a more profound understanding of B cell function might reveal new opportunities for disease modification. Again, the targeted transfer of knowledge from 'classical' neuroinflammatory disease would be useful (103).

2.4 Anti-Inflammatory Disease Modification

Since proinflammatory changes have been consistently observed in PD, several studies analyzed whether suppressing inflammation might modify the disease course of PD. A population-based, case–control study found that patients receiving immunosuppressive therapy had a decreased risk of developing PD (106). Interestingly, the greatest risk reduction was conveyed by drugs affecting T cells such as the inosine monophosphate dehydrogenase inhibitors, azathioprine and mycophenolat mofetil (106), while a protective effect was revealed for corticosteroids. However, this finding might be biased by smoking behavior. Smoking is described as a possible protective factor for PD, and smokers frequently need corticosteroids due to pulmonary maladies (106, 107). Moreover, cigarette smoke induces chronic inflammation, which results in immune cell exhaustion and generally attenuates the function of many immune responses (108). Therefore, smoking can be considered an immune-inhibitory activity (109, 110). The potential effectiveness of anti-inflammatory treatment is further exemplified by a lowered risk for developing PD in patients receiving immunosuppressive therapy (111).

Accordingly, patients with inflammatory bowel disease (IBD) receiving an anti-TNF treatment had a 78% reduction in the incidence of PD compared to IBD patients who did not receive this therapy (112). However, the lack of direct comparison between the incidence of PD in treated IBD patients compared with non-IBD patients (112) means that the ability of anti-TNF treatment to reduce PD incidence in non-IBD patients remains unanswered. Other research has indicated that severity of IBD is inversely associated with the risk of developing PD (-15%) (113). However, although patients suffering from severe IBD are more likely to receive anti-inflammatory drugs, immune-modulatory treatment was not directly assessed. Accordingly, further studies are necessary to verify a positive or negative correlation between PD and anti-inflammatory treatment, both for IBD and for non-IBD patients.

One of the most frequently used anti-inflammatory drugs is ibuprofen, a reversible cyclooxygenase-2 (COX-2) inhibitor and non-steroidal anti-inflammatory drug (NSAID) (114). Intriguingly, ibuprofen users showed a lower risk for PD, whereas no protective effect could be demonstrated for acetylsalicylic acid (ASA) (114). In a prospective study of subjects without PD who were assessed at baseline for NSAID intake, 0.2% developed PD during a 6-year follow-up (115). A significant dose-response relationship between ibuprofen intake (tablets/week) and reduced PD risk was found. Again, this effect was not observed for ASA, other NSAIDs (e.g., indomethacin), or acetaminophen. Although all NSAIDs and ASA are known to inhibit COX-2, only ibuprofen was shown to have some impact on PD prevention. Pre-clinical findings from *in vitro* models

indicate that ibuprofen prevents oxidative damage induced by LPS injection and better protects dopaminergic neurons against glutamate neurotoxicity than ASA (116, 117). The neuroprotective effects of ibuprofen have been confirmed in studies that considered confounder variables of PD, such as age, smoking, and caffeine intake (115, 118). However, the putative neuroprotective effect of ibuprofen in PD remains controversial; other research focusing on all NSAIDs rather than on ibuprofen alone found that NSAIDs had no neuroprotective effects (107). In this context, it is noteworthy that ibuprofen is known to reduce inflammation *via* inhibition of COX-2, but also *via* modulation of the angiotensin pathway by shifting the ACE/ACE-2 (angiotensin converting enzyme) receptor ratio towards ACE-2 (119, 120) (discussed in detail in Section 4.2).

Drugs such as β-agonists and β-antagonists have been shown to affect the incidence of PD in opposing directions, with β-agonists seeming to be somewhat protective (121, 122). However, further evidence is needed to confirm whether β-agonists might act directly on immune cells and are thus capable of modulating PD-related inflammation (122). It should be noted that β-agonists are often used in patients suffering from chronic obstructive pulmonary disease, a common consequence of frequent smoking. The fact that smoking was reported to lower the incidence of PD in one study represents an epidemiological bias (121).

Given that observational studies have yielded controversial data regarding the neuroprotective effects of different drugs and immunomodulators (123–128), randomized, controlled trials are needed to better decipher which drugs have distinct neuroprotective effects in PD.

2.5 Current Clinical Trials for Anti-Inflammatory Disease Modification in PD

Current clinical trials on anti-inflammatory therapy in PD focus on the treatment of glucose metabolism, improvement of oxidative stress, and regulation of gut microbiota.

2.5.1 Regulation of Glucose Metabolism

Impaired insulin signaling resulting in altered energy balance and impaired cell repair mechanisms has been observed in PD patients (129–131). Glucagon-like peptide-1 (GLP-1) is a growth factor and insulin-stimulating hormone that can activate the same effector molecules as insulin, and its receptor (GLP1R) is expressed in neurons. In addition to the known effects of GLP1R on blood sugar regulation and stimulation of the hypothalamus to regulate appetite, studies have shown that GLP1R agonists have neuroprotective effects in different animal models of PD. One agonist, exendin-4, can enhance cognitive function (132) and attenuate neurodegeneration in 6-hydroxydopamin (6-OHDA) rats (133). In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, the newer GLP-1 mimetics liraglutide and lixisenatide can improve motor impairment and reduce dopaminergic cell loss in the SN (134, 135). In a human A53T αSYN (hA53T-αSYN) transgenic mouse model, a long-acting GLP1R agonist reduced behavioral deficits and neurodegeneration (136). It is noteworthy that activation of GLP1R can inhibit the production of proinflammatory cytokines in microglia, such as TNF and IL-1B (137, 138). The neuroprotective effect of semaglutide treatment is currently under investigation (139).

2.5.2 Regulation of Gut Microbiota

The gastrointestinal (GI) tract and CNS have complex mutual interactions (140) that are affected by the intestinal flora (141–143). Therefore, a healthy and stable intestinal flora is important for immunity, homeostatic balance of barrier integrity, and metabolism (144). Interestingly, changes in the intestinal flora may alter nerve development and even cause neurodegenerative disorders (145). Conversely, traumatic brain injury may lead to impaired intestinal barrier function (146, 147). GI dysfunction in PD patients, particularly obstipation, is commonly known to be one of the initial symptoms that precedes motor impairment (148). Changes in intestinal microbial flora can alter the barrier function and permeability of the gut and subsequently influence the immune system (140, 149), and have been linked to PD (149). It is noteworthy that pαSYN can be found in the enteric nervous system (150).

A speculative hypothesis is that the methylation status of the SNCA gene might be influenced by the gut microbiome. It is accepted that duplications and triplications of the SNCA gene confer a higher risk of developing PD (151, 152). Decreased methylation of the SNCA gene, which would lead to increased transcription, could likewise affect αSYN expression and influence the risk of PD (153). Postulating a potential role of the gut microbiota as an epigenetic factor for DNA methylation, an ongoing clinical study is investigating the composition of the GI bacteria in the stool of PD patients and healthy controls by extracting total bacterial DNA from the samples (154).

2.5.3 Regulation of Oxidative Stress

Oxidative stress in the brain plays a key role in the development of PD (155, 156), and results from an imbalance between reactive oxygen species (ROS) and the ability to scavenge reactive intermediates (157). ROS, which include superoxide, nitric oxide, hydroxyl radical, hydrogen peroxide, and peroxynitrite, can be generated from various sources including the electron transport chain or activated immune cells. Interestingly, increased oxidative stress seems to be target-specific and, in the case of PD, related to LP and the pαSYN contained in Lewy bodies, where pαSYN nitration (an oxidation marker) was found to be elevated (158–160). Furthermore, the SN of early PD patients has been found to contain reduced amounts of the antioxidant glutathione (GSH), which correlated with PD severity (159).

Increasing the availability of reduced GSH N-acetylcysteine (NAC) can potentially reduce the damage to neurons caused by oxidative stress. In a current clinical trial, NAC is being used to protect PD patients from oxidative damage (161). Aside from NAC, other supplements with antioxidative properties are under investigation. In the CNS, vitamin B_3 (niacin) is considered a key mediator of neuronal development and survival (162). Niacin is a cofactor of nicotinamide adenine dinucleotide and NADPH, which are necessary for the scavenging of oxidants and needed for GSH regeneration (157). Niacin can improve the integrity of mitochondria and hence the energy supply of neurons. By enhancing antioxidant defense

mechanisms, niacin can protect the normal structure and function of neurons exposed to oxidative stress, thereby delaying the progression of PD (163). Furthermore, niacin binds to the G-protein-coupled hydroxycarboxylic acid receptor 2, which is upregulated in a proinflammatory environment and which may downregulate inflammation (164–166). Since PD patients are known to have low levels of niacin due to mitochondrial malfunctioning, supplementation of niacin could be beneficial (166). A clinical trial is evaluating the effectiveness of niacin or niacinamide supplementation on inflammation and severity of PD symptoms (167).

3 ANIMAL MODELS AND THEIR CONTRIBUTION TOWARDS UNDERSTANDING PD PATHOMECHANISMS

Animal models play an important role in understanding PD pathophysiology, including neuroinflammatory processes. Next to the 6-OHDA model (168), one of the most often used PD models is induced by MPTP delivery (169), which persists extensively in the SN and results in PD symptoms with inhalation and cutaneous contact (169). Immune responses in the various MPTP models used are, however, quite different.

3.1 Innate Immune Responses in Animal Models for PD

Toxins such as MPTP, rotenone, and 6-OHDA act as DAMPs and initiate a strong innate immune response with microglia activation and subsequent neurodegeneration in the SN (170–172). This immune response resembles the microglia activation found in human brain autopsies (21, 173). In the 6-OHDA model, neurodegeneration is accompanied by a gradual repolarization of microglia from an anti-inflammatory M2 to a pro-inflammatory M1 phenotype (174). Cytokine production by M1 cells is intracellularly initiated by NFKB (53, 175, 176), which in turn induces interleukin and procaspase-1 transcription. Together with the inflammasome NLRP3, caspase-1 activates IL-1B. Moreover, other proinflammatory proteins such as iNOS and TNF are also released from M1 cells (53, 175, 176) and contribute to neurodegeneration in PD (177). Consequently, inhibition of NFKB protects MPTP mice from neurodegeneration (178). M2 macrophages, in contrast, contribute to neuroprotection and release neurotrophic factors (176) (**Figure 1**). Since TLR-4 knock-out reduced the number of MHC-II$^+$ microglial cells and partially protected MPTP-treated mice from dopaminergic degeneration in the SN, MPTP triggers also neuroinflammation *via* TLR-4 (179). However, the canonical ligand for TLR-4 is LPS, which is commonly found on cell walls of gram-negative bacteria (180) In LPS-driven models, LPS is either systemically administered or focally injected into the SN (181). Since microglia activation after delivery of LPS or another of the aforementioned toxins results in the same downward cascades, all of these models lend themselves to studying innate immune responses (181–183). However, in mice lacking the mitophagy inducers PINK and Parkin, LPS triggers the release of mitochondria-derived vesicles (184, 185). This can prime CD8$^+$ T cell responses against mitochondrial antigens (184, 185). External stressors such as LPS can thus also induce adaptive immune responses, in particular in transgenic models with impaired mitochondrial functions.

For MPTP, but not 6-OHDA, a Lewy body-like pathology has been found in non-human primates (186). An even more prominent Lewy body-like histopathology was observed in viral vector-based models overexpressing human mutations of αSYN (e.g., A53T) (187). Furthermore, viral vector-based models demonstrate a similar innate immune response, as seen in toxin-based models. pαSYN, which is believed to enter the cell *via* TLR-2 to unleash its toxic potential (53, 188, 189), thus apparently acts like a DAMP (190). Interestingly, orthotopic injection of an αSYN-encoding AAV into the SN only induced neurodegeneration in the presence of Fc gamma receptors (191), which implies an antibody-dependent activation of the complement cascade and/or antibody-dependent cellular cytotoxicity that may be mediated by microglia, for example (192). However, unlike astrocytes, microglia do not directly contribute to a proinflammatory environment after exposure to pαSYN (193). Nevertheless, progressive neurodegeneration can be induced by microglia-specific overexpression of pαSYN in the absence of pαSYN in dopaminergic cells (194). The critical role of CD4$^+$ T cells was confirmed by showing that virus-based αSYN-overexpression can only induce microglia activation and neurodegeneration when MHC-II is present (195). Therefore, viral vector-based models require a complex interplay between different types of immune cells, which may also best reflect observations made in human PD.

Since animal research in PD has focused mostly on alterations within the CNS, our understanding of peripheral innate and adaptive immune processes in PD pathology is still limited. Recent studies have provided evidence that increased gut permeability and gut microbiota are likely to contribute to PD development (149, 196). This has resulted in PD becoming more and more accepted as a systemic disease (197). For instance, overexpression of human TNF (hTNF) in a mouse model leads to microglia activation in various regions of the brain (198, 199), while immunomodulation with infliximab could attenuate microglia activation (199). Since infliximab cannot penetrate the blood-brain barrier, this finding indicates that effective treatment of neuroinflammation could be achieved by modulating peripheral immune mechanisms.

3.2 Adaptive Immune System in Animal Models for PD

Several animal models support the hypothesis that the adaptive immune response in PD is mainly T cell driven, whereas the role of B cells remains enigmatic (41, 42, 73, 75, 88, 200). Nevertheless, the mechanisms of T cells vary across different animal models (42, 201).

3.2.1 MPTP Model

Variations in frequency and dosage of MPTP injections in mice result in different PD models with diverse disease kinetics. The subacute MPTP model is generated by administering MPTP once daily over five consecutive days, whereas an acute model is

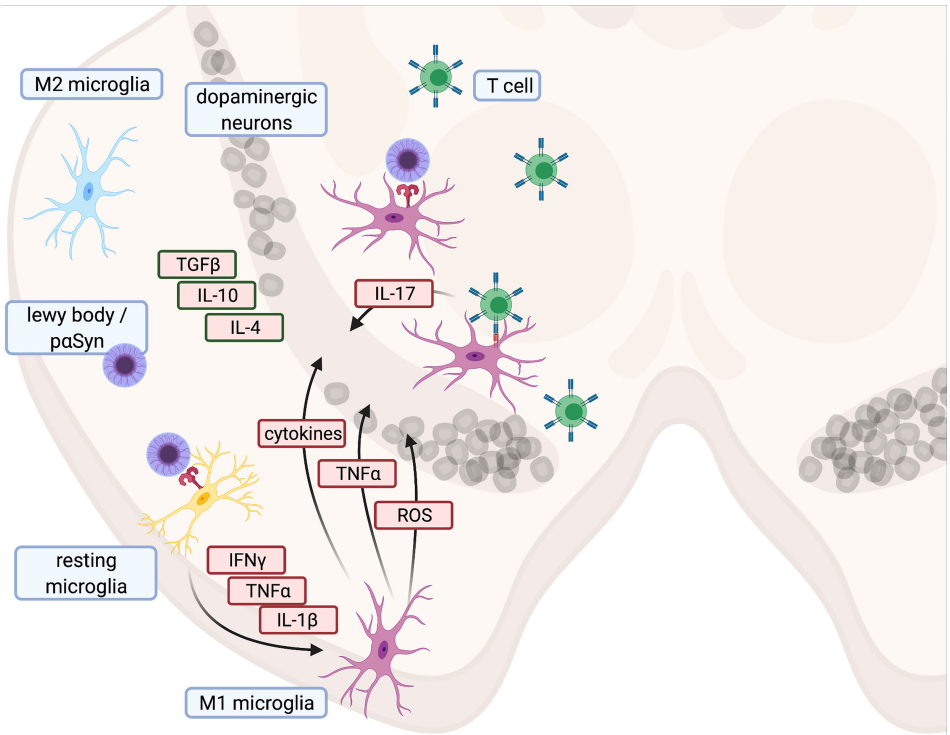

FIGURE 1 | Illustration of proinflammatory cascade and main cytokines contributing to cell death in Parkinson's disease - Created with BioRender.com.

induced by delivering MPTP every two hours for four times in only one day (202). For the acute model, it is important to note that in contrast to humans, the initial immune response decreases significantly within 30 days, and MPTP-injected mice may recover over time (203). Moreover, pαSYN is not found in this model *per se*. However, if the adaptive immune response in the acute model is maintained by peripherally administering cytokines such as RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and eotaxin twice a week, the inflammation is sustained and pαSYN accumulates in the SN of the rodents (203). In this model, pαSYN formation is linked to a sustained proinflammatory environment.

$CD4^+$ and $CD8^+$ T cell infiltration in the SN of acute MPTP mice supports the idea that T cells contribute to neurodegeneration in this PD model (42). $CD4^+$ or recombinant activating gene 1 (RAG1)-deficient mice, which lack mature lymphocytes, show attenuated neurodegeneration upon treatment with MPTP. In contrast, neurodegeneration is unrestrained in $CD8^+$ T cell-deficient mice (42). Blockade of RANTES and eotaxin in the MPTP mouse and non-human primate model reduces microglia activation, $CD4^+$ and $CD8^+$ T cell infiltration, and neurodegeneration (204, 205). Continuous T cell trafficking and induction of pαSYN in the SN of MPTP mice is also driven by supplementation of RANTES and/or eotaxin, but not by TNF and IL-1B (203). Furthermore, MPTP-treated mice show altered and dysfunctional $CD4^+CD25^+$ T_{reg} cells. Administration of natural T_{reg} cells leads to attenuation of SN degeneration (206). As Th17 cells appear to contribute to nigrostriatal degeneration (206, 207), the beneficial effect of T_{reg} may be due to their ability to inhibit TH17 cells. However, while mechanistic insights from the aforementioned studies are based on the acute MPTP model, $CD4^+$ T cell infiltration occurs earlier in the subacute model (208). Given the differences between the models, the choice of the most appropriate model may determine the outcome (209).

3.2.2 6-OHDA Model

This model is generated by injecting 6-OHDA either directly into the SN, the medial forebrain bundle, or the striatum (168, 210, 211). The initial cytotoxic effect of 6-OHDA is known to be mediated *via* oxidative stress (212) and impaired mitochondrial function (213). Data on the role of the adaptive immune response in the 6-OHDA model are still scarce. But it is known that 6-OHDA administration leads to infiltration of $CD4^+$ and T_{reg} cells in the CNS (76, 174). In contrast to MPTP, 6-OHDA must only be administered once to induce continuous neuroinflammation and neurodegeneration (174, 201). Apart from neuroinflammation, 6-OHDA-treated rats also show decreased circulating T_{reg} cells, indicating that this model can reflect aspects of human PD (174). However, while $CD4^{-/-}$ or $Rag1^{-/-}$ knock-out mice injected with MPTP were partly protected from severe neurodegeneration (42), 6-OHDA-treated $Rag1^{-/-}$ mice showed even more pronounced neurodegeneration in the SN than

immune-competent mice (201) or rats (214). Accordingly, the adaptive immune system is mostly neuroprotective in the 6-OHDA rodent model. Thus, toxin-based models provide contradictory data on the role of the adaptive immune system. Based on the epidemiological data linking PD and adaptive immune responses in humans, these models do not seem to adequately recapitulate the pathomechanisms underlying the human disease.

3.2.3 Viral Vector and Preformed Fibrils Models

Creating a model that shows a large overlap with the human phenotype and pathophysiology is the main objective when generating new animal models. Regarding chronic disease progression, mild-to-moderate nigrostriatal degeneration, Lewy-like pathology, and neuroinflammation, the AAV1/2-A53T-αSYN model resembles human PD much more closely than conventional toxin-induced models (75, 187, 215). Recently, $CD4^+$ and $CD8^+$ cells infiltrating the SN were found in the AAV1/2-A53T-αSYN model (75). Ten weeks after AAV1/2-A53T-αSYN injection, $CD4^+$ and $CD8^+$ cells in the spleen were also activated. Moreover, RAG deficiency protected dopaminergic SN neurons, indicating that $CD4^+$ and $CD8^+$ crucially contribute to neurodegeneration in this model (75). This reflects findings made in PD patients, thereby demonstrating a higher immune-related face validity of the AAV1/2-A53T-αSYN mouse model compared to the toxin-induced MPTP and 6-OHDA models. Comparable results, including gradual dopaminergic cell loss and $CD3^+$ infiltration in the SN, were also achieved by viral vector-based application of human αSYN in the SN of mice (216, 217) or by directly injecting preformed αSYN fibrils (αSYN-PFF) into the striatum of mice (62). The immune response triggered by the presence of pαSYN is thus conserved and comparable between different models. This is in line with the recently revealed role of αSYN as a critical mediator of inflammatory and immune responses (18).

Aside from PD models with direct injection of pαSYN or αSYN-PFF into the brain, there are also models based on peripheral injection of αSYN-PFF. Intriguingly, spread of pαSYN from the periphery to the brain is observed, accompanied by motor deficits (218, 219). However, in a more recent study where strong involvement of the adaptive immune response in the brain was confirmed, further inflammatory stress by LPS was required to achieve spreading of pαSYN into the brain (220). Accordingly, ongoing neuroinflammation contributes to the progression and maintenance of PD (197, 221–225).

3.2.4 Conclusion for the Role of the Immune System in PD Animal Models

These animal models provide substantial support for the hypothesis that the immune system participates in the neurodegeneration observed in PD. However, for investigating adaptive immunity, choosing the right animal model is key. Central and peripheral immune responses must both be considered. Finally, since the overall role of the immune response in PD animal models is still understudied, interactions between pαSYN, microglia, astrocytes, the adaptive immune system, and peripheral inflammation need to be explored.

4 SIGNALING CASCADES RELATED TO PD AND NEUROINFLAMMATION AS POSSIBLE THERAPEUTIC TARGETS

Pathophysiology of PD is manifold and includes several well-investigated pathways, while others may still be unknown. There are several important signaling pathways related to inflammation and neurodegeneration.

4.1 COX-2 and Disease Modification With NSAIDs

ASA is a standard drug for secondary prophylaxis of ischemic stroke and a potent non-competitive inhibitor of COX-2. Since COX-2 is expressed in many cell types, including platelets and microglia, it is involved in many physiological processes. Intriguingly, the protective role of COX-2 inhibitors on neuroprotection remains controversial. An early administration of high ASA doses showed a neuroprotective effect in MPTP mice, while other COX-2 inhibitors including ibuprofen did not (226). In contrast, the protective effect of ASA in PD patients is comparatively low, while ibuprofen is associated with decreased disease risk compared to other NSAIDs (114, 115). Furthermore, some studies have shown that NSAID intake has no effect on PD development, but instead correlates with an increased risk for PD (107, 227). It is important to note that none of these studies compared equivalent dosages. In MPTP mice, however, COX-2 is upregulated and known to be involved in the oxidation of dopamine, forming dopamine quinone (DAQ) (202). Interestingly, COX-2-deficient mice are protected against induction of PD, which was not explained by reduced inflammation but by prevention of DAQ formation (202, 228, 229). DAQ conjugates with GSH to form GSHDAQ. After conversion to 5cysDAQ, GSHDAQ inhibits mitochondrial complex I. In turn, this leads to decreased degradation of ROS and increased DAQ levels. Subsequently, this vicious circle results in microglia and astrocyte activation and a shift towards a proinflammatory state (175), accompanied by exhaustion of the pentose-phosphate pathway (PPP). However, there are multiple pathways that result in upregulation of COX-2 in brain neurons, including increased N-methyl-D-Aspartate (NMDA) receptor-dependent activity (230), cerebral ischemia, seizures, and diseases such as AD or PD (231). Another important function of COX-1 and COX-2 is the further processing of arachidonic acid. COX-1 induction leads to a substantial increase in thromboxane A2 (TXA_2) in human endothelial cells, while induction of COX-2 by IL-1B leads to a minor increase in TXA_2 but a substantial induction of the proinflammatory proteins prostacyclin (PGI_2) and prostaglandin E2 (PGE_2) (232).

PGI_2 and PGE_2 are physiologically involved in body temperature regulation. PGI_2 and TXA_2 have important roles in the maintenance of vascular homeostasis and can mainly be described as agonist and antagonist (233). Among the different PGE_2 receptors (EP1-4), EP2 contributes to neurodegeneration in the presence of pαSYN in PD (234). EP2-deficient microglia from mice showed increased clearance of pαSYN on brain slices from Lewy-body patients. Furthermore, $EP2^{-/-}$ MPTP mice were spared from neurodegeneration in the SN (234). However, this

mechanism only seems to apply for neurotoxicity associated with chronic inflammatory processes. In acute pathological processes such as brain ischemia or NMDA-induced toxicity, EP2 activation exerts protein kinase A-dependent protective effects (231). Interestingly, EP2 is also neuroprotective in the 6-OHDA primary cell culture (235). The cause of this dichotomous action of EP2 is not yet understood (231).

Beside possible neuroprotective effects, COX-2 inhibitors increase intestinal permeability that correlates with their potency to inhibit COX-2, thus ultimately leading to local inflammatory side effects (236, 237). Furthermore, since increased intestinal permeability is discussed as an important pathological axis of PD development, prolonged treatment of PD with potent NSAIDs is not advisable, which could explain the controversial results (149, 196).

4.2 Oxidative Stress, PPP, and Renin-Angiotensin System

4.2.1 Oxidative Stress and PPP

Under steady-state conditions, ROS are maintained in a stable range and participate in the regulation of cell growth, differentiation, apoptosis signals, and enzyme activity by modulating the production of ceramide, kinase regulatory proteins, and activation of transcription factor NFKB (238, 239). Oxidative stress is a potent and highly conserved defense mechanism against intruders such as bacteria or fungi (240). By stimulating the production of inflammatory factors, ROS play an essential role in pathogen clearance (238). Free radical scavengers such as GSH act as a counterpart to ROS and protect cells from excessive oxidative stress. Maintaining the reduced state of GSH by oxidation of NADPH is therefore important for tissue homeostasis. Under pathological conditions, such as in PD, the level of ROS outpaces the compensatory mechanisms. ROS are located upstream of many inflammatory signaling pathways, upregulate the expression of other inflammatory factors, interfere with signaling pathways that regulate growth factors, and often alter signal transduction. High levels of ROS can be generated (241–244) by M1 microglia, but also the renin-angiotensin system (RAS) or NADPH-oxidase and its homologues, the so-called NOX enzymes (240). High ROS concentrations may cause apoptosis, structural damage, and cell death (159). ROS can therefore directly exert neurotoxicity and amplify neurotoxic inflammatory responses (157, 159). Understanding the role of pathways such as the PPP, which expurgate excessive amounts of ROS, might be crucial for finding an effective treatment against PD. The PPP connects glucose metabolism with NADPH production and nucleotide precursor synthesis. In this pathway, glucose-6-phosphate (G6P) is metabolized to produce NADPH and ribose-5-phosphate. In nearly all cells, the main task of PPP is to provide ribose-5-phosphate. In neurons, however, its main task is to provide NADPH to eliminate ROS (245). The definite role of PPP in PD remains still uncertain (245), even though there is increasing evidence that PD is associated with disorders of glucose metabolism (246–248).

The rate-limiting enzyme of the oxidative part of the PPP is G6P dehydrogenase (G6PD), which catalyzes the reaction between G6P and $NADP^+$. Through consumption of NADPH, oxidative stress leads to the accumulation of large amounts of $NADP^+$ and subsequently to the activation of G6DP due to the increased concentration of oxidants (249). In MPTP mice, neurodegeneration was shown to correlate with NADPH oxidase upregulation (242). Moreover, G6PD expression was positively associated with microglial activation and dopaminergic neurodegeneration (245), indicating that oxidative stress is closely linked to neurodegeneration.

Another important protein related to oxidative stress is GSH, an important antioxidant in brain neurons. The level of GSH in cultured cells is directly related to the production of NADPH by PPP (250). Increased ROS and decreased GSH levels have been described in many PD models and patients (155, 251–254).

Maintaining or adding NADPH in therapeutic dosages might help to overcome high levels of ROS, keep a stable level of GSH in the brain, and finally result in neuroprotection.

4.2.2 Renin-Angiotensin(-Aldosterone) System

The renin-angiotensin[-aldosterone] system (RA[A]S) is well-known in the context of blood pressure regulation (255). Stimuli such as increased activity of kidney baroreceptors, low plasma sodium levels, and activation of β1-receptors result in release of renin from the kidney into the blood circulation. Renin then cleaves liver-synthesized angiotensinogen into angiotensin I (AngI). Subsequently, AngI is cleaved *via* ACE to angiotensin II (AngII). Binding of AngII to the G-protein-coupled AngII receptor type 1 (AT_1R) is essential for blood circulation. AT_1Rs are commonly expressed in the vascular system, the heart, and the brain. Aside from binding to AT_1R, AngII also induces release of aldosterone from the adrenal cortex (256). Drugs for the treatment of arterial hypertension therefore interfere with the RA[A]S by inhibiting renin, ACE, AT_1R, or the aldosterone receptor (257). Investigations into the role of the RA[A]S in the brain, and more specifically in dopaminergic neurons, have deepened our understanding of neurodegeneration in PD. Different groups have demonstrated in both 6-OHDA and MPTP mice that neurodegeneration can be accelerated or decelerated through AT_1R activation or blockade, respectively (243, 255, 258, 259). However, the RAS includes many complex crosslinks with pro- and anti-inflammatory endpoints (**Figure 2**). The main axis consists of core substrates including angiotensinogen, AngI/II, and renin. AngII mainly binds AT_1R, but also the AngII receptor (AT_2R), which initiates anti-inflammatory and anti-oxidative cascades (123, 260). This contrasts with AT_1R, which initiates and maintains neuroinflammation and oxidative stress (260–262). AngII can be cleaved to AngIII by aminopeptidase-A, and further to AngIV and Ang3-7 by aminopeptidase N or carboxypeptidase P and prolyl-oligopeptidase, respectively. AngIII binds to AT_1R and AT_2R, whereas AngIV has a low affinity to AT_1R and AT_2R but a high affinity to the AngIV receptor (**Figure 2**). AngIV and analogues were shown to have a positive effect on synaptogenesis and improved clinical outcomes after stroke and subarachnoid hemorrhage by increasing cerebral blood flow (263–266).

The neurodegenerative effect mediated by AngII and the AngII-AT_1R complex are mediated by an increased release of ROS *via* the

NADPH-oxidase complex in microglia and dopaminergic neurons, resulting in a vicious proinflammatory circle (123). The increased release of ROS is mediated by increased nuclear expression of AT_1R and NOX in dopaminergic neurons of PD patients, resulting in oxidative DNA damage and cell loss (267). In contrast, the cytoplasmic and membrane expression of AT_1R is reduced (267). Under normal conditions, the AT_1R-NOX interaction maintains cell homeostasis and physiological levels of dopamine. The pathological shift of AT_1R expression from the membrane/cytoplasm to the nucleus reflects the disease process and contributes to secondary neurodegeneration due to increased oxidative stress. Interestingly, this shift can be observed relatively early. An altered ratio of membrane/cytoplasm to nuclear AT_1R expression has already been observed in patients with a neuropathological diagnosis of PD who had not yet developed clinical manifestations or significant dopaminergic neurodegeneration (267). Yet to date, a potential re-distribution of other AT receptors in the brain of human PD patients has rarely been explored. However, ACE2 cleaves AngII and AngI to Ang1-9 and Ang1-7, respectively, and thereby shifts the RAS away from the proinflammatory main axis to the more anti-inflammatory side axis. Altogether, the RAS is a complex system that may be amenable to therapeutic modulation. The potential of targeting the RAS in PD and AD has therefore been discussed in considerable detail in dedicated review articles (123, 261).

Of note, unwanted alterations in the subtle balance between the pro- and the anti-inflammatory axis of the RAS occur during COVID-19 infections, where ACE2 is the main entry receptor for the pathogenic SARS-CoV-2 virus (268). Neurological consequences of the strong inflammation observed in severe COVID-19 remain to be studied.

5 CONCLUSIONS/OUTLOOK

Even 200 years after the first description of PD, its pathophysiology is still far from being fully understood. Fortunately, research towards a causal treatment of PD has made substantial progress in the last 15 years. Realizing that (systemic) inflammation plays an essential role of PD, at least in its maintenance, might pave the way for new therapeutic pathways. Knowledge transfer from classical inflammatory diseases or diseases that lead to inflammatory processes will likely have a major impact on the therapy of PD, and allow new

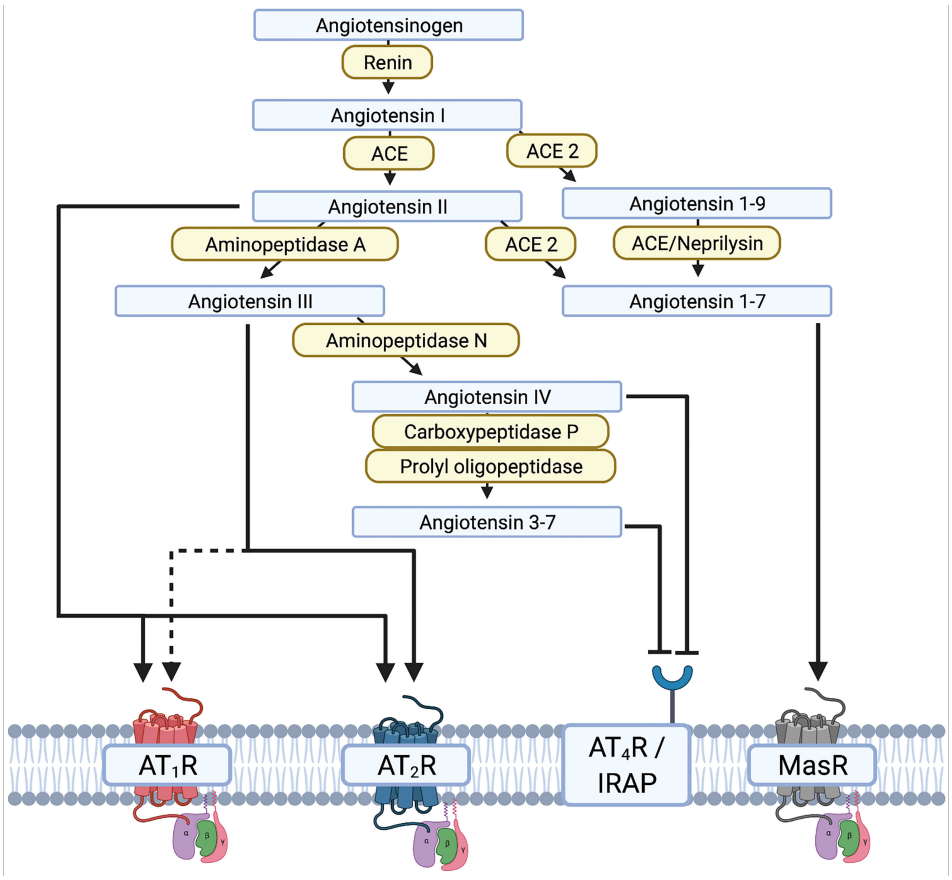

FIGURE 2 | Illustration of the renin-angiotensin system (RAS) and the different interactions of the renin-angiotensin-[aldosterone] system RAAS substrates on receptors. - Created with BioRender.com.

development and/or a repurposing of drugs that are already available. In addition, research in early diagnostics for PD is important. Ideally, neurodegeneration can be halted by initiating treatment before severe symptoms have developed. Skin biopsy or sleep analysis, along with new serum/liquor parameters, might lead to a sensitive and specific early diagnosis of PD. In turn, this should open up an earlier treatment window for future PD patients. Treating early with well-tolerated neuroprotective/anti-inflammatory agents certainly holds great promise for this devastating disease.

AUTHOR CONTRIBUTIONS

AG - Conceptualization, Visualization, Writing - original draft, Writing – review & editing. RM - Writing - review & editing. JWu - Writing - original draft. JWi - Writing - review & editing. CWI - Conceptualization, Supervision, Writing - review & editing. All authors contributed to the article and approved the submitted version.

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EDITED BY
Amy Lovett-Racke,
The Ohio State University,
United States

REVIEWED BY
John R. Lukens,
University of Virginia, United States
Ted M. Dawson,
Johns Hopkins Medicine, United States

*CORRESPONDENCE
Miao Sun
miaosunsuda@163.com
Bin Wang
binwang2233@suda.edu.cn

†These authors have contributed equally to this work

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Th17 cells and inflammation in neurological disorders: Possible mechanisms of action

Yajun Shi†, Bin Wei†, Lingjun Li, Bin Wang* and Miao Sun*

Institute for Fetology, The First Affiliated Hospital of Soochow University, Suzhou City, China

Neurological disorders (NDs) are one of the leading causes of global death. A sustained neuroinflammatory response has been reported to be associated with the pathogenesis of multiple NDs, including Parkinson's disease (PD), multiple sclerosis (MS), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and major depressive disorder (MDD). Accumulating evidence shows that the recruitment of abundant lymphocytes in the central nervous system may contribute to promoting the development and progress of inflammation in neurological disorders. As one subset of T lymphocytes, $CD4^+$ T cells have a critical impact on the inflammation of neurological disorders. T helper (Th) 17 is one of the most studied $CD4^+$ Th subpopulations that produces cytokines (e.g., IL-17A, IL-23, IL-21, IL-6, and IFN-γ), leading to the abnormal neuroinflammatory response including the excessive activation of microglia and the recruitment of other immune cell types. All these factors are involved in several neurological disorders. However, the possible mechanisms of Th17 cells and their associated cytokines in the immunopathology of the abovementioned neurological disorders have not been clarified completely. This review will summarize the mechanisms by which encephalitogenic inflammatory Th17 cells and their related cytokines strongly contribute to chronic neuroinflammation, thus perpetuating neurodegenerative processes in NDs. Finally, the potential therapeutic prospects of Th17 cells and their cytokines in NDs will also be discussed.

KEYWORDS

Th17 cells, neurological disorders, IL-17A, neuroinflammation, immune system, microglia

1 Introduction

Neurological disorders (NDs) are highly prevalent and have become the second most frequent cause of mortality, with approximately 276 million cases worldwide to date (1). With the global population aging, NDs are recognized as a significant public health challenge today (1, 2). At the same time, the causes and mechanisms behind most NDs are still vague. Though both genetic and environmental factors had been suggested in the

etiology of NDs (3, 4), increasing evidence indicates that neuroinflammation is one of the defining characteristics of NDs.

As a pivotal part of the central nervous system (CNS) innate immunity, neuroinflammation initially restrains infection and eliminates pathogens, cell debris, and aggregated or misfolded proteins in a generic manner. While in chronic NDs, neuroinflammation becomes continuous and is harmful to neuronal cells. Increasing evidence has suggested that neuroinflammation contributes to various NDs, including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Alzheimer's disease (AD), and major depressive disorder (MDD) (5–7). One of the common traits of these NDs is their neuropathological interactions with the microglia (the resident macrophages in the brain) and astroglia, which trigger an innate immunological response, contributing to the disease's severity and course (8). The pathogenesis of these diseases was not only restricted to the immunological mechanisms in the brain but also had a strong interaction with the systemic immune system.

Additionally, various pieces of evidence demonstrate that the communication between the CNS and systemic immune systems is possible through immune cells and cytokines breaking the integrity of the blood–brain barrier (BBB) (9, 10). It has been demonstrated that blood-borne immune cells (e.g., $CD4^+$ T cells, Th cells) are highly neurotoxic and represent an additional crucial mediator of neuroinflammation. Systemic inflammation, induced by oxidative stress damage, environmental stressors, gut microbiota imbalance, etc., may trigger microglial activation and ultimately contribute to the development of neurological disease processes, according to abundant evidence (8, 11, 12).

Among the critical neuroinflammatory cells in NDs, Th cells, especially Th17 cells, have already been known to be involved. At the same time, various sources of evidence have indicated that Th17 cell levels in both serum, cerebrospinal fluid (CSF), and brain were elevated in the laboratory model animals of NDs (13–15). However, the accurate role of neuroinflammation mediated by Th17 cell and their cytokines in the etiology of the abovementioned NDs is still unclear. With a focus on the roles of Th17 cells and their cytokines in NDs, we have attempted to unravel the precise mechanism of neuroinflammatory disorder in this study. In addition, we provide an overview of the most recent developments in targeting Th17 cells and their cytokines, as well as their prospective clinical applications.

2 Th17 cells

2.1 Definition and differentiation of Th 17 cells

After encountering specific pathogens, the innate immune system will promote the development of naive $CD4^+$ T cells into effector Th cells, which are fundamental regulators of adaptive immunity. Different subtypes of $CD4^+$ Th cells are distinguished according to cytokines, transcription factors, and effector immune regulatory functions. Initially, activated $CD4^+$ Th cells were frequently differentiated into one of two fates: Th1 or Th2 cells. Th1 cells, generating interleukin (IL)-2, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), mainly take part in delayed-type hypersensitivity reactions and cell-mediated immune responses, such as autoimmune disorders. Th2 cells, renowned for their generation of the cytokine IL-4, mediate host defense against helminths and link with the pathogenesis of allergic diseases. In 2005, a newly identified subtype of $CD4^+$ Th cells, so-called Th17 cells, was discovered that represent a diverse population, which underwent differentiation in the presence of transforming growth factor-beta (TGF-β) and IL-6 (16, 17). Now, it was recognized that Th17 cells might be more significant than Th1 cells in the development of some models of autoimmune illness, including PD, AD, MS, ALS, and MDD (5–7).

Th17 cells perform crucial roles in pro-inflammatory properties, inflammation, and essential tasks to defend the host against extracellular bacterial and fungal infections. Th17 cells are defined as $CD4^+$ Th lymphocytes that secrete large amounts of IL-17A and express the transcription factor retinoic acid-related orphan receptor gamma-T (RORγt), which possibly acts as a molecular determinant for their polarization (18, 19). Th17 cells have two most distinct characteristics: strong plasticity and prominent capability to boost other immune cells (e.g., Th1 cells, neutrophils, and immunosuppressive head box P3 Treg cells).

Initially, the differentiation of Th17 cells was shown to be induced by the combination of IL-6 and TGF-β (20). Subsequently, Korn and colleagues found that IL-21, which was induced by signal transducer and activator of transcription 3 (STAT3)/IL-6 signaling, could affect Th17 cell development and response amplification (21). IL-21 acts in a loop of autocrine amplification to culminate in the differentiation and proliferation of Th17 cells and the production of the IL-23 receptor (IL-23R). IL-23 plays a key role in the late growth and maturation of Th17 cells by activating its receptor IL-23R *via* IL-6 and/or IL-21 (Figure 1) (22). Additionally, Th17 cells differentiated in the $IL\text{-}1^+/IL\text{-}23^+/TGF\text{-}\beta^-$ environment show greater encephalitogenic activity after adoptive transfer, which highlights the importance of IL-23 and IL-1 in the differentiation and pathogenicity of Th17 cells (23). Nonetheless, IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) can inhibit the differentiation of Th17 cells from naive $CD4^+$ Th cells as long as the Th17 cells have not been fully developed (24, 25).

RORγt, which was encoded by the *RORC* gene and generally expressed in Th17 cells, is the major regulator of the Th17 cell differentiation. *Rorc* knockout mice cannot produce Th17 cells (26). Meanwhile, overexpression of RORγt, triggered by the

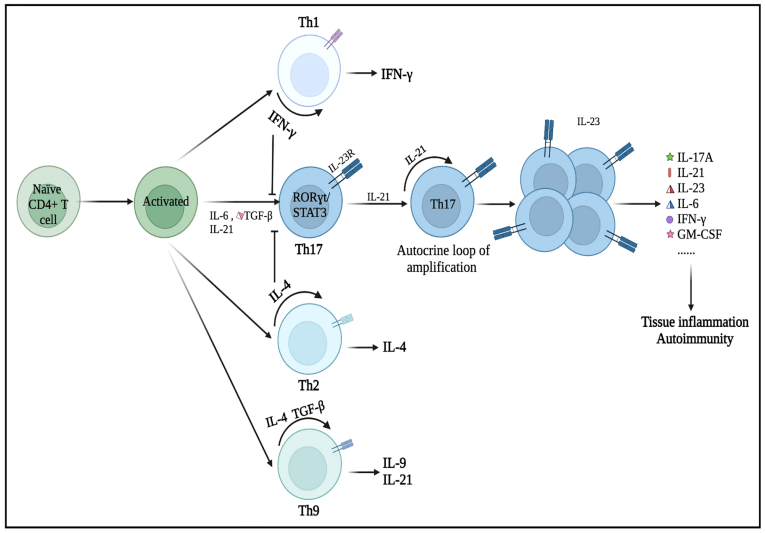

FIGURE 1
Differentiation of Th17 cells and its secretion of inflammatory cytokines. Under the microenvironment of pro-inflammatory cytokines (IL-6, IL-21, and/or TGF-β), naive CD4$^+$ T precursors can be induced to differentiate into Th17 cells, which have crucial roles in the immunopathogenesis of neurological disorders. Nonetheless, IFN-γ (a Th1 cell-related cytokine) and IL-4 (a Th2 cell-related cytokine) can inhibit the differentiation of Th17 cells from naive CD4$^+$ T cells as long as Th17 cells have not been fully developed. These primary differentiated Th17 cells will express specific transcription factors, such as RORγt and STAT3. IL-21 is produced by Th17 cells in an autocrine manner and other cells then promote Th17 cell proliferation and differentiation (expansion stage). IL-23 promotes the terminal differentiation, development, and survival of expanded Th17 cells in their final stable state. IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; STAT, signal transducer and activator of transcription.

activity of IL-6 and TGF-β, can lead to Th1- and Th2-independent differentiation of naive CD4$^+$ Th cell progenitors into the Th17 subtype (27, 28). Moreover, RORγt may cooperate with other transcription factors during the differentiation of Th17 cells (29). When activated by IL-6, IL-21, or IL-23, STAT3 is the upregulator of RORγt in response to TGF-β and IL-6. STAT3 can bind to plenty of Th17-related loci and is essential for the induction of the Th17 transcriptional program (30). In the absence of STAT3, overexpression of RORγt cannot restore Th17 cell development (31). Numerous essential transcription factors, such as STAT5, granulocyte monocyte-colony stimulating factor (GM-CSF), aryl hydrocarbon receptor (Ahr), activating transcription factor-like runt-related transcription factor-1 (RUNX1), transcriptional coactivator with postsynaptic density 65-discs large-zonal occluder 1-binding motif (TAZ), and interferon regulatory factor 4 (IRF4), regulate Th17 cell differentiation (29, 32, 33). Th17 cells are regarded as strongly pathogenic because they drive autoinflammation by producing a unique set of cytokines (e.g., IL-17A, IL-21, IL-23, IL-6, IFN-γ, and GM-CSF) (Figure 1).

It has been shown that IL-17A, mainly produced by Th17 cells, can induce cytokine secretion and has functions in immune inflammation (34). Numerous studies signify that IL-17A, stimulating glial cells and enhancing neuroinflammatory responses, performs a pathogenic role in the CNS (35, 36). IL-17 signaling also induces the activation of NF-κB cascade response to control the corresponding physiological function (37).

2.2 Environmental factors that affect the pathogenic potential of Th17 cells

Increasing evidence indicates that multiple environmental factors will contribute to the development of NDs. It is not difficult to think that different host environmental factors may also accelerate the pathogenic potential of Th17 cells and that immune responses may promote autoimmunity, therefore causing NDs.

2.2.1 Peripheral inflammation

Numerous studies indicated that Th17 cells and their cytokines would compromise the integrity of the BBB. Thus, the migration of immune cells from the periphery into the central nervous system has become one of the major contributors to numerous NDs (38, 39). For instance, *Porphyromonas gingivalis* stimulates the production of Th17-supportive cytokines (IL-1, IL-6, and IL-23), which may permeate the BBB to endotoxin and expose the brain cells to

endogenous and exogenous inflammatory mediators (40). Th17 cells and their related cytokines are also associated with abnormally aggregated plaques of the protein amyloid-beta (Aβ) in neurons (41). In a mouse model of PD, Vitola and colleagues demonstrated for the first time that peripheral injection of lipopolysaccharide could trigger a long-lasting increase in the pro-inflammatory cytokine TNF-α, induce neuroinflammation, influence the action of α-synuclein (αSyn) oligomers, and potentiate the detrimental effects (42). Moreover, Th17 cells, immune cells, and macrophages are abundant in the peripheral blood of MS patients, which would infiltrate the CNS and collaborate to cause tissue damage (43). The entry of peripheral immune cells from the periphery into the CNS can also be viewed as a precursor to the breakdown of the BBB, thereby heightening brain inflammatory responses (44). Rodent models suggested that vagal stimulation can increase the levels of brain cytokines in response to prolonged peripheral inflammation (45). Interestingly, the response to acute peripheral inflammation of the microglia is not associated with long-term neuronal damage. Only in chronic and slowly progressive diseases (such as AD, MDD, and PD) are the microglia activated by peripheral (cytokines) and/or brain [Aβ, oxidative stress (OxS), etc.] pro-inflammatory stimulus to prime for further neurotoxicity (8). As evidence, ND patients and animal models with persistent inflammatory neurodegeneration exhibited an acceleration of the neurodegeneration process in response to peripheral inflammatory stimulus (46, 47).

2.2.2 Enhanced oxidative stress

Indeed, one of the mechanisms underlying the pathogenesis of NDs is the vicious cycle between OxS and neuroinflammation, which is reflected in the brain and peripheral immune system (8). As elegantly discussed by Cobley and colleagues, the brain is highly sensitive to oxidative damage for a variety of reasons, including hypoxia/ischemia, glutamate, mitochondrial dysfunction, endogenous neurotransmitter metabolism (e.g., dopamine), and abnormal activation of microglia (48). Under the condition of chronic oxidative stress, mitochondrial hyperpolarization, nitric oxide production, and Ca^{2+} influx were enhanced, and the overexpression of the mechanistic target of rapamycin complex 1 (mTORC1) was facilitated (49). Furthermore, the activity of mTORC1 promotes the expansion of Th17 pro-inflammatory lymphocytes from $CD4^+$ T cells, which constitute a critical CNS mechanism of immune regulation. After activation, $CD4^+$ T cells can easily cross the BBB. As a result of the presence of IL-23, $CD4^+$ T cells differentiate into Th17 cells when they reach the damaged site. In intestinal immunity, Th17 cells can contribute to neuroinflammation and neurodegeneration by activating the apoptotic Fas/FasL pathway (50). OxS has been ascribed as a major contributor to the pathogenesis and clinical progression of NDs (11, 50).

2.2.3 The gut–brain axis

Considerable evidence suggests that the gut microbiota is essential for normal host metabolism and physiological function, influencing not only the immune system but also the nervous system (51, 52). The gut microbiota is also related to brain development and cognitive function. The gut microbiota affects the gut–brain axis *via* direct and indirect (*via* systemic circulation) pathways, including immune (cytokines), endocrine (cortisol), and neural (enteric nervous system) pathways (53). Diet-induced alterations to the gut microbiome can result in a compromised lamina propria, where Th17 cells are the predominant Th cells (26). Recently, Th17 cells, inducing the production of IL-17A and IL-22, have been shown to play distinct roles during fungal infection and colonization (54, 55). The importance of gut microbiota, particularly segmented filamentous bacteria (SFB), in the generation of mucosal Th17 cells was first demonstrated by Littman and colleagues (56). Surprisingly, colonization of the gut with SFB alone can induce the differentiation of Th17 cells in the gut and CNS, thereby accelerating the progression of NDs (57). Chen et al. have proven that the major metabolite of clostridia species, short-chain fatty acid butyrate, is sufficient to restore BBB dysfunction in patients with AD (58). The disruption of the gut microbiota stimulates the activation of astrocytes and microglia, which in turn influence a variety of neuropsychological processes (e.g., neuronal development, BBB integrity, CNS immune system activation) (59). Various environmental factors modulate the immune system's response, strongly accelerating the pathogenic potential of Th17 cells. Indeed, alterations of Th17 cells in the gut microbiota have been documented in several diseases such as AD (60), PD (61), and MS (62). Under high-salt conditions, a highly pathogenic Th17 cell population activated by the p38/MAPK pathway has been observed (63). Furthermore, the mice on a high-salt diet developed cognitive impairment due to cerebral endothelial dysfunction (64), which was associated with an increase in Th17 polarization and IL-17A plasma level in the small intestine. Moreover, hypoxia promoted Th17 differentiation by activating the hypoxia-inducible factor 1 (HIF-1), a critical metabolic sensor that directly regulates the expression of RORγt and IL-17A at the transcriptional level (65). The gut–brain axis appears to be a potent druggable target for the immunotherapy of IL-17A in NDs.

3 Th17 cells and its cytokines in neurological disorders: Possible mechanisms of action

3.1 Th17 cells in Parkinson's disease

PD, the second most prevalent form of neurodegenerative disease, is characterized by motor symptoms, such as tremors,

rigidity, and bradykinesia (66). In PD, there is a progressive degeneration of neurons in various regions of the brain, including dopaminergic neurons in the substantia nigra pars compacta (67). A critical pathological hallmark of PD is the accumulation of αSyn (so-called Lewy bodies). In addition to protein aggregation, inflammatory responses play a crucial role in the etiology and pathogenesis of PD, as evidenced by the elevated expression of IFN-γ, IL-17A, and IL-6 in the brain (68). PD patients also have a disruption of BBB, which allows peripheral immune cells to infiltrate into the brain and potentially influences other mechanistic pathways associated with neurodegeneration, such as oxidative stress and mitochondrial dysfunction potentially (68). Therefore, the enhanced peripheral inflammation may initiate or exacerbate PD pathology. Recently, Sommer et al. provided direct evidence that the neurotoxic effect of Th17 cells, expanded in an autocrine loop, was increased in PD patients (69). This model also revealed a direct contact between Th17 cells and neurons leading to dopaminergic neuronal apoptosis. In a mouse model with a deficiency of IL-17A, motor impairment, dopaminergic neurodegeneration, and BBB disruption were alleviated (70). Furthermore, Gate and colleagues provided the exact mechanism for Th17 cell-mediated dopaminergic neuron death *via* secretion of IL-17A in PD (71). Th17 cells secrete pro-inflammatory cytokines that were associated with the activation of other detrimental inflammatory factors (like TNF-α, IL-1β, and IL-6), which were released from brain microglial cells (the most numerous types of brain cell). Ultimately, Th17 cells and inflammatory factors promote inflammatory reactions and neuronal apoptosis (**Figure 2**). On the other hand, abnormally accumulated αSyn becomes an autoimmune antigen that activates microglia into the microglia type 1 subtype, which promotes the differentiation of Th17 subtypes and activates intracellular inflammatory pathways (72). Although the direct effects of Th17 cells on neurons have been described in these studies, additional work

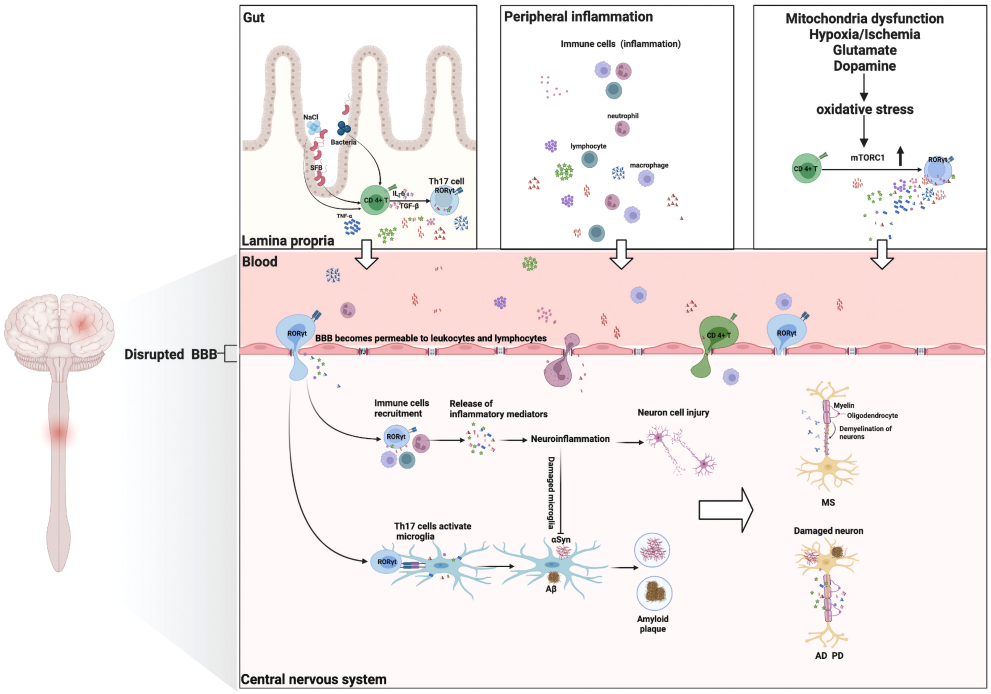

FIGURE 2
Th17 cells and their cytokines in neurological disorders: possible mechanisms of action. Multiple environmental factors (peripheral inflammation, enhanced oxidative stress, gut–brain axis) induce a pro-inflammatory microenvironment that modifies the $CD4^+$ T-cell phenotype and then differentiates into encephalitogenic Th17 cells, producing inflammatory cytokines (IL-17A, IL-6, IL-21, IFN-γ, GM-CSF, and IL-23). These Th17 cells are capable of entering the CNS. They proliferate and produce cytokines that are conducive to BBB disruption and recruitment of other immune cells (lymphocytes, macrophages, and neutrophil cells) into the CNS, ultimately leading to myelin damage (multiple sclerosis). Th17 cells and their cytokines can cause neuronal damage through direct cytotoxic effects or through recruitment of immune cells and induction of neuroinflammation, resulting in deposition of Aβ fibrils or the aggregation of αSyn (Alzheimer's disease/Parkinson's disease). Additionally, Th17 cells can activate the microglia and phagocytose amyloid fibrils, but neuroinflammation will induce microglia damage, thereby exacerbating amyloid β deposition or αSyn aggregation (Alzheimer's disease/Parkinson's disease). GM-CSF, granulocyte monocyte-colony stimulating factor; Aβ, amyloid β.

is still needed to comprehend the activity of PD-related Th17 neurotoxicity in a more complex intracellular environment to open up novel therapeutic development avenues.

3.2 Th17 cells in Alzheimer's disease

AD is the most frequent form of late-onset dementia and has progressive and irreversible pathogenesis with a complex molecular disease etiology, which affects over 40 million people worldwide (73). AD is a multifactorial neurological disorder, in which the extracellular deposits of amyloid β (Aβ) peptides and fibrillary tangles of intraneuronal hyperphosphorylated tau protein are the major histopathological hallmarks. Aβ is obtained by the frequent and serial activation of the γ-secretase enzymes and β-secretase 1 (BACE1) on a larger precursor protein (APP) (74). Highly insoluble Aβ peptides are considered an important factor in AD pathogenesis. They motivate the microglia to secrete pro-inflammatory cytokines and chemokines to activate the complement pathway (75), thus inducing the cumulation of inflammatory cells into the CNS that leads to neurodegeneration (76). Indeed, Aβ peptides have been shown to enhance the manufacture of reactive oxygen species (ROS) and nitric oxide (NO) by microglia, leading to OxS development and stimulating the inflammation of the Th17/IL-17A axis, and then impair microglia-mediated Aβ phagocytosis and contribute to Aβ accumulation and neuronal damage. Additionally, the primary roles of IL-17A in the pathogenesis of AD are to attract neutrophils and then stimulate their function. Zenaro et al. showed that Aβ aggregates could mediate the recruitment and chemotaxis of neutrophils to produce IL-17A, which is directly toxic to neurons and the BBB and may amplify neutrophils in the CNS, thus contributing to a vicious circle that leads to exacerbating pathology (77). Since neutrophils are the primary targets and crucial sources of IL-17A in the CNS, they may play a significant role in the development of AD pathology by promoting inflammation and neuron autophagy. The presence of extremely high levels of IL-17A, TNF-α, IL-2, and GM-CSF in the brain of a triple transgenic mouse model of AD indicates that neurodegeneration in these mice is related to Th17 polarization. Another study found a notable increase of IL-17A, RORγt, and IL-22 in the serum, hippocampus, and CSF of Aβ-42 peptide-injected rats (78). In addition, Tian et al. indicated a correlation between postoperative cognitive impairment and elevated levels of IL-17A in the hippocampus (79). Recently, a study has reported that the administration of blocking anti-IL-17A antibodies could rescue Aβ-induced cognitive impairment and neuroinflammation (80). These findings provide evidence for the synergistic roles of Th17 cells and their associated cytokines in promoting neuroinflammation and degeneration in AD.

The number of $CD4^+$ and $CD8^+$ T cells in AD patients' brain parenchyma and vascular endothelium is significantly higher than normal (81). The activated Th17 cells and their inflammatory cytokines (e.g., IL-17A, IL-21, and IL-23) in the brain may jointly promote AD neuropathology (82). Furthermore, a number of studies found that AD patients had elevated levels of the Th17 transcription factor RORγt in their lymphocytes (83, 84). Recently, other studies have demonstrated the relationship between Th17 cells and early AD (85). Therefore, some scholars proposed that the optimal AD vaccine should inhibit Th17 immune responses to Aβ to prevent neuroinflammation and subsequent neurodegeneration (86).

3.3 Th17 cells in multiple sclerosis

MS is a widespread demyelinating disease of the CNS resulting in substantial neurological disability, in which immune cells and related cytokines are involved in the degradation of myelin sheaths (87). Inflammatory processes in these foci led to myelin damage and oligodendrocyte rupture, followed by axonal loss and transient or permanent loss of neurologic functions, resulting in varying degrees of disability (88). Until now, the pathophysiology of MS has not been elucidated clearly. Still, the prevailing view of MS pathogenesis is the breach of immunological tolerance and the active infiltration of myelin antigen-sensitive immune cells into brain tissue through the BBB. Emerging data from clinical and animal studies have revealed that myelin-specific immune cells (such as B cells, Th1 cells, and Th17 cells) are activated in lymph organs of the periphery. These myelin-specific immune cells develop encephalitogenic potential and infiltrate the CNS, where they are reactivated and expanded by the IL-23 and IL-1β (produced by resident microglia and infiltrating inflammatory monocytes) (62, 89). Furthermore, multiple studies have indicated that Th1 and Th17 cells are enhanced in the brain parenchyma in the acute phase of MS patients (90, 91). Th17 cells mainly cause brain damage, but Th1 cells induce spinal cord inflammation. In the attempt to clarify the role of Th1 and Th17 cells in the pathogenesis of MS, Langrish et al. showed that Th17 cells induce more severe experimental autoimmune encephalomyelitis (EAE) than Th1 cells (92). Th17 cells, especially Th1-like Th17 cells, may participate in EAE pathology by producing IFN-γ and IL-17A. Th17 cells result in oligodendrocyte death, axonal degeneration, and neuronal dysfunction (91, 93). Multiple studies in various EAEs, animal models of MS, have demonstrated that Th1-like Th17 cells can across the BBB by stimulating the IL-17A and C-C chemokine receptor 6 (CCK6) and enhance neuroinflammation (87, 94, 95). In the CSF and peripheral blood of patients with MS relapse, Durelli and colleagues detected that a higher IL-17A expression was positively associated with disease activity (96). Mice that lack Th17 and its characteristic cytokines, including IL-23, IL-21, and IL-22, are also at risk of developing EAE (97, 98).

Furthermore, Hartlehnert and colleagues identified the induction of the B-cell-supporting meningeal microenvironment by Bcl6 in Th17 cells as a mechanism controlling neuroinflammation (99). Therefore, IL-17A and B cells have a considerable but non-essential function in EAE. Additionally, mice lacking RORγt, a crucial transcription factor for Th17 differentiation, exhibit a delayed onset or mild progression of EAE (100). Unlike other Th17 cytokines, GM-CSF, regulated by IL-23, RORγt, and IL-1β, has an encephalitogenic profile and a non-redundant role in active myeloid cell infiltration leading to sustained neuroinflammation in MS (101). Although the absence of GM-CSF had no effect on Th cells infiltrating into the CNS, it severely inhibited the accumulation of tissue-invading phagocytes, which are the primary drivers of immunopathology and are capable of initiating tissue damage (101). Consequently, Th17 cells and their related factors have a significant role in the pathogenesis of MS.

3.4 Th17 cells in ALS

ALS is a progressive neurodegenerative disease, which is characterized by the clustering and accumulation of ubiquitylated, proteinaceous inclusions in the upper and lower motoneurons (MN), resulting in dramatic muscle paralysis, extramotor abnormalities, or death (102). Although the pathophysiological mechanisms of ALS are still unresolved, multiple studies corroborated that the pathological processes of ALS are involved in neuroinflammation, mainly attributed to the activation of CNS innate immune cells, and may precede MN cell death (35, 103). Neuroinflammation in ALS is characterized by the accumulation of massive astrocytes and activated microglia, which increases the production of potentially cytotoxic molecules (such as ROS, inflammatory mediators, and pro-inflammatory cytokines) (104). Interestingly, recent studies provide evidence that Th17 cells and their associated cytokines (e.g., IL-17A, TNF-α, IFN-γ) are enhanced in the CSF, peripheral blood, and serum of ALS patients (35, 104). In clinical studies with ALS, Meng et al. showed that peripheral pro-inflammatory Th17 cell shift was linked to disease severity (13). These findings proved that therapeutic interventions on Th17 cells and/or their cytokines may be a promising treatment strategy in ALS patients.

3.5 Th17 cells in MDD

MDD is one of the most commonly recognized mental disorders globally. The number of patients receiving antidepressant treatment is increasing yearly, which is a severe medical, social, and economic problem. According to the Composite International Diagnostic Interview version of the World Mental Health Survey, MDD is a chronic psychiatric disorder with long-term depressive symptoms (e.g., anxiety, loss of pleasure, and low sense of self-worth). With the steadily rising number of young patients with MDD over the past decade, there is an urgent need for more effective therapeutic strategies and the identification of the molecular mechanisms underlying chronic MDD. Although the etiology of MDD has yet to be determined, mounting evidence supports a link between depression and elevated levels of IL-17A, suggesting that inflammation exacerbates depressive symptoms. Indeed, numerous pro-inflammatory cytokines (e.g., IL-1β, IL-23, IFN-γ, and TNF-α) and Th17 cells are elevated in the peripheral blood of depressed patients (105, 106). Alvarez-Mon et al. clearly showed an increased propensity for Th17 differentiation in the circulating population of $CD4^+$ T lymphocytes in adult patients with MDD (105). In addition, several studies have also demonstrated that Th17 cells were increased in the brains of the rodent models of MDD (107). Interestingly, studies showed that administration of Th17 cells would result in increased sensitivity to depression in two different mouse models, giving a direct correlation between Th17 cells and depression sensitivity (108, 109). The absence of RORγt and the neutralization of IL-17A both conferred resistance to the induction of learned helplessness in mice (108). Nevertheless, the levels of IL-17A are not always correlated with depression, and there is no evidence indicating that Th17 cells cause neuron damage in MDD. Similar to psoriasis and MS surveys, patients with rheumatoid arthritis who have elevated levels of IL-17A also have an increased risk of depression and anxiety disorders (110, 111). These findings suggest that Th17 cells may not be sufficient to cause depression on their own, but they have additional roles to promote depression. To clarify whether IL-17A is directly involved in neuroimmune interactions, further research is required.

4 Prospective and effective therapeutic strategies targeting Th17 cells and their cytokines in neurological disorders

As demonstrated previously, Th17 cells and their cytokines play crucial roles in the pathogenicity of the immune system in neurological diseases. Consequently, they may be prospective therapeutic targets for NDs due to their pathogenic functions. At present, numerous methods target Th17 cells and their cytokines, including anti-GM-CSF antibodies, anti-IL-17A monoclonal antibodies, and RORγt

TABLE 1 Drugs targeting Th17 cells and their cytokines.

Drug name	Target cytokine	Mechanism of action	Clinical development	Mouse model	References
Secukinumab	IL-17A	Full human monoclonal antibody targeting IL-17A	Rheumatoid arthritis, spondylarthrosis, psoriasis, MS	NA	(112)
Ixekizumab	IL-17A	Humanized anti-IL-17A monoclonal antibody	Psoriasis, rheumatoid arthritis	NA	(113)
Brodalumab	IL-17R	Full human monoclonal antibody targeting the IL-17 receptor	Psoriasis	NA	(114)
Anti-GM-CSF antibody	GM-CSF	Suppresses microglia activation in the brains of C57/BL6 mice	NA	AD, MS	(115–117)
TAK-828F SR1001	RORγt	RORγt inverse agonists	MS	NA	(118, 119)
Ursolic acid, oleanonic acid	RORγt	RORγt inverse agonists	NA	MS	(120, 121)

inhibitors (Table 1). Although the efficacy of these techniques in the treatment of NDs involving pathogenic Th17 cells has not yet been demonstrated and is still in the developmental stages, strategies that target Th17 and their cytokines remain promising.

4.1 Targeting the IL-17A/IL-17AR axis

IL-17A is the critical effector cytokine secreted by Th17 cells. Only three monoclonal antibodies of IL-17A and IL-17AR were approved, namely, secukinumab, ixekizumab, and brodalumab. Secukinumab is a fully human monoclonal antibody, which was labeled as first-line therapy for moderate to severe cases of active or stable psoriasis (122). Macaluso and colleagues first demonstrated secukinumab's clinical efficacy on neurological manifestations in patients with concomitant ankylosing spondylitis (AS) and MS, supporting the fact that IL-17A blockade may become a potential therapeutic target in MS (112). Cortese et al. also suggested that secukinumab was efficacious for treating CNS demyelination in AS patients, but this topic still needs further studies (123). Moreover, the restoration of neuronal cell death produced by Th17 cells by secukinumab in PD patient-generated pluripotent stem cell-derived neurons motivated us to look for additional possible immunotherapies for PD (69). Ixekizumab, another human monoclonal antibody, inhibits the inflammatory response mediated by IL-17A binding to the IL-17A receptor (IL-17AR), whereas brodalumab can specifically block the IL-17 receptor. Extensive clinical studies have shown that ixekizumab and brodalumab are beneficial and used by the FDA in adults with moderate to severe plaque psoriasis (114, 124). Emerging evidence hints that the therapeutic interventions on IL-17A may be a promising treatment strategy in ND patients. To confirm the efficacy of anti-IL-17A/IL-17AR antibodies in the treatment of NDs, additional research is required.

4.2 Targeting other Th17 cytokines and Th17 cell development

In addition to IL-17A, Th17 cells release numerous additional cytokines, including GM-CSF, IL-23, and RORγt. GM-CSF was reported to play a pathogenic role in NDs, such as AD, PD, MS, and MDD. Several studies in AD and PD animal models have demonstrated that anti-GM-CSF antibodies would inhibit the activation of the microglia and astrocytes in the CNS, suggesting that it may be a potential therapeutic target (113, 115). In support of this finding, Manczak and colleagues observed that animals injected with anti-GM-CSF antibodies had decreased Aβ deposition and microglia expression (116). Since IL-23 and RORγt are involved in the differentiation and activation of Th17 cells, targeting the IL-23 or RORγt in the treatment of NDs is exciting and an effective approach (117). Currently, several RORγt inverse agonists, including oleanonic acid, ursolic acid, and TAK-828F, have been identified (118–120). For instance, SR1001 could alleviate the severity and delay the onset of EAE in distinct mechanisms, including limiting Th17 cell differentiation and reducing the expression of Th17 cytokines (117).

5 Conclusions and future directions

Since the discovery of the link between Th17 cells and the development of neurological disorders, the world of Th17 cells and their cytokines in NDs has experienced a boom of research and discoveries. Th17 cells have been described to play crucial roles in NDs, but more work is needed to clarify the exact mechanisms of their function in the future. This knowledge is essential for determining the importance of Th17 cells and finding prospective therapies for patients with neurological disorders. Existing data and future directions suggest that Th17 cells and their cytokines and Th17-related signaling pathways will be

potentially effective therapeutic neuroprotective targets in the treatment of NDs (121).

Author contributions

YJS and BW wrote the manuscript with support from LJL, BW, and MS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Rui Li,
University of Pennsylvania,
United States

REVIEWED BY
Ayman Rezk,
University of Pennsylvania,
United States
Linlu Tian,
Medical College of Wisconsin,
United States

*CORRESPONDENCE
Jian-Ting Chen
chenjt@smu.edu.cn

†These authors have contributed equally to this work

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Bioinformatics analysis identified apolipoprotein E as a hub gene regulating neuroinflammation in macrophages and microglia following spinal cord injury

Xin-Qiang Yao[1†], Jia-Ying Chen[2†], Zi-Han Yu[1], Zu-Cheng Huang[1], Regan Hamel[3], Yong-Qiang Zeng[1], Zhi-Ping Huang[1], Ke-Wu Tu[1], Jun-Hao Liu[4], Yan-Meng Lu[5], Zhi-Tao Zhou[5], Stefano Pluchino[3], Qing-An Zhu[1] and Jian-Ting Chen[1*]

[1]Division of Spine Surgery, Department of Orthopaedics, Nanfang Hospital, Southern Medical University, Guangzhou, China, [2]Department of Comprehensive Medical Treatment Ward, Nanfang Hospital, Southern Medical University, Guangzhou, China, [3]Department of Clinical Neurosciences, University of Cambridge, Cambridge, United Kingdom, [4]Division of Spine Surgery, Department of Orthopaedics, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou, China, [5]Center of Electron Microscopy, Central Laboratory, Southern Medical University, Guangzhou, China

Macrophages and microglia play important roles in chronic neuroinflammation following spinal cord injury (SCI). Although macrophages and microglia have similar functions, their phagocytic and homeostatic abilities differ. It is difficult to distinguish between these two populations *in vivo*, but single-cell analysis can improve our understanding of their identity and heterogeneity. We conducted bioinformatics analysis of the single-cell RNA sequencing dataset GSE159638, identifying apolipoprotein E (APOE) as a hub gene in both macrophages and microglia in the subacute and chronic phases of SCI. We then validated these transcriptomic changes in a mouse model of cervical spinal cord hemi-contusion and observed myelin uptake, lipid droplets, and lysosome accumulation in macrophages and microglia following SCI. Finally, we observed that knocking out APOE aggravated neurological dysfunction, increased neuroinflammation, and exacerbated the loss of white matter. Targeting APOE and the related cholesterol efflux represents a promising strategy for reducing neuroinflammation and promoting recovery following SCI.

KEYWORDS

apolipoprotein E, neuroinflammation, macrophages, microglia, spinal cord injury, bioinformatics analysis

Introduction

Traumatic spinal cord injury (SCI) can lead to permanent neurological disorders (1). The dynamic SCI environment has three phases: the acute phase, occurring up to three days post injury (dpi), is characterized by hemorrhage, cell death, and cytokine release; the subacute phase, occurring 3-14 dpi, is characterized by angiogenesis, immune cell infiltration, and phagocytosis of myelin debris; and the chronic phase, occurring more than 14 dpi, is characterized by glial scar formation, remyelination, and neural remodeling (2). Persistent inflammatory processes and neuronal damage are associated with failed functional recovery. Macrophages are mainly immersed in the injury core and are associated with both pro- and anti-inflammatory effects, whereas microglia are mainly located in the injury rim and are associated with a pro-inflammatory role (3). Macrophages and microglia act as "professional phagocytes" following SCI (4). Although macrophages have stronger phagocytic activity, they are less efficient at processing phagocytosed material (5). Therefore, a better understanding of the mechanisms underlying processes such as microglial uptake and digestion is essential. Unfortunately, distinguishing macrophages from microglia is difficult (5).

Single-cell RNA sequencing (scRNA-seq) has facilitated the study of macrophage and microglial complexity following SCI (6). scRNA-seq analysis has been conducted for almost all cell types involved in angiogenesis, gliosis, and fibrosis in a mouse model of SCI (7). Macrophages and microglia are divided into different transcriptional profiles and subpopulations, depending on their functions and tasks (8). A disease-associated microglial subtype was potentially protective against wound healing following SCI (9). FABP5+ macrophages and microglia are regarded as proinflammatory myeloid cells with neurotoxic effects (10). A previous proteomic analysis of a rat SCI model showed that the protein cluster continuously upregulated in the acute and subacute phases was enriched in markers of myeloid cells, lipid regulation pathways, and lysosomes (11). Lipid droplet formation occurs in macrophages and requires lysosomal degradation following SCI (5, 12). Excessive lipid levels lead to the formation of foam cells, which have pro-inflammatory effects (13, 14). Macrophages are more prone to cell death than microglia during the phagocytic response that occurs following SCI (15). The phagocytic mechanism in macrophages and microglia is still not fully understood, and single-cell analysis may help us differentiate these cells (16).

In this study, we analyzed the scRNA-seq dataset GSE159638 to explore differentially expressed genes (DEGs) and pathways that are activated in macrophages and microglia during the subacute and chronic phases of SCI. We then employed histological analysis of a mouse model of cervical spinal cord hemi-contusion to experimentally validate the results of the bioinformatics analysis. Finally, we analyzed the effect of apolipoprotein E (APOE) knocked out on neurological function.

Abbreviations: SCI, spinal cord injury; APOE, apolipoprotein E; DPI, days post injury; WPI, weeks post injury; WT, wild-type; scRNA, single-cell RNA; DEGs, differentially expressed genes; GEO, gene expression omnibus; UMAP, uniform manifold approximation and projection; AUC, under the curve; GO, gene ontology; GSVA, gene set variation analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; PPAR, peroxisome proliferator-activated receptor; MBP, myelin basic protein.

Materials and methods

Data acquisition

The GSE159638 count matrices were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The dataset contains 25 spinal cord samples; five in the one dpi group, four in the two dpi group, seven in the three dpi group, five in the 10 dpi group, two in the 21 dpi group, and two in the sham group. The dataset contains a total of 30,958 cells. The 10X Genomics Chromium™ 3' single cell solution was used as the single-cell capture platform while GPL24247 (Illumina NovaSeq 6000, Mus musculus) was used as the sequencing platform. Each sample contained either resident microglia or infiltrating myeloid cells, including infiltrating macrophages. These populations were isolated *via* FACS, based on fate mapping labels described in the original article (10).

Data processing

Downstream data analysis was performed using the "Seurat" package, version 4.10, in R version 4.1.2 (17). Cells containing more than 15% mitochondrial genes were filtered out and the remaining data was normalized using the "LogNormalize" function. Dimensionality reduction was conducted using "FindVariableFeatures" and "RunPCA," functions and visualized using uniform manifold approximation and projection (UMAP) analysis. Clusters were identified using the following settings: FindNeighbors (dims=1:13), FindClusters (resolution=0.5), and RunUMAP (reduction = "pca," dims = 1:13). Clusters with fewer and dispersed cells were excluded from further analyses. Cell marker genes and four myeloid cell subtypes were identified using "FindAllMarkers (min. pct = 0.25, logfc.threshold= 0.25)".

Group-specific macrophage and microglia markers

A standard area under the curve (AUC) classifier was used to identify macrophage and microglia gene markers expressed at

different time points using "FindAllMarkers (min.pct = 0.1, logfc.threshold = 0.25)". Gene Ontology (GO) analysis of marker genes was performed using the "clusterProfiler" package, version 4.2.2 (18), and three GO terms representing the main functions at each time point were selected from the top 30 terms.

Differential gene expression analysis

Since macrophages were not detected in the sham group, we used the mean value of macrophage gene expression for the first three days to represent the transcriptional profile of the acute phase. Genes expressed by macrophages 10 and 21 dpi were compared with those expressed over the first three days using the "FindAllMarkers (logfc.threshold=0.5, p_val_adj=0.05)" function. DEGs in microglia were identified by comparing each experimental group with the sham group. GO analysis of overlapping genes expressed 10 and 21 dpi was performed in macrophages and microglia.

Identification of time-dependent gene expression modules

Macrophage and microglia modules with consistent expression patterns at different time points were categorized using fuzzy c-mean clustering implemented in the "Mfuzz" package, version 2.54.0 (19). First, the average expression of each gene at each time point was calculated for macrophages and microglia. An analysis workflow based on "filter.std (min.std = 0)", "standardise ()", and "mestimate ()" functions was then used. Third, clusters containing upregulated genes at 10 and 21 dpi were analyzed against c2 Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets using gene set variation analysis (GSVA) implemented using the "GSVA" package (version 1.42.0) and "Msigdbr" package (version 7.4.1). Finally, the gene clusters were compared with the DEGs, and genes overlapping the two datasets were subjected to protein-protein interaction (PPI) network analysis using STRING software (20).

Animal experiments

The animal experiments were approved by the Laboratory Animal Care and Use Committee of Nanfang Hospital, Southern Medical University, and performed according to the National Guidelines for the Care and Use of Animals. Eight-week-old wild-type and $APOE^{-/-}$ male mice were obtained from the Laboratory Animal Center of Southern Medical University and housed at the Laboratory Animal Center of Nanfang Hospital with ad libitum access to food and water.

Cervical spinal cord hemi-contusion injury

The mice were anesthetized using isoflurane (3% for induction and 1.5%–2% for maintenance) and C5 hemi-contusion injuries were induced as previously described (21). Briefly, the C5 lamina was exposed and removed. A contusion SCI was induced at C5 using an impactor tip (diameter=1.0 mm) with a preset displacement of 1.2 mm at 300 mm/s, controlled by an electromagnetic servo material testing machine (Instron E1000, Instron, United States). Only C5 laminectomy was performed as part of the sham surgery.

Behavioral assessment

Behavioral assessments were performed as previously described (22). Behavioral assessment was conducted by two independent researchers at different time points before and after surgery. Cylinder rearing test was used to evaluate the use of forelimbs (23). Mouse activity in a 20 cm-diameter transparent cylinder was recorded for 15 min and the first 20 climbing movements (left forelimb touch, right forelimb touch, and both forelimbs touch) or all climbing movements within 15 min were recorded. The grooming test is mainly used to evaluate the motion of the shoulder and elbow joints, with scores ranging from 1 to 5 based on the position of the foreclaws contacting the head and face (24).

Immunofluorescence staining

Animals were anesthetized with sodium pentobarbital and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Spinal cords (5 mm rostral to 5 mm caudal to the epicenter) were dissected, postfixed overnight with paraformaldehyde, and dehydrated in 12%, 18%, and 24% sucrose solutions at 4°C. The samples were embedded in optimal cutting temperature compound (Tissue-Tek, 4583, Sakura), and sliced transversely into 20 μm sections using a Leica CM1950 cryostat. For myelin basic protein (MBP) staining, slices were washed once with PBS and then permeabilized in a graded series of ethanol solutions (50%, 70%, 90%, 95%, 100%, 100%, 95%, 90%, 70%, and 50%). The slices were blocked with 0.01 M PBS containing 0.1% Triton X-100 and 10% normal donkey serum for 30 min. The slices were then incubated overnight at room temperature with the following primary antibodies: anti-F4/80 (1:200, Cell Signaling Technology 71299S), anti-APOE (1:400, Abcam ab183597), anti-CD68 (1:400, Abcam ab125212), anti-GFAP (1:800, Cell Signaling Technology 3670S), anti-MBP (1:400, Abcam, ab4039), anti-SMI312 (1:800, Covance, SMI-312R-100), and anti-LAMP2 (1:200, Abcam, ab13524). The following day, the

slices were washed three times in PBS and incubated at room temperature for two hours with the following secondary antibodies: donkey anti-mouse Alexa Fluor 488 (1:200, Abcam ab150105), donkey anti-rabbit Alexa Fluor 555 (1:200, Abcam ab150062), and donkey anti-rat Alexa Fluor 647 (1:200, Abcam ab150155). To stain lipid droplets, the slices were incubated with BODIPY (1:400, Invitrogen D3922) for 20 min following their incubation with primary antibodies. They were then washed thrice in PBS and mounted with Fluoromount-G (0100-20, Southern Biotech). Images were captured and analyzed using a Zeiss confocal microscope (LSM980, Zeiss, Germany) and ZEISS ZEN 3.3 software.

Transmission electron microscopy (TEM)

The mice were transcardially perfused with PBS and 4% paraformaldehyde, and the injury epicenters harvested, postfixed in 2% glutaraldehyde, and incubated overnight at 4°C. The samples were then rinsed in PBS, incubated with osmium tetroxide for 1 h, rinsed in PBS, dehydrated in 30%, 50%, 70%, 90%, and 100% ethanol solutions, and permeabilized in a graded series of acetone-Epon mixtures (1:1 for 1 h, 1:2 for 2 h, 1:2 for 3 h, and pure Epon overnight). Spinal cords were embedded in Epon, sliced into 0.8 μm semi-thin and 60–90 nm ultrathin sections using ultramicrotome Leica UC7. The sections were subsequently stained with uranyl acetate and lead citrate and analyzed under a transmission electron microscope (HITACHI H-7500, Japan).

Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 8.2.1). Unpaired *t*-test was used to analyze injury-related biomechanical parameters of wild-type and $APOE^{-/-}$ mice. Behavioral assessments and weight changes were investigated using two-way ANOVA with repeated measurement, followed by Bonferroni multiple comparison test between groups at each time point. Kruskal-Wallis test followed by Dunn's test *post hoc* was used to analyze the immunofluorescence results of F4/80, APOE and BODIPY at different time point. Mann-Whitney test was used to analyze the immunofluorescence results of GFAP, CD68, F4/80, MBP and SMI312 between wild-type and $APOE^{-/-}$ mice. Statistical significance was set at $p< 0.05$.

Results

Identification of macrophage and microglia clusters

The GSE159638 dataset introduced a mouse spinal contusion model using an impactor tip (1.3 mm) and an impact of 70 kilodyne force. Transcriptional profiles of 30,768 cells and 15,731 genes were obtained after quality control. The UMAP algorithm was used to convert the multidimensional data into a two-dimensional plot (Figure 1B). Four myeloid cell subtypes were identified in each population using multiple marker genes (Figures 1A, C). The distribution and proportion of myeloid cell subtypes at different time points are shown in Figure 1D and Figure 1E. Macrophages increased rapidly in the first three days but decreased in the subacute and chronic phases. Microglia were the only myeloid cells in the sham group and were the major cell type in the subacute and chronic phases, consistent with previous studies (25).

Unique marker genes and signatures of macrophages and microglia at different time points

To investigate the biological functions of macrophages and microglia at different time points, differential analysis was conducted based on the AUC classifier and GO analysis. Macrophages and microglia had different DEGs and pathways 10 and 21 dpi compared with the first three days post injury but were indistinguishable between 10 dpi and 21 dpi. For example, GO terms specific to macrophages included cytoplasmic translation pathway, regulation of cellular amide metabolic processes, and antigen processing and presentation at 10 dpi, and lymphocyte-mediated immunity pathway, positive regulation of endocytosis, and neuroinflammatory response at 21 dpi (Figure 2A). GO terms specific to microglia included lysosome organization, lipid transport, and autophagy pathways at 10 dpi, and response to lipoprotein particles, myelination, and lipid storage pathways at 21 dpi (Figure 2B). Interestingly, the biological function of microglia indicates that microglia may produce lipid droplets and degrade them through autophagy to maintain lipid metabolism balance.

APOE is the top upregulated gene in macrophages and microglia in the subacute and chronic phases

We identified genes that were differentially expressed in macrophages during the subacute and chronic phases (Figure 3A). Compared to the acute phase, a total of 642 genes were differentially expressed in macrophages at 10 and 21 dpi (Figure 3D). The top 10 upregulated and downregulated DEGs are shown in the bar graphs in Figure 3B and Figure 3C, respectively. GO analysis showed that co-expressed genes, including *APOE*, *TREM2*, *CST3*, and *AIF1* were enriched for phagocytosis, ERK1 and ERK2 cascades, oxidative stress response, lysosomes, and positive regulation of lipid localization (Figure 3E). Notably, the TREM2-APOE pathway

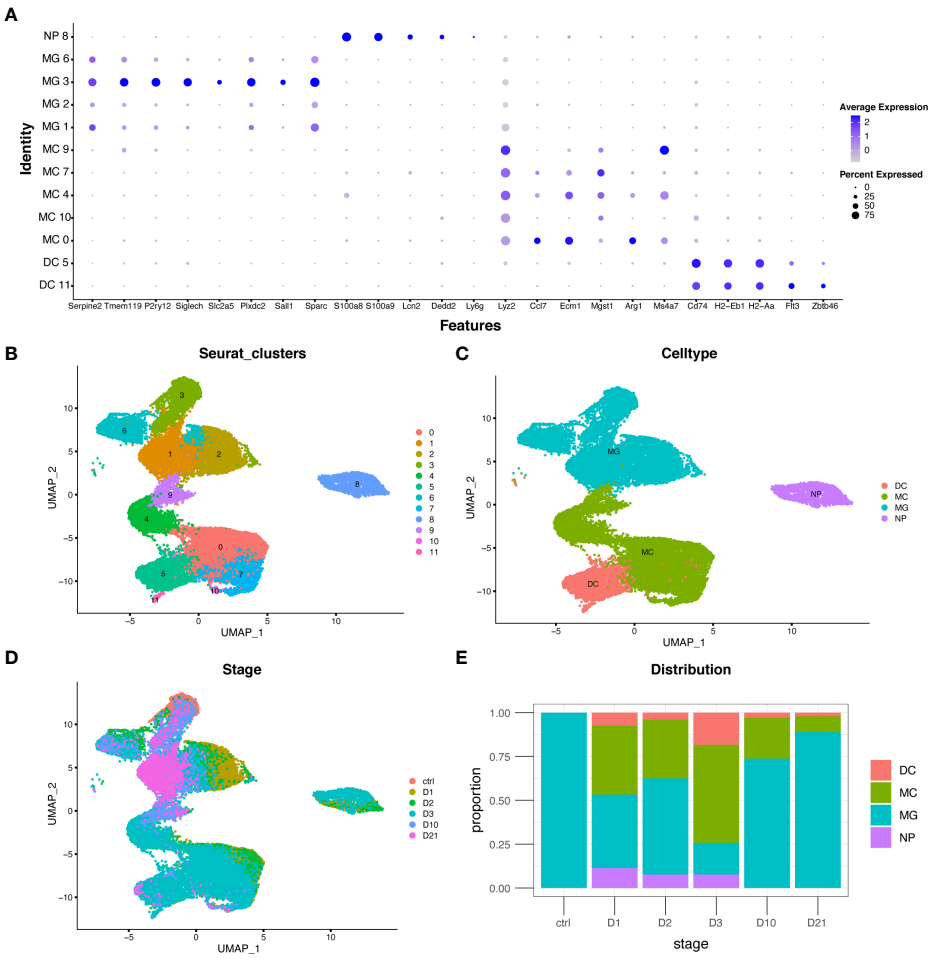

FIGURE 1
Distribution and proportion of myeloid cell subtypes following spinal cord injury (SCI). **(A)** Dot plot of marker genes in each population. **(B–D)** Uniform manifold approximation and projection (UMAP) plot of each population, four myeloid cell subtypes, and cells from different time points. **(E)** Bar graph showing the proportion of myeloid cell subtypes from different time points. DC, dendritic cells; MC, macrophages; MG, microglia; NP, neutrophils.

has been identified as a major regulator of dysfunctional microglia and as a therapeutic target for neurodegenerative diseases (26).

A total of 1059 genes were differentially expressed at 10 and 21 dpi in microglia compared to the acute phase (Figures 4A, D). Of the top 10 upregulated genes (Figure 4B), the *FABP5* is thought to be neurotoxic as it promotes immune cell infiltration and the release of inflammatory factors (10). Additionally, LGALS3/Galectin-3 was recently identified as a critical factor in microglia-mediated neuroinflammation (27). Most of the top 10 downregulated genes were homeostatic microglial genes, including *JUN, P2RY12, SIGLECH, TMEM119*, and *TGFBR1* (Figure 4C). GO analysis showed that overlapping genes such as *APOE, APOC1, CCL3, LPL, IGF1, MIF*, and *FABP3* were involved in the regulation of phagocytosis, positive regulation of lipid localization, response to oxidative stress, production of tumor necrosis factor, regulation of inflammatory response, and metabolism of glycerolipids (Figure 4E).

Temporal clustering analysis

This study focused on the analysis of gene expression in macrophages and microglia during the subacute and chronic phases of SCI. Of the nine time-dependent expression patterns observed in macrophages, cluster five contained 1493 genes with upregulated expression at 10 dpi and 21 dpi (Figure 5A). GSVA showed that the enrichment scores of lipid metabolism-related

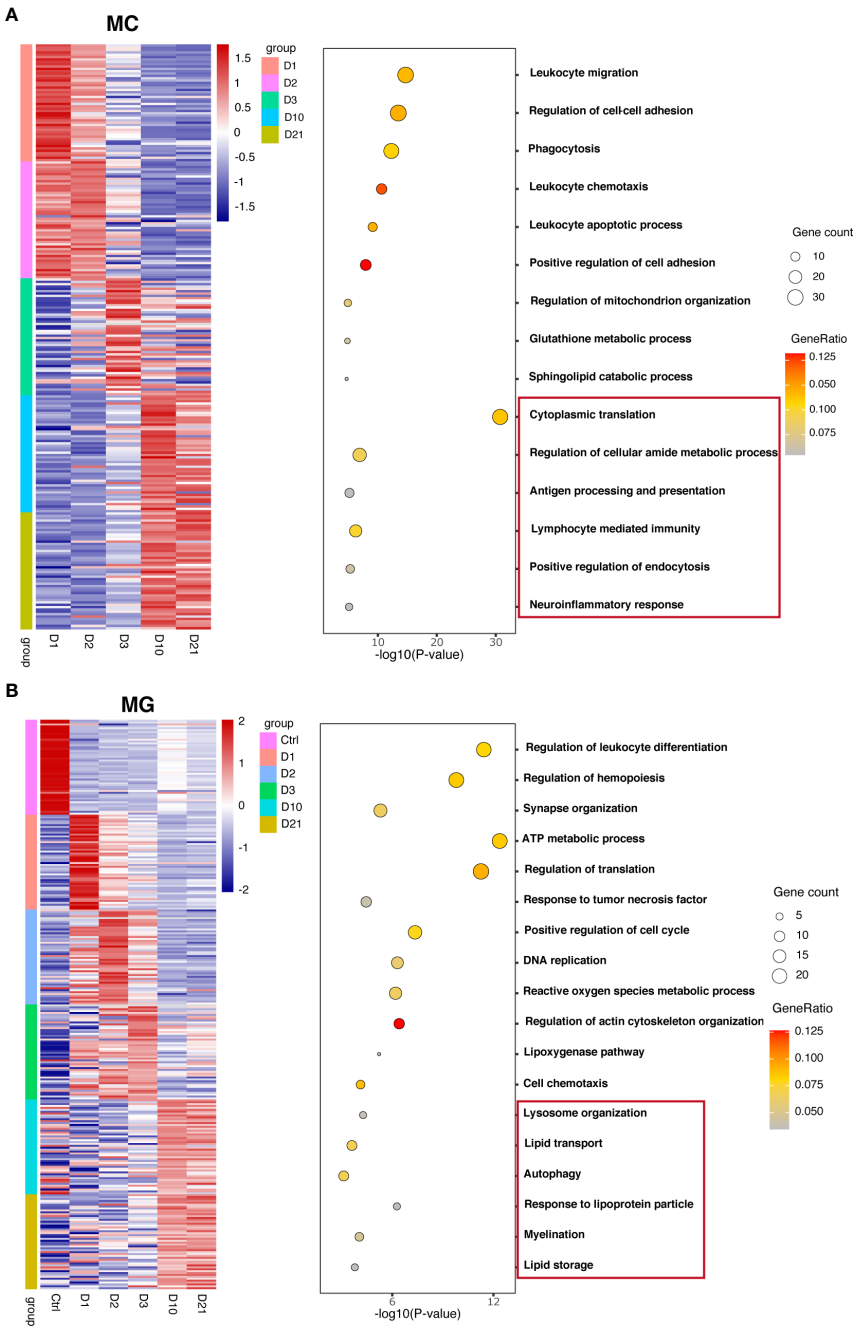

FIGURE 2
Unique marker genes and related gene ontology (GO) of biological processes in macrophages **(A)** and microglia **(B)** collected at different time points.

pathways were higher at 10 dpi and 21 dpi, including arachidonic acid, ether lipid, and glycerophospholipid metabolism (Figure 5B). A total of 146 genes in cluster five overlapped with 642 genes that were differentially expressed between 10 dpi and 21 dpi (Figure 5C). PPI network analysis in macrophages identified APOE as a hub gene intersecting with DEGs such as *C1QA, AIF1, TREM2*, and *TMEM119* (Figure 5E). Six time-dependent expression patterns were identified in microglia (Figure 5G). Cluster three contained 1552 genes whose expression was upregulated at 10 and 21 dpi. GSVA showed that the pathways associated with neurodegenerative diseases, lysosomes, peroxisome proliferator-activated receptor

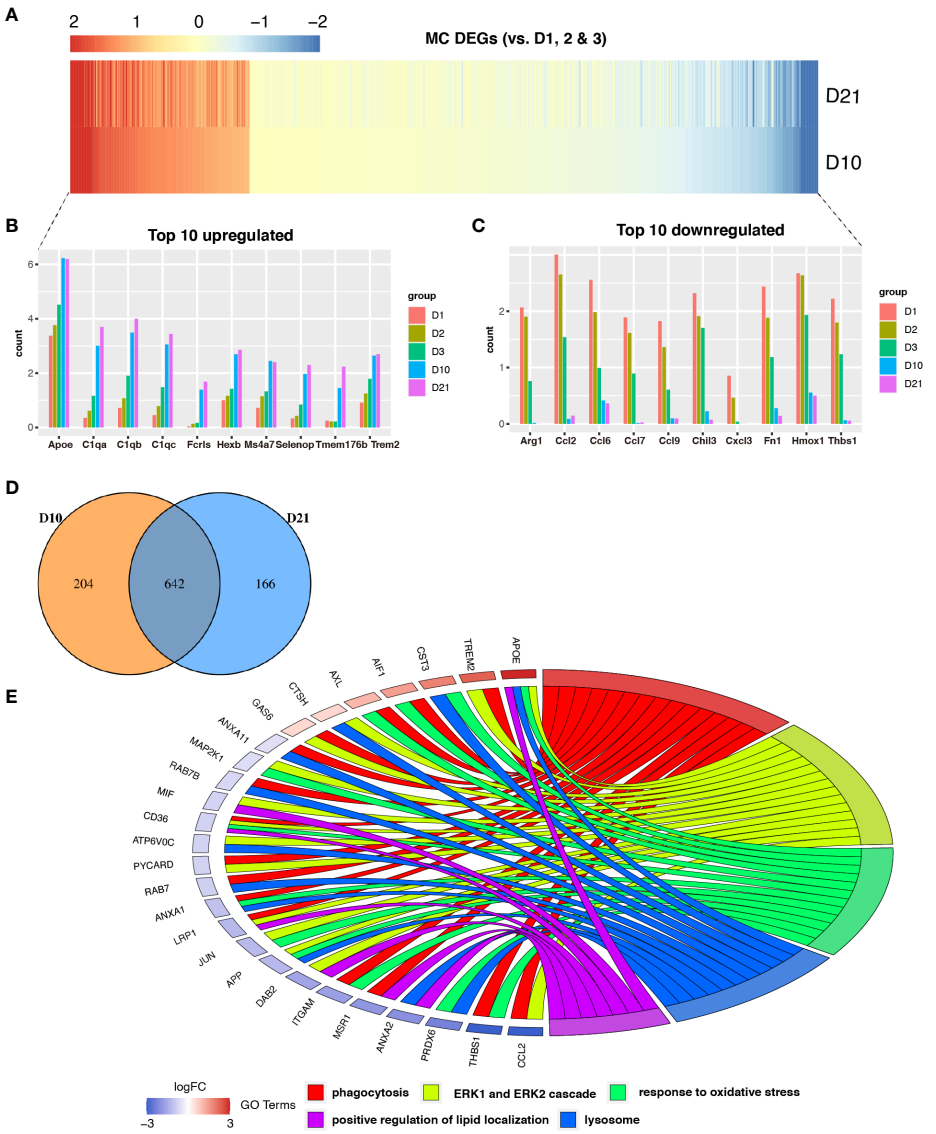

FIGURE 3
Analysis of differential gene expression in macrophages following spinal cord injury (SCI). **(A)** Heatmap showing differentially expressed genes (DEGs) between macrophages sampled 10 days post injury (dpi) and 21 dpi compared with macrophages sampled in the first three days. **(B, C)** Bar graph showing the top 10 upregulated and downregulated genes in macrophages sampled 10 dpi and 21 dpi. **(D)** Venn diagram showing an overlap between genes that were differentially expressed between macrophages sampled 10 dpi and those sampled 21 dpi. **(E)** Chord plot showing the gene ontology (GO) of biological processes associated with overlapping DEGs in macrophages sampled 10 dpi and 21 dpi.

(PPAR) signaling pathway, and autophagy had the highest scores at 21 dpi (Figure 5H). A total of 123 genes in cluster three overlapped with the 1059 genes that were differentially expressed at 10 dpi and 21 dpi in microglia (Figure 5D). PPI network analysis in microglia also identified APOE as a hub gene intersecting with DEGs such as *ABCA1, ABCG1, APOC1, CD68*, and *CTSB* (Figure 5F). These results demonstrate the essential role of APOE in macrophages and microglia during the subacute and chronic phases following SCI.

Accumulation of lipid droplets in macrophages and microglia following SCI

To explore the reason for the transcriptional changes in macrophages and microglia after SCI, we analyzed the changes in the ultrastructure of a mouse model of spinal cord contusion injury using TEM. Healthy microglia had a small body, thin cytoplasm, and bean-shaped nuclei containing heterochromatin

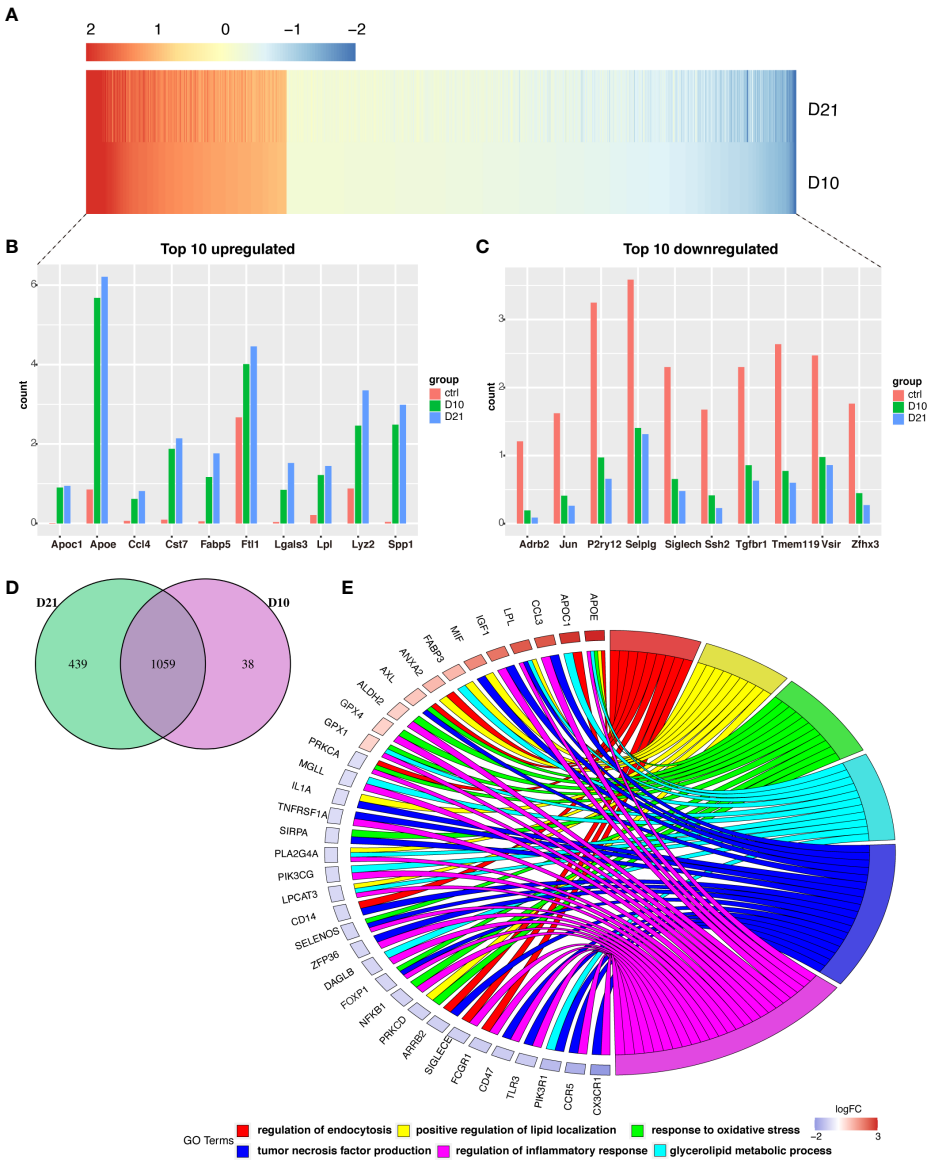

FIGURE 4
Analysis of differential gene expression in microglia following spinal cord injury (SCI). **(A)** Heatmap showing differentially expressed genes (DEGs) in microglia sampled 10- and 21-days post injury (dpi) compared with those sampled from the sham group. **(B, C)** Bar graph showing the top 10 upregulated and downregulated genes in microglia sampled 10 dpi and 21 dpi. **(D)** Venn diagram showing overlap between DEGs in microglia sampled 10 dpi and 21 dpi. **(E)** Chord plot showing the gene ontology (GO) of biological processes associated with overlapping DEGs in microglia sampled 10 dpi and 21 dpi.

(Figure 6E) (28). Seven days after SCI, both macrophages and microglia contained myelin debris, had increased levels of lipid droplets and lysosomes, and the "wrapping lysosomes" engulfing the lipid droplets were similar to macrophage foam cells (Figures 6A–C) (29). Demyelination is a pathological hallmark of preclinical models of SCI (2). Six weeks after SCI, prominent Wallerian degeneration and chronic demyelination were observed in the lesions (Figure 6D). Similar to the lipid droplet-accumulating microglia in the aging brain (30), the microglia contained lipofuscin granules and lipid droplets (Figures 6F, G). It is difficult to distinguish macrophages from microglia, since they share several markers, such as F4/80 and iba-1. Immunofluorescence staining showed upregulated APOE expression with abundant lipid droplets in F4/80+ microglia/macrophages (Figures 6H–N).

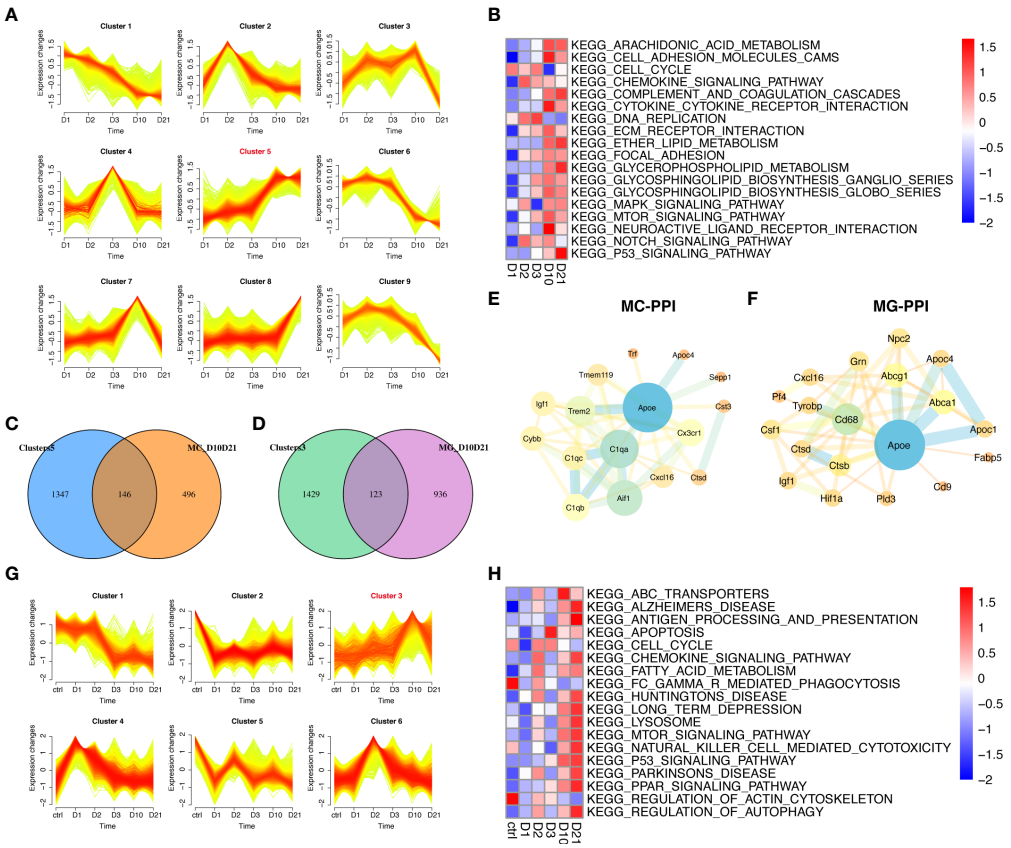

FIGURE 5
Temporal clustering analysis of macrophages and microglia following spinal cord injury (SCI). **(A)** Temporal clustering analysis of macrophages following SCI. **(B)** Heatmap showing gene set variation analysis (GSVA) of genes in cluster five and c2 KEGG gene sets. **(C)** Venn diagram showing an overlap between genes in cluster five and genes that were differentially expressed between macrophages sampled 10 days post injury (dpi) and those sampled 21 dpi. **(D)** Venn diagram showing an overlap between genes in cluster three and genes that were differentially expressed between microglia sampled 10 dpi and those sampled 21 dpi. **(E)** Protein-protein interaction (PPI) network showing an overlap of genes in cluster five and genes that were differentially expressed in macrophages sampled 10 dpi and those sampled 21 dpi. **(F)** PPI network showing the overlap between genes in cluster three and genes that were differentially expressed between microglia sampled 10 dpi and those sampled 21 dpi. **(G)** Temporal clustering analysis of microglia following SCI. **(H)** Heatmap showing GSVA of genes in cluster three and the c2 KEGG gene sets.

Deletion of *APOE* aggravates neuroinflammation and reduces recovery

To investigate the function of APOE, we performed spinal cord contusion injury in APOE$^{-/-}$ mice. Typical changes in the biomechanical parameters are shown in Figure 7A. There were no significant between-group differences in contusion displacement, speed, or peak force (Figures 7B–D). The cylinder rearing test on the ipsilateral forelimb revealed worse motor functional recovery in APOE$^{-/-}$ mice, four and six weeks post-SCI (Figure 7E). Both groups showed similar ipsilateral grooming scores and changes in body weight post-SCI (Figures 7F, G). The reduction in the rate of utilization of the ipsilateral forelimb in APOE$^{-/-}$ mice indicated the benefits of APOE in fostering recovery following SCI. Neuroinfiammation is a hallmark of SCI (31). Six weeks after SCI, however, APOE$^{-/-}$ mice displayed aggravated neuroinflammation in the lesion rim (Figures 7H–K). Furthermore, APOE$^{-/-}$ mice showed increased F4/80+ microglia/macrophage infiltration and white matter loss (Figures 7L–O). Although non-significant, these results were worthy of further investigation in subsequent studies.

Myelin uptake in astrocytes and lysosome accumulation in macrophages and microglia in APOE$^{-/-}$ mice

Astrocytes phagocytose myelin debris and recruit immune cells during acute demyelination of brain tissue (32). We observed MBP in GFAP-positive cells in lesion rims of

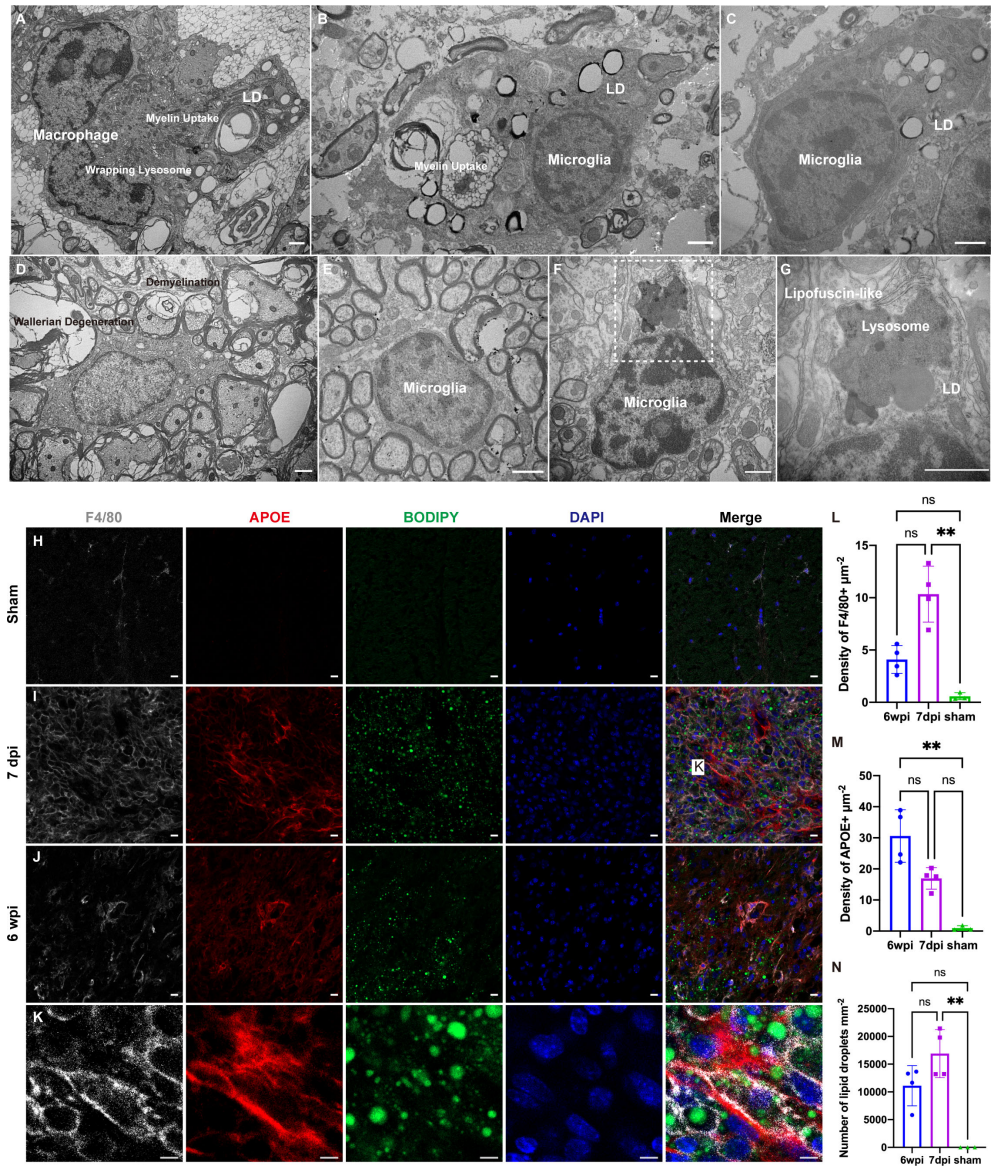

FIGURE 6
Transmission electron microscopy (TEM) analysis and immunofluorescence staining of a mouse model of cervical spinal cord hemi-contusion. **(A)** TEM image of macrophages sampled seven days post injury (dpi) showing myelin uptake, lipid droplet accumulation, and "wrapping lysosomes". **(B, C)** Representative images of microglia sampled seven dpi showing myelin uptake, lipid droplet accumulation, and "wrapping lysosomes". **(D)** TEM image of lesions sampled six weeks post injury (wpi) showing Wallerian degeneration and demyelination. **(E)** Representative image of healthy microglia in the sham group. **(F, G)** TEM images of microglia sampled six wpi showing lipofuscin granules and lipid droplets. The boxed area is shown in **(G)** at higher magnification. **(H–J)** Immunofluorescence staining of spinal cord samples showing F4/80+ microglia/macrophages (white), APOE (red), BODIPY (green) and DAPI (blue). Box is the approximate area where **(K)** was imaged. **(K)** High-magnification representative images of the APOE+ macrophages/microglia containing BODIPY+ lipid droplets. **(L)** Quantitation of F4/80+ densities showed that F4/80+ densities were significantly increased at 7dpi. **(M)** Quantitation of APOE+ densities showed that APOE+ densities were significantly increased at 6 wpi. **(N)** Quantitation of lipid droplets showed that the number of lipid droplets were significantly increased at 7dpi. LD, lipid droplets. $^{ns}P > 0.05$, $^{**}P < 0.05$. Scale bar **(A–G)** = 1 µm. Scale bar **(H–J)** = 10 µm. Scale bar **(K)** = 5 µm.

APOE$^{-/-}$ mice (Figures 8A, B). TEM confirmed that the hypertrophic astrocytes contained degraded myelin debris (Figures 8C, D) and lysosomes accumulated in astrocytes (Figure 8E). Furthermore, APOE$^{-/-}$ mice had increased numbers of lipid droplets and dense lysosomal material in macrophages and microglia (Figures 8F, G), suggesting that more lysosomes are activated in APOE$^{-/-}$ mice following SCI. Interestingly, the TEM images of lesions sampled 16 weeks post injury showed the formation of needle-like cholesterol crystals (Figures 8H, I), which was similarly observed in the aged central

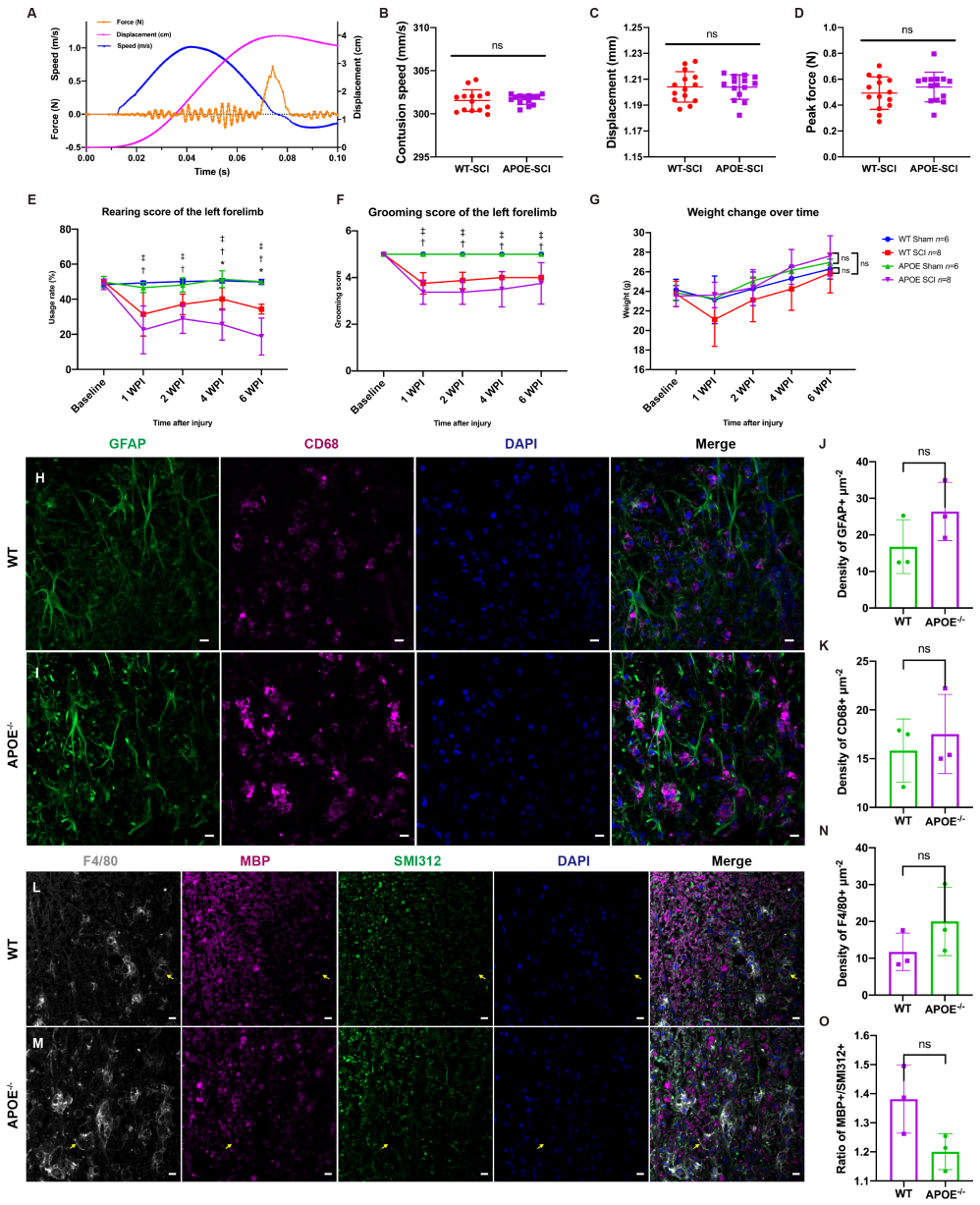

FIGURE 7
Apolipoprotein E (APOE) knockout aggravated dysfunction, increased neuroinflammation, and exacerbated white matter loss following spinal cord injury (SCI). **(A)** Typical changes in biomechanical parameters during contusion. Orange, contusion force; magenta, contusion displacement; blue, contusion speed. **(B–D)** There were no significant between-group differences in contusion displacement, speed or peak force. **(E)** Cylinder rearing test showed worse motor functional recovery in APOE$^{-/-}$ mice following SCI. **(F, G)** There were no significant differences in grooming scores and body weight between the two injured groups. **(H–K)** APOE$^{-/-}$ mice displayed aggravated neuroinflammation in the lesion rim. Green, GFAP+ astrocytes; purple, CD68+ macrophages/microglia; blue, DAPI+ cell nuclei. **(L-O)** APOE$^{-/-}$ mice showed increased white matter loss and microglia/macrophage infiltration following SCI. The myelin debris was engulfed by microglia/macrophages (yellow arrows). White, F4/80; purple, myelin basic protein (MBP); green, SMI312+ axons; blue, DAPI. $^{ns}P > 0.05$, $^{*}P < 0.05$ between the APOE-SCI and wild-type (WT)-SCI groups; $^{\dagger}P < 0.05$ between the APOE-SCI and APOE-Sham groups; $^{\ddagger}P < 0.05$ between the WT-SCI and WT-Sham groups. Scale bar **(H, I, L, M)** = 10 μm..

nervous system (33). This confirmed chronic accumulation of cholesterol in microglia in APOE$^{-/-}$ mice following SCI.

Discussion

We identified APOE as a hub gene in both macrophages and microglia during the subacute and chronic phases of SCI. Histopathological analysis revealed the accumulation of lipid droplets and lysosomes in both macrophages and microglia. Furthermore, APOE$^{-/-}$ mice showed worse functional recovery associated with increased neuroinflammation and white matter loss. APOE is the major cholesterol carrier involved in cellular lipid efflux in the central nervous system (33). Our study indicates that APOE and the associated cholesterol efflux might be therapeutically targeted to promote recovery following SCI.

Myelin and cellular debris are mainly cleared by monocyte –derived macrophages and resident microglia, creating a pro-regenerative environment (5). Macrophages are attracted to the injury site at around 3 days after SCI and reach a peak at 7 days after SCI (16). Macrophages play an important role in debris clearance, but excessive myelin debris uptake leads to the formation of "foamy macrophages" and subsequent death (6, 15). The primary injury causes glial necrosis within the lesion epicenter. Microglia are rapidly activated and proliferate after SCI (34). The efficiency of myelin phagocytosis and proliferation rate of microglia is much higher than those of macrophages (5). Therefore, microglia were the major myeloid cell type in the subacute and chronic phases of SCI. We found that microglial marker genes expressed 10 and 21 dpi were associated with lipid transport, autophagy, and lipid storage. Furthermore, autophagy regulation and the PPAR signaling pathway had high enrichment scores in microglia sampled at 21 dpi. Lipid droplets are organelles that store lipids and play a central role in cellular metabolism and lipid homeostasis (35). Lipid droplets have variable protein and lipid composition and their size, and the number of lipid droplets, are regulated *via* autophagy (36). PPAR-γ can upregulate the expression of *ABCA1* and *ABCG1* to boost lipid efflux (5). Stimulating the autophagy of lipid droplets and promoting lipid efflux in macrophage foam cells is an attractive therapeutic strategy for atherosclerosis (29). Thus, promoting macrophage autophagy and lipid efflux may reduce secondary damage in SCI.

Upregulated APOE expression in microglia is common during development, damage, and disease (37). In this study, we found that APOE is involved in multiple pathways and interacts with multiple genes in macrophages and microglia. The different effects of APOE depend on its cellular origin, binding lipid molecules, and microenvironment (38). APOE$^{-/-}$ mice showed impaired remyelination and increased phagocyte infiltration in a demyelination model (33). We found that APOE$^{-/-}$ mice showed worse functional recovery following SCI, consistent with previous reports (39, 40). The uptake of myelin debris by macrophages and microglia was confirmed using TEM. Myelin has a high lipid composition, particularly cholesterol (41). Our results suggest that cholesterol overload in macrophages and microglia may induce a maladaptive immune response that aggravates secondary damage. Further studies are required to explore the stimulation of reverse cholesterol transport.

Traumatic SCI leads to progressive cord atrophy and neurodegeneration (42). We observed clear demyelination and Wallerian degeneration on the ipsilateral side of the injury epicenter six weeks post injury (wpi) using TEM. Furthermore, we identified lipofuscin granules and lipid droplets in the microglia 6 wpi. Lipid droplet-rich microglia have recently been implicated in the release of large quantities of reactive oxygen species and proinflammatory cytokines in the aging brain, and nearly half of the constituents of lipid droplets are glycerolipids, in line with the transcriptomics data (10), although few of these are cholesteryl esters (30). Likewise, lipid droplets accumulate in microglia in patients with Alzheimer's and Parkinson's diseases (43, 44). Interestingly, the number and morphology of lipid droplets differed in microglia sampled seven dpi and six wpi. The effects of lipid droplets on microglia may depend on their composition, which is affect by different environmental conditions (30). It will be worth analyzing the content of lipid droplets in macrophages and microglia collected at different time points following SCI.

This study has several limitations. First, the numbers of samples and cells collected 21 dpi were small and no macrophages were present in samples used in the sham operation as expected, potentially introducing bias. Second, the cause of transcriptional changes and the effects of APOE were validated and explored using wild-type and APOE$^{-/-}$ spinal cord contusion injury mouse models. Although we did not use exogenous APOE treatment or other methods to increase cholesterol efflux in the present study, previous *in vivo* study using COG112, an APOE mimetic peptide combined a protein transduction domain antennapedia to improve blood-brain barrier and cell membrane penetration, suggest neuroprotective roles of endogenous APOE in reducing neuroinflammation and white matter loss after SCI (40). Third, the APOE$^{-/-}$ knockout is not myeloid-specific, and the molecular mechanisms underlying the phenotypes of APOE$^{-/-}$ mice have not been fully elucidated. Since APOE has multiple functions in different environmental conditions (38), high-throughput assays can be used to elucidate differences in molecular mechanisms between wild-type and APOE$^{-/-}$ mice following SCI.

Taken together, our study demonstrated that APOE is a hub gene in both macrophages and microglia in subacute and chronic phases of SCI. APOE knockout aggravates neurological dysfunction, increases neuroinflammation, and exacerbates white matter loss. Targeting APOE and related cholesterol efflux may be a promising strategy for reducing neuroinflammation and promoting recovery following SCI.

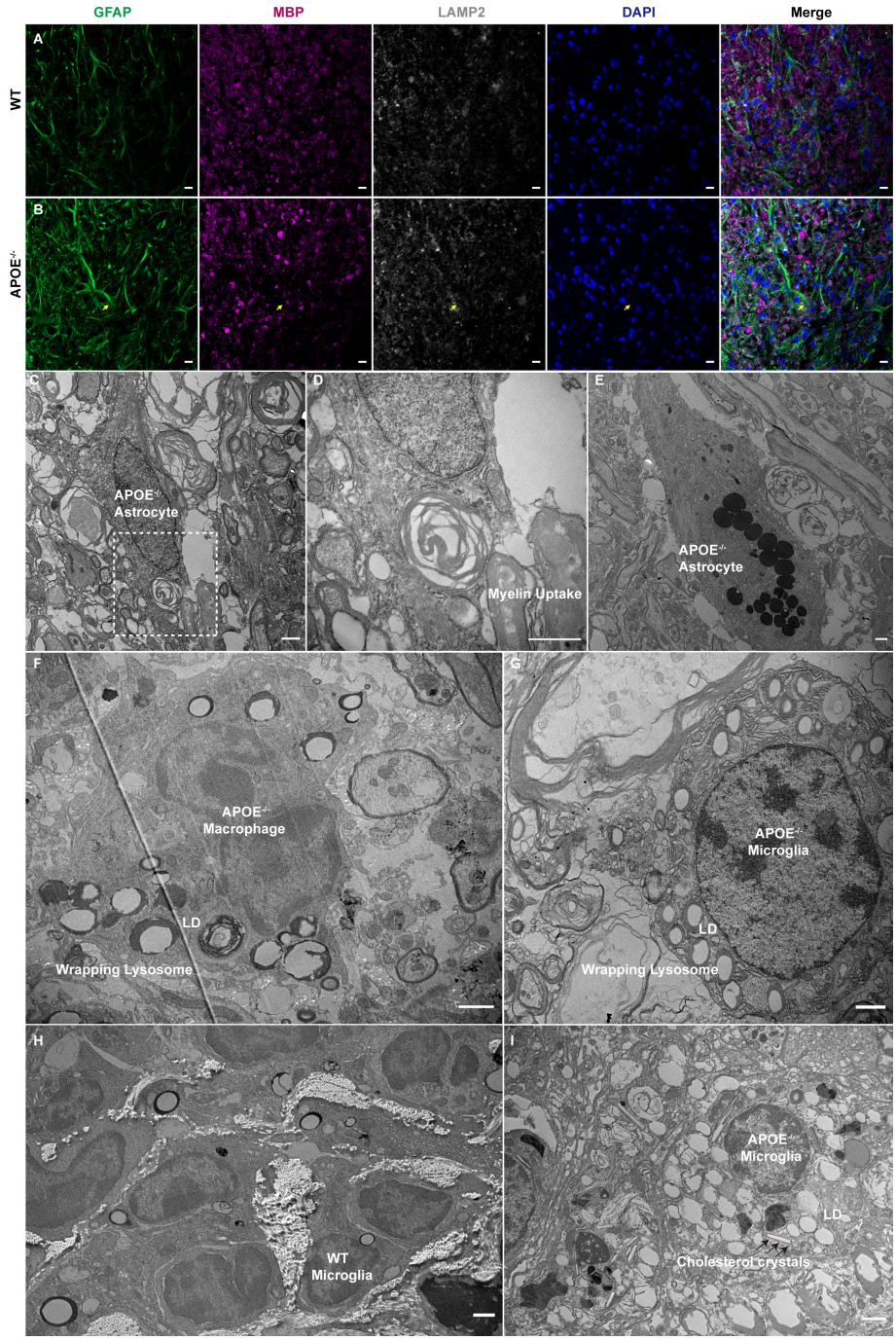

FIGURE 8
Myelin uptake in astrocytes and lipid droplets and lysosome accumulation in macrophages and microglia in APOE$^{-/-}$ mice following spinal cord injury (SCI). **(A, B)** Myelin debris was engulfed by astrocytes in the lesion rim of APOE$^{-/-}$ mice six weeks post injury (wpi) (yellow arrows). Green, GFAP; purple, MBP; white, LAMP2; blue, DAPI. **(C, D)** Transmission electron microscopy (TEM) images of astrocytes in APOE$^{-/-}$ mice six wpi confirmed that the hypertrophic astrocytes contained degraded myelin debris. The boxed area is shown in **(D)** at higher magnification. **(E)** TEM image of astrocytes in APOE$^{-/-}$ mice six wpi shows markedly elevated lysosomes. **(F, G)** TEM image of macrophages and microglia in APOE$^{-/-}$ mice taken seven days post injury (dpi) shows increased number of lipid droplets and dense lysosome material. **(H)** Representative image of microglia in wild-type (WT) mice 16 wpi. **(I)** TEM image of microglia in APOE $^{-/-}$ mice 16 wpi shows markedly elevated lipid droplets and the formation of needle-like cholesterol crystals (black arrows). LD, lipid droplets. Scale bar **(A, B)** = 10 μm. Scale bar **(C–I)** = 1 μm.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by Laboratory Animal Care and Use Committee of Nanfang Hospital, Southern Medical University.

Author contributions

X-QY, J-YC, Q-AZ, and J-TC conceived and designed the research. X-QY, Z-HY, Z-CH, Y-QZ, Z-PH, K-WT, and J-HL performed the experiments. RH collected the transcriptomics dataset under the supervision of SP and performed the pre-processing analysis. Y-ML and Z-TZ helped with the transmission electron microscopy analyses. X-QY, J-YC, Z-HY, and K-WT analyzed the data. X-QY, J-YC, and J-TC wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Pedro A. Reche,
Complutense University of Madrid, Spain

REVIEWED BY
Youhei Egami,
Kagawa University, Japan
Roland Nau,
University Medical Center Göttingen, Germany
Akira Monji,
Saga University, Japan

*CORRESPONDENCE
Michael K. E. Schäfer
michael.schaefer@unimedizin-mainz.de

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Microglia subtypes show substrate- and time-dependent phagocytosis preferences and phenotype plasticity

Shuailong Li[1], Isa Wernersbach[1], Gregory S. Harms[2,3] and Michael K. E. Schäfer[1,4,5]*

[1]Department of Anesthesiology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany, [2]Cell Biology Unit, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany, [3]Departments of Biology and Physics, Wilkes University, Wilkes Barre, PA, United States, [4]Focus Program Translational Neurosciences (FTN), Johannes Gutenberg-University Mainz, Mainz, Germany, [5]Research Center for Immunotherapy (FZI), University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

Microglia are phagocytosis-competent CNS cells comprising a spectrum of subtypes with beneficial and/or detrimental functions in acute and chronic neurodegenerative disorders. The heterogeneity of microglia suggests differences in phagocytic activity and phenotype plasticity between microglia subtypes. To study these issues, primary murine glial cultures were cultivated in the presence of serum, different growth factors and cytokines to obtain M0-like, M1-like, and M2-like microglia as confirmed by morphology, M1/M2 gene marker expression, and nitric oxide assay. Single-cell analysis after 3 hours of phagocytosis of *E.coli* particles or IgG-opsonized beads showed equal internalization by M0-like microglia, whereas M1-like microglia preferably internalized *E.coli* particles and M2-like microglia preferably internalized IgG beads, suggesting subtype-specific preferences for different phagocytosis substrates. Time-lapse live-cells imaging over 16 hours revealed further differences between microglia subtypes in phagocytosis preference and internalization dynamics. M0- and, more efficiently, M1-like microglia continuously internalized *E.coli* particles for 16 hours, whereas M2-like microglia discontinued internalization after approximately 8 hours. IgG beads were continuously internalized by M0- and M1-like microglia but strikingly less by M2-like microglia. M2-like microglia initially showed continuous internalization similar to M0-like microglia but again discontinuation of internalization after 8 hours suggesting that the time of substrate exposure differently affect microglia subtypes. After prolonged exposure to *E.coli* particles or IgG beads for 5 days all microglia subtypes showed increased internalization of *E.coli* particles compared to IgG beads, increased nitric oxide release and up-regulation of M1 gene markers, irrespectively of the phagocytosis substrate, suggesting phenotype plasticity. In summary,

microglia subtypes show substrate- and time-dependent phagocytosis preferences and phenotype plasticity. The results suggest that prolonged phagocytosis substrate exposure enhances M1-like profiles and M2-M1 repolarization of microglia. Similar processes may also take place in conditions of acute and chronic brain insults when microglia encounter different types of phagocytic substrates.

KEYWORDS

brain, inflammation, immune response, microglia, phagocytosis, polarization, plasticity, live imaging

Introduction

Microglia are central nervous system (CNS) resident cells that originate from fetal macrophages and play an essential role in innate immune responses and CNS homeostasis, both in the healthy and diseased brain (1, 2). Furthermore, it has become clear that microglia can be efficiently targeted by genetic and pharmacological tools (3). As a result, they are considered suitable targets for modulating CNS diseases (4).

Traditionally, two opposing phenotypes of activated microglia, M1-and M2-like, have been described as pro-and anti-inflammatory subtypes analogously to macrophages (5). However, pathological conditions induce different phenotypes of microglia that are unique and distinct from other macrophage cell types, more diverse than an M1/M2 classification, and more heterogeneous than previously anticipated (2, 6–9). This is reflected by the various roles of microglia in CNS homeostasis, comprising beneficial and detrimental actions in CNS diseases after a bacterial infection or acute injuries such as traumatic brain injury and stroke (5, 10–12).

In vitro stimulation of microglia with individual growth factors and cytokines can induce M1-like and M2-like microglia with distinct phenotypes and functional properties (8). This also applies to phagocytosis, the main established functional feature of microglia in development, homeostasis, and pathology (13). For example, M2-like microglia induced by anti-inflammatory cytokines IL-4 and IL-10 show overall higher activity in phagocytosis of microbeads than M1-like microglia generated by the pro-inflammatory cytokine IFN-γ (14). Phagocytosis of zymosan, a yeast cell wall component, is increased in IL-4-induced M2-like microglia compared to bacterial endotoxin lipopolysaccharide LPS-induced M1-like microglia (15). M2-like microglia induced by IL-4, IL-13, and IL-10 were also more efficient than LPS/IFN-γ-induced M1-like microglia in the phagocytosis of myelin (16). Likewise, M2-like microglia induced by stem cell factor (SCF) show increased phagocytosis of FITC-IgG opsonized beads compared to GM-CSF-induced M1-like microglia (17). However, other studies reported that GM-CSF-induced M1-like microglia had significantly higher phagocytic activities for FITC-IgG beads than IL-4-induced M2-like microglia (8). Controversial results were also obtained for the phagocytosis of amyloid-β (Aβ). While microglia pretreated with the M1-like-inducing bacterial endotoxin lipopolysaccharide (LPS) display enhanced phagocytosis (18), pro-inflammatory cytokines known to promote an M1-like phenotype inhibited Aβ phagocytosis (19). More recently, treatment of primary mouse microglia either with LPS or synthetic double-stranded RNA poly(I: C) was shown to differently affect phagocytosis of synaptosomes, *E.coli* particles, or IgG beads (20). Microglia also phagocytose stressed or apoptotic neurons which contributes to brain pathology after ischemic injury (21).The receptor tyrosine kinases *Axl* and *Mertk* control the phagocytic specialization of microglia, for example for apoptotic cells generated during neurogenesis (22). Comparative studies on phagocytosis of neurons by different microglia subtypes are scarce. It was shown that LPS- or Aβ-mediated pro-inflammatory stimulation of BV2 microglia induced neuronal loss and death by phagocytosis of neurons (23), while fractalkine (CX3CL1), which promotes M2 polarization (24), increased the phagocytosis of apoptotic neuron-like SY5Y cells *via* Milk Fat Globule Factor-E8 MFG-E8 (25). Overall, these and other studies using distinct microglia subtypes show differences in phagocytic activity *in vitro* but few studies directly compared different microglia subtypes.

In addition, only a few studies have also shown that phagocytosis of specific substrates is associated with changes in microglia phenotypes. For instance, pathogenic oligomeric Aβ shows a more potent induction of M1-like microglia than the fibrillar form (26). Somewhat controversial data were provided for microglia phenotype plasticity after myelin phagocytosis. Myelin enhanced the M1-like profile and dampened the M2-like profile of primary rat microglia (27) but also induced a switch of M1-like microglia to an M2-like state (28). The latter finding may relate to the up-regulation of the scavenger receptor CD36 in macrophages/microglia after myelin phagocytosis (29).

Another study using *E.coli* particles, cell debris or Aβ as phagocytosis substrates showed that *E.coli* particles but not the other substrates encountered by microglia triggered secretion of the pathophysiologically relevant matrix metalloproteinase MMP-9 (1). Furthermore, interaction with apoptotic neurons shifts microglia toward distinct remodeling states (30), which share features with disease-associated microglia (31). Finally, phagocytosis of astrocyte- or neuron-derived exosomes may influence microglia polarization due to the transcellular transfer of miRNAs (32, 33)

Taken together, microglia play various roles in innate immune responses, CNS homeostasis and disease, which likely reflect both their heterogeneity and plasticity. However, while their function extends well beyond removing pathogens, dead cells and cell debris, there is still little knowledge about the relationships between microglia subtypes, different types of phagocytic substrates and phenotype plasticity. To address this, we performed *in vitro* experiments with distinct microglia subtypes subjected to phagocytosis assays for different time periods using two types of phagocytic substrates, *E.coli* particles and IgG-opsonized beads, followed by immunocytochemistry, gene expression analyses, nitric oxide assays, and time-lapse live-cell imaging.

Methods and materials

Approval of animal experiments

Newborn mice to obtain primary glia were handled in accordance with the institutional guidelines of the Johannes Gutenberg University Mainz, and Rhineland-Palatine, Germany.

Primary mixed glial culture and differentiation of microglia subtypes

Mixed glial cultures were prepared from cerebral cortices of 1-5 days-old C57BL/6 mice sacrificed by decapitation. Brains were extracted, the meninges were carefully removed, and the cerebral cortex was dissected under a stereomicroscope. Cells were dissociated using the Neural Tissue Dissociation Kit-P according to the manufacturer`s protocol (#130-092-628; Miltenyi Biotec). $3x10^5$ cells/ml were seeded into T25 cell culture flasks in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad) containing 10% fetal calf serum (FCS, Life Technologies, Carlsbad), 1% penicillin/streptomycin (P/S, 100 U/ml, Life Technologies, USA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (Heraeus® HERAcell® CO_2 Incubators, Thermo Fisher Scientific, DE). The cultures were maintained for 14 days *in vitro* (div) and the medium was changed every 3 days. Next, cells were detached using Trypsin/EDTA solution (Sigma, Steinheim) and dissociated in medium (DMEM/10% FCS/1% P/S) and 6-$7x10^4$ cells per well were seeded in 24-well plates onto Poly-D-Lysine-coated (0.1% PDL Sigma, Steinheim) glass coverslips (13 mm, SCHOTT, Mainz). For the differentiation of microglia subtypes, cells were incubated in medium (DMEM/10% FCS/1% P/S) supplemented with granulocyte colony-stimulating factor and interferon-gamma (GM-CSF: 20 ng/ml; IFNy: 40 ng/ml, PeproTech GmbH, Hamburg), or macrophage colony-stimulating factor and interleukin-4 (M-CSF, IL-4; 20 ng/ml each, PeproTech GmbH, Hamburg) for 7 div to obtain M1-, or M2-like microglia, respectively. Non-supplemented medium was used to obtain M0-like microglia. Medium was replaced by fresh (non-) supplemented medium at 3 div and 7 div. Different treatment conditions of each experiment were run in parallel on the same 24-well plates.

Immunocytochemistry and morphological assessment

For immunocytochemistry and morphological assessment of microglia subtypes, cultures were fixed with 4% paraformaldehyde (PFA) for 10 min, blocked (5% normal goat serum, 0.5% BSA, 0.1% Triton X-100 in PBS) for 1 h at room temperature (RT), and incubated with primary antibodies specific to Iba1, MHC-II, and MRC1 (Supplementary Table 1) overnight at 4°C. The next day, cells were washed with PBS, incubated with fluorophore-conjugated secondary antibodies (Supplementary Table 1) for 1 h at RT, washed, and mounted. Images were taken using a confocal laser scanning microscope (LSM5 Exciter; Carl Zeiss DE) with equal acquisition parameters for different microglia subtypes from five independent cell culture preparations (n=5, biological replicates) and five regions of interest (ROIs, n=5) from each coverslip. Morphological parameters (cell size and circularity) of single cells (20-30 cells per ROI) were analyzed using ImageJ (NIH Image, RRID: SCR_003070) with appropriate threshold settings based on anti-Iba1 immunostaining in a blinded and unbiased fashion and data were expressed as mean values from biological replicates (n=5).

Nitric oxide assay

The Griess assay was used for colorimetric detection of NO_2^- anions which is proportional to nitric oxide (NO) production (34). 200 µL of cell culture supernatants were mixed with 50 µl of 1% sulfanilic acid (Sigma Cat#S9251). Then, 50 µl of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich; Cat#222488) was added, and the absorbance at 540 nm was detected after 10 min using a microplate reader (Sunrise™, Tecan, Switzerland). The nitrite concentration in each sample was interpolated

from a standard curve generated from a series of NaNO2 samples (Sigma, Cat#237213) of known concentration.

Quantitative PCR

The cell culture medium was removed, the cells were rinsed once with PBS, and the RNeasy and QuantiScript Reverse Transcription Kits (Qiagen) were used to extract RNA and transcribe mRNA into cDNA according to the manufacturer's instructions. The cDNA was amplified and quantified by real-time detection of SYBR Green (Thermo Scientific) with oligonucleotide primers (Supplementary Table 2) purchased from Eurofins using the Light Cycler 480 (Roche). For absolute quantification, a target-specific standard curve was generated and the copy numbers of target genes were normalized to the copy numbers of the reference gene *Ppia* (Cyclophilin A) essentially as described (35, 36).

Phagocytosis assays

Phagocytosis assays were performed using the Red *E.coli* Phagocytosis Assay Kit (PromoKine, Cat#PK-CA577-K964) or the Phagocytosis Assay Kit (IgG FITC complex, Cayman Chemicals, Cat#500290). Phagocytosis substrates were added at dilutions of 1:50 for Red *E.coli* or 1:100 IgG-FITC beads for 3 h or 5 days, respectively, and processed for immunocytochemistry using anti-Iba1or qPCR as described above. To determine the number of microglia with phagocytosis activity, ImageJ was used to outline Iba1-immunolabelled cells followed by counting the number of cells containing Red *E.coli* particles or IgG-FITC beads above a constant threshold level and the percentage of microglia subtypes containing Red *E.coli* or IgG-FITC beads was calculated. To determine microglia phagocytosis capacity in single cells (25 cells per condition), the relative occupancy of Iba1 immunostaining by the fluorescent phagocytosis substrates was calculated. Images were taken in a blinded and unbiased fashion and data were expressed as mean values ± SEM from independent experiments (biological replicates, n=5).

For time-lapse live imaging of microglial cells, primary mixed glial cultures were detached with Trypsin/EDTA solution and $3x10^5$ cells per well were seeded onto 8-well slides (µ-slide, Ibidi GmbH, Germany). Cells were treated with growth factors and cytokines for 7 div as described above to obtain M0-, M1- and M2-like microglia subtypes. A Leica TSP8 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with an incubator module (20% O_2, 5% CO_2, and 75% N2, at 37°C) (Oko Labs, Italy) and a motorized position stage were used for time-lapse live imaging experiments. Since CD68 is not involved in binding bacterial/viral pathogens, innate, inflammatory or humoral immune responses (37), we performed live immunolabelling of microglia with rat anti-mouse CD68-BV421 (dilution: 1:125, clone FA-11, BD biosciences, RRID: AB_2744447). The antibody was applied 30 minutes before the random selection of 5 regions of interest (ROI) per condition, followed by the separate addition of the phagocytosis substrates (Rhodamine-*E.coli*: 1:50. IgG-FITC beads: 1:100). The multi-position confocal images were acquired at an interval of 12 mins over 16 hours using a 20x (0.75 NA) planapochromat objective with differential interference contrast imaging. The automated quantification was based on fluorescent particle tracking of the E. coli-rhodamine or IgG-FITC substrates in single cells labelled with BV421-anti-CD68 using Imaris software (version 9.3.1, BitPlane, Zurich, Switzerland). A total of 1-3 x 10^3 cells were acquired from 5 ROIs of each condition over the 16-hours live imaging period in each of two independent experiments.

Statistical analysis

Data analyses were performed with GraphPad Prism® (RRID: SCR_002798). Data outliers were identified and removed using ROUT's test followed by the Shapiro-Wilk test to determine data distribution as specified in the figure legends. Comparisons between two groups were performed dependent on data distribution by Student's t-test or Mann-Whitney-U test. Multiple comparisons were performed dependent on data distribution by one-way ANOVA (*post-hoc* correction Holm-Šídák) or Kruskal-Wallis test (*post-hoc* correction Dunnett), if F achieved the necessary level of statistical significance $p<0.05$. Data are expressed as mean ± SEM. Individual data points represent biological replicates or means from biological replicates as specified in figure legends, $p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$, $p^{****}<0.0001$.

Results

Microglia subtypes differ in morphology, expression of M1/M2 markers and metabolism

Neural cells were isolated from the cerebral cortex of 3-5 days-old newborn mice, cultivated for 14 days, dissociated and further cultivated in 24 well plates for 7 days in medium (DMEM/10% FCS/1% P/S) containing GM-CSF/IFNy or M-CSF/IL-4 to stimulate proliferation and polarization of microglia into M1-like or M2-like phenotypes, respectively. Heat-inactivated serum was present in all conditions to mimic pathophysiological conditions of BBB breakdown (38, 39) but to prevent complement-enhanced phagocytosis (40). Cultures without the addition of growth factors and cytokines yielded M0-like microglia. First, anti-Iba1 immunostaining to compare

microglia morphology between microglia revealed a smaller cell size of M1- than M2-like microglia and a higher circularity of M1-like microglia compared to M0- and M2-like microglia (Figures 1A–C). We next assessed the extent of differentiation towards the M1- or M2-like microglia subtype using triple-immunostainings with antibodies specific to the pan-microglia marker Iba1, the M1 marker MHC2, and the M2 marker MRC1 (Figure 1D). We found that Iba1$^+$/MHC2$^+$ cells showed the highest abundance in M1-like microglia (Figure 1E, 66.00% ± 3.797%, SEM), an intermediate abundance in M0-like microglia (Figure 1E, 34.12% ± 3.797%, SEM), and the lowest abundance in M2-like microglia (Figure 1E, 17.65% ± 3.797%, ± SEM). Conversely, the abundance of Iba1$^+$/MRC1$^+$ cells was highest in M2-like microglia (Figure 1F, 78% ± 2.195%, SEM), intermediate in M0-like microglia (Figure 1F, 27.52% ± 2.195%, SEM), and almost absent in M1-like microglia (Fig, 1F, 1.651% ± 2.195%, SEM).

To examine whether microglia subtypes showed corresponding gene expression levels, we performed qPCR using primers specific for the established microglia markers *Aif1* (encoding for Iba1), *Mhc2* and *Nos2* (M1 marker) as well as *Arg1* and *Mrc1* (M2 marker). In agreement with previous studies using purified microglia (41, 42), *Nos2* and *Mhc2* were highly expressed in cultures containing M1-like microglia, whereas *Arg1* and *Mrc1* were highly expressed in cultures containing M2-like microglia (Figures 2A–E).

To verify that our differentiation protocol induced subtype-specific alterations in cell metabolism, i.e., arginine metabolism, we determined nitrite levels in cell culture supernatants as a measure for the release NO. Cultures containing M1-like microglia released significantly more NO than those containing M0- or M2-like microglia (Figure 2F), and the NO release was correlated with the *Nos2* expression (Figure 2G). Taken together, these results confirmed the differentiation of M0-, M1-, and M2-like microglia in our primary mixed glia model.

M1- and M2-like microglia show substrate-specific phagocytosis preference and capacities

We next examined phagocytosis activity and capacity of M0-, M1-, and M2-like microglia in mixed glial cultures. Cells were incubated for 3 h in the presence of two phagocytic substrates, rhodamine-E. coli particles or IgG-FITC beads, and then processed for anti-Iba1 immunocytochemistry and confocal microscopy (Figures 3A–C).

Determining the percentage of Iba1$^+$ M0-like microglia containing E. coli particles or IgG-beads revealed that approximately 75% of M0-like microglia showed phagocytic activity, regardless of substrate identity (Figure 3A *E.coli*: 74.32% ± 2.85%; IgG: 73.86% ± 2.85%, SEM). In contrast, the percentage of M1-like microglia containing *E.coli* particles was higher than the percentage of M1-like microglia containing IgG-beads (Figure 3B, *E.coli*: 73.06% ± 2.84%; IgG: 27.37% ± 2.84%, SEM). However, we observed opposing internalization ratios for M2-like microglia. The percentage of M2-like microglia containing IgG-beads was increased compared to the percentage of M2-like microglia containing *E.coli* particles (Figure 3C, *E.coli*: 81.49% ± 4.92%; IgG: 51.74% ± 4.96%, SEM).

To assess the phagocytic capacity of the microglia subtypes, we determined the relative occupancy of the Iba1-immunostained cell area by either *E.coli*-rhodamine or IgG-FITC beads in individual microglia. In M0-like microglia, no differences were observed in the cell area occupancy between *E.coli*-rhodamine and IgG-FITC beads, both of which show a cell area occupancy of less than 20% (Figure 3D, *E.coli*: 17.68% ± 3.15%; IgG: 19.37% ± 2.97, SEM). In M1-like microglia, the cell area occupancy by *E.coli*-rhodamine was about 35% of the cell area, and the occupancy by IgG-FITC was less than 10% (Figure 3D, *E.coli*: 37.12% ± 2.31%; IgG: 6.57% ± 2.07%, SEM). In contrast, M2-like microglia displayed a cell area occupancy by *E.coli*-rhodamine of about 20%, whereas the occupancy by IgG-FITC was more than 40% (Figure 3D, *E.coli*: 22.16% ± 0.95%; IgG: 42.53% ± 2.18%, SEM). Together, these results indicated that M1- and M2-like microglia subtypes show substrate-specific phagocytosis activities.

Microglia subtypes show substrate-specific phagocytosis capacities and dynamics over 16 hours

Our experiments to study differences in phagocytosis by microglia subtypes after fixative treatment and immunocytochemistry at a predetermined time point did not allow examination of microglial phagocytosis continuously over time. Therefore, we performed multicolour time-lapse live imaging for 16 hours. To identify microglia, we added BV421-fluorophore-conjugated anti-CD68 to the cultures 30 min before the imaging experiments started with the addition of *E.coli*-rhodamine particles or IgG-FITC beads. The subsequent analysis was based on fluorescent particle tracking of the *E.coli*-rhodamine or IgG-FITC substrates in single cells labelled with BV421-anti-CD68 and expressed as the mean number of particles per ROI using Imaris software (see methods for details).

Images taken shortly after the addition of *E.coli*-rhodamine particles at 0 h and 16 h after their addition indicated high phagocytosis rates of this substrate by M0- and M1-like microglia over time (Figure 4A. Higher magnifications demonstrated the internalization of *E.coli* particles into vesicle-like structures and substantial intracellular accumulation of the particles at 16 h (Figure 4B). However, M2-like microglia showed less internalized *E.coli* particles than M0- or M1-like

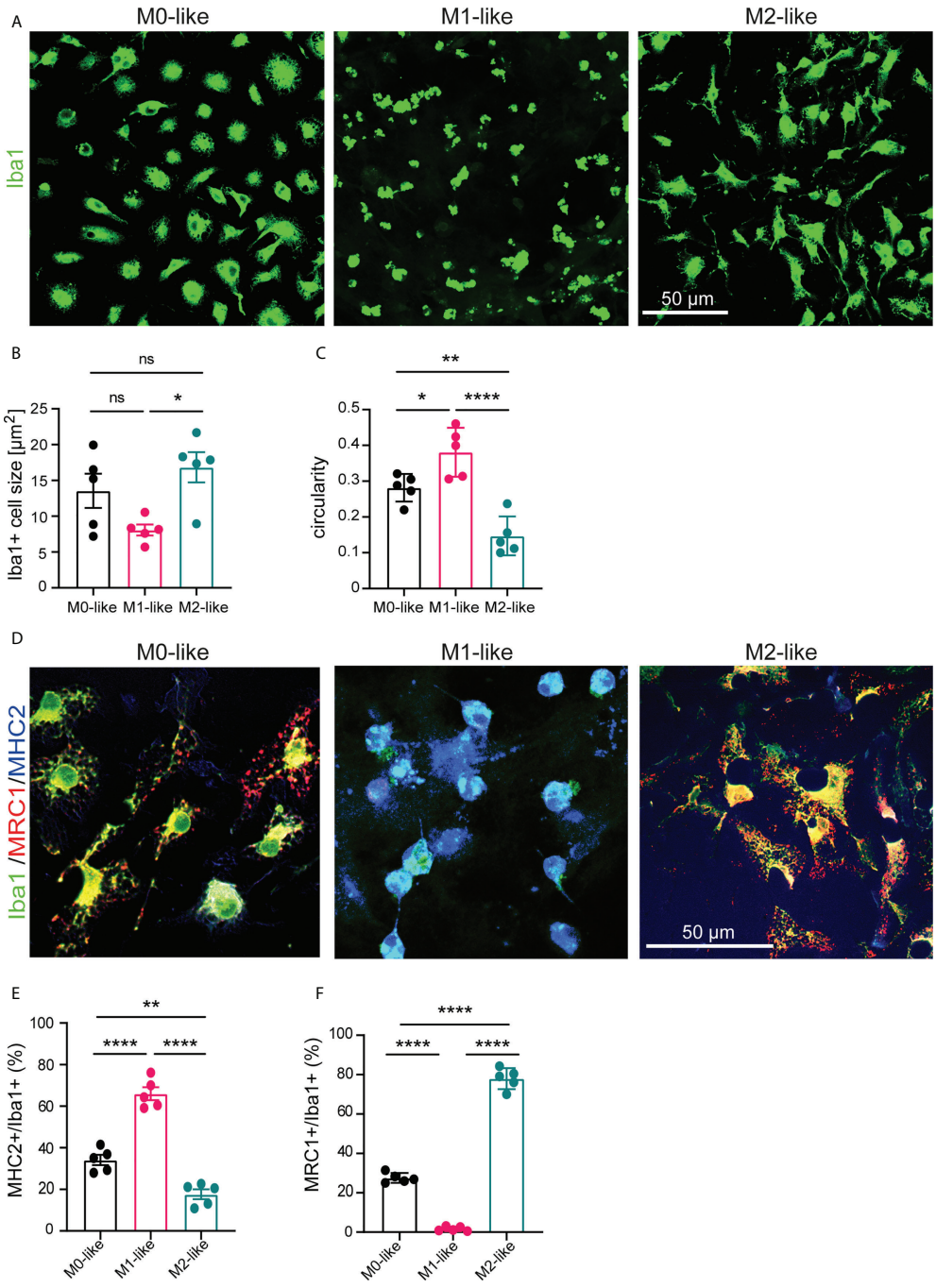

FIGURE 1
Microglia subtypes differ in morphology and expression of M1/M2 protein markers. **(A)** Confocal images showing anti-Iba1 immunostaining of M0-, M1-, and M2-like microglia subtypes in primary glia cultures at 7 div, cultivated the presence of serum and/or growth factors and cytokines (GM-CSF/IFNγ for M1-like or M-CSF/IL-4 for M2-like) **(B)** Histograms showing differences in mean cell size and **(C)** circularity of microglia subtypes. **(D)** Confocal images showing triple-immunostaining using antibodies specific to the pan-marker Iba1, the M1-marker MHC2, or the M2-marker MRC1. M1- or M2-like microglia show increased expression of MHC2 or MRC1, respectively. **(E, F)** Histograms showing the percentage of Iba1+ microglia expressing MHC2 or MRC1 as determined by cell counts after triple immunostaining using antibodies specific to Iba1, MHC2, or MRC1. Data are expressed as mean ± SEM (n = 5, independent biological replicates are shown) and were tested for significant differences by one-way ANOVA (*post-hoc* correction Holm-Šídák) or Kruskal-Wallis test (*post-hoc* correction Dunnett), *p < 0.05, **p < 0.01, ****p < 0.0001 ns, not significant.

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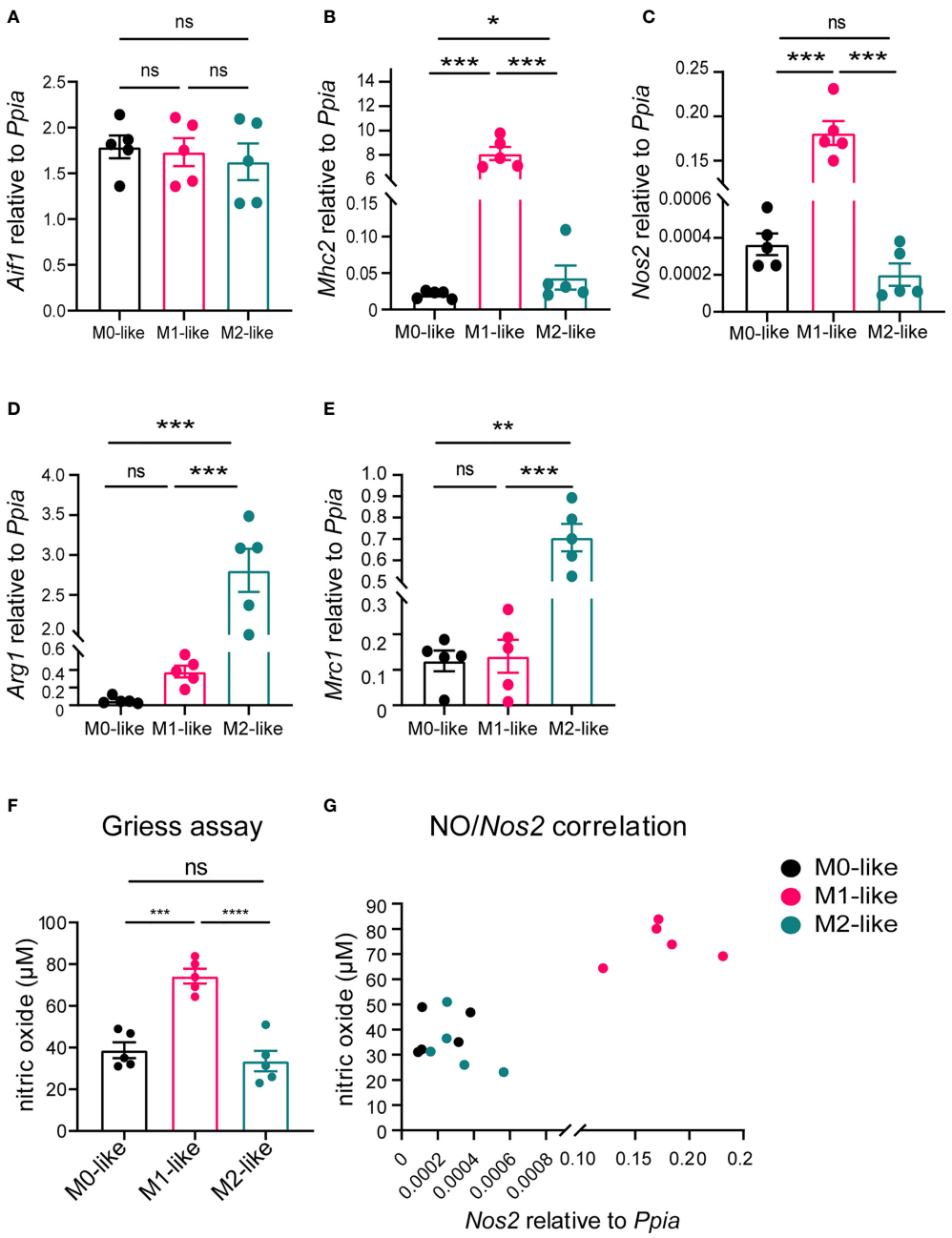

FIGURE 2
Microglia subtypes differ in M1/M2 gene marker expression and metabolism. **(A–E)** Gene expression analyses of the pan-marker *Aif1*, M1-markers *Mhc2*, *Nos2* and M2 markers *Arg1*, and *Mrc1* demonstrating differential expression by microglia subtypes. *Ppia* was used as a reference gene. **(F)** Alterations in cellular arginine metabolism were detected by colorimetric Griess assay, with M1-like microglia releasing significantly more NO than M0- or M2-like microglia. **(G)** Scatter plot showing positive correlation between NO and *Nos2* expression (non-parametric Spearman correlation, r = 0.8842, p < 0.0001). Values are expressed are mean ± SEM from 5 individual experiments (biological replicates), one-way ANOVA (*post-hoc* correction Holm-Šídák), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. ns, not significant.

microglia (Figures 4A, B), suggesting reduced phagocytosis rates over the 16 hours period

E.coli particle tracking in CD68+ microglia revealed that M0- and M1-like microglia continuously internalized *E.coli* particles over 16 h. However, internalization by M1-like microglia occurred more steadily (Figure 4C). M2-like microglia showed internalization rates similar to M0- and M1-like subtypes until approximately 8 h after adding *E.coli* particles, but then

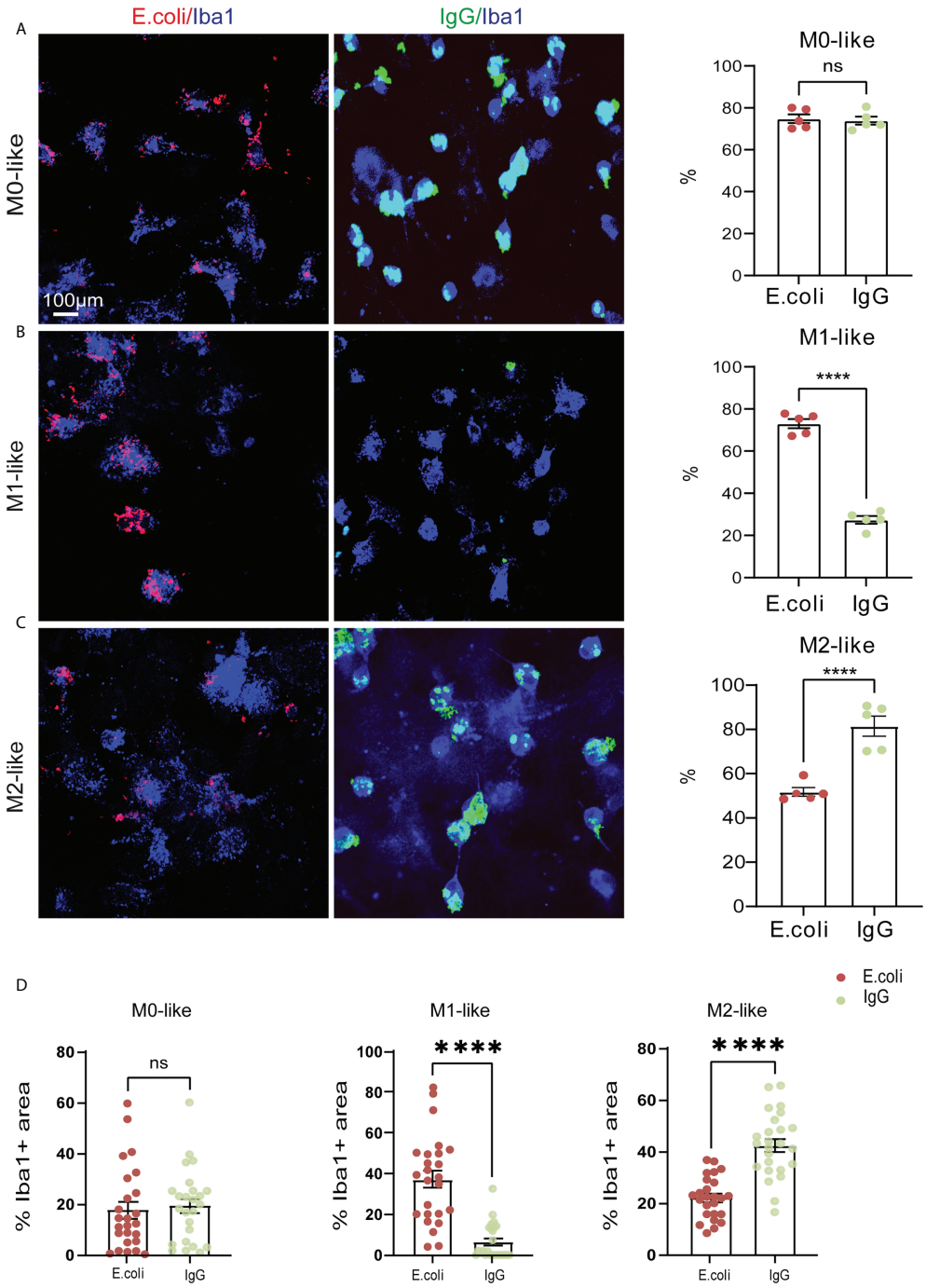

FIGURE 3
M1- and M2-like microglia show substrate-specific phagocytosis preference and capacities. **(A–C)** Confocal images showing anti-Iba1 immunostaining of M0-, M1- or M2-like microglia subtypes along with rhodamine-E.coli particles (red, E.coli) or IgG-FITC beads (green, IgG) after 3 hours of phagocytosis substrate exposure. Histograms showing percentage of microglia with internalized E.coli particles or IgG beads. M0-like microglia showed no phagocytosis preference, whereas M1- and M2-like microglia showed opposing phagocytosis preferences for E.coli particles or IgG beads, respectively. **(D)** Co-localization analyses showing the relative occupancy of Iba1 immunostained cell areas by E.coli particles or IgG beads. M1- and M2-like microglia displayed opposing phagocytosis capacities for E.coli particles or IgG beads, respectively. Data are expressed as mean ± SEM from 5 independent biological replicates **(A–C)** or 5 cells from each of 5 independent biological replicate **(D)**. Data are means ± SEM. ****$p < 0.0001$, ns, not significant, Student's t-test.

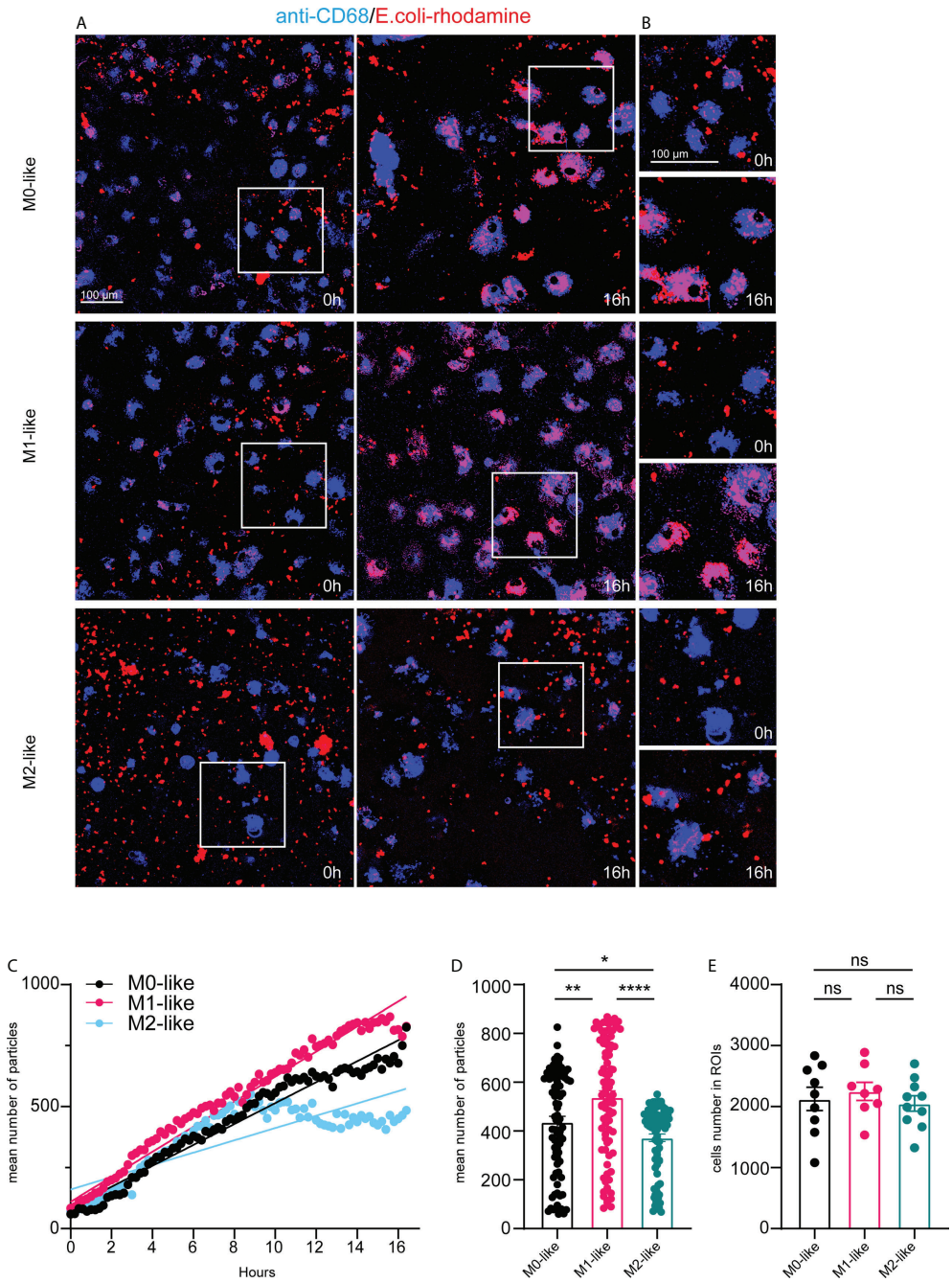

FIGURE 4
Microglia subtypes show substrate-specific phagocytosis capacities and dynamics for *E.coli* particles over 16 hours **(A)** Single frame images and **(B)** image enlargements from live imaging movies of anti-CD68 live-immunolabelled microglia (blue) after addition of E.coli-rhodamine particles (red) at time-points 0h and 16h, scale bar, 100µm. Note pronounced E.coli particle accumulation in M0- and M1- but not in M2-like microglia at 16h after addition of E.coli particles. **(C)** Time-series plot showing the mean number of E. coli-rhodamine particles internalized (averaged from 8-10 ROIs for each time interval) by microglia subtypes over 16 hours. Simple linear regression calculation indicate different slopes of phagocytic capacities of M0- (black) (r^2: 0,6902, $p < 0,0001$), M1- (pink) (r^2: 0,5364, $p < 0,0001$), and M2-like (cyan) (r^2: 0,3691, $p < 0,0001$), respectively. Note that M2-like microglia discontinued internalization at about 9 hours after addition of E.coli particles. **(D)** Mean number of internalized particles over 16 hours (averaged from 8-10 ROIs for each time interval). **(E)** Number of imaged anti-CD68 immunolabelled microglia encountering E.coli particles over 16 hours. Data are expressed as means ± SEM, one-way ANOVA (*post-hoc* correction Holm-Šídák test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. ns, not significant.

discontinued internalization (Figure 4C). These differences were also evident when comparing the mean number of internalized *E.coli* particles (averaged from 3-5 ROIs for each time interval) over 16 h (Figure 4D, M0: 445.3 ± 25.80, SEM; M1: 531.9 ± 31.49, SEM; M2: 400.8 ± 18.05, SEM). The mean particle number was significantly higher in M1-like microglia than in M0-like or M2-like microglia, and M0-like microglia showed overall higher internalization than M2-like microglia (Figure 4D). These differences were independent of the number of imaged CD68+ cells encountering *E.coli* particles over the 16 h imaging period (Figure 4E, M0: 2126 ± 191.7, SEM, M1: 2250 ± 148.9, SEM, M2: 2052 ± 127.8, SEM), which further confirms that the differences between the mean particle numbers per ROI reflect differences at the single cell level.

Images were taken shortly and 16 h after the addition of IgG-FITC beads to microglia subtypes, suggesting that the phagocytosis of IgG-FITC beads by M0- and M2-like microglia was more efficient than by M1-like microglia (Figure 5A).Furthermore, higher magnifications demonstrated the vesicle-like appearance of IgG beads and pronounced accumulation in M0- and M2-like microglia and relatively low accumulation in M1-like microglia (Figure 5B).

IgG bead tracking in CD68+ microglia over 16 hours revealed different internalization rates and dynamics by microglia subtypes. M1-like microglia showed continuous internalization but internalized overall, clearly fewer IgG beads than M0- or M2-like microglia (Figure 5C). M0-like microglia showed an almost steady increase of IgG bead internalization over 16 h. M2-like microglia initially showed continuous internalization, but discontinuation of internalization occurred approximately 8 hours after adding the phagocytosis substrate (Figure 5C). The low phagocytosis activity of M1-like microglia was also reflected by a significantly reduced mean number of internalized beads (averaged from 4-5 ROIs for each 12 min interval) over 16 h, whereas no significant differences were observed between M0-like and M1-like microglia (Figure 5D, M0: 443.2 ± 24.81, SEM; M1: 87.27 ± 5.238, SEM, M2: 364.2 ± 17.27, SEM). The number of microglia subtype cells encountering the substrate was statistically not different, albeit the mean number of M1-like cells was lower than in the other conditions (Figure 5E, M0: 2273 ± 132.8, SEM, M1: 1532 ± 282.0, SEM, M2: 2231 ± 217.3, SEM).

Altogether, the results indicate that microglia subtypes display substrate-specific phagocytosis over time.

Microglia subtypes show M1-like features after prolonged phagocytosis substrate exposure

The phagocytic activity of microglia in neurological conditions may exceed well beyond the time-course examined in our live imaging experiments. To mimic this situation, we subjected microglia to prolonged exposure to phagocytosis substrate for 5 days. Determining the percentage of Iba1+ microglia containing *E.coli* particles after 5 days revealed that approximately 75% of all microglia subtypes showed phagocytosis activity (Figures 6A–C, M0: 74.28% ± 4.39%, SEM, M1: 80.6% ± 3.76%, SEM, M2: 72.81% ± 5.4%, SEM). However, the percentage of Iba1+ cells containing IgG beads was significantly lower (Figures 6A–C, M0: 27.58% ± 4.16%, SEM, M1: 27.56% ± 6.13%, SEM, M2: 27.55% ± 4.01%, SEM). Analysis of colorimetric NO assays and qPCR results showed that NO release and *Nos2* mRNA expression correlated for all microglia subtypes and that both increased relative to the control microglia subtypes not exposed to phagocytosis substrates (Figure 6D). Next, the relative occupancy of the Iba1-immunostained cell area by either *E.coli*-rhodamine or IgG-FITC beads was determined. We found that all microglia subtypes encountering the phagocytosis substrates showed a higher phagocytosis capacity for *E.coli* than for IgG beads (Figures 6E–G, *E.coli*: M0: 32.02% ± 4.2%, SEM, M1: 39.22% ± 3.19%, SEM, M2: 37.63% ± 4.06%, SEM; IgG: M0: 6.903% ± 1.9%, SEM, M1: 20.04% ± 2.04%, SEM, M2: 9.307% ± 2.32%, SEM).

Since this result was in stark contrast to the substrate preferences of microglia subtypes observed in our experiments after 3 h of substrate exposure, we tested whether changes in microglial subtype identity might have contributed to this result. We determined gene expression levels of microglia subtype markers by qPCR using primers specific for *Aif* (*Iba1*), *Mhc2*, *Nos2*, *Arg1* and *Mrc1* (Figure 7). In M0-like microglia cultures, the M1-like markers *Mhc2* and *Nos2* were both strongly up-regulated after 5 days of exposure both to *E.coli* particles or IgG beads as compared to M0-like cells cultured without phagocytic substrate (Figures 7B, C), while the pan-microglia marker Aif1 was not significantly different between the three conditions (Figure 7A). Determination of M2-like marker expression revealed up-regulation of *Mrc1* after IgG bead exposure and *Arg1* expression did not alter in response to substrate exposure (Figures 7D, E). In M1-like microglia, we found decreased *Aif1* expression but increased expression of M1-like markers *Mhc2* and *Nos2* after *E.coli* particle exposure as well as increased *Nos2* expression after IgG bead exposure (Figures 7F–H). No significant differences were found for *Arg1* or *Mrc1* expression (Figures 7I, J). In M2-like microglia, both *E.coli* particles and IgG beads caused up-regulation of M1-like markers *Mhc2* and *Nos2* (Figures 7L, M). M2-marker *Arg1* was down-regulated after *E.coli* exposure and *Mrc1* was downregulated both after *E.coli* particle and IgG bead exposure as compared to M2-like cells cultured without phagocytosis substrates (Figures 7N, O).

Thus, microglia subtypes subjected to prolonged exposure to phagocytosis substrate for 5 days displayed changes in M1/M2 marker gene expression indicating a shift towards M1-like phenotypes. Recently, it was shown that phagocytosis of *E.coli* particles by M0-like microglia leads to delayed release of the

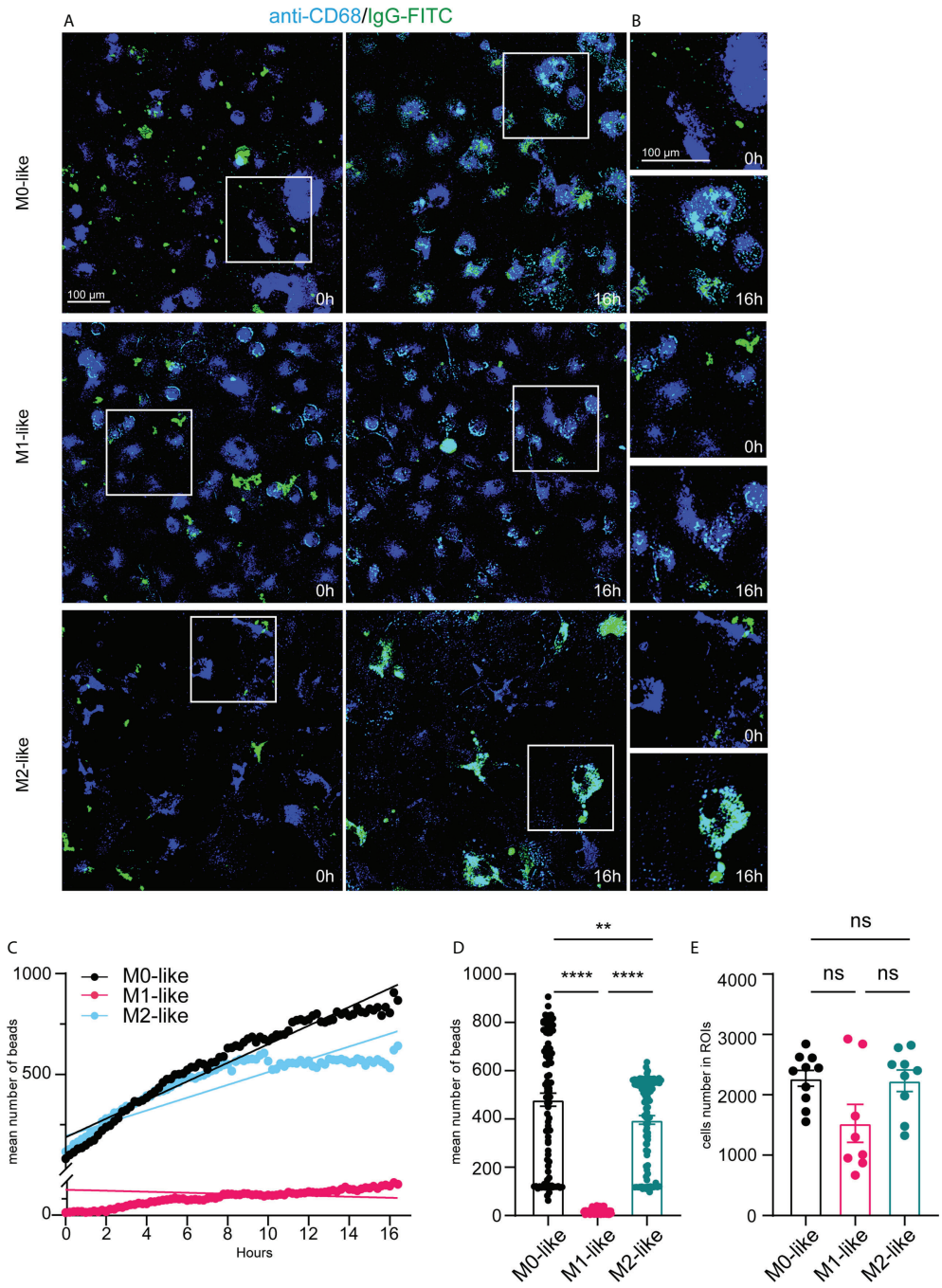

FIGURE 5
Microglia subtypes show different phagocytosis capacities and dynamics for IgG-FITC beads over 16 hours. **(A)** Single frame images and **(B)** image enlargements from live imaging movies of anti-CD68 live-immunolabelled microglia (blue) after addition of IgG-FITC beads (green) at time-points 0h and 16 h, scale bar, 100µm. Note pronounced IgG bead accumulation in M0- and M2- but not in M1-like microglia at 16h after addition of E.coli particles. **(C)** Time-series plot showing the mean number of IgG beads internalized (averaged from 8-10 ROIs for each time interval) by microglia subtypes over 16 hours. Simple linear regression calculation indicate different slopes of phagocytic capacities of M0- (black) (r^2: 0,1637, $p < 0,0001$), M1- (pink) (r^2: 0,2733, $p < 0,0001$), and M2-like (cyan) (r^2: 0,07045, $p < 0,0001$), respectively. Note that M2-like microglia discontinued internalization at about 9 hours after addition of IgG-FITC beads. **(D)** Mean number of internalized particles over 16 hours (averaged from 8-10 ROIs for each time interval). **(E)** Number of imaged anti-CD68 immunolabelled microglia encountering IgG-FITC beads over 16 hours. Data are expressed as means ± SEM, one-way ANOVA (*post-hoc* correction Holm-Šídák test, ****$p < 0.0001$. ns, not significant.

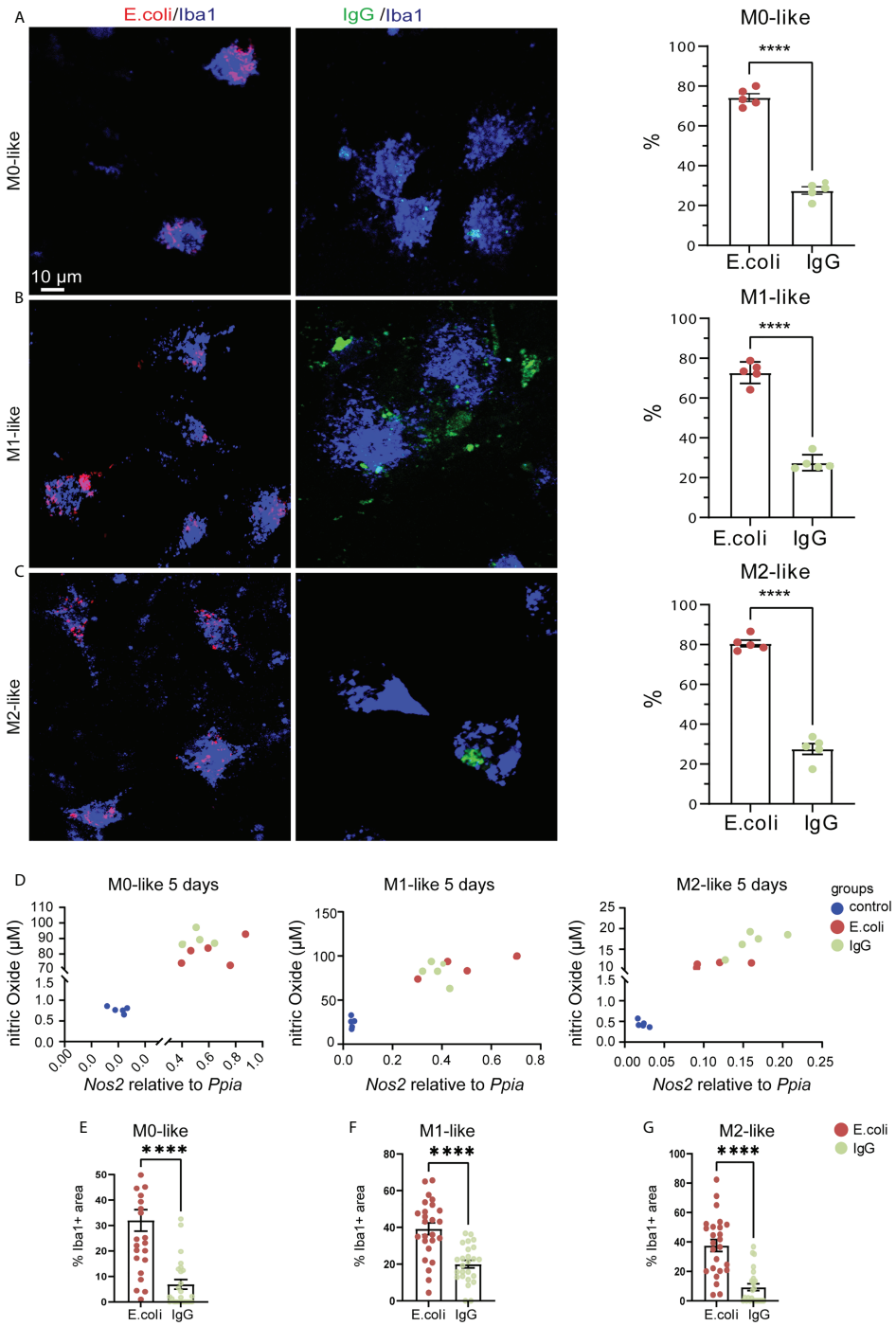

FIGURE 6
Microglia subtypes show M1-like features after prolonged phagocytosis substrate exposure **(A–C)** Confocal images showing anti-Iba1 immunostaining of M0-, M1- or M2-like microglia subtypes along with rhodamine-E.coli particles (red, E.coli) or IgG-FITC beads (green, IgG) after 5 days of phagocytosis substrate exposure. Histograms showing percentage of microglia with internalized E.coli particles or IgG beads. Microglia subtypes showed phagocytosis preference for E.coli particles rather than IgG beads. **(D)** Scatter plots showing similar correlation (non-parametric Spearman correlation) between NO levels and *Nos2* gene expression for M0- (r = 0.6536, p=0.0099), M1- (r = 0.8214, p = 0.0003), and M2-like (r = 0.8857, p < 0.0001) microglia. Ppia was used as a reference gene. **(E–G)** Co-localization analyses showing the relative occupancy of Iba1 immunostained cell areas by E.coli particles or IgG beads. Microglia subtypes showed phagocytosis preference for E.coli particles rather than IgG beads. Data are expressed as mean ± SEM from 5 independent biological replicates **(A–C)** or 4-6 cells from each of 5 independent biological replicate **(D)**. Data are means ± SEM. ****p < 0.0001, ns, not significant, Student's t-test.

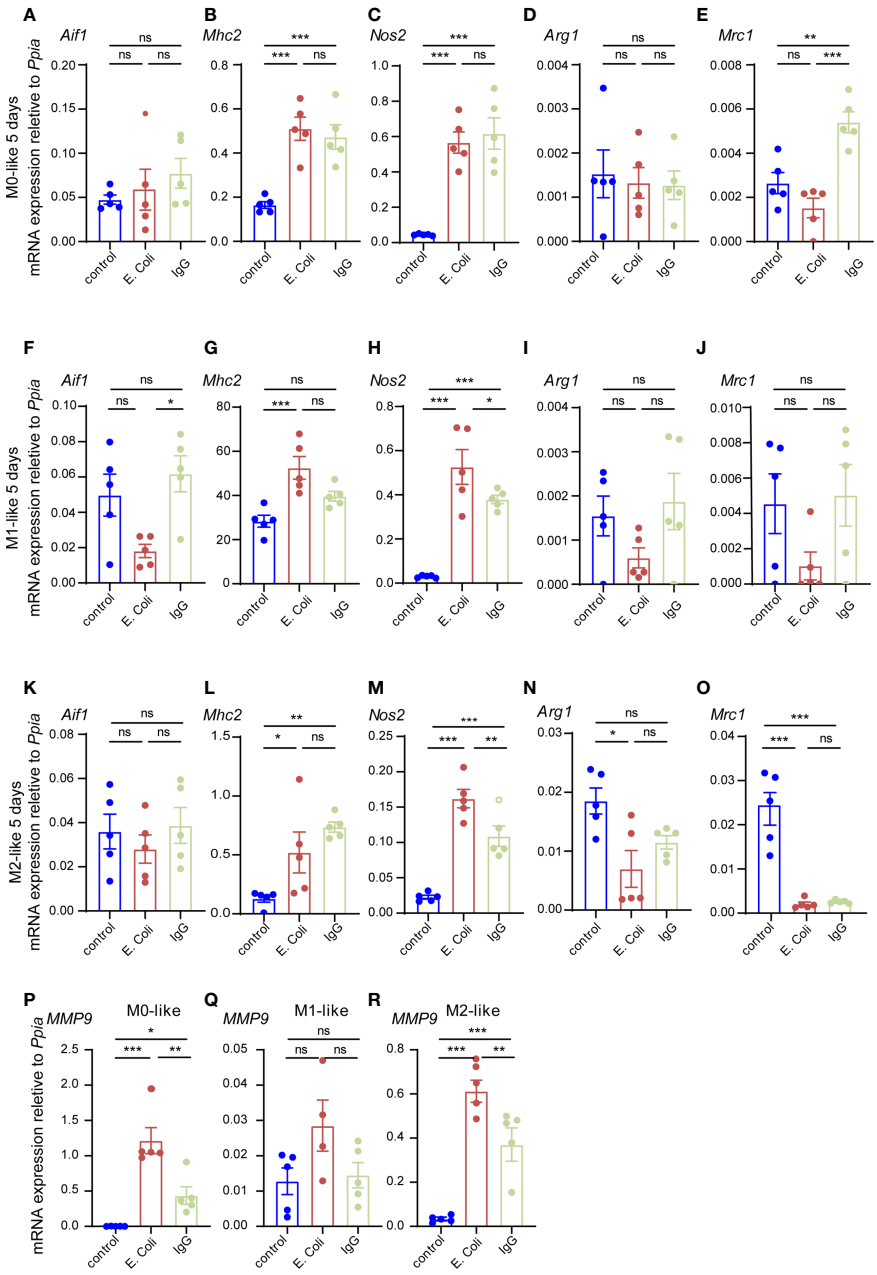

FIGURE 7
Microglia subtypes show M1-like gene expression after prolonged phagocytosis substrate exposure **(A–R)** Gene expression analyses of microglia subtypes after prolonged phagocytosis substrate exposure (E.coli-rhodamine particles, IgG-FITC beads) for the pan-marker *Aif1*, M1-markers *Mhc2*, *Nos2* and M2 markers *Arg1*, *Mrc1* as well as the glia activation marker *Mmp9*. *Ppia* was used as a reference gene. **(A–E)** In M0-like microglia, conditions with E.coli particles or IgG beads show up-regulation of M1 markers *Mhc2* and *Nos2* as well as up-regulation of M2 marker *Mrc1* as compared to M0-like microglia not exposed to phagocytosis substrate (control). **(F–J)** In M1-like microglia, conditions with E.coli particles show downregulation of microglia pan-marker *Aif1* and up-regulation of M1 markers *Mhc2* and *Nos2* but down-regulation of M2 markers *Arg1* and *Mrc1* and conditions with IgG show up-regulation of *Nos2* as compared to M1-like microglia not exposed to phagocytosis substrate (control). **(K–O)** In M2-like microglia, conditions with E.coli particles or IgG beads show up-regulation of M1 markers *Mhc2* and *Nos2* as compared to M2-like microglia not exposed to phagocytosis substrate (control). Down-regulation of M2 marker *Arg1* was observed in conditions with E.coli particles as well as down-regulation of *Mrc1* in conditions with E.coli particles or IgG beads as compared to M2-like microglia not exposed to phagocytosis substrate (control). **(P–R)** The glia activation marker *Mmp9* was up-regulated in M0- and M2-like microglia both in conditions with E.coli particles of IgG beads as compared to microglia subtypes not exposed to phagocytosis substrate (control). Multiple comparisons were performed dependent on data distribution by one-way Anova (*post-hoc* correction Holm-Šídák) or Kruskal-Wallis test (*post-hoc* correction Dunnett), if F achieved the necessary level of statistical significance $p < 0.05$. Data points are shown for biological replicates and expressed as mean ± SEM, $p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$.

matrix metalloproteinase MMP-9 (43) suggesting that *Mmp9* gene expression may serve as a marker for microglia plasticity in response to phagocytosis substrate exposure. Indeed, *Mmp9* expression was up-regulated in M0- and M2-like microglia after 5 days exposure to *E.coli* particles or IgG beads as compared to control cultures without phagocytosis substrate. However, *Mmp9* expression did not increased significantly in M1-like microglia (Figures 7P–R) suggesting that *Mmp9* gene expression was stronger induced in microglia undergoing shifts towards a M1-like phenotype.

Discussion

This study examined phagocytosis efficiencies of M0-, M1-, and M2-like microglia for *E.coli* particles and IgG-opsonized beads over different time periods *in vitro*. These substrates were chosen due to their pathological relevance, their common use in phagocytosis research and better detection and quantification properties compared to soluble fluorophore-conjugated molecules. Cell cultivation and phagocytosis assays were carried out in the presence of serum to mimic pathophysiological conditions of BBB breakdown such as meningitis, trauma or stroke (38, 39). We found that microglia subtypes differ in phagocytosis efficiencies for the two types of substrates in a time-dependent manner and long-term substrate exposure enhanced or induced M1-like profiles of M0-, M1-, and M2-like microglia, respectively. Our results suggest that phagocytosis substrates can trigger phenotype plasticity of microglia including M2 to M1 repolarization which may also take place in neurological conditions when microglia encounter different types of phagocytic substrates.

In general, our results showing substrate-specific phagocytosis efficiencies of distinct microglia subtypes are consistent with previous *in vitro* studies examining phagocytosis of diverse substrates such as zymosan, IgG-opsonized beads or Aβ by M1- and M2-like microglia (44–48). The prevailing view is that M2-like microglia show higher phagocytic activity than M1-like microglia. However, there are also conflicting observations on the phagocytic preference and efficiency of different microglia subtypes suggesting a context dependency (8, 14, 19, 20, 49, 50).

In the present study, when microglia were exposed to *E.coli* particles or IgG beads for 3 hours, we found that a higher proportion of M1-like than M2-like microglia phagocytose *E.coli* particles. This result is in agreement with findings that M1-like microglia, showing augmented release of NO and expression of pro-inflammatory cytokines, more efficiently phagocytose pathogenic bacteria than unstimulated microglia, but M2-like microglia were not examined (51, 52). Studies comparing *in vitro* phagocytosis dynamics between different microglia subtypes over time are scarce (53). Therefore, we conducted live imaging experiments over 16 hours in anticipation of gaining new insights. Similar to the 3 h exposure time, M1-like microglia internalized more *E.coli* particles over 16 h than M2-like microglia, and *vice versa*, M2-like microglia internalized more IgG beads than M1-like microglia. The strongest differences in substrate preference were found for M1-like microglia, which clearly preferred *E.coli* particles over IgG beads. This result supports and extends the aforementioned findings that M1-like microglia show high efficiency in the phagocytosis of pathogenic bacteria, which holds potential for novel therapeutic approaches e.g. in bacterial meningoencephalitis and sepsis (51, 52, 54, 55). In this context, factors have been identified, i.e. palmitoylethanolamide and activin A, to enhance phagocytosis of *E.coli* by M1-like microglia while preventing excessive and potentially harmful release of NO and pro-inflammatory cytokines (56–58). Interestingly, similar mechanisms may underlie therapeutic benefit after treatment of Alzheimer`s Disease model mice with the non-pyrogenic LPS-derivative monophosphoryl lipid A (59), which promotes phagocytosis of Aβ after 3 h of substrate exposure by pretreated microglia *in vitro* (18). To expand existing research, live cell imaging of microglia over a longer observation time might be useful to characterize this and other phagocytosis-enhancing drugs in terms of optimal stimulation protocols to achieve sufficient phagocytosis activity.

We further found that M2-like microglia internalized IgG beads more efficiently than M1-like microglia. These results are in line with previous studies showing efficient phagocytosis of substrates from other sources than *E.coli* by M2-like microglia (14–16). Likewise, M2-like microglia induced by SCF show increased phagocytosis of FITC-IgG opsonized beads as compared to GM-CSF-induced M1-like microglia (17, 48). However, we found time-dependent changes in substrate internalization of M2-like microglia, almost discontinuing both *E.coli* particle and IgG bead phagocytosis approximately 8 h after addition of the phagocytosis substrates. We have not addressed the question of whether stalled phagocytosis activity by M2-like microglia is a transient or permanent effect beyond the 16 h live imaging period. Interestingly, biphasic phagocytosis activities have been reported for bone marrow-derived macrophages with peaks at 4 h and 24 h and an intervening period of no internalization (60), but the underlying mechanisms are elusive. Rate-limiting factors reported for phagocytosis comprise scavenger receptors, Fcγ and/or complement receptors, the myosin/actin network, second messengers such as phosphoinoside, but also physical and metabolic constraints might play a role (61–64). Further studies using genetic, pharmacological and single-cell transcriptomics approaches are required to modulate key factors of phagocytosis and elucidate the molecular mechanisms underlying this and other observations of our study.

We found that after prolonged substrate exposure for 5 days all microglia subtypes showed a higher preference for *E.coli*

particles than IgG beads. Notably, prolonged exposure resulted in increased expression of the M1 markers *Mhc2* and *Nos2* irrespectively of the substrate. Gene expression changes after prolonged IgG bead exposure were similar to those after prolonged *E.coli* particle exposure and in support of a shift towards M1-like phenotypes. These results suggest that phagocytosis associated with the cellular environment can be considered a key factor in the phenotype transformation of microglia. Indeed, it has become clear that phagocytosis is not an isolated cellular response and may represent a source of cellular heterogeneity and plasticity in different tissues (65). Compelling evidence was provided that this is also true for microglia in the CNS. Regional changes in epigenetic regulation of microglia transcriptomes have been connected to the basal phagocytic activity of microglia (66) and phagocytosis-induced transcriptional changes were demonstrated to support the long-term maintenance of hippocampal neurogenesis in mice (67). However, in mouse models of acute or chronic neurodegeneration, phagocytosis of apoptotic cells caused a microglia phenotype shift from a homeostatic to a neurodegenerative phenotype (68). Our results suggest that shifts towards pro-inflammatory M1-like phenotypes occur irrespectively of the pre-established microglia subtype thereby providing another piece of evidence for high microglia plasticity in response to environmental factors. We did not explore whether the presence or phagocytosis of *E.coli* particles or IgG beads were decisive for this phenotype shift but we favor the possibility that both phagocytosis, the environmental presence of substrate as well as the duration of substrate exposure is critical. Indeed, experimental evidence from macrophages shows time-dependent phenotypic switches in response to LPS (69). Dynamic changes in pro-inflammatory cytokine gene expression were also observed in M1-like microglia after myelin phagocytosis (70) and chronic myelin phagocytosis induces a disease-associated transcriptional state in microglia (71). Another non-mutually exclusive possibility is that increased pro-inflammatory cytokine expression mediates feedback loops that enhance or drive a phenotype shift towards M1-like profiles (72–75).

The same could apply to the metalloproteinase MMP-9, which is expressed by LPS-activated microglia in primary neuron-glia cultures (76) and secreted in a delayed manner by primary microglia in response to phagocytosis of *E.coli* particles (43). In support of a role of MMP-9 in microglia responses after phagocytosis, we found robust up-regulation of *Mmp9* expression after long-term substrate exposure for 5 div. Interestingly, *Mmp9* up-regulation was found in cultures showing shifts towards the M1-like phenotype whereas no up-regulation was observed in cultures of pre-differentiated M1-like cells. This finding suggests that MMP-9 may serve as a marker for microglia plasticity including M2 to M1 repolarization, consistent with observations on the positive regulation of MMP-9 expression by M1-like phenotype inducers IL-1, TNFα, and LPS and negative regulation by the M2-like phenotype inducers IL-4 and IL-10 (77). In addition, paracrine/autocrine loops involving MMP-9 have been suggested to amplify microglia activation, whereas deletion of MMP-9 maintained microglia in a resting phenotype in an animal model of spinal cord injury (78). These findings suggest a broader functional spectrum of MMP-9 and other matrix metalloproteinases (MMPs) released by microglia beyond established physiological roles in synaptic plasticity and extracellular matrix modeling (79) or pathological roles in neuroinflammation or gliomas, for example (77, 80, 81).

Our results further support the hypothesis that M1/M2 microglia can shift between functional phenotypes depending upon environmental signals, here *E.coli* particles or IgG beads. Similar processes may also take place in conditions of acute and chronic brain insults when microglia encounter different types of phagocytic substrates. Indeed, M2-like to M1-like shifts in microglia populations were also observed in models of ischemic stroke (82, 83), spinal cord injury (28), and traumatic brain injury (84, 85). Furthermore, phagocytosis by microglia can play an important role in chronic neurodegeneration as well as neurodevelopmental and neuropsychiatric disorders (86–88). Reprogramming patient-derived cells to microglia-like cells and testing for their phenotype plasticity and phagocytosis function may help to gain insights into pathological mechanisms. For example, schizophrenia patient-derived microglia-like cells show higher rates of synaptic phagocytosis and elimination and targeting microglia by the immunomodulatory drug minocycline reduced abnormal synapse elimination by phagocytosis (89). Interestingly, minocycline was proposed to act *via* inhibition of MMPs in the autism spectrum disorder fragile X syndrome (90) suggesting that better understanding the role of MMPs for microglia activation, phenotype plasticity, and phagocytic function may provide novel immunomodulatory treatment options.

Some limitations of this study should be considered. Our *in vitro* approach does not reproduce the brain environment, and many factors influencing microglial morphology, polarization and function are absent. To partially compensate for these limitations, we used primary glia cultures containing a substantial number of astrocytes in combination with microglia-specific immunolabelling as well as microglia-specific qPCR assays. The presence of astrocytes under the different experimental conditions likely influenced microglia responses as compared to pure microglia cultures since astrocytes modulate microglia polarization, activation and function. Conversely, activated microglia can trigger changes in the inflammatory profile of astrocytes both *in vitro* and *in vivo* (91, 92). As LPS-activated microglia can induce a neurotoxic A1 astrocyte phenotype (92), prolonged exposure of the primary glial cultures to *E.coli* particles affects microglia but also astrocytes. Therefore, further studies are required to examine possible alterations in astrocytes and their influence on microglia

under the experimental conditions of our study. Another limitation in this study is the use of two different phagocytic substrates and fluorophore conjugates, which likely undergo different lysosomal processing and fluorescent decay after phagosomal acidification. This may particularly play a role for the long-term experiments and the results of the phagocytic uptake should be interpreted with caution. Importantly, regardless of this limitation, data on gene expression and nitric oxide levels demonstrate microglia plasticity and phenotype shifts after long-term substrate exposure. Finally, non-defined serum proteins in the, however heat-inactivated, culture media can trigger microglia activation as well as phagocytosis by microglia (38, 39, 93, 94). As indicated by previous findings, serum-derived IgG likely influenced phagocytosis by microglia in the present studies. It has been also shown that the opsonization of *E.coli* with human serum or murine IgG increases the phagocytic ability of macrophages to clear *E.coli* (60). However, given that the presence of serum mimics neuropathological conditions involving BBB damage in our *in vitro* model, the findings of this study may more closely resemble pathological *in vivo* conditions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval were not required for the animal study because we used newborn mice as a source for primary neurons which does not require an ethical review. Newborn mice to obtain primary glia were handled in accordance with the institutional guidelines of the Johannes Gutenberg University Mainz, and Rhineland-Palatine, Germany.

Author contributions

MS conceptualized and designed the study. SL performed the experiments, data collection, and analysis. IW assisted in experiments and data analysis. GH conducted microscopy methodology and advice on data analysis and interpretation. All authors contributed to the manuscript writing and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.945485/full#supplementary-material

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EDITED BY
Sundararajan Jayaraman,
University of Illinois, United States

REVIEWED BY
Anthony T. Reder,
The University of Chicago, United States
Maria Chiara Gemma Monaco,
National Institute of Neurological Disorders and Stroke, United States

*CORRESPONDENCE
Markus J. Hofer
markus.hofer@sydney.edu.au

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Breaking down the cellular responses to type I interferon neurotoxicity in the brain

Barney Viengkhou and Markus J. Hofer*

School of Life and Environmental Sciences and the Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia

Since their original discovery, type I interferons (IFN-Is) have been closely associated with antiviral immune responses. However, their biological functions go far beyond this role, with balanced IFN-I activity being critical to maintain cellular and tissue homeostasis. Recent findings have uncovered a darker side of IFN-Is whereby chronically elevated levels induce devastating neuroinflammatory and neurodegenerative pathologies. The underlying causes of these 'interferonopathies' are diverse and include monogenetic syndromes, autoimmune disorders, as well as chronic infections. The prominent involvement of the CNS in these disorders indicates a particular susceptibility of brain cells to IFN-I toxicity. Here we will discuss the current knowledge of how IFN-Is mediate neurotoxicity in the brain by analyzing the cell-type specific responses to IFN-Is in the CNS, and secondly, by exploring the spectrum of neurological disorders arising from increased IFN-Is. Understanding the nature of IFN-I neurotoxicity is a crucial and fundamental step towards development of new therapeutic strategies for interferonopathies.

KEYWORDS

type I interferons, cerebral interferonopathies, neurotoxin, neurodegenerative diseases, aging, multiple sclerosis, Aicardi-Goutières syndrome, traumatic brain injury

Introduction

Central nervous system (CNS) inflammation is involved in a wide range of neurological disorders and diseases, from pathogen-driven encephalitis and autoimmune disorders to trauma, aging, and neurodegeneration (1–4). The complex nature of inflammation is typically portrayed as either beneficial, such as pathogen elimination, or detrimental, like induction of cell death. Yet in many cases, these processes occur simultaneously and are driven by multiple mediators. The type I interferons (IFN-Is) are master regulators of inflammation. They include the IFN-α subtypes and IFN-β and were originally identified due to their ability to interfere with viral replication (5). However, a vast amount of research over the past 60 years has revealed that IFN-Is have a wide range of roles in addition to regulating inflammation and immunity.

There are three main mechanisms by which IFN-I production and signaling can be increased. Firstly, activation of innate immune sensors by pathogens or cellular danger signals

triggers increased expression of IFN-I genes. For example, cytosolic dsDNA from viruses, damaged mitochondria, or improperly processed self-nucleic acids are recognized by cyclic GMP–AMP synthase (cGAS), which in turn activates the stimulator of interferon genes (STING) (6). Activated STING then triggers a signaling cascade resulting in the upregulation of IFN-I expression (6). In addition to STING, there are multiple other immune sensors that upregulate IFN-I expression in similar ways (7, 8). Secondly, genetic changes can result in increased IFN-I signaling such as in trisomy 21 due to an extra copy of IFN-I receptor 1 (IFNAR1) (9), or reduced negative regulation of the IFN-I pathway such as in patients with mutations in *USP18* or *ISG15* (10, 11). Thirdly, IFN-Is are used as treatment for a range of diseases including chronic viral infections (12), multiple sclerosis (MS), and several cancers and tumors (13–16).

All IFN-Is mediate their cellular effects through binding to a single heterodimeric cell surface receptor consisting of the IFNAR1 and IFNAR2 chains. Activation of the receptor complex triggers two distinct signaling phases (Figure 1). The first phase induces rapid and widespread changes to protein phosphorylation and affects multiple signaling pathways including mitogen-activated protein kinase, cyclin-dependent kinase, and AKT (17). While still not fully understood, it appears that this widespread change in protein phosphorylation prepares the cell for the second phase, which modulates the expression of several hundreds of IFN-regulated genes (IRGs). To make matters more complex, this transcriptional phase mediates its effects through several signaling pathways. Of these, the best understood is the activation of the interferon-stimulated gene factor 3 (ISGF3) complex, which consists of the transcription factors signal transducer and activator of transcription (STAT1) 1, STAT2, and interferon regulatory factor 9 (IRF9). The ISGF3 pathway is often also called the canonical IFN-I signaling pathway and is critical to activate the antiviral response. By contrast, all other pathways are termed 'non-canonical' and are thought to modulate the antiviral response in a cell- and stimulus-dependent context (18–22). Moreover, the signaling components in the IFN-I pathway and can be activated by other cytokines, which complicates defining the precise contribution of IFN-Is in inflammation and immunity *in vivo*. In particular, while IFN-IIIs bind to their unique cell surface receptor, they also mediate their effects through the ISGF3 complex. Recent findings suggest that IFN-IIIs, which consist of the IFN-λs, contribute to neuroinflammation, however, many aspects remain unclear. It appears that IFN-Is are more potent than IFN-IIIs (23, 24) and that the expression of the IFN-III receptor is restricted (25) with very low transcript levels in the brain (23, 24). Thus, while

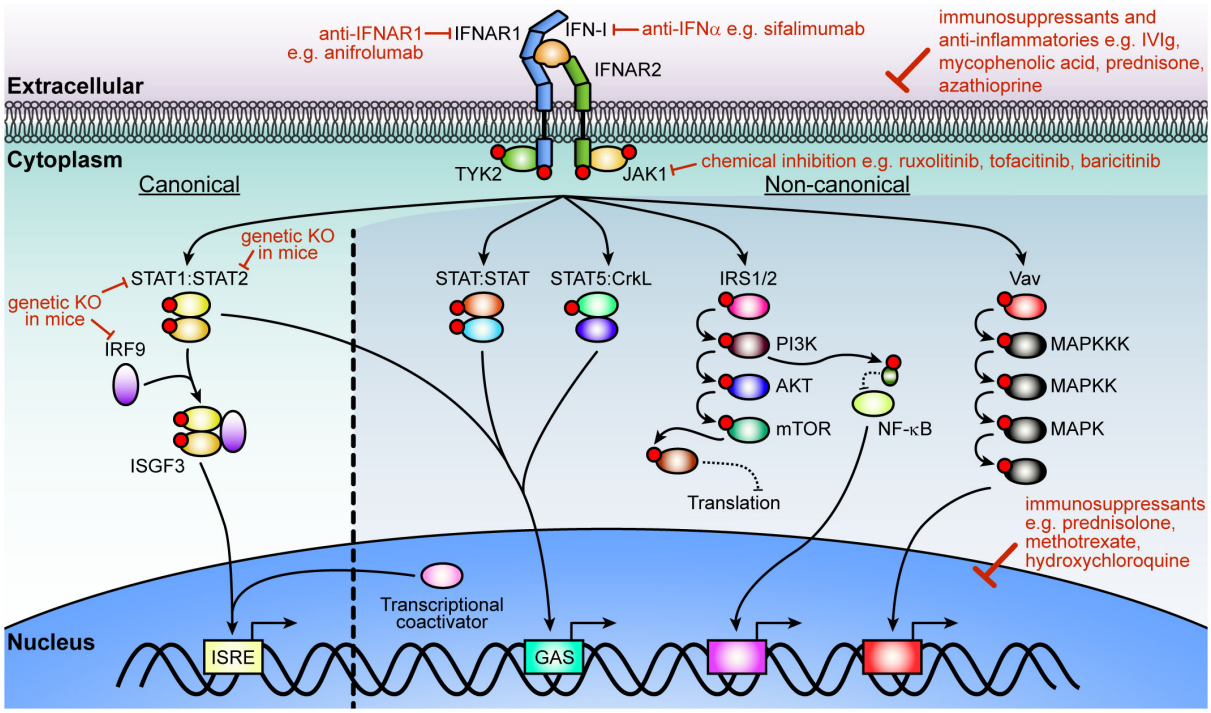

FIGURE 1
IFN-I signaling pathway and strategies of inhibition. After ligation of IFN-Is with its cognate receptor chains, IFNAR1 and IFNAR2, JAK1 and TYK2 transphosphorylate each other before phosphorylating the receptors. In the canonical pathway, STAT1 and STAT2 dock at the receptor to become phosphorylated by the JAKs. Phosphorylated STAT1 and STAT2 then form a trimolecular complex (ISGF3) with IRF9 and translocate into the nucleus to bind ISREs to regulate the expression of hundreds of interferon-regulated genes (IRGs). Non-canonical signaling involves homodimers or heterodimers of STATs, STAT5 binding to CrkL, or recruitment of transcriptional coactivators to regulate ISRE or GAS elements. Additional kinases are activated (PI3K, NF-κB and MAPK pathways) which modulate the cellular response to IFN-Is that includes translation of a subset of genes, regulation of transcription or a range of cellular functions. Multiple strategies have been employed to target IFN-I signaling including inhibition or elimination of proteins in the pathway or its overall effects with immunosuppressants and anti-inflammatories that act on the cell or affect the expression of genes associated with inflammation. Red circles indicate phosphorylation of a protein. IFN-I, type I interferon; IFNAR, IFN-α/β receptor; JAK1, Janus kinase 1; TYK, tyrosine kinase 2; STAT, signal transducer and activator of transcription; IRF9, interferon regulatory factor 9; ISFG3, interferon-stimulated gene factor; ISRE, interferon-stimulated response-elements; GAS, γ-activated sequence; CrkL, Crk like proto-oncogene, adaptor protein; IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase.

we will not discuss the role of IFN-IIIs in detail, it is important to keep in mind that synergism and antagonism of signaling pathways between IFN-Is and other cytokines influences the outcomes of IFN-I-induced cellular and tissue responses.

Although IFN-Is are critical for the physiological regulation of inflammation, they are associated with a range of adverse effects. These adverse effects manifest often as neurological deficits and are commonly observed when IFN-Is are used as a drug or in patients with chronically elevated IFN-I production in the brain (26). Importantly, the cellular and molecular basis for this IFN-I neurotoxicity remains unclear and its study is complicated by the presence of multiple cell types in the CNS (e.g., neurons, glia, and vascular cells), each of which shows unique cell type-specific responses (17, 27–30). Accordingly, in this review, we dissect the complexity of IFN-I neurotoxicity at two levels: firstly, by analyzing the cell-type specific responses to IFN-I in the CNS, and secondly, exploring the spectrum of diseases and symptoms of neurological disorders with increased IFN-Is.

Cellular responses to IFN-Is in the brain

The existence of a homeostatic level of IFN-I signaling in the brain is demonstrated by the presence of IRG products in the healthy brain (31, 32) and reduced expression of IRGs in unstimulated IFNAR1-deficient mice (18) and cells lacking IFN-I signaling proteins (33). The role of homeostatic IFN-I signaling in the brain is diverse and ranges from priming cells for detection and response to pathogens to roles in learning and memory. For example, several studies have shown that neutralization of IFNAR1 results in synapse reduction and impaired synaptic plasticity (34) and ablation of IFN-β leads to defective neuronal autophagy (35). In addition to homeostatic production, IFN-I expression can be markedly increased in most if not all brain-resident cells in response to a range of stimuli. Recent progress in omic analyzes, particularly at the single-cell level, has demonstrated that within the diseased brain, a spectrum of cellular response states occurs simultaneously rather than a uniform response (36–42). Moreover, while all cell types in the CNS can respond to IFN-Is, each cell type mounts its specific response to IFN-Is. Consequently, the sum of the individual responses determines the local tissue response. In the following sections, we will summarize these cell-type specific responses.

Neurons

Neurons require IFN-I signaling for normal development. Homeostatic IFN-β signaling in neurons is involved in the formation of dendritic spines, neurite branching, and neuronal autophagy, while loss of IFNAR1 signaling in neurons leads to formation of protein aggregates or Lewy bodies (35). However, IFN-β injected into the brain also causes a reduction of synapses (43), demonstrating the importance of balanced IFN-I signaling for neuronal function. In response to viral infections, neurons show limited production of IFN-Is (44, 45).

Importantly, while they mount a robust response to IFN-Is, neurons only regulate the expression of a limited set of IRGs (46, 47). This comparatively (to other CNS cell types - see below) narrow response provides antiviral protection and may serve to limit adverse or detrimental effects of IFN-I signaling in these delicate cells. The need to protect neurons from damage is also supported by the elevated basal expression of some IRGs like *ISG15* in neurons compared with other cells contributing to an intrinsic antiviral resistance (48). IFN-I mediated neurotoxicity manifests in neurons after IFN-α treatment with fewer dendrites (49, 50), decreased neuronal neurogenesis (51), reduced neurotrophic signaling (52), and increased apoptosis of precursor cells (53). In addition, IFN-α alters glutamate-induced excitatory potentials in hippocampal neurons and inhibitory post synaptic potentials in pyramidal neurons (47, 54–56). This in turn may increase epileptiform discharges associated with seizures and inhibit long term potentiation, a process important in memory formation (47, 54–56). Moreover, antagonizing the glutamate receptor, N-methyl D-aspartate receptor (NMDAR), reduces the neurotoxicity of IFN-α, indicating a toxic role of IFNAR and NMDAR coactivation (50). IFN-β also modulates ion channels to increase the number of action potentials elicited after activation of protein kinase C (56) and is in line with IFN-β altering glutamatergic neurotransmission (57). In addition, increased cerebral IFN-α levels in transgenic mice with CNS-targeted overproduction of IFN-α (termed GFAP-IFN mice) results in a progressive loss of neurons (58), impaired learning (59), and changes in phosphoproteins that are associated with various neuronal functions (17). Thus, increased IFN-I signaling has detrimental effects on neuronal health and survival.

Astrocytes

Astrocytes are the most abundant glia cell and tile the CNS. Similar to neurons, basal IFN-I signaling in astrocytes is required for a healthy brain. Astrocyte-specific deletion of IFNAR1 results in impaired learning, reduced synapse plasticity, and fewer synapses (34). Following infection with neurotropic viruses, astrocytes are the main producers of IFN-β in mice (44, 60). Their response to IFN-Is is required to limit pathogen replication (61) and to promote blood–brain barrier (BBB) integrity following virus infection (23). Astrocytes alter morphology in response to IFN-Is as observed in brains of patients with increased cerebral IFN-I production (62–64) and GFAP-IFN mice (17, 59). Treatment of astrocytes with IFN-α or IFN-β reduces astrocytic process complexity and domain range and also upregulates genes involved in antiviral responses, metabolism, apoptosis, and major histocompatibility complex (MHC) (17, 27, 39, 59, 62–64). Of note, increased levels of MHC on astrocytes negatively impact neuronal function, activate microglia, and are correlated with social and cognitive deficits in mice (65). Astrocytes can facilitate leukocyte infiltration by increasing chemokine expression after IFN-α treatment (66). In line with this, a subset of astrocytes located around outer cortical blood vessels, and thought to regulate leukocyte access, has been identified as being highly responsive to IFN-Is (39). This highly IFN-I-responsive subset has also been identified in mouse models of Alzheimer's disease (AD), MS, and acute cortical trauma (39). Hypertrophic astrocytes and increased parenchymal leukocytes are also observed in brains of GFAP-IFN mice, supporting a role for astrocytes in mediating leukocyte infiltration (58, 59). While these findings suggest an inflammation-promoting role of IFN-Is on astrocytes, IFN-I signaling in astrocytes can also limit neuroinflammation through the production of the aryl hydrocarbon

receptor and suppressor of cytokine signaling 2, dampening activation of proinflammatory signaling pathways (67). Specifically, mice with astrocyte-restricted *Ifnar1*-knockdown show exaggerated neuroinflammation in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (67). In addition, IFN-α but not IFN-β treatment of human astrocytes reduces proliferation and glucose uptake (68) which impacts the metabolic heath of the CNS. Thus, while the contribution of astrocytes to IFN-I neurotoxicity is not clear, these findings suggest a complex role for astrocytes in modulating IFN-I responses, one that is of increasing interest.

Microglia

Unlike neurons or astrocytes, microglia do not originate from the neuroectoderm. They are derived from the yolk sac and colonize the brain early during embryonic development (69). Microglia are highly plastic and sensitive to the local environment and are considered the key immunoresponsive cell type in the CNS. Microglia produce IFN-α and IFN-β in a wide range of neurological diseases ranging from viral infection to autoimmune disorders (44, 70, 71). Microglia show a more rapid and diverse response to IFN-α compared with astrocytes and neurons (17, 27, 46). Similar to astrocytes, microglia morphology has been used as an indicator of their functional state (72). However, rather than changing into an amoeboid morphology, which is typically observed of microglia in inflammatory situations, in response to IFN-Is, microglia become hyper-ramified with increased process complexity (73). This is also observed in AD and aging (74), indicating microglia are responding to IFN-Is in these conditions. In response to IFN-α, microglia upregulate expression of IRGs, cytokines and chemokines and increase antigen presentation (27), enabling them to act as antigen-presenting cells, propagate inflammation, and promote leukocyte infiltration. This transcriptomic response has been similarly identified in microglia in the aged brain, AD or demyelination in humans or mouse models (38, 40–42). Although most microglia upregulate IRGs, there is a small subset of microglia that are IFN-I-hyperresponsive as identified by single-cell sequencing of a large number of microglia (36, 38, 40, 41). It has been suggested that this hyperresponsive subset contributes to age-dependent cognitive decline and increased synaptic stripping (75–77). In support, minocycline inhibition of microglia activation reduced features of depression and impaired learning of fear extinction in mice injected with IFN-α (78) and use of anti-IFNAR1 treatment in a mouse model of AD demonstrated that IFN-Is promote microglial engulfment of synapses (79). Additionally, minocycline has been used in various neurodegenerative diseases with varied outcomes in animal and human studies (80). However, a recent study using GFAP-IFN mice has demonstrated that depletion of microglia exaggerated disease (81), suggesting that the role of these cells in IFN-I-driven disease may be both beneficial and detrimental.

Oligodendrocytes

Oligodendrocytes have limited responses to IFN-α and IFN-β. In viral infections, oligodendrocytes have low production of IFN-Is and show less expression of IRGs, compared with microglia (82). Additionally, IFN-α or IFN-β have no effect on oligodendrocyte proliferation or survival (31, 51, 83). This suggests on the one hand a partial refractory state of oligodendrocytes to IFN-Is, and on the other hand, that the loss of myelin in neurodegenerative diseases may be an indirect response due to actions from surrounding cells or other mediators rather directly through IFN-I signaling. In support of this, a study using single-cell transcriptomics in a mouse model for MS identified a subset of oligodendrocytes that actively recruit T cells, driving the loss of myelin (37). However, data on oligodendrocyte responses to IFN-Is remains limited and further studies are needed to provide a deeper understanding how IFN-Is affect these cells.

Blood–brain barrier and endothelial cells

The BBB is critical for maintaining CNS homeostasis and brain function (84) and plays crucial roles in neuroinflammation by regulating the migration of leukocytes and diffusion of plasma proteins into the brain parenchyma (85). This separation between blood and brain tissue differs from most other vascular barriers, resulting in vascular cells of the BBB adopting a comparatively distinct phenotype (86). The vascular cells forming the BBB include endothelial cells, pericytes, and mural cells. In particular, cerebral endothelial cells may contribute more to IFN-I signaling in the murine CNS than other cell types as single-cell transcriptomics indicate expression of *Ifnar1* and *Ifnar2* is higher in these cells than in microglia, astrocytes, and neurons (87, 88). Similarly, in humans, *IFNAR2* expression is higher in endothelial cells than glia and neurons (89). This responsiveness of the vasculature is also evident from reports of systemic vasculitis and loss of BBB integrity in patients receiving IFN-Is (55, 90, 91). This vasculopathy is amplified in patients with cerebral interferonopathies and in GFAP-IFN mice, where aneurysms and perivascular calcification are hallmarks of the disease (58, 62, 63, 91). However, the mechanisms leading to these pathologies are unclear, and studies suggest opposing actions of IFN-Is. IFN-α blocks angiogenesis and is toxic to endothelial progenitor cells, contributing to irregular vasculogenesis, abnormal repair and increased atherosclerosis (92). IFN-I therapy can also cause thrombotic microangiopathy and aneurysms (91). The response of endothelial cells in the BBB to IFN-β leads to the secretion of C-X-C motif chemokine 10 resulting in compromised neuronal function and sickness behavior (30). *In vitro* studies support the BBB-damaging effects of IFN-Is, showing that IFN-α and IFN-β enhance endothelial apoptosis and reduce angiogenesis (93–96). Yet, other studies found that IFN-α induces endothelial proliferation (97, 98) and that IFN-β signaling in endothelial cells has anti-inflammatory roles by inhibiting intracellular signaling of proinflammatory pathways and promoting BBB integrity in the host response to viruses and in MS (23, 99, 100). While the basis for these reported differences in endothelial responses to IFN-Is remains unclear, it points to the importance of the subtype of IFN-Is involved and also the context in which IFN-I signaling occurred. Nevertheless, the impact of IFN-Is on the cerebral vasculature has an active role in disease progression of patients with cerebral interferonopathies and in other neurodegenerative diseases. Accordingly, should further studies demonstrate a direct pathogenic role for the brain's vasculature, this would open new therapeutic avenues as in contrast to the brain's parenchyma, the vessels are easily targeted by peripheral drugs.

Neurological disorders with increased IFN-I

There is growing evidence that inflammatory processes and, in particular, IFN-Is, are involved in a wide range of neurological diseases (Table 1) (1–3). The symptomatic overlap between these diseases, as well as the reported adverse effects of IFN-I therapy, suggests a causal contribution of increased IFN-I signaling to their pathogenesis (Figure 2). However, the specific contribution of IFN-Is to the pathogenesis of these diseases is often not well understood.

Type I interferons directly induce neurotoxicity

The direct neurotoxic effects of IFN-Is are well documented due to their clinical use (53, 138–143). Common (>20%) adverse neurological reactions in patients include flu-like symptoms, fatigue, and depression. Less commonly (<5%) observed adverse events include personality changes, cognitive dysfunction, memory loss, mood disorders, psychomotor slowing, and rare (<1%) but severe reactions including psychosis, mania, and seizures. Nature and severity of adverse reactions is dose dependent and generally worsens over time. Fortunately, cessation of treatment leads to an eventual recovery in most cases (140), indicating that these reactions are mediated by IFN-Is rather than the underlying condition for which IFN-Is have been used as treatment. Importantly, the requirement of basal IFN-I signaling for normal brain development suggests a threshold above which IFN-Is become neurotoxic. This is further supported by findings in glioblastomas. In a subset of glioblastoma, stem cells that display elevated cell-intrinsic IFN-I signaling, which contributes to tumor growth, IFN-β treatment can induce cell death, but not in tumor stem cells that have lower cell-intrinsic IFN-I signaling (144, 145). Several mechanisms by which IFN-Is mediate neurotoxicity have been proposed. For example, IFN-α-induced neuropsychiatric symptoms have been associated with changes in glucose metabolism and neuronal circuitry activity in the basal ganglia and prefrontal cortex (146–148), decreased tryptophan availability with altered serotonergic signaling (149–152) and increased presence of proinflammatory cytokines (141, 149, 152–154). IFN-α treatment can also cause retinopathy (30–86% occurrence) (90, 155) and focal BBB leakage which potentially induces seizures in patients (55). Although rare, IFN-α and IFN-β can prompt extensive vascular changes including thrombotic microangiopathy which encompasses endothelial dysfunction, microvascular ischemia, and microangiopathic hemolytic anemia with vascular microaneurysms and stenoses (91).

Effects of chronically elevated type I interferon signaling in the CNS

Diseases associated with chronically elevated levels of IFN-I in the CNS are collectively termed 'cerebral interferonopathies'. This diverse group of diseases may be genetic/hereditary (e.g., Aicardi-Goutières Syndrome (AGS), ISG15 deficiency, and USP18 deficiency), autoinflammatory [e.g., systemic lupus erythematosus (SLE) with neurological manifestation], caused by congenital and chronic viral infections (e.g., infections with *Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus, hepatitis B and C virus, and human immunodeficiency virus), or without known etiologies such as Degos disease (156–158). Given their many shared symptoms and pathological features, cerebral interferonopathies provide valuable insights into the long-term biological effects of increased IFN-I signaling in the CNS.

AGS is the commonly exemplified cerebral interferonopathy whereby mutations in genes involved in nucleic acid detection and metabolism lead to increased intrathecal IFN-α production (159, 160). So far, mutations in nine genes have been identified to cause AGS: *TREX1*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *SAMHD1*, *ADAR1*, *IFIH1*, *LSM11*, and *RNU7-1* (26, 161). It is proposed that loss-of-function mutations in TREX1, RNASEH2, and SAMHD1 lead to the accumulation of immunostimulatory nucleic acid species derived from endogenous retroviral element expression which activate sensors that induces the expression of IFN-Is (162). Similarly, loss of function in ADAR1 results in lack of posttranscriptional modification of endogenous retroviral element transcripts, resulting activation of MDA5, PKR, and ZBP1, which induces IFN-Is and cell death (163–165). Gain-of-function mutations in *IFIH1* cause an overactive gene product, MDA5, and consequently abnormal induction of IFN-Is (166). In contrast to aberrant IFN-I induction through sensing or regulating endogenous retroviral elements, mutations in *LSM11* and *RNU7-1* result in disrupted histone packing of DNA leading to the activation of cGAS/STING to induce IFN-Is (161).

Clinically, AGS has an early onset that mimics transplacental-acquired infections and includes increased mortality before adulthood, irritability, slowed cognitive growth, abnormal movements that develop into ataxia, and epileptic seizures (26, 156, 162, 167, 168). Neuroimaging reveals features including microcephaly, white matter disease, intracranial calcification, necrosis, and vasculopathy with stenosis, moyamoya (small and inadequate vessels formed due to the narrowed cerebral artery), aneurysms, infarcts, and hemorrhage (26, 162, 167, 168). Neuropathological brain examinations show demyelination, perivascular calcification, T-cell infiltration, and apoptotic cells (62–64, 169, 170). Consequently, the clinical and neuropathological observations have led to the proposal of AGS being either a leukodystrophy (171, 172) or a microangiopathy (63, 173). Notably, while vessel disease is a common feature in brains from patients with AGS, whether it mediates pathology or is a consequence of disease has not been clarified. Further, immunohistochemistry has revealed that astrocytes are the main source of IFN-α in the CNS in patients with AGS (62–64) and AGS has thus also been classified as an astrocytopathy by some authors (174). Similar to IFN-I therapy, elevated IFN-α plasma and CSF levels correlate with clinical severity in patients with AGS (160). However, there is a lack of knowledge regarding which cell types and molecular mechanisms mediate disease pathology in AGS, a deficit that also extends to other cerebral interferonopathies. This lack of knowledge stems in large parts from the fact that mouse or zebrafish models that mimic the genetic mutations of patients with AGS, do not recapitulate the human disease (175). By contrast, transgenic mice with increased cerebral IFN-I production (GFAP-IFN mice) – recapitulating the one feature common to of all cerebral interferonopathies – develop closely overlapping clinical and pathological changes also present in patients (Figure 3) (58, 59).

TABLE 1 IFN-I signaling and its inhibition in neurological disorders.

Disorder	Cause of increased IFN-I signaling	Blocked IFN-I induction or signaling	Consequence
Aicardi-Goutières syndrome (AGS)	Genetic mutation in genes associated with nucleic acid regulation	JAK inhibitor	(H) Improvement of symptoms including some neurologic features (101–107)
USP18 deficiency	Mutation in *USP18* that reduces negative regulation of IFN-I signaling	JAK inhibitor	(H) Remission of symptoms (10)
		USP18$^{-/-}$ x IFNAR1$^{-/-}$ mice	(M) Normal phenotype (31)
Systemic lupus erythematosus (SLE)	Unknown	Anti-IFNAR1	(H) Improvements of some symptoms (108, 109) (M) No change phenotypic change (110) (M) Rescue of some autoimmunity features, no change, or worsen survival dependent on model (111)
		Anti-IFN-α	(H) Improvements of symptoms (112)
		JAK inhibitor	(H) Improvements of some symptoms (113)
		x IFNAR$^{-/-}$ mice	(M) Attenuated disease phenotype (114–117)
Chronic viral encephalopathy	Chronic response to viruses	IFNAR1 deficiency	(H) Lethal infection (118)
Aging	Unknown	Anti-IFNAR1	(M) Improved cognitive function, reduced gliosis, and reduced age-related neuroinflammation (119)
		JAK inhibitor	(M) Improved physical functions and coordination (120)
Trisomy 21	Increased expression of IFNAR	JAK inhibitor	(H) Improvement in peripheral symptoms, central symptoms not reported (121–123) (M) Improved survival and reduced loss of weight when immunologically challenged (124)
Alzheimer's disease (AD)	Microglia response to nucleic acid containing plaques (43)	Anti-IFNAR	(M) Restored microglia activity (43) (M) Rescued cognitive function (79)
		$APP_{SWE}/PS1_{\Delta E9}$ x IFNAR1$^{-/-}$	(M) Reduced cognitive decline and anti-inflammatory glia response (125)
Parkinson's disease (PD)	α-synuclein aids in neuron-specific IFN-I responses (126)	MPTP-treated IFNAR1$^{-/-}$ mice MPTP-treatment and anti-IFNAR1	(M) Reduced neuroinflammation and reduced loss of dopaminergic neurons (127)
Huntington's disease (HD)	Activation of cGAS/STING which indues IFN-Is (128) Mutant huntingtin leads to mitochondrial dysfunction which induces IFN-Is (129)	cGAS deletion	(M) Reduced expression of proinflammatory genes and reduced autophagy (128)
Amyotrophic lateral sclerosis (ALS)	Accumulated TDP-43 activates cGAS/STING to induce IFN-Is (130)	SOD1 x IFNAR1$^{-/-}$	(M) Prolonged survival (131)
		x STING$^{-/-}$ mice STING inhibitor	(M) Reduced IFN-I gene expression, prevented loss of neurons, and improved motor function (130)
Prion	STING mediated IFN-I induction (132)	IFNAR1$^{-/-}$ mice	(M) Reduced neuroinflammation and prolonged survival from slowed disease progression (132)
Traumatic brain injury (TBI)	STING-mediated IFN-I induction (4)	STING$^{-/-}$ mice	(M) Reduced neuroinflammation, reduced lesion size, and completion of autophagy process (4)
		IFN-β$^{-/-}$ mice	(M) Reduced proinflammatory response, improved motor and cognitive functions, and reduced neurodegeneration (133)
		Anti-IFNAR1	(M) Improved motor and cognitive functions and no change in lesion volume (133) (M) Reduced infarct volume, reduced inflammatory response, and improved behavioral outcomes (134)
		IFNAR1$^{-/-}$ mice	(M) Reduced infarct volume and reduced inflammatory response (134)
Multiple sclerosis (MS)	Increased around lesions	EAE in IFNAR1$^{-/-}$	(M) More severe disease, increased neuroinflammation, and increased demyelination (28)
		EAE in IFNAR1$^{-/-}$ EAE in IFN-β$^{-/-}$	(M) Increased myelin debris accumulation (71)

(Continued)

TABLE 1 Continued

Disorder	Cause of increased IFN-I signaling	Blocked IFN-I induction or signaling	Consequence
GFAP-IFN mice	Transgenic overproduction of IFN-α in the brain	x IFNAR1$^{-/-}$ mice	(M) WT-like phenotype (91)
		x STAT1$^{-/-}$ mice	(M) Exacerbated disease (91, 135)
		x STAT2$^{-/-}$ mice	(M) Different disease pathology (135, 136)
		x IRF9$^{-/-}$ mice	(M) Exacerbated disease (136, 137)

cGAS, cyclic GMP-AMP synthase; EAE, experimental autoimmune encephalomyelitis, a model for MS; IFN-I, type I interferon; IFNAR, type I interferon receptor; IRF, interferon regulatory factor; JAK, Janus kinase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, used to model PD; STAT, Signal transducer and activator of transcription; STING, Stimulator of interferon genes; USP18, Ubiquitin specific peptidase 18; WT, wild-type; (H) indicates findings in humans and (M) indicates findings in mice.

Chronic infectious encephalopathy

A key feature of the host immune response to pathogens is the rapid production of IFN-Is that activate and regulate both the innate and adaptive immune response (176). The ultimate aim of this immune response is to limit damage to the host, eliminate the pathogen, and re-establish organismal homeostasis. However, in situations where pathogen elimination is not achieved, chronic production of IFN-Is occurs. This is evident in a range of congenital and chronic infections of the CNS including toxoplasmosis, syphilis, rubella, cytomegalovirus, Zika virus, herpes simplex virus and human immunodeficiency virus (177). Many of the clinical and neuropathological findings mirror those observed in patients with AGS (157) including cognitive and motor dysfunction, microcephaly, leukodystrophy, cerebral calcification, loss of neurons, and gliosis (178, 179) (Figure 2). Importantly, these changes are paralleled by elevated cerebral IFN-α levels (180). Further, increased IFN-α levels detected in patients with human immunodeficiency virus are linked to developing neurocognitive disorders (50, 181). Together, these findings indicate a direct association between increased chronic cerebral IFN-I and disease.

Aging

Aging of the brain concurrently occurs with cognitive decline, reduced neurogenesis, cerebral atrophy, waning of cerebral vascular function, and increased neuroinflammation (182, 183), symptoms which are also seen in patients with cerebral interferonopathies (Figure 2). The mechanisms of aging are not well understood and are made more complex by the presence of comorbidities like BBB breakdown (184, 185), dementia, cerebral small vessel disease and neurodegenerative disorders (182, 186). Notably, IFN-β protein and IFN-I signaling are increased in the choroid plexus in the aged CNS of humans and mice (75, 119). Antibody-mediated neutralization of IFNAR1 in mice reversed the aged transcriptomic phenotype while increased IFN-β expression in the choroid plexus of young mice resulted in a transcriptome that reflected that of aged mice (75, 119).

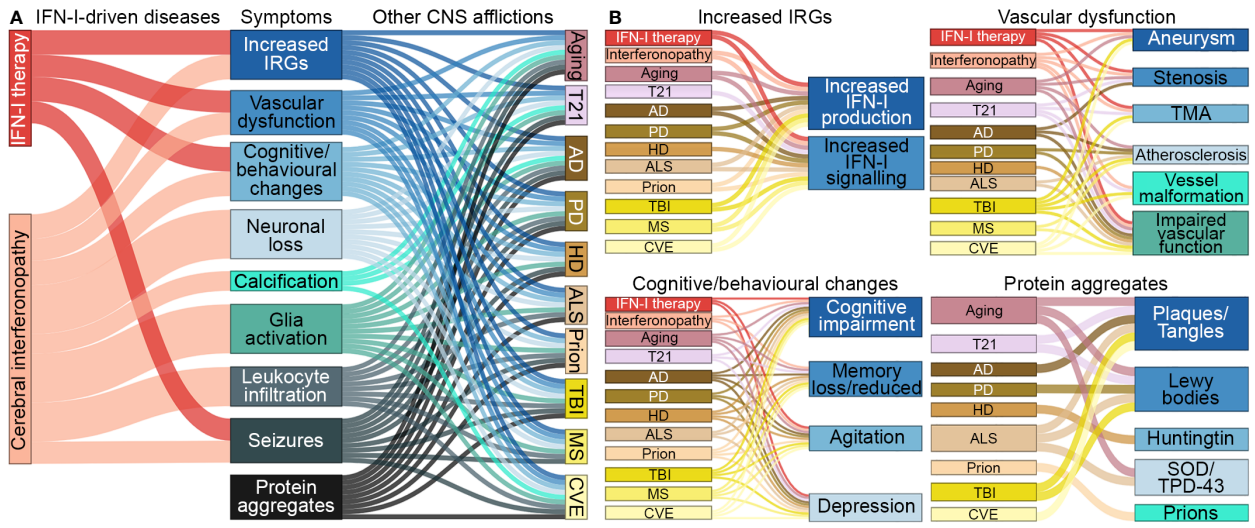

FIGURE 2
Symptomatic links between IFN-I-driven diseases and CNS afflictions. **(A)** Symptoms that arise in diseases driven by IFN-Is overlap with symptoms that occur in aging, trisomy 21 and several neurodegenerative diseases, trauma, autoimmune diseases and chronic viral infections, CNS afflictions found to have increased IFN-I signaling. **(B)** Further breakdown of symptoms linked to each of the CNS afflictions. Note, protein aggregates can lead to increased IFN-Is and expression of IRGs. CNS-centric symptoms were compared and linked if there was prevalence in several human cases. Size of nodes and links are arbitrary. T21, Trisomy 21; AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; TBI, traumatic brain injury; MS, multiple sclerosis; CVE, chronic viral encephalopathy; TMA, thrombotic microangiopathy; SOD, superoxide dismutase; TPD-43, transactive response DNA binding protein 43 kDa.

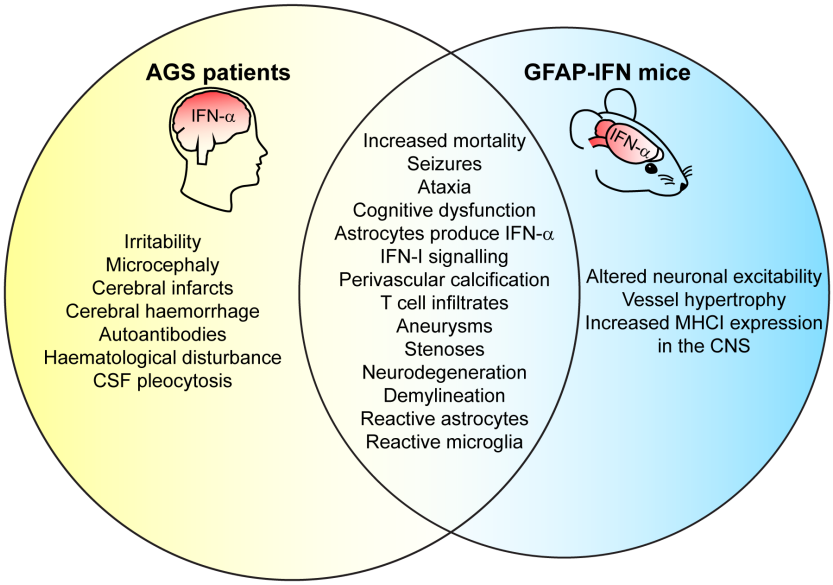

FIGURE 3
GFAP-IFN mice recapitulate clinical and pathological features of patients with AGS. Venn diagram showing overlap between clinical CNS symptoms and neuropathology observed in the GFAP-IFN mice and patients with AGS. Features that do not overlap and/or have yet to been shown in mice or in patients are also indicated.

Additionally, JAK inhibition reduced cellular senescence and improved physical functions in aged mice (120). Thus, aging and increased IFN-I signaling in the CNS appear to be interlinked, with implications for the further study of age-related cognitive decline.

Diseases with abnormal protein aggregation

One important and so far, understudied aspect of neurodegenerative diseases is the co-occurrence of inflammation and increased IFN-I signaling. To date, this has probably been best studied in AD. In brain tissue from patients with AD, expression of IFN-Is and IRGs is increased (32, 187, 188), which is supported by similar findings in mouse models of AD (43, 79, 188). A recent study demonstrated that the induction of IFN-I is due to nucleic acid contained in amyloid-beta plaques that stimulates IFN-β production and IFN-I signaling in microglia (43). A role for increased IFN-Is in AD pathogenesis (rather than just being a bystander effect) has been demonstrated in mouse models, where IFNAR1 deletion or neutralization resulted in downregulated expression of proinflammatory cytokines, attenuated microgliosis, increased complement-mediated synapse engulfment, enhanced astrogliosis, and partial improvement in learning (43, 79, 125). Likewise, patients with mild cognitive impairment had increased blood IFN-I signaling compared with healthy controls, which was further increased in those with AD (189). Of note, in a rat model of AD, IFN-β treatment improved memory and reduced inflammatory markers (190), and in humans with subtle cognitive decline, a preclinical feature of AD, reduced blood IFN-I signaling levels is linked to an increased risk of progression to mild cognitive impairment (189). Thus, IFN-Is display protective and damaging properties in AD.

In Parkinson's disease (PD), increased IFN-Is and IRG products surround Lewy bodies (32, 127, 187), the disease-defining pathological hallmark of PD. Additionally, the protein α-synuclein, that form into Lewy bodies, enhances the signaling of IFN-Is in neurons (126). Ablation of IFNAR1 in a mouse model of PD reduced neuroinflammation and decreased dopaminergic neuronal death (127). The increased IFN-I signaling in the vicinity of protein aggregation, pathological hallmarks of AD and PD, indicates that protein aggregation facilitates localized IFN-I production in surrounding cells. This is supported by studies in mouse models on prion disease, which also involves abnormal protein aggregation. Here, robust IFN-I signaling is seen in microglia (132), and in mice lacking IFNAR1 or STING, disease pathology was delayed (132). Furthermore, increased IFN-I signaling is also observed in the CNS of mouse models of Huntington's disease (HD) (128, 129, 191) and amyotrophic lateral sclerosis (ALS) (130, 131), other disorders with prominent protein aggregates. Together, these findings suggest that protein aggregates are strong inducers of IFN-I signaling and may contribute to disease progression (Table 1).

Traumatic brain injury

Unlike the previous CNS conditions, traumatic brain injury (TBI) involves external physical disruption to the CNS. Symptoms reflect both trauma severity and impact location and may include depression, memory problems, anxiety, agitation, and motor coordination problems (192, 193). The pathological features around the CNS injury site include necrosis, glial cell activation, BBB leakage, neuron degeneration, neuroinflammation, and leukocyte infiltrates (194), features that also occur in cerebral interferonopathies (Figure 2). In response to TBI, chronic local upregulation of IRGs occurs at the injury site, persisting for several months post-injury in both humans and mice (4, 133, 195, 196). Additional increase in IFN-β or IFN-I signaling, for example, in the case of traumatic infection or an aged brain, exacerbates disease outcomes in patients and mice,

whilst loss of *Ifnb* and anti-IFNAR1 treatment in mice attenuates the damage from TBI (133, 134, 196–198), further demonstrating the neurotoxic capacity of IFN-Is.

Trisomy 21

An extra copy of chromosome 21 in humans (trisomy 21) results in diverse symptoms affecting many organs including the CNS. Although symptoms may not all manifest together (9), they include cognitive dysfunction, moyamoya, craniofacial abnormalities, autoimmunity, hematological disorders, intracranial calcification, and early-onset AD (9, 199–203). Some degree of resistance to the development of solid tumors has been observed (9, 199). *IFNAR1* and *IFNAR2* are located on chromosome 21 and their levels are elevated in trisomy 21 (9, 204–206), possibly rendering cells hyperresponsive to IFN-Is. In support, both transcriptomic and proteomic studies of various cell types from trisomy 21 patients show elevated IFN-I signaling and IRG products (9, 204). Notably, many CNS-associated symptoms mirror those observed in cerebral interferonopathies (Figure 2) indicating that increased cerebral IFN-Is may contribute to disability in these patients, and trisomy 21 has been suggested to be an interferonopathy by some authors (9, 204). This in turn opens new therapeutic options for patients with trisomy 21 and accordingly, JAK inhibitors, which block formation of the ISGF3 signaling complex, have been used with some success in case studies and mouse models showing improvements in disease (121–124) and is in a clinical trial (ClinicalTrials.gov Identifier: NCT04246372).

Multiple sclerosis

MS is a demyelinating disease with unclear etiology (207). Patients exhibit a diverse range of symptoms which are largely associated with the location of lesions that occur in the CNS (207). These lesions contain inflammatory leukocytes that presumably mediate oligodendrocyte damage, loss of myelin (208), and local disruption of the BBB (209). IFN-I serum and CSF levels in MS patients do not differ from healthy controls (210). However, there is a focal increase of IFN-I production and IRGs in brain lesions of MS patients and mouse models of MS (71, 211). This mirrors the increase in IFN-I around abnormal protein aggregates and TBI lesions described above, indicating that local production of IFN-I to cellular damage is a common response in the brain. Further, pathological overlaps with AGS/leukodystrophies (212) and MS (Figure 2) such as cerebral small vascular disease exist (213).

Although IFN-Is are produced locally in MS and some mouse models, overall, IFN-I signaling appears to be protective. Genetic ablation of IFNAR1 or IFN-β in mice, results in more severe EAE (28). IFN-β is highly effective for the treatment of MS (IFN-α, although effective, is less well tolerated due to adverse effects including increased occurrence of depression) (16, 214, 215). However, the mechanisms by which IFN-Is are beneficial in MS remain unclear and there is variability in the responses to IFN-β, with some MS patients showing improvement, while others having no change or worsening of disease (216, 217). It has been suggested that some MS patients with IFN-I-induced worsening of disease may have been misdiagnosed; MS and neuromyelitis optica spectrum disorder (NMOSD) can cause very similar symptoms, but in contrast to most MS patients, IFN-Is exacerbate disease in NMOSD (218). In addition, variations in responses to IFN-Is could be due to subnormal serum responses to IFN-Is (219, 220). Thus, it is possible that IFN-β treatment rebalances host IFN-I signaling activity in these patients, rather than being excessive or detrimental.

Therapeutic potential of blocking IFN-I signaling

Currently, there is no cure for cerebral interferonopathies, such as AGS and SLE, and available treatments are primarily aimed at managing symptoms. Treatment is complicated by differences in etiologies, disease progression, severity, and symptoms and importantly by a lack of knowledge regarding the vulnerable and disease-mediating cell types (162). Anti-inflammatory and immunosuppressant drugs (Figure 1) such as corticosteroids or methotrexate are often given to dampen inflammation and reduce infiltrating immune cells, while antiepileptics are used to manage seizures (101, 158, 162, 221–223). Careful consideration is required when devising therapeutic strategies as inactivating canonical signaling factors STAT1, STAT2, or IFR9 in GFAP-IFN mice results in exacerbated disease (135–137), demonstrating that maintaining balanced IFN-I signaling is critical.

Recently, targeting the IFN-I signaling pathway has shown some promise. Treatments with anti-interferon, anti-IFNAR, or JAK inhibitors (Figure 1, Table 1) results in dramatic improvements in some patients with AGS, SLE, and even recovery of patients with peripheral interferonopathies (10, 101–106, 108–112, 221, 224–226). However, these treatments lack support from larger clinical trials, especially in regards to changes in neurological symptoms (162). Importantly, the ability of these treatments to bypass the BBB and improve CNS pathology is yet to be confirmed. Furthermore, the safety profiles of the therapies are noted to include an increased risk of opportunistic infections due to the generalized immunosuppression, as well as an increased risk of major adverse cardiovascular events (227–230). Currently, several clinical trials are underway for patients with AGS (ClinicalTrials.gov Identifier: NCT03921554, NCT04517253, and NCT01724580) and their outcomes will hopefully provide the necessary rationale for the wider use of these treatments. The therapeutic potential of IFN-I signaling inhibition is less clear in the other discussed neurological disorders, with evidence suggesting it may be beneficial in some cases and detrimental in others (Table 1).

Discussion

IFN-Is are a double-edged sword in the CNS. While they are critical for normal brain function and antimicrobial immunity, chronically elevated levels of IFN-Is can be highly neurotoxic. In addition to both the level and signaling duration of IFN-Is, these opposing effects of IFN-Is are in part due to cell-type specific responses, disease-specific contexts, and biological differences between IFN-I subtypes. These parameters modulate the overall tissue response to IFN-Is in the brain. The detrimental effects of IFN-Is are most evident in cerebral interferonopathies which can serve as a paradigm of IFN-I

neurotoxicity, providing valuable insight into a broad spectrum of neurological diseases. Recent advancements with single-cell technologies have provided us with a glimpse of the diversity of the IFN-I responses in the CNS. These studies have provided novel insights into the cell-type specificity of the responses to IFN-Is and demonstrated their variability within a single-cell type. Together, this evidence points to a complex coordination to IFN-Is resulting in a highly stimulus- and time-specific response of CNS-resident cells.

Author contributions

BV wrote the review with revisions by MH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Rajnikant Mishra,
Banaras Hindu University, India

REVIEWED BY
Jinming Han,
Capital Medical University, China
Sachin Tiwari,
Indian Institute of Technology Roorkee, India

*CORRESPONDENCE
Lin Li
lilin.xtt@163.com

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Mechanisms underlying the beneficial effects of physical exercise on multiple sclerosis: focus on immune cells

Boyi Zong[1,2], Fengzhi Yu[1,2,3], Xiaoyou Zhang[4], Wenrui Zhao[5], Shichang Li[1,2] and Lin Li[1,2]*

[1]College of Physical Education and Health, East China Normal University, Shanghai, China, [2]Key Laboratory of Adolescent Health Assessment and Exercise Intervention of Ministry of Education, East China Normal University, Shanghai, China, [3]School of Exercise and Health, Shanghai Frontiers Science Research Base of Exercise and Metabolic Health, Shanghai University of Sport, Shanghai, China, [4]School of Physical Education, Hubei University, Wuhan, China, [5]College of Physical Education and Health Sciences, Zhejiang Normal University, Jinhua, China

Multiple sclerosis (MS) is a prevalent neuroimmunological illness that leads to neurological disability in young adults. Although the etiology of MS is heterogeneous, it is well established that aberrant activity of adaptive and innate immune cells plays a crucial role in its pathogenesis. Several immune cell abnormalities have been described in MS and its animal models, including T lymphocytes, B lymphocytes, dendritic cells, neutrophils, microglia/macrophages, and astrocytes, among others. Physical exercise offers a valuable alternative or adjunctive disease-modifying therapy for MS. A growing body of evidence indicates that exercise may reduce the autoimmune responses triggered by immune cells in MS. This is partially accomplished by restricting the infiltration of peripheral immune cells into the central nervous system (CNS) parenchyma, curbing hyperactivation of immune cells, and facilitating a transition in the balance of immune cells from a pro-inflammatory to an anti-inflammatory state. This review provides a succinct overview of the correlation between physical exercise, immune cells, and MS pathology, and highlights the potential benefits of exercise as a strategy for the prevention and treatment of MS.

KEYWORDS

multiple sclerosis, exercise, immune cell, adaptive immunity, innate immunity

1 Introduction

Multiple sclerosis (MS) is a disease characterized by neuroinflammation, demyelination, and axonal damage, with lesions that involve both the brain and spinal cord. It is estimated that MS affects approximately 2.8 million individuals worldwide, with a higher prevalence in women (1). Symptoms of MS, such as vision loss, numbness, tingling,

motor paralysis, cognitive impairment, and bladder dysfunction, significantly diminish the quality of life for patients (2, 3). In general, the course of MS disease manifests in three main forms: primary progressive MS (PPMS), secondary progressive MS (SPMS), and relapsing-remitting MS (RRMS) (4). Initially, most patients with MS (PwMS) experience the neurological symptoms of RRMS. Within a decade of disease onset, approximately 30-40% of PwMS transition into SPMS, which is characterized by an irreversible and progressive accumulation of neurological disability (3). The disability status of PwMS can be assessed on a scale of zero to ten using the Expanded Disability Status Scale (EDSS), with zero representing a normal neurological examination, and ten representing MS-caused death (5). There is evidence that the disease is associated with genetic, lifestyle and environmental risk factors (6, 7), but the exact cause of MS remains unclear.

The myelin sheath is a protective lipoprotein coating that surrounds axons and is composed mainly of oligodendroglial cell membranes, which help to protect nerves and ensure the normal conduction of nerve impulses. The normal formation of myelin depends on the process of myelination (8). Oligodendrocytes (OLs) are glial cells responsible for myelination, and these cells differentiate from oligodendrocyte progenitor cells (OPCs) (9). However, in MS, dysfunction of OLs and pathology of myelin lead to severe demyelination, impaired remyelination, and axonal degeneration (10). Over the years, the interactions between the immune cell, glial cell, and neuronal cell in the pathology of MS have been extensively studied. In the early stages, pathogenesis is primarily driven by peripheral immune cell responses targeting the CNS (11–13). The peripheral immune cells, such as T cells, B cells, and myeloid cells, infiltrate the CNS and interact with microglia and astrocytes, causing damage to OLs and inhibiting myelin formation (14–19). In the progressive stages, immune responses mediated by CNS-resident microglia and astrocytes predominate (20). In the inflammatory state, microglia generate pro-inflammatory cytokines and chemokines, and increase the expression of costimulatory molecules that facilitate the recruitment and activation of peripheral leukocytes (21, 22). Furthermore, microglia stimulate pro-inflammatory and neurotoxic responses in astrocytes that exacerbate demyelination, neurodegeneration, and atrophy of both grey and white matter (23, 24) (Figure 1). The autoimmune response directed against neuronal axons or synapses interferes with proper neurotransmission, resulting in a variety of motor and non-motor symptoms (25, 26).

Nowadays, pharmacotherapy is considered the primary treatment for MS; however, its efficacy falls short for a significant number of patients. Furthermore, the side effects and exorbitant costs linked with pharmacotherapy may result in reduced patient compliance (27). Non-pharmacological treatments, such as physical exercise, have gained attention as potential disease-modifying therapies for PwMS (28, 29). Physical exercise has been shown to be effective in rehabilitating PwMS, effectively alleviating symptoms, enhancing functionality, improving quality of life, and increasing engagement in daily activities (30–32). Mechanistically, physical exercise provides some protection to the CNS from disease-related atrophy and dysfunction. Structurally, objective research has demonstrated that several months of exercise in PwMS can preserve cortical thickness (33), pallidum (34) and hippocampal volume (35), as well as the microstructural integrity of the insula (36) and motor-related tracts and nuclei (37). Functionally, research has found that exercise can improve functional connectivity between the caudate and the left inferior parietal, bilateral frontal, and right insula regions (38). In addition, exercise can also increase functional connectivity within the hippocampus and the default-mode network (39). Notably, the utilization of animal models is of great value in investigating cellular and molecular mechanisms. Commonly used models include myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) and toxin and/or virus-induced demyelination models, such as cuprizone (CPZ) and lysophospholipid, among others (40). In animal studies, there is evidence that regular exercise training can effectively promote the process of remyelination, alleviate demyelination, and enhance neuroplasticity by modulating the activity and function of OLs and neurons (41–45), and exert neuroprotective effects by reducing oxidative stress (46–49), maintaining the integrity and permeability of the blood-brain barrier (BBB) (48, 50), and adjusting the physiological levels of various exercise metabolites (51, 52). Moreover, it is imperative to recognize the anti-inflammatory benefits of physical exercise, as it not only regulates OLs and neurons, but also influences numerous immune cell types. This review will focus on the effect of physical exercise on neuroimmune regulation in MS, specifically regarding T cells, B cells, dendritic cells, neutrophils, macrophages, microglia, and astrocytes.

2 Effect of physical exercise on immune cells in multiple sclerosis

2.1 Adaptive immune cells

2.1.1 T cells and B cells

Lymphocytes, particularly T cells and B cells, are integral components of the adaptive immune system and are required for immune surveillance of the CNS. They can induce significant immunopathological responses in the presence of viral infections and autoimmune disorders (53, 54). T cells are mainly classified into $CD4^+$ T cells and $CD8^+$ T cells based on distinct cell surface differentiation antigens (55). Aberrant activation of autoreactive $CD4^+$ T cells is considered a primary factor in the development of MS (56, 57). Upon activation, naive $CD4^+$ T cells differentiate into different T helper (Th) cell subsets, including Th1, Th2, Th17, and T regulatory (Treg) cells. These subsets have distinct cytokine profiles and effector functions (58). Th17 cells can release several pro-inflammatory cytokines, such as interleukin 17A (IL-17A), interferon γ (IFN-γ), and IL-22 (57). In EAE, the number of peripheral Th1/Th17 cells increases significantly, as do the levels of IFN-γ and IL-17. These immune cells and their associated cytokines, infiltrate the CNS to exacerbate autoimmune neuroinflammation (59). In addition to neuroinflammation, excessive inflammatory cytokines (such as members of the IL-17 family) can initiate other malignant events. Within the CNS of the

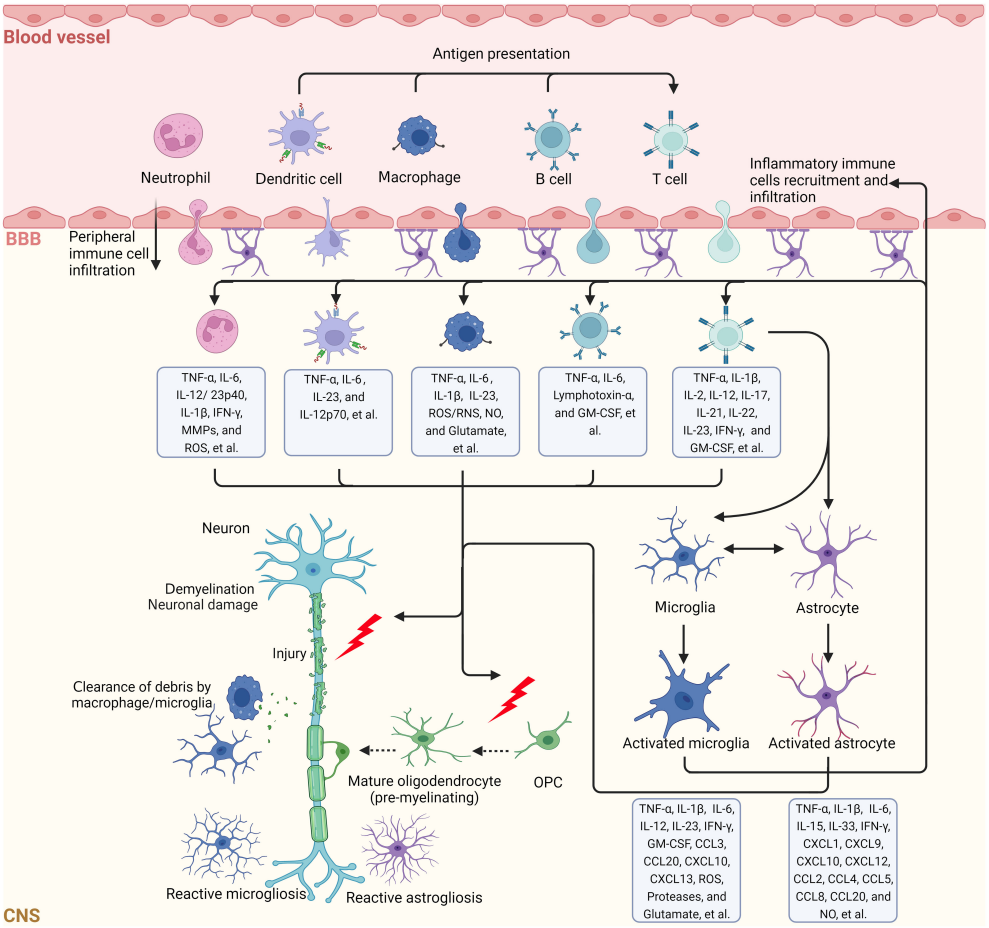

FIGURE 1
Schematic diagram of immune cells-driven multiple sclerosis pathology. In multiple sclerosis, peripheral immune cells, including lymphocytes and monocytes, infiltrate into the central nervous system and secrete pro-inflammatory and neurotoxic substances. These cells, particularly T lymphocytes, possess the ability to interact with CNS-resident microglia and astrocytes, leading to microglial and astrocyte activation and the subsequent release of pro-inflammatory and neurotoxic substances. These substances contribute to the demyelination and neuronal damage, and erode oligodendrocytes, preventing them from forming myelin. Meanwhile, some of the pro-inflammatory substances released by microglia and astrocytes promote the recruitment, infiltration and activation of peripheral immune cells, further enhancing the autoimmune response in the CNS. The figure was created using BioRender. BBB, blood-brain barrier; CCL, chemokine (C-C motif) ligand; CNS, central nervous system; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; MMP, matrix metallopeptidase; NO, nitric oxide; OPC, oligodendrocyte progenitor cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; Th, T helper cells; TNF-α, tumor necrosis factor-α.

EAE model, IL-17 is involved in pain modulation as an upstream regulator of Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) (60). During EAE, overexpression of IL-17A results in impaired long-term potentiation (LTP) and synaptic plasticity in the hippocampus. This leads to cognitive decline through activation of the IL-17A receptor and the p38 mitogen-activated protein kinase (MAPK) signaling pathway, as reported by Di Filippo et al. (26). In contrast, Treg cells possess the ability to release anti-inflammatory cytokines such as IL-10, transforming growth factor β (TGF-β), and IL-35 (61). The beneficial effects of natural Treg cells, which express $CD4^+$ forkhead box protein 3 (FoxP3), and T regulatory type 1 (Tr1) cells, which produce IL-10, on autoimmune neuroinflammation have been demonstrated in both MS patients (62, 63) and experimental animal models (64, 65). A crucial aspect contributing to tissue inflammation in CNS autoimmunity is the impaired functionality of Th17 and Treg cells. It is noteworthy that modulation of the Th17/Treg balance, as well as the functional state of the intrasubsets, can attenuate CNS autoimmunity (66, 67). Different substances, compound 21 (68) and ACDT (69), have demonstrated inhibition of the infiltration of pathogenic Th1/Th17 cells into the CNS in the EAE model. Furthermore, the administration of propionic acid to PwMS resulted in a significant and sustained increase in functional Treg and a significant decrease in Th1/Th17 cells (70). Ultimately, the disease severity of EAE is minimized, or the clinical symptoms of PwMS are reduced.

Alongside $CD4^+$ T cells, some of the cytotoxic $CD8^+$ T cells, such as IL-17-producing $CD8^+$ T (Tc17) cells, have been identified as possible drivers of localized autoimmune damage to the CNS in the EAE model (71). Intriguingly, while in most animal models this is not the case, studies examining human patients have revealed that $CD8^+$ T cells are the main type of T cells present in the CNS of these

individuals (72–74). Inflammatory active lesions in MS are populated by $CD8^+$ tissue-resident memory T cells, exhibiting indications of reactivation and infiltration into the brain parenchyma (73). The $CD8^+$ T cells could serve various functions, as they have been assigned both pathogenic and regulatory roles. On one hand, $CD8^+$ T cells could act as pathogenic effectors that lead to the breakdown of the BBB (75) and promote pathogenic $CD4^+$ T cell activity (71), damage OLs (76) and OPCs (77), and/or direct damage axons (78). On the other hand, $CD8^+$ T cells may regulate pathogenic $CD4^+$ T cells by directly modulating antigen-presenting cells and/or through releasing immunoregulatory cytokines such as IL-10, IFN-γ, and TGF-β (79, 80). Moreover, the efficacy of several therapeutic interventions that selectively deplete B cells (rituximab, ocrelizumab and ofatumumab) highlights the importance of B cells in the pathogenesis of the disease (81). B cells contribute to the pathology of MS through multiple mechanisms. They present antigens to T cells, driving the auto-proliferation of brain-homing T cells (82). Additionally, B cells secrete pro-inflammatory cytokines, such as TNF-α, IL-6, IL-15, and granulocyte-macrophage colony stimulating factor (GM-CSF) (83), and produce extracellular vesicles and antibodies (84). It should be noted that there are distinct functional differences within subpopulations of $CD8^+$ T cells and B cells, emphasising the need for the development and implementation of therapies that target specific pathogenic cell subsets.

Physical exercise has been proven to improve systemic autoimmune inflammation mediated by lymphocytes, in addition to pharmacological treatment, and is generally secure for individuals with autoimmune disorders like systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel diseases and MS, among others (85). Since 2018, numerous studies conducted by Einsteina et al. have investigated the effect of different exercise programs on T cell-mediated autoimmunity from the proteolipid protein (PLP)-induced transfer EAE model in animals. By transferring T cells from lymph nodes (LN-T cells) obtained from mice that underwent six weeks of treadmill running, or from sedentary donor mice, to naive recipients and recipient mice that were either trained prior to EAE induction or sedentary, researchers confirmed that physical exercise limits immune responses to an auto-antigen to weaken EAE, instead of suppressing the immune system in general (86). Further studies have confirmed the superior effect of high-intensity continuous training (HICT) in preventing T cell-induced autoimmunity in EAE through treadmill running, compared to moderate-intensity continuous training (MICT) (87). Remarkly, variations were found in the mechanisms by which continuous and intermittent exercise, performed at the same high intensity, alleviated systemic autoimmunity and T cell encephalitogenicity. Specifically, HICT impeded PLP-induced T cell proliferation without affecting T cell differentiation, while high-intensity intermittent exercise (HIIT) had no noticeable impact on T cell proliferation but hindered T cell polarization into Th1 and Th17 pro-inflammatory phenotypes (88). Taken together, because of the significant variation observed across different disease trajectories, it is essential to implement effective intervention programs that are customized to suit the specific characteristics of each phase of the disease.

In other previous animal studies, mice that underwent regular swimming exercise before EAE induction showed suppressed infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and B cells into the spinal cord. Meanwhile, the proliferation of antigen-specific T cells was halted and the proliferation of Treg cells was promoted, while restricting the secretion of IFN-γ and IL-17 and enhancing the secretion of IL-10 and TGF-β. Furthermore, regular swimming exercise also alleviated damage to myelin and axons and reduced clinical scores (89, 90). Notably, research suggests that high-intensity swimming (4% body weight) may prove more effective than moderate-intensity swimming (0% body weight) (90). It seems that swimming exercise represents a noteworthy non-pharmacological intervention for improving chronic inflammation or autoimmunity; however, the success of this intervention could be modified by the intensity of the exercise. In addition, it is probable that the efficacy of exercise interventions is also reliant on the type of exercise employed. Over a four-week period, it was observed that both strength and endurance training programs impeded the development and progression of disease, improved genomic antioxidant defense-nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response elements (ARE) pathway, lowered the production of IFN-γ, IL-17, and IL-1β, reduced the expression of adhesion molecules, such as platelet and endothelial cell adhesion molecule 1 (PECAM-1), and reinstated the expression of tight junction proteins such as occludin and claudin-4 in the spinal cord after EAE induction. However, only strength training significantly increased the expression of Treg cell markers, specifically CD25 and IL-10, obtained from spleen cells, and inhibited the production of IL-6, monocyte chemotactic protein 1 (MCP-1), and TNF-α (48). Further analyses revealed that while endurance exercise was superior in delaying disease progression and lowering clinical scores as well as antioxidants, strength training was more effective in improving immune system function. Voluntary wheel running, as a rehabilitation approach, has been demonstrated as an effective intervention for promoting motor recovery. Regular voluntary wheel running had a significant positive effect on demyelination and axonal damage in EAE mice, in comparison to their sedentary counterparts. However, the impact of lymphocyte infiltration was insignificant (47, 91). Additionally, gender of the subjects must be taken into consideration as it may have an influence on the exercise intervention's efficacy (47, 92). Further, a study has investigated the potential of combined interventions and has discovered a substantial positive interaction between exercise and galantamine medication. The outcome of this interaction led to a notable rise in the quantity of $Foxp3^+$ T cells in the brainstem of rats affected by EAE (93). The animal studies' collective findings suggest that physical exercise could potentially suppress lymphocyte infiltration, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells. Additionally, it could modulate the Th cell phenotype and regulate related cytokine levels, eventually leading to a reduction in autoimmune responses in the CNS, an improvement in MS pathology, and a decrease in disease severity (Table 1). Nonetheless, the effects of exercise may be impacted by different aspects of the exercise intervention, such as the type and intensity of the exercise, as well as the heterogeneity of the subjects.

TABLE 1 Effect of exercise on adaptive immune cells in animal models and human patients of MS.

Subjects		Exercise intervention program			Region	Mode of action	References
Model	Characteristics	Type	Intensity	Duration			
MOG_{35-55}-induced EAE model	C57BL/6 mice; female; 8 weeks old	Voluntary wheel running	ND	60 min/ session for 40 consecutive days	Brain and spinal cord	$CD4^+$ T cells, and $CD8^+$ T cells infiltration →; Synaptic plasticity ↑; Clinical scores ↓	(91)
	C57BL/6 mice; female; 7 weeks old	Prior swimming	7% BW	30 min/ session, 5 sessions/ week for 6 weeks	Spinal cord	B cells, $CD4^+$ T cells, and $CD8^+$ T cells infiltration ↓; Myelin and axonal damage ↓	(89)
	C57BL/6 mice; female; 6~12 weeks old	Strength training (ST): Climbing the ladder Endurance training (ET): Treadmill running	(i) ST: 25%, 50%, and 75% BW (ii) ET: 13~17 m/ min	30 min/ session, 5 sessions/ week for 4 weeks	Spinal cord	(i) ST: Treg cell markers: CD25 and IL-10 ↑; IFN-γ, IL-17, and IL-1β ↓; Clinical scores ↓; Protein oxidation and NO levels ↓; GPx activity ↑; TJPs ↑; CAMs ↓ (ii) ET: IFN-γ, IL-17, and IL-1β ↓; Clinical scores and weight loss ↓; Lipid peroxidation, protein oxidation and NO levels ↓; GPx activity, GSH content and Nrf-2 expression ↑; TJPs ↑; CAMs ↓	(48)
	C57BL/6 mice; male and female; 6~8 weeks old	Voluntary wheel running	ND	60 min/ session for 30 consecutive days	Spinal cord	(i) Male: $CD4^+$ T cells infiltration →; Demyelination and axonal loss ↓; Oxidative stress ↓; Clinical scores → (ii) Female: No effect	(47)
	C57BL/6 mice; female; 6~8 weeks old	Prior swimming	(i) HE: 4% BW (ii) ME: 0% BW	50 min/ session, 5 sessions/ week for 6 weeks	Spinal cord	(i) HE: Treg proliferation ↑; Antigen-specific T cell proliferation ↓; Th1 and Th17 populations ↓; IFN-γ and IL-17 ↓; IL-10 and TGF-β ↑; BDNF ↑; Demyelination ↓; Clinical scores ↓ (ii) ME: No effect	(90)
	C57BL/6 mice; male and female; 6~8 weeks old	Voluntary wheel running	ND	60 min/ session, 6 sessions/ week for 1 week	Spleen	(i) Male: T cell proliferation ↑; IFN-γ, TNF-α, IL-17A ↑; Dorsal root ganglia excitability and calcium responses →; Nociceptive behaviour →; (ii) Female: T cell proliferation ↑; IFN-γ, TNF-α, IL-17A ↓; Dorsal root ganglia excitability and calcium responses ↓; Nociceptive behaviour ↓	(92)
	C57BL/6 mice; female; 3~4 weeks old	Stair climbing	ND	20, 40, and 60 min/ session, 6 sessions/ week for 4 weeks	Intestine lymphoid tissues and spinal cord	Th17 responses ↓; Treg responses ↑; IL-17A and IFN-γ ↓; Firmicutes/Bacteroidetes ratio and intestinal mucosal permeability ↓; Microbial abundance and diversity ↑; Demyelination and axonal damage ↓	(94)
	SD rats; male; 2~3 months old	Walking on a rotating metallic rod with galantamine	ND	30 min/ session for 30 consecutive days	Brain stem and cerebrospinal fluid	$Foxp3^+$ T cells ↑; TNF-α and IL-6 ↓; Demyelination ↓; BDNF and Bcl-2/Bax ratios ↑; Motor performance ↑	(93)
CPMS and RRMS patients	12 female/10 male; (46.0 ± 2.0) years old; EDSS score < 6	Endurance training (walking and bicycling) combined with strength training (resistance exercise)	(i) ET: 65% HRR (ii) ST: 70% 1RM	A single bout	Blood	(i) Immediate post-exercise: Lymphocytes number ↑; $CD25^{hi}Foxp3^+$ Treg and antigen-induced IL-10-producing Tr1 number ↑; Th3 cells number → (ii) 2 hours post-exercise: Lymphocytes number ↑; $CD25^{hi}FoxP3^+$ Treg and antigen-induced IL-10-producing Tr1 number ↑; Th3 cells number →	(95)
	16 female/13 male; (46.0 ± 2.0) years old; EDSS score (3 ± 0.2)	Endurance training (cycling and treadmill walking or running) combined with strength	ND	5 sessions/2 weeks for 12 weeks	Blood	Treg cells number and proportion →; CD25hi$Foxp3^+$, Tr1, and Th3 cells →	(96)

(Continued)

TABLE 1 Continued

Subjects		Exercise intervention program			Region	Mode of action	References
Model	Characteristics	Type	Intensity	Duration			
		training (resistance exercise)					
RRMS patients	7 female/1 male; (41.1 ± 12.9) years old; EDSS score < 2	Strength training combined with bicycling	ST: <35%, 35%~65%, >65% 1RM; AT: 60% AC_{max}	60 min/ session, 12 weeks	Blood	TNF-α and IL-6 ↓; IL-22 ↓; IFN-γ and IL-17 →; IL-10 ↑; Fatigue ↓	(97)
	7 female/12 male; 20~60 years old; EDSS score < 4.5	Normoxic (N) or hypoxic (H) treadmill training	65% HR_{max}	60 min/ session, 3 sessions/ week for 4 weeks	Blood	(i) N: $CD39^+$ Treg cells ↑; $CD31^+$ Treg cells ↓; IL-17A-producing $CD4^+$ T cells ↓; Fitness and mood ↑ (ii) H: $CD39^+$ Treg cells, $CD31^+$ Treg cells, and IL-17A-producing $CD4^+$ T cells →; Fitness and mood ↑	(98)
PwMS treated with either ATZ, FTY, or NAT	17 female/13 male	Climbing stairs at normal speed (CN) or fast (CF) or cycling (C)	C: 1, 2 Watt per kilogram BW	CN: ND CF: ND C: 20 min	Blood	(i) CN: Absolute lymphocyte number ↑ (ii) CF: Absolute lymphocyte number ↑; $CD19^+$ B cell, and $CD3^+$ T cell number ↑ (iii) C: Absolute lymphocyte number ↑	(99)

1RM, repetition maximum; AC_{max}, maximal aerobic capacity; ATZ, alemtuzumab; Bax, BCL2-associated X; Bcl2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; BW, body weight; CAMs, cell adhesion moleculars; CPMS, chronically progressive MS; EAE, experimental autoimmune encephalomyelitis; EDSS, Expanded Disability Status Scale; Foxp3, forkhead box protein 3; FTY, Fingolimod; GPx, glutathione peroxidase; GSH, glutathione; HE, high-intensity exercise; HR_{max}: maximal heart rate; HRR, heart rate reserve; IFN-γ, interferon-γ; IL-10, interleukin-10; ME, moderate-intensity exercise; MOG, myelin oligodendrocyte glycoprotein; NAT, natalizumab; ND, not determined; NO, nitric oxide; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PwMS, patients with MS; TGF-β, transforming growth factor-β; Th, T helper cells; TJPs, tight junction proteins; TNF-α, tumor necrosis factor-α; RRMS, relapsing-remitting MS. ↑, significantly increased or improved; ↓, significantly decreased or reduced; →, no significant change.

In human studies, lymphocyte proliferation has been observed to be suppressed after acute exercise in healthy individuals. This effect is more pronounced during exercise sessions exceeding an hour in duration, regardless of exercise intensity (100). However, some studies have reported inconsistent findings. For example, individuals who are healthy controls and those with PwMS receiving alemtuzumab, fingolimod, or natalizumab displayed an increase in the absolute number of lymphocytes and specific subsets following exercise. The degree of response was impacted by the intensity of the exercise program (99). In Deckx's study, naturally occurring $CD25^{hi}Foxp3^+$ Treg cells and antigen-induced IL-10-producing Tr1 cells increased in the peripheral blood of patients with chronically progressive MS and RRMS following a single session of moderate-to-high-intensity endurance with resistance exercise. The number of Tr1 cells remained elevated for up to two hours after exercise (95). This increase in Treg cells may serve as a negative feedback mechanism to the immune system's capacity to elicit tissue damage and inflammation when responding to exercise. Moreover, the findings of regular exercise intervention studies require careful observation. A four-week experiment of treadmill running in normoxic conditions (rather than hypoxic conditions) caused modifications in circulating Treg subpopulations among patients with RRMS. These alterations comprised of an increase in $CD39^+$ Treg cells and a decrease in $CD31^+$ Treg cells, as well as a reduction in IL-17A-producing $CD4^+$ T cells. These results imply that treadmill running has a vital function in adjusting the adaptive immune response in MS through impacting distinct T cell subsets (98). Remarkably, conflicting results have also emerged. As early as 2012, a cross-sectional study revealed that there were no discernible differences in the proportions of circulating $CD4^+$ T cells (including $Foxp3^+$ Treg cells), $CD8^+$ T cells, and B cells in the peripheral blood between physically active and inactive PwMS, and no correlation with physical performance parameters (101). These findings suggest that prolonged physical activity may not have a significant impact on the adaptive immune cells in PwMS. In accordance with this, Deckx et al. (96) discovered that 12 weeks of endurance and strength training had no effect on the circulating Treg subsets, including $CD25^{hi}Foxp3^+$, Tr1, and Th3 cells in PwMS. These inconsistent findings in human patients have significant implications for experimental and clinical research, particularly regarding the development of interventions to address autoimmune factors in MS.

The BBB is a dynamic interface linking the blood with the brain parenchyma. It comprises capillary endothelial cells (ECs) from the brain and spinal cord, and perivascular cells including smooth muscle cells, microglia, pericytes, and astrocytes. Of note, the ECs have adherens junctions and tight junctions between cells and lack fenestration (102). It has been suggested that the destruction of BBB integrity and permeability may be the initial pathological features of MS. This results in the infiltration of immune cells from the periphery into the brain parenchyma (103). This is indicated by changes in biomarker levels, such as enzymes gelatinase A/MMP-2 (104), gelatinase B/MMP-9 (105), S100 calcium-binding protein B (S100B) and neuron-specific enolase (NSE) (106), among others. Although it is unclear whether the destruction of the BBB is the cause or the result of MS, several studies have confirmed that MS-related neuroinflammation has an impact on the structure and function of the BBB (107). Physical exercise has been shown to

regulate BBB permeability through various pathways, including systemic inflammation, the brain renin-angiotensin and noradrenergic systems, central autonomic function, and the kynurenine pathway (108). In human studies, Mokhtarzade et al. (106) found that acute cycling causes a significant increase in circulating S100B, but has no effect on NSE in RRMS patients. Proschinger et al. (109) showed that a 12-month combination of functional resistance and endurance training programs reduce serum MMP-2 concentration in RRMS patients. Furthermore, Zimmer et al. (104) discovered that patients with RRMS or SPMS who participated in HIIT or MICT programs for three weeks reported a significant decrease in serum MMP-2 levels, while the level of MMP-9 remained stable. Therefore, exercise can partially ameliorate the disruption of the BBB in PwMS, as evidenced by circulating biomarkers. Tight junction proteins, consisting mainly of transmembrane and cytoplasmic proteins, are essential components of the BBB. The transmembrane structure of tight junctions is comprised primarily of three classical proteins: claudins, occludins, and junction adherence molecules. Furthermore, the support structure of tight junctions is established by cytoplasmic attachment proteins such as zonula occludens (ZO) and cingulin, among others (110). During the development of neuroinflammation, certain chemokines and cytokines may induce the expression of EC adhesion molecules, specifically intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, and PECAM-1, among others. As a result, peripheral immune cells could cross the BBB (111). Abnormal expression of tight junction proteins has been observed in animal models of MS and in human studies. For instance, the permeability of the BBB to Evans blue in the brain homogenate of mice with EAE significantly increased, accompanied by a reduction in claudin-5, occludin and ZO-1, while ICAM-1 and VCAM-1 expression increased (112). Similar findings were attained by other researchers in their evaluation of the degree of loss or redistribution of tight junction proteins, and the expression of ICAM-1 and VCAM-1 in the brains of EAE models (113). Another animal research demonstrated that after four weeks of strength or endurance training programs, the expression levels of tight junction proteins, including occludin and claudin-4, were restored in the CNS, and the expression of PECAM-1 was significantly suppressed, thus preserving the BBB from injury in EAE (48). A recent study conducted by Hamdi et al. (114) has implemented a PLP-induced transfer EAE model. The results show that HICT has an impact on T cell migration and invasion and is linked to a decrease in interactions between very late antigen 4 (VLA-4)/VCAM-1 and lymphocyte function antigen 1 (LFA-1)/ICAM-1. Thus, physical exercise could indirectly regulate lymphocyte infiltration by modifying BBB integrity and permeability.

In light of emerging evidence on the disruption of gut microbiota in PwMS, the mechanism by which gut microbiota disorder exacerbates the condition is progressively becoming more apparent (115–118). Studies have shown that the intestinal microbiome can promote the development of CNS-reactive pathogenic T cells in both EAE (119, 120) and MS (121). Aberrant alterations in colony patterns were noted in PwMS. These changes were accompanied by an increase in *Desulfovibrionaceae*, *Akkermansia muciniphila*, and *Acinetobacter calcoacetius* levels, among others, as well as a decrease in *Faecalibacterium prausnitzii*, *Parabacteroides*, *Prevotella*, and *Bacteroides fragilis* (122). There is an increasing body of evidence that suggests physical exercise could positively influence the composition and function of the gut microbiota (123–125). The implementation of a four-week strength training program, performed six times per week, led to significant outcomes in EAE. Specifically, this intervention resulted in increased abundance and diversity of gut microbiota, a decrease in the *Firmicutes* to *Bacteroidetes* ratio, and improvement in intestinal mucosal permeability. Various bacteria including *Akkermansia*, *Clostridium*, *Parabacteroides*, *Christensenella*, *Dorea*, *Roseburia*, and *Paraprevotella* can produce short-chain fatty acids (SCFAs). The training program efficiently decreased Th17 responses and increased Treg responses in lymphoid tissues of the small intestine. It is noteworthy that after completing four weeks of strength training, with each session lasting up to 60 minutes, there was a significant improvement in disease severity and neuropathology in EAE. Moreover, the microbiome fecal transplantation of trained mice into microbiota-depleted mice alleviated disease severity and neuropathology scores in microbiota-depleted mice relative to controls. However, shorter training durations, either 20 or 40 minutes per session, do not appear to affect T cell-mediated autoimmunity in EAE (94). These observational data indicate that the modulation of gut microbiota through exercise represents a mechanism that can improve T cell-mediated autoimmunity in MS. The beneficial effects of exercise on the pathology of EAE mice may be affected by the duration of training sessions, except for exercise type and intensity. For human patients, a brief high-impact multidimensional rehabilitation program that incorporates physical activity in a leisurely setting has demonstrated a decrease in proportions of pathobionts, such as *Collinsella* and *Ruminococcus*, while increasing amounts of SCFA producers, such as *Coprococcus*, *Bacteroides*, and *Oscillospira*. The alterations in the colony were associated with a reduction in the quantity of pro-inflammatory T lymphocyte subpopulations, especially $CD4^+$/IFN-γ^+ Th1 cells and $CD4^+$/ROR-γ^+ and $CD4^+$/IL-17^+ Th17 cells, as well as a decrease in circulating lipopolysaccharide (LPS). Simultaneously, the rehabilitation program also improved physical performance and relieved fatigue (126). In a separate study, a six-month home-based exercise training program held with a frequency of five sessions per week exhibited a significant increase in *Prevotella* populations and a reduction in *Akkermansia muciniphila* populations among PwMS. Furthermore, this intervention had a positive effect on adverse psychological states such as anxiety and depression. However, no substantial influence was observed on fatigue, *Faecalibacterium prausnitzii* and *Bacteroides* counts, or the presence of anti-inflammatory cytokines in the serum. Nonetheless, changes in *Akkermansia muciniphila*, *Prevotella*, and *Bacteroides* counts in response to the intervention were correlated with changes in IL-10 (127). The above results strongly indicate that exercise can elicit neuroimmunomodulatory effects by regulating the gut microbiome.

2.2 Innate immune cells

2.2.1 Dendritic cells

Dendritic cells (DCs) are specialized antigen-presenting cells and are vital regulators of innate and adaptive immune responses (128). They have the ability to express many molecules associated with antigen presentation that interact with T cells, including major histocompatibility complex-I (MHC-I), MHC-II, and CD1, as well as co-stimulatory molecules, including CD80, CD86, and CD40 (129). Moreover, upon activation, DCs also produce multiple cytokines, such as GM-CSF, IL-23 (130, 131), and IL-27 (132), which direct the differentiation of naive T cells. A human study compared the phenotypes and cytokine secretion of DCs among PwMS, individuals with other neurological disorders, and healthy controls. The research discovered that the number, morphology, and phenotype of DCs were comparable in PwMS and healthy controls. The phenotypic features included immature myeloid lineages such as $CD1a^+$ and $CD11c^+$. However, PwMS showed a higher proportion of $CD1a^+$ DCs and a lower proportion of $CD86^+$ DCs compared to controls (133). It is evident that alterations in the surface molecules of DCs, which have functional significance, are related to MS. In the EAE model, dysfunctional or deficient DC genes result in abnormal responses from effector T cells. For instance, researchers have identified that mammalian sterile 20-like kinase 1 (MST1) to be an essential regulator of EAE, promoting Th17 differentiation depending on DCs. The absence of MST1 in DCs causes $CD4^+$ T cells to produce higher quantities of IL-17, whereas the amplification of MST1 in DCs restrains IL-17 production. Mechanically, activation of p38 MAPK signaling occurs in DCs lacking MST1, resulting in increased IL-6 secretion in Th17 differentiation induction and the activation of IL-6 receptor α/β and signal transducer and activator of transcription 3 (STAT3) in $CD4^+$ T cells (134). Additional *in vivo* research with rodents revealed worsened autoimmune neuroinflammation with increased Th17 cell polarization during EAE induction in REGγ-deficient mice. Moreover, ex vivo experiments have confirmed that a REGγ deficit enhances integrin αvβ8 expression in DCs, which stimulates TGF-β1 maturation and promotes Th17 cell development. The process is supported by REGγ proteasome-dependent degradation of IRF8 (135). DCs play an important role in immune regulation initiation and maintenance of inflammatory events. It is essential to conduct further research on DC genes that affect T cell-mediated pathology in MS. This will improve our basic understanding of MS pathogenesis and support the creation of more effective treatments for this disease. Bilirubin nanomedicine (136), urolithin A (137), and optineurin (138) have already been demonstrated to be effective in impacting disease progression by regulating the activity and function of DCs.

Furthermore, it should be noted that DCs may also exhibit heterogeneity in the pathogenesis of MS. DCs are generally classified into two main subsets, referred to as myeloid/conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Interestingly, cDCs and pDCs obtained from PwMS manifested significant tolerogenic (139) or regulatory effects (140) in comparison with control groups. The cDCs are further categorized into cDC1 and cDC2 cells, which exhibit distinct ontogenies, surface markers, localizations, and immunological functions (141). In a stable condition, the cDCs commonly reside in the meninges, brain, and spinal cord of the CNS. They are capable of stimulating the activation and secretion of pro-inflammatory cytokines directly ex vivo from naive, effector, myelin-specific T cells. The population of cDCs increases in the meninges and CNS parenchyma during the development of EAE. Upon selective depletion of cDCs, the quantity of myelin-primed donor T cells in the CNS decreased, resulting in a 50% reduction in the incidence of clinical presentation (142). The pDCs can be subdivided into pDC1 and pDC2. The former displays increased levels of CD123 expression, while demonstrating decreased expression of CD86 and Toll-like receptor 2 (TLR2). It also facilitates the secretion of IFN-α and IL-10. Conversely, the latter subtype, pDC2, exhibits reduced expression of CD123 but higher expression of CD86 and TLR2. It promotes the secretion of TNF-α and IL-6 (143). Thewissen et al. (144) reported that circulating DCs in PwMS demonstrate a pro-inflammatory state and possess a migratory phenotype. DCs derived from MS patients exhibited increased production of IL-12p70 following TLR ligation. Additionally, these DCs had heightened expression levels of the migratory molecules C-C chemokine receptor 5 (CCR5) and CCR7, as well as improved *in vitro* chemotaxis when compared to healthy controls. Another study showed a significant alteration in the pDC1/pDC2 ratio, with a ratio of approximately 4.4:1 observed in healthy controls and 0.69:1 observed in PwMS. This shift towards pDC2 may contribute to the preferential activation of IL-17-secreting cells in MS, over IL-10-secreting $CD4^+$ T cells (145). The concurrent occurrence of various DC subpopulations suggests their dual function in MS pathology.

In 2007, research conducted on DCs in Sprague-Dawley rats showed that progressive endurance exercise for five weeks modified the development of DCs and directed them towards a more mature state (146). However, in studies of animal disease models, Mackenzie et al. (147) found that four weeks of treadmill running led to a reduction in DC activation. This was shown by a decrease in production of the inflammatory markers IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1)/KC, IL-12p70, and TNF-α, as well as a decrease in MHC-II expression, indicating a decrease in DC maturation (147). It also appears that the effects of exercise on different DC subtypes may vary considerably. In a study of an asthma model, a four-week treadmill exercise program led to a reduction of co-stimulatory molecules, CD80, CD86, and inducible T-cell costimulator ligand (ICOSL), in cDCs located in the lymph nodes that drained from the affected areas, and an increase in ICOSL expression in pDCs (148). Human studies have demonstrated that acute exercise causes a transient increase in DCs in the blood and a greater mobilization of pDCs than cDCs (149). In patients suffering from chronically progressive MS or RRMS, an increase in the numbers of cDC and pDC, along with the expression of the cell adhesion molecule CD62 ligand (CD62L) and CCR5, were noticed after a session of endurance and resistance training, and most of the markers did not return to their resting state within two hours of exercising. This increase may be mediated by FMS-like tyrosine kinase 3 ligand (FLT3L)- and MMP-9-dependent DCs mobilization. Acute exercise can potentially

reduce the responsiveness of circulating DCs to TLR, thus establishing a negative feedback regulatory mechanism to counteract the heightened inflammatory state resulting from acute exercise (95). In the chronic exercise intervention program, a 12-week training program that combining endurance with resistance exercise significantly increased the absolute number of pDCs in patients with chronically progressive or RRMS. This increase was observed specifically in those pDCs expressing CD80 and CD62L, whereas there were no significant changes in cDCs. Further analysis demonstrated a positive correlation between the quantity of $CD80^+$ pDCs and IL-10-producing Tr1 cells. These findings suggest that regular exercise may enhance the immunomodulatory function of circulating pDCs. Moreover, the exercise program suppressed the production of TNF-α and MMP-9 by DCs in response to TLR activation, indicating that the program could reduce inflammation in individuals (96). Although acute exercise resulted in an elevation of cDCs and pDCs, that is not indicative of an exercise-induced response of DCs contributing to the advancement of an inflammatory state. Additionally, a regular exercise program in PwMS can result in an increase in activated pDCs, and is associated with the occurrence of Tr1 cells. However, only two human investigations have studied the influence of exercise on DCs in MS; further research is necessary on this issue.

2.2.2 Neutrophils

Neutrophils, which originate from the bone marrow, are the most prevalent leukocyte in peripheral blood and are crucial for non-specific host defense. They are responsible for phagocytosis of microbial, bacterial, and viral pathogens, while also producing and releasing cytokines that regulate T cell and B cell activities (150). Several studies have shown that neutrophils in PwMS exhibit a higher quantity and activated phenotype compared to healthy controls. This phenotype is distinguished by an elevated surface expression of TLR-2, N-Formyl-methionyl-leucyl-phenylalanine (fMLP) receptor, IL-8 receptor, and CD43, an increased granule release and oxidative burst, and also higher serum levels of neutrophil extracellular traps (NETs) (151–153). Multiple mechanisms exist through which neutrophils promote MS, including the secretion of inflammatory mediators and enzymes such as IL-1β (154, 155), myeloperoxidase (156), and various proteinases (157, 158), the production of reactive oxygen species (ROS) (159, 160), and antigen presentation to T cells (161). In EAE, a lineage tracing study has demonstrated a significant increase in myelopoiesis in the bone marrow resulting in the enhanced production and subsequent invasion of neutrophils in the CNS (162). The regulation of neutrophil-associated factors, specifically granulocyte colony-stimulating factor (G-CSF) and CXCL1, plays a crucial role in this process (163). Deficiency of the G-CSF receptor and obstruction of CXCL1 lessened myeloid cell accumulation in the bloodstream and ameliorated the clinical outcomes of mice that received injections of myelin-reactive Th17 cells (163). Additionally, the presence of CXCL1, CXCL2, and CXCL6 was essential for the recruitment of neutrophils in the CNS. These chemokines exert their effects via activation of the G protein-coupled receptor CXCR2, which is predominantly expressed on mature neutrophils (164). The existence of neutrophils positive for CXCR2 has been shown to contribute to the process of inflammatory demyelination in demyelination models, such as EAE and CPZ intoxication. In contrast, CXCR2-deficient mice exhibit greater resistance to CPZ-induced demyelination (165). It can be inferred that CXCR2 may be a pivotal molecular target for MS therapy. Additionally, the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) are frequently observed in clinical practice as dependable indicators of inflammation related to various pathologies (166, 167). In PwMS, the NLR has been proposed as a marker of disease activity, with elevated levels displaying a positive association with the severity of MS symptoms (168, 169). Therefore, it may be crucial to monitor neutrophil activity and function to gain understanding of the progression of MS.

The evidence clearly shows that acute exercise affects neutrophil response. At the gene expression level, a study discovered that a brief bout of intense exercise modifies neutrophil gene expression, including the janus kinase (Jak)/STAT pathway involved in apoptosis, and genes linked to inflammation, such as IL-32, TNF receptor superfamily member 8 (TNFSF8), CCR5 and Annexin A1 (ANXA1), in addition to genes related to growth and repair, such as Amphiregulin (AREG) and fibroblast growth factor receptor 2 (FGFR2) genes (170, 171). In terms of activity and function, physical exercise typically induces an initial activation of neutrophils. This is demonstrated through the release of enzymes (172, 173) and subsequent changes in crucial effector functions, including phagocytosis and respiratory burst activity (174, 175). Acute exercise has been shown to attenuate neutrophil apoptosis, possibly by its action on the inducible nitric oxide synthase (iNOS)-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-myeloid cell leukemia 1 (Mcl-1) pathway (176), as well as calcium and redox signaling (177). Furthermore, although acute aerobic exercise was able to increase the number of total circulating neutrophils, the number of neutrophils expressing CXCR2 decreased during the recovery period (178). Previous research indicates that regular, chronic exercise can have a positive impact on neutrophil-mediated immune function in both physiological and pathological conditions. A cross-sectional study involving older adults found that increasing habitual physical activity can potentially enhance neutrophil-mediated immunity (179). Moreover, several months of exercise training not only reduce individual neutrophil chemotaxis and lower IL-8 and noradrenaline concentrations (180), but also enhance deoxyribonuclease (DNase) activity, increasing the ability to degrade NETs (181). In the case of EAE and MS, one study conducted with animals suggested that EAE mice that underwent six weeks of voluntary wheel running prior to the disease had a lower rate of neutrophil infiltration in the spinal cord and lesser severity of EAE in the chronic period (49). Furthermore, three weeks of HIIT programs during inpatient rehabilitation of patients with RRMS or SPMS resulted in a greater decrease of NLR compared to MICT. This could be attributed to the repetitive inflammatory status and compensatory anti-inflammatory balance after each high-intensity exercise, as suggested by Joisten et al. (182). The research shows that regular exercise has the potential to

ameliorate the clinical symptoms of MS by modulating the activity of neutrophils.

2.2.3 Microglia/macrophages

Microglia and macrophages are integral components of the mononuclear phagocytic system. They accumulate at the sites of active demyelination and neurodegeneration in the CNS of MS and are believed to be central to the disease process. Evidence suggests an increase in macrophage infiltration into the CNS and exaggerated activation of resident microglia and pathological microgliosis (183, 184). Microglia and macrophages can be classified into two subtypes: the classically activated M1 phenotype, which is associated with inflammatory and degenerative processes, and the alternatively activated M2 phenotype, which has protective properties. In addition to these two subtypes, there may exist intermediate polarization phenotypes (185). Classical activation can be induced by various stimuli such as IFN-γ and LPS. This activation results in the increased expression of antigen presentation related molecules, specifically CD80, CD86, and CD40, which demonstrate a significant ability to present antigens. Furthermore, M1 microglia/macrophages can produce pro-inflammatory cytokines like TNF-α and IL-6, and chemokines such as CCL2 and CCL3, as well as neurotoxic NO. In contrast, M2 microglia/macrophages lack cytotoxicity and can be stimulated by IL-4 and IL-13. They could exhibit raised levels of CD14 and CD163, among other markers, and release anti-inflammatory cytokines such as IL-10 and TGF-β (21, 186). It should be noted that microglia and macrophages play a dual role in the pathology of MS. In the early stages of demyelination and neurodegeneration present in active lesions, microglia with a pro-inflammatory phenotype were observed. They expressed molecules involved in phagocytosis, oxidative injury, antigen presentation, and T cell co-stimulation. In later stages, the microglia and macrophages in active lesions shifted to a phenotype that was intermediate between pro- and anti-inflammatory activation (187). Activated microglia have the ability to directly drive demyelination and are necessary for it (188). Conversely, microglia and monocyte-derived macrophages play a significant role in facilitating efficient remyelination by secreting growth factors and eliminating inhibitory myelin debris (189). Genetic fate mapping and multiphoton live imaging demonstrate that administering niacin at therapeutically relevant doses to demyelinated aged mice assists in clearing myelin debris in lesions through the action of both peripherally-derived macrophages and microglia (190). Moreover, M2 microglia and macrophages were found to drive OLs differentiation during CNS remyelination (191). Notably, the triggering receptor expressed on myeloid cells 2 (TREM2) is believed to play a significant part in the remyelination process. Research indicates that TREM2 is highly expressed on myelin-laden phagocytes in active demyelinating lesions in the CNS of PwMS. Gene expression research indicates that macrophages in individuals with genetic deficiency in TREM2 lack phagocytic pathways (192). Additionally, when TREM2 is deficient, the capability of microglia to phagocytose myelin debris is significantly diminished. These microglia also display impaired mobility and are unable to metabolize cholesterol, leading to deficient remyelination in TREM2-deficient mice (193). However, TREM2 activation in microglia led to increased OPC density in demyelinated regions, contributed to the development of mature OL, which subsequently improved remyelination and axonal integrity (192). Furthermore, regulation of neuroinflammation can be attained by adjusting the dynamic alterations in two phenotypes of microglia/macrophages. It has been recommended that to alleviate clinical symptoms in EAE mice, M1 microglia/macrophage polarization should be suppressed and shifted towards the protective M2 phenotype (194–196). As a result, the regulation of the activation and polarization of microglia/macrophages may be an effective approach to MS pathology.

Accumulated evidence over the past decades suggests that exercise have a considerable impact on macrophage chemotaxis, antigen presentation, phagocytosis, inflammatory cytokine release, antiviral capability, and antitumor activity (197–202). These effects could be attributed to exercise's regulation of immunometabolism and macrophage polarization. Murugathasan et al. (203) conducted a study which revealed bone marrow-derived macrophages (BMDMs) obtained from mice that underwent eight weeks of moderate-intensity treadmill running exhibited reduced LPS-induced NF-κB activation, decreased expression of pro-inflammatory genes (such as *Il-1β* and *Tnfα*), and increased M2-like-associated genes (such as *Arg1* and *Hmox-1*) in contrast to BMDMs from sedentary mice. This was linked to improved mitochondrial quality and higher dependence on oxidative phosphorylation, accompanied by reduced mitochondrial ROS production. Similarly, physical exercise has a wide range of effects on microglia activity and function by modulating the expression of cytokines and their receptors (204) and attenuating oxidative stress (205). Recently, mounting evidence has confirmed the influence of exercise on microglia in the physiology of the CNS and various conditions, such as AD (206–208), PD (209), and cerebral ischemia (210). In MS, the effects of physical exercise on microglia/macrophages can be summarized in three key ways: (i) inhibiting macrophage infiltration into the CNS, (ii) constraining atypical microglia activation and microgliosis at lesion sites, and (iii) inhibiting M1 polarization and promoting M2 polarization. Specifically, the results of a pre-training program, involving either a three-week voluntary wheel running or six-week treadmill running, demonstrated the capacity to restrain the infiltration of macrophages into the spinal cord, which was induced by EAE (49, 211). Regarding CNS-resident microglia, Rizzo et al. (212) showed that engaging in voluntary wheel running for three weeks alleviated microgliosis and reduced the expression of TNF-α and IL-1β in the hippocampal CA1 area of EAE mice. In CPZ-induced mice, six weeks of voluntary wheel running alleviated microgliosis in the striatum and corpus callosum (42). Additionally, regular exercise may lower the number of neurotoxic M1-like phenotype cells while increasing the number of M2-like phenotype cells. Before the induction of EAE by injecting PLP-reactive T-cells, the mice underwent six weeks of HICT, which reduced the number of neurotoxic microglia expressing the ionized calcium binding adapter molecule 1 ($Iba1^+$) and the M1-like marker inducible nitric oxide synthase ($iNOS^+$). The content of pro-inflammatory

cytokines IL-6 and MCP-1 secreted by microglia in response to PLP and LPS stimulations also decreased (213). Meanwhile, a daily one-hour voluntary exercise on a wheel reduces the number of Iba1$^+$ microglia/macrophages expressing iNOS in the spinal cord of EAE mice (46). In mice with lysolecithin-induced demyelination, voluntary wheel running for a duration of time augments the M2-like phenotype in the myelin lesions and enhances the phagocytic function of myelin fragments. This reduction in inhibitory lipid debris likely facilitates the prolonged proliferation of OPCs with exercise to produce increased numbers of OLs, ultimately promoting the remyelination process (214). However, other studies have reported negative and contradictory results regarding macrophage infiltration and microgliosis (47, 51, 91) (Table 2). Although there is no data available for humans, research on animals confirms that exercise elicits a response in MS-afflicted microglia/macrophages. In addition, it is noteworthy that several studies have demonstrated the impact of exercise on microglia activation, microglial glucose metabolism, and morphological plasticity by modifying the TREM2 pathway (207, 218). Xu et al. (219) propose that physical exercise can assist in the regeneration of OLs to protect against white matter damage after a stroke. This is primarily achieved by increasing TREM2 and microglia-generated factors. Due to TREM2's regulatory function in microglia, and its impact on myelin regeneration and neuroinflammation, it is imperative to investigate whether TREM2 can assist physical exercise in mitigating MS pathology in an animal model of MS.

2.2.4 Astrocytes

Astrocytes represent the most prevalent type of glial cells in the mammalian brain and perform various physiological functions, including regulating ion homeostasis, neurotransmitter clearance, synapse formation and removal, and neurovascular coupling, among others (220–222). It is noteworthy that astrocyte dysfunction can lead to the development of MS, including neuroinflammation and demyelination. In MS/EAE, the excess activation of astrocytes may foster innate inflammation and neurodegeneration via the production of cytokines such as IL-6, IL-15, and TNF-α, chemokines such as CXCL1, CXCL10, CCL2, and CCL20, and neurotoxic metabolites such as NO (19). Despite being neither immune progenitors nor strictly classified as innate immune cells, astrocytes can perceive inflammatory signals and regulate neuroinflammation. Some studies have suggested a possible connection between abnormal gene expression in astrocytes or metabolic abnormalities and increased neuroinflammation (223–225). Wheeler et al. (226) utilized single-cell RNA sequencing combined with cell-specific Ribotag RNA profiling, assay for transposase-accessible chromatin with sequencing, chromatin immunoprecipitation with sequencing, genome-wide analysis of DNA methylation and *in vivo* CRISPR-Cas9-based genetic perturbations to examine astrocytes in MS and EAE. The results showed that astrocytes in both EAE and MS exhibit reduced expression of Nrf2 and an upregulation of V-maf musculoaponeurotic fibrosarcoma oncogene homolog G (MAFG). MAFG collaborates with methionine adenosyltransferase II alpha (MAT2α) to propagate DNA methylation and impede antioxidant and anti-inflammatory transcriptional programs. GM-CSF signaling in astrocytes induces the expression of MAFG and MAT2α, as well as pro-inflammatory transcriptional modules, which potentially lead to CNS pathology in both EAE and MS. In general, astrocytes experience persistent and extensive activation in response to pathological stimuli, resulting in a reactive state that encompasses two subtypes: A1, characterized by a pro-inflammatory function, and A2, which exerts a protective effect (227). Additionally, numerous studies have identified the beneficial and detrimental roles performed by astrocytes in the process of remyelination. Molina-Gonzalez et al. (228) employed unbiased RNA sequencing, functional manipulation, and rodent models *in vivo*/ex vivo/*in vitro*, as well as human brain lesion analyses, to investigate the interaction between astrocytes and OLs during remyelination. The investigation has revealed that astrocytes can promote the survival of regenerating OLs by suppressing the Nrf2 pathway and stimulating the cholesterol biosynthesis pathway. This finding highlights the importance of astrocyte-OL interaction in myelin repair. In contrast, demyelinating lesions exhibit an augmented degree of reactive astrogliosis. Such reactive astrocytes present a hypertrophic phenotype and generate astroglial scars that can create an inhibitory milieu, ultimately obstructing tissue repair (229). Moreover, it has been discovered that irregular copper transportation in astrocytes may lead to demyelination in MS (230). The regulation of reactive astrocytes could hold significant therapeutic potential in the context of inflammation and myelin damage associated with MS.

Similar to microglia and OLs, the effect of physical exercise on the activity and function of astrocytes in the CNS has been widely researched. Appropriate exercise can alter astrocyte activation (231), phenotype (232, 233), remodeling (234, 235), tropic factor release (236), and energy metabolism (237), among others. Furthermore, it can regulate astrocyte-mediated neuroinflammatory responses (234) and intercellular interactions of astrocytes with other cells (238). In mouse models of MS, Bernardes et al. (211) found that a pre-exercise program involving six weeks of treadmill running contributed to a further reduction in astrocyte responses in the dorsal horn of the spinal cord, induced by GA drug therapy in EAE mice after the first relapse. This reduction was demonstrated using glial fibrillary acidic protein (GFAP) immunofluorescence. In addition, simultaneous voluntary wheel running during CPZ-induced demyelination alleviated astrogliosis in the striatum and corpus callosum, while decreasing CXCL10 expression and ameliorating axonal pathology in CPZ-treated mice (42). These findings imply that physical exercise has the potential to mitigate the pathophysiological features of MS through the reduction of the astrocytic response. However, it is important to acknowledge that the human studies have yet to provide concrete evidence for the consistency of these findings. GFAP is released into the cerebrospinal fluid and blood in disorders associated with astrocyte activation and astrogliosis following inflammation and neurodegeneration and therefore is highly expressed in MS lesions (239). In 2021, Ercan et al. (216) conducted a study observing a decrease in serum levels of GFAP and neurofilament light (NFL) after eight weeks of cycling in

TABLE 2 Effect of exercise on innate immune cells in animal models and human patients of MS.

Subjects		Intervention program			Region	Mode of action	References
Model	Characteristics	Type	Intensity	Duration			
MOG_{35-55} -induced EAE model	C57BL/6 mice; female; 8 weeks old	Voluntary wheel running	ND	60 min/ session for 40 consecutive days	Brain and spinal cord	Macrophages infiltration →; Synaptic plasticity ↑; Clinical scores ↓	(91)
	C57BL/6 mice; male and female; 6~8 weeks old	Voluntary wheel running	ND	60 min/ session for 30 consecutive days	Spinal cord	Microgliosis →	(47)
	C57BL/6 mice; male and female; 4~6 weeks old	Prior treadmill running	11 m/min	30 min/ session, 5 sessions/ week for 6 weeks	Spinal cord	Microglial reactivity and macrophages infiltration ↓; Astrocyte reactivity ↓; Synaptic plasticity ↑; Demyelination ↓	(211)
	C57BL/6 mice; female; 9~10 weeks old	Prior voluntary wheel running	ND	60 min/ session, 5 sessions/ week for 6 weeks	Spinal cord	Neutrophils and macrophages infiltration ↓; Nrf-2 and IL-10 ↑; IL-17 ↓; Clinical scores ↓	(49)
	C57BL/6 mice; female; 9 weeks old	Voluntary wheel running	ND	3 weeks	Hippocampus	Microgliosis ↓; Microglial TNF-α ↓; IL-1β ↓; Cognition ↑; Synaptic plasticity ↑; Clinical scores ↓	(212)
CPZ-induced toxic-demyelinating model	C57BL/6 mice; male; 4 weeks old	(i) Interval treadmill running (IT) (ii) Continuous treadmill running (CT)	(i) IT: 50%, 90% EC_{max} (ii) CT: 70% EC_{max}	5 sessions/ week for 4 weeks	Hippocampus	(i) IT: Microglial number ↑; Oligodendrocytes number ↑; BDNF, GDNF, and NGF ↑ (ii) CT: Microglial number →; Oligodendrocytes number ↑; BDNF, GDNF, and NGF ↑	(51)
	C57BL/6N mice; female; 8 weeks old	Voluntary wheel running	ND	6 weeks	Corpus callosum and striatum	Microgliosis ↓; Astrogliosis ↓; CXCL10 ↓; TNF-α, IL-1β, TGF-β, and CXCL12→; Demyelination ↓; Remyelination ↑; Axonal damage ↓; Weight loss ↓; Motor and neuromuscular function ↑	(42)
Lysolecithin-demyelinating model	C57BL/6 mice; female; 8~12 weeks old	Voluntary wheel running	ND	14 consecutive days	Spinal cord	CD206 anti-inflammatory phenotype macrophage and microglia ↑; CD16/32 pro-inflammatory phenotype macrophage and microglia ↓; Phagocytic Clearance of Lipid Debris ↑; Remyelination ↑; Axonal degeneration ↓	(214)
RRMS patients	10 female; (32.15 ± 7.57) years old; EDSS score ≤ 4	Stretch training, aerobic exercises, endurance and resistance training	ND	55~65 min/ session, 3 sessions/ week for 8 weeks	Blood	PBMC-derived INF-γ and IL-17 ↓; IL-4 →; Fitness ↑; Clinical scores ↓	(215)
	31 female/7 males; 19~40 years old; EDSS: 1~4.5	Cycing	60%~70% VO_{2max}	30 min/ session, 3 sessions/ week for 8 weeks	Blood	GFAP and NFL ↓	(216)

(Continued)

TABLE 2 Continued

Subjects		Intervention program			Region	Mode of action	References
Model	Characteristics	Type	Intensity	Duration			
CPMS and RRMS patients	12 female/10 male; (46.0 ± 2.0) years old; EDSS score < 6	Endurance training (ET) (walking and cycling) combined with strength training (ST) (resistance exercise)	ET: 65% HRR; ST: 70% 1RM	A single bout	Blood	(i) Immediate post-exercise: Monocytes and granulocyte number ↑; cDC and pDC number ↑; $CD62L^+$ cDC and $CD62L^+$ pDC number ↑; $CCR5^+$ cDC and $CCR5^+$ pDC number ↑; MIP-1α →; Flt3L ↑; MMP-9 → (ii) 2 hours post-exercise: Monocytes number →; Granulocyte number ↑; cDC number →; pDC number ↑; $CD62L^+$ cDC ↑; $CD62L^+$ pDC number →; $CCR5^+$ cDC and $CCR5^+$ pDC number ↑; MIP-1α →; Flt3L →; MMP-9 ↑	(95)
	16 female/13 male; (46.0 ± 2.0) years old; EDSS score: (3 ± 0.2)	Endurance training (cycling and treadmill walking or running) combined with strength training (resistance exercise)	ND	5 sessions/2 weeks for 12 weeks	Blood	pDC number and proportion ↑; cDC number →; $CD80^+$ pDC number and proportion ↑; $CCR7^+$ pDC number ↓; $CD62L^+$ pDC number ↑; $CD62L^+$, $CCR5^+$, and $CCR7^+$ cDC number →; The fold change of $CCR5^+$ cDC and TNF-α and MMP-9 secreation upon LPS and IFN-γ stimulation ↓; The fold change of $CD86^+$ and $HLA\text{-}DR^+$ cDC and IL-1β, IL-6, IL-12p70, IFN-α, and caspase-1 secreation upon LPS and IFN-γ stimulation ↓; The fold change of $HLA\text{-}DR^+$ pDC upon IQ stimulation ↑; The fold change of $CCR5^+$ and $CD86^+$ pDC and IL-6, IL-12p70, TNF-α, IFN-α, caspase-1, and MMP-9 upon IQ stimulation →	(96)
SPMS and RRMS patients	42 female/26 male; (50.3 ± 10.2) years old; EDSS score: 3~6	High-intensity interval training (HIIT) or moderate continuous training (MCT)	(i) HIIT: 95%~100% HR_{max} (ii) MCT: 65% HR_{max}	3 sessions/ week for 3 weeks	Blood	(i) HIIT: NLR ↓; Systemic immuneinflammation index ↓ (ii) MCT: NLR→; Systemic immuneinflammation index ↑	(182)
PwMS	22 female/8 male; 43.5 ± 10.1) years old; EDSS score: 2~3	High-intensity aerobic training	40%, 60%, and 80% peak power	3 session/ week for 16 weeks	Blood	GFAP, BDNF, and NFL →	(217)
PwMS treated with either ATZ, FTY, or NAT	17 female/13 male	Climbing stairs at normal speed (CN) or fast (CF) or cycling (C)	C: 1, 2 Watt per kilogram BW	CN: ND CF: ND C: 20 min	Blood	(i) CN: Absolute NK cells number ↑ (ii) CF: Absolute NK cells number ↑ (iii) C: Absolute NKT cells and NK cells number ↑	(99)

1RM, repetition maximum; ATZ, alemtuzumab; BDNF, brain-derived neurotrophic factor; CCR, chemokine C-C-Motif receptor; cDC, conventional dendritic cells; CPMS, chronically progressive MS; CPZ, cuprizone; CXCL, chemokine (C-X-C motif) ligand; EAE, experimental autoimmune encephalomyelitis; EC_{max}: maximal exercise capacity; EDSS, Expanded Disability Status Scale; Flt3l, FMS Like Tyrosine Kinase 3 Ligand; FTY, Fingolimod; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidase; HLA-DR, human leukocyte antigen DR; HR_{max}: maximal heart rate; HRR, heart rate reserve; IFN, interferon; IL-10, interleukin-10; IQ, imiquimod; LPS, lipopolysaccharide; MIP-1α: macrophage inflammatory protein 1 α; MMP, matrix metallopeptidase; MOG, myelin oligodendrocyte glycoprotein; NAT, natalizumab; ND, not determined; NFL, neurofilament light; NGF, nerve growth factor; NLR, neutrophil to lymphocyte ratio; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PBMC, peripheral blood mononuclear cell; pDCs: plasmacytoid DCs; PwMS, patients with MS; TGF-β, transforming growth factor-β; Th, T helper cells; TNF-α, tumor necrosis factor-α; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS.
↑, significantly increased or improved; ↓, significantly decreased or reduced; →, no significant change.

patients with RRMS. However, a subsequent investigation by Gravesteijn et al. (217) found no statistically significant changes in serum levels of GFAP, brain-derived neurotrophic factor (BDNF), and NFL in PwMS following a 16-week cycling intervention. The amount of relevant research available is restricted and there is a lack of consistency in the results obtained from human trials. Additional research is necessary to obtain a more comprehensive understanding of this issue.

3 Conclusions and perspectives

Both peripheral and CNS immunity are essential for maintaining the proper CNS function. Physical exercise provides direct neuroprotective benefits and induces immunomodulatory effects. However, additional research is necessary to fully understand the impact of physical exercise on autoimmune diseases. In animal models and PwMS, the aberrant functioning of immune cells has been identified as a significant pathological mechanism. The implementation of a moderate exercise program has been shown to effectively limit the infiltration of various peripheral immune cell types, including T lymphocytes, B lymphocytes, neutrophils, dendritic cells, and macrophages, into the CNS. This physiological phenomenon can be attributed to the fact that physical exercise can modify the quantity, functionality, and migratory potential of immune cells and contribute to the establishment of immune cell homeostasis from a pro-inflammatory phenotype to an anti-inflammatory phenotype. For instance, studies have indicated that exercise can promote the differentiation of Treg cells, while inhibiting the differentiation of Th1/Th17 cells, thereby leading to a reduction in IFN-γ and IL-17 production, and an increase in IL-10 and TGF-β production. Additionally, physical exercise has been observed to modulate the structure of the BBB, consequently improving integrity and decreasing permeability. In addition, physical exercise has an impact on resident innate immune cells, specifically microglia and astrocytes in the CNS. This impact mainly manifests as a reduction in the activation of microglia and astrocytes induced by pathological stimuli, as well as a decrease in microgliosis and astrogliosis and the synthesis of pro-inflammatory cytokines (Figure 2). The immunomodulatory responses elicited by exercise may constitute a vital mechanism by which exercise ameliorates myelin and axonal damage, alleviates disease symptoms, and abates clinical scores.

The regulation of immune cells in MS through exercise has attracted growing attention. However, some immune cells, including γ-δT cells, MAIT cells, and natural killer cells, among others, have not yet been investigated. Additionally, current research has some potential limitations. Firstly, so far, most studies have only described alterations in cellular phenotype. Few studies have been undertaken regarding the molecular mechanisms that underlie the effects of exercise on immune cells in MS. Although it has been proposed that exercise could modulate immune cell function by altering immunometabolism in MS (240), the current evidence is insufficient. Furthermore, many

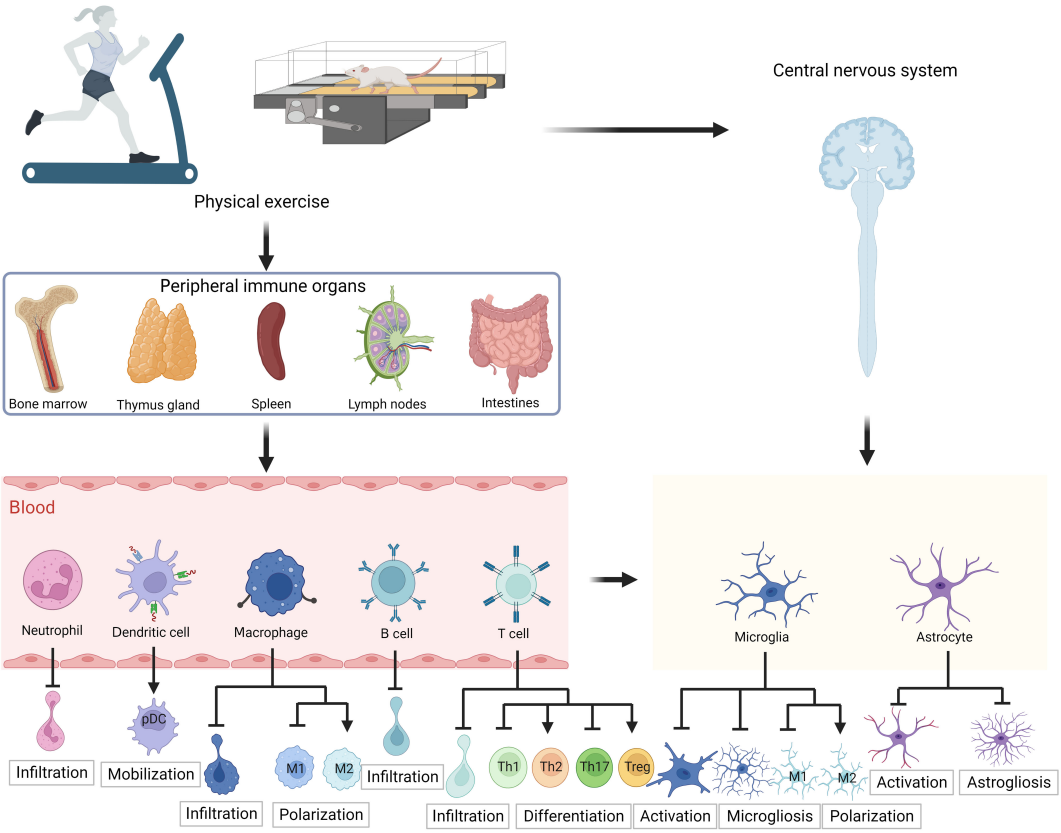

FIGURE 2
Schematic diagram of the effect of exercise on immune cells in multiple sclerosis. In human patients with MS or animal models, moderate exercise inhibits infiltration of peripheral immune cells including lymphocytes and monocytes, increases anti-inflammatory Th cell differentiation, decreases pro-inflammatory Th cell differentiation, promotes pDC mobilization, and induces macrophage polarization toward the M2 anti-inflammatory phenotype. In addition, exercise also inhibits microglia and astrocyte hyperactivation in the CNS, limits microgliosis and astrogliosis, and promotes microglia polarization toward the M2 anti-inflammatory phenotype. The figure was created using BioRender. pDC, plasmacytoid dendritic cell; Th, T helper cells; Treg, regulatory T cells.

transcription factors, including peroxisome proliferator-activated receptor γ (PPAR-γ) (241), and regulators of signaling pathways, such as nuclear factor kappa-B (NF-κB) (242) are involved in regulating immune cell plasticity but have not been explored in MS. Secondly, it is essential to give more consideration to the interaction between the immunomodulatory mechanisms linked to exercise improvement and other mechanisms, such as the release of neurotrophic factors, mitochondrial dysfunction, and oxidative stress. Thirdly, exploration of the disparities in immunomodulatory mechanisms induced by varied experimental protocols in animal studies, such as disease prevention via pre-training, disease progression inhibition via concurrent training, functional improvement through training during remission, presents a fascinating future research topic. Additionally, the effects of exercise alone and exercise combined with other interventions should be actively explored. Finally, despite extensive research and notable advancements in studying animal models, it is important to acknowledge that these models cannot fully replicate the entire spectrum of MS and its clinical manifestations due to significant heterogeneity observed in various disease courses. Therefore, further empirical studies are imperative to validate the efficacy of exercise interventions in ameliorating the disease across diverse types, durations, intensities, and cycles. During the clinical translational phase, it is crucial to provide personalized exercise programs to PwMS to improve functional recovery.

Author contributions

BZ: Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Investigation. FY: Writing – original draft, Writing – review & editing. XZ: Investigation, Writing – review & editing. WZ: Investigation, Writing – review & editing. SL: Writing – review & editing. LL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Estela Maris Muñoz,
CONICET Dr. Mario H. Burgos Institute of Histology and Embryology (IHEM), Argentina

REVIEWED BY
Maria Silvia Di Genaro,
National University of San Luis, Argentina
Shusaku Hayashi,
University of Toyama, Japan

*CORRESPONDENCE
Sarah J. Spencer
sarah.spencer@rmit.edu.au

†These authors have contributed equally to this work

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Macrophage regulation of the "second brain": CD163 intestinal macrophages interact with inhibitory interneurons to regulate colonic motility - evidence from the *Cx3cr1-Dtr* rat model

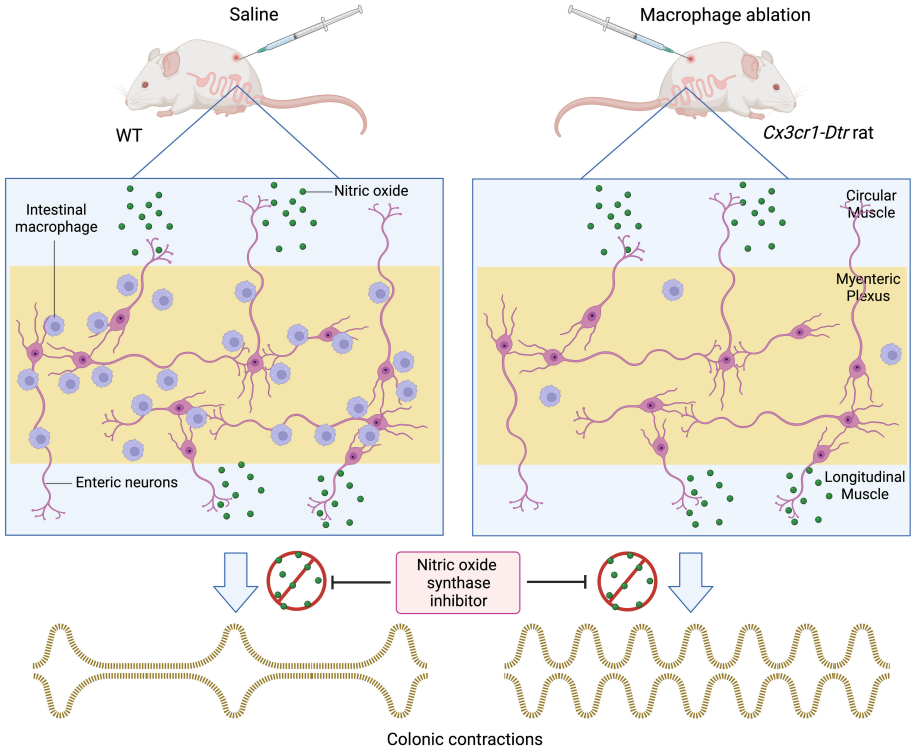
Jackson L. K. Yip[1], Soniya Xavier[1], Gayathri K. Balasuriya[1,2], Elisa L. Hill-Yardin[1†] and Sarah J. Spencer[1*†]

[1]School of Health and Biomedical Sciences, RMIT University, Bundoora, Melbourne, VIC, Australia, [2]Department of Physiology and Cell Biology, Kobe University School of Medicine, Kobe, Japan

Intestinal macrophages are well-studied for their conventional roles in the immune response against pathogens and protecting the gut from chronic inflammation. However, these macrophages may also have additional functional roles in gastrointestinal motility under typical conditions. This is likely to occur via both direct and indirect influences on gastrointestinal motility through interaction with myenteric neurons that contribute to the gut-brain axis, but this mechanism is yet to be properly characterised. The CX3CR1 chemokine receptor is expressed in the majority of intestinal macrophages, so we used a conditional knockout *Cx3cr1-Dtr* (diphtheria toxin receptor) rat model to transiently ablate these cells. We then utilized *ex vivo* video imaging to evaluate colonic motility. Our previous studies in brain suggested that *Cx3cr1*-expressing cells repopulate by 7 days after depletion in this model, so we performed our experiments at both the 48 hr (macrophage depletion) and 7-day (macrophage repopulation) time points. We also investigated whether inhibitory neuronal input driven by nitric oxide from the enteric nervous system is required for the regulation of colonic motility by intestinal macrophages. Our results demonstrated that CD163-positive resident intestinal macrophages are important in regulating colonic motility in the absence of this major inhibitory neuronal input. In addition, we show that intestinal macrophages are indispensable in maintaining a healthy intestinal structure. Our study provides a novel understanding of the interplay between the enteric nervous system and intestinal macrophages in colonic motility. We highlight intestinal macrophages as a potential therapeutic target for gastrointestinal motility disorders when inhibitory neuronal input is suppressed.

KEYWORDS

gastrointestinal, macrophages, nitric oxide, myenteric plexus, colonic motility



GRAPHICAL ABSTRACT

We used a conditional knockout *Cx3cr1-Dtr* rat model to decipher the role of intestinal macrophages in colonic motility. Transgenic rats injected with diphtheria toxin (DT) showed a significant reduction of ionized calcium-binding adaptor molecule 1 (Iba-1)-positive intestinal macrophages in the myenteric plexus 48 hours post-injection. We demonstrated that in the presence of the nitric oxide synthase inhibitor, Nω-nitro-L-arginine (NOLA), macrophage ablated rats had a greater increase in contraction frequency than controls. We then examined the macrophage population 7 days after DT injection and found that cluster of differentiation 163 (CD163) tissue resident macrophages do not repopulate whereas Iba-1 positive macrophages do. A greater increase in contraction frequency in macrophage-ablated rats in the presence of NOLA indicates that CD163 positive macrophages are crucial in regulating colonic motility in the absence of nitric oxide.

Highlights

- Intestinal macrophages regulate intestinal motility but the mechanisms by which this occurs are largely unknown.
- We utilized a *Cx3cr1-Dtr* (diphtheria toxin receptor) rat model to transiently deplete macrophages and thus investigate the macrophage contribution to colonic motility in the context of enteric nervous system inhibitory input.
- We show that tissue-resident CD163 intestinal macrophages regulate colonic motility, particularly in the absence of the main inhibitory drive in the gut which occurs via nitric oxide-dependent input.
- These findings allow us to better understand how intestinal macrophages regulate colonic motility and provide insights to support the development of macrophage-specific therapeutic targets for gut motility disorders.

Introduction

As the most abundant immune cell type of the gastrointestinal tract, intestinal macrophages play a key role in maintaining homeostasis (1), including resistance to invasion by foreign antigens and commensal bacteria. It is accepted that intestinal macrophages generally maintain an anti-inflammatory (M2) profile to prevent chronic inflammation and promote tissue repair (2, 3). They secrete the anti-inflammatory cytokine interleukin (IL)-10, which is constitutively expressed in the gut in the healthy individual (3). During inflammation, intestinal macrophages differentiate into pro-inflammatory macrophages (M1) and secrete pro-inflammatory cytokines, including IL-1β, IL-6, and tumour necrosis factor (TNF)-α (1). The release of pro-inflammatory cytokines helps combat pathogens by further recruitment of inflammatory cells or by stimulating production of inflammatory response proteins such as serum amyloid A and C-reactive protein (4, 5).

In addition to an immunological role, intestinal macrophages regulate intestinal motility via interaction with the enteric nervous

system (ENS), specifically with myenteric neurons. Muller and others have demonstrated that colony stimulating factor 1 (CSF-1)/bone morphogenetic protein 2 (BMP-2) bi-directional signaling between enteric neurons and intestinal macrophages is crucial in maintaining intestinal peristalsis in healthy mice (6). However, the mechanisms by which this neuroimmune crosstalk affects intestinal muscle contractions, and thereby digestion, are still poorly understood (3). Here we hypothesized that gastrointestinal motility would be broadly maintained in the absence of intestinal macrophages but that motility responses to ENS input would lack the coordinated contractile patterns seen typically in rodents, highlighting the interplay between the gut-brain axis and its immune component.

Different myenteric neuron populations, as identified by their neurochemical coding, play different roles in regulating intestinal motility (7). The major inhibitory myenteric neurons express neuronal nitric oxide synthase (nNOS) (8). nNOS neurons stimulate relaxation of the smooth muscle (9). A recent study has suggested that colonic migrating motor complexes (CMMCs), responsible for initiating colonic contractions, originate from the blockade of the inhibitory nitrergic cyclic guanosine monophosphate (cGMP)-dependent pathway (10). The nitrergic pathway is therefore likely to play a principal role in regulating colonic motility (10). As such, loss of nNOS has been implicated in several gastrointestinal disorders, such as oesophageal achalasia, gastroparesis and Hirschsprung's disease (11–13). In mice, transplanting healthy enteric neural stem cells into nNOS-deficient mice can rescue impaired colonic motility (7).

Although colonic motility is regulated by the neurons of the myenteric plexus, additional factors contribute to the detailed contraction profile (14). Apart from input by myenteric neurons, pacemaker interstitial cells of Cajal also generate myogenic rhythmicity (14). Depending on the distance that motor complexes travel, the resultant neurogenic and myogenic contractions in the proximal colon can be characterized into different patterns (15). Therefore, in this study we focused on defining differences in contraction patterns occurring in the rat proximal to mid colon following the ablation of intestinal macrophages.

One of the main identifiers of intestinal macrophages is the CX3C chemokine receptor 1 (Cx3cr1). *Cx3cr1* expression is low in circulating monocytes but increases as monocytes differentiate into resident intestinal macrophages (3, 16). Therefore, a transgenic model targeting *Cx3cr1*-expressing cells allows us to directly investigate the role of intestinal macrophages in the gut. In previous work, we used a conditional diphtheria toxin receptor (Dtr) knock-in *Cx3cr1-Dtr* rat model to target Cx3cr1-containing cells and study their roles in satiety control, circadian rhythms, neuroimmune responses and cognitive function (17–21). These studies suggest that the effects of ablating Cx3cr1-cells (i.e., microglia, monocytes, macrophages) are not due to sickness, withdrawal, anxiety, or nausea (17). Here, we utilized this transgenic rat model to investigate the role of intestinal macrophages in gastrointestinal motility and their interactions with the ENS. We measured colonic motility patterns at the mid-point of the proximal colon, since previous studies demonstrated that only a subset of contractions generated from the beginning of the rat proximal colon are propagated into mid-colon and beyond (22–25). We found that the loss of intestinal macrophages in this model led to shortening of the small intestine and colon. Furthermore, intestinal macrophage depletion increased motility in the proximal colon only when nNOS was inhibited. The difference in motility was not caused by changes in the number of nNOS neurons in the myenteric plexus. Notably, spontaneous repopulation of ionized calcium binding adaptor molecule 1 (Iba-1)-positive but not cluster of differentiation 163 (CD163)-positive intestinal macrophages ensued after 7 days, and this was sufficient to rescue some aspects of the phenotype, including intestine length. However, we observed increased motility upon nNOS inhibition that persisted even after Iba-1-positive macrophages had repopulated the tissue. Our findings indicate that CD163-positive macrophages are crucial in regulating gut motility when the major inhibitory neural input is blocked.

Methods

Animals

All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with approval from the RMIT University Animal Ethics Committee (AEC #1920). The chemokine receptor Cx3cr1 is exclusively expressed in microglia and monocytes (26). To specifically ablate Cx3cr1-expressing cells, we generated a *Cx3cr1-Dtr* knock-in rat model on a Wistar Han background using CRISPR/Cas9 technology, as previously described (17).

In the present experiments, we used female rats aged between 13 and 17 weeks. Initial analyses suggested females perform similarly to males in terms of microglial and weight responses to the DT (17) and so we selected one sex only to first establish mechanistic insight into how macrophages affect gut motility before proceeding to sex-comparison studies. The rats were kept under standard laboratory housing conditions, with a 12 hr light cycle (7 am to 7 pm), an ambient temperature of 22 °C, with humidity between 40 and 60%, and free access to water and standard rat chow except where stated. We administered DT as two separate injections, 8 hr apart, of 25 ng/g DT in sterile saline, subcutaneously (s.c.), according to our previous studies (17, 27). Our previous work has shown that depletion of microglia and monocytes is maximized at 48 hr, and that spontaneous repopulation is in progress around 7 days after depletion (17). Thus, basal and post-DT tissue collection was performed 48 hr or 7 days after the first injection, after the rats were euthanized with overdose of ketamine and xylazine, 20 mg/mL ketamine (Cenvet Australia, Lynbrook, VIC, Australia), 5 mg/mL xylazine (Cenvet). All experiments were completed between 9 am and 1 pm to limit potential effects of circadian rhythms on any parameters measured.

Colon collection and wholemount tissue preparation for immunofluorescence

The proximal colon (the first 3-5 cm of colon measured from the caecum and visualized by colonic striation patterns) from each animal was opened, stretched, pinned with the mucosa facing upwards, and submerged in 0.1 M phosphate buffered saline (PBS) on a Petri dish lined with Sylgard (Sylgard Silicone Elastomer, Krayden Inc., Denver, CO, USA). To obtain a longitudinal muscle-myenteric plexus (LMMP) preparation, the mucosa, submucosal plexus and circular muscle were peeled away from the remaining colonic tissue under a dissecting microscope. A small area of tissue containing the LMMP was transferred to a Petri dish (35 mm), submerged in 0.1 M PBS, and stored at 4°C before assessment of neuronal populations by immunofluorescence.

Wholemount immunofluorescence for neuronal populations and identification of intestinal macrophages

We have previously described the myenteric plexus wholemount immunofluorescence for mouse tissues (28). Here, immunofluorescence was performed on wholemount rat colonic tissue samples to assess for potential differences in neuron numbers and intestinal macrophage populations between saline- and DT-treated *Cx3cr1-Dtr* rats. Wholemount LMMP samples were incubated at room temperature (RT) for 30 min in 0.01% Triton X-100 (Sigma Aldrich, St Louis, MO, USA) with 10% CAS-block™ (Invitrogen Australia, Mt Waverley, VIC, Australia) to reduce non-specific binding of antibodies. Then, tissues were incubated with three primary antisera for neuronal populations: human anti-Hu (1:5,000, a pan-neuronal marker; a gift from Dr. V. Lennon, Mayo Clinic, Rochester, MN, USA), sheep anti-nNOS (1:400; Millipore, RRID: AB_90743) and rabbit anti-Iba-1 (1:400; Wako Chemicals USA Inc., Richmond, VA, USA, RRID: AB_839504) and stored at 4°C overnight in a sealed container. For assessing macrophage populations, tissues were incubated with two primary antisera: rabbit anti-Iba-1 (FUJIFILM Wako Shibayagi, RRID: AB_839504; 1:400) and mouse anti-CD163 (Bio-Rad Laboratories, RRID: AB_2074558; 1:100). After incubation, colonic tissues were washed with 0.1 M PBS (three washes of 10 min each). Secondary antisera corresponding to the host of the primary antibody were applied to the samples and left for 2.5 hr at RT on a shaker incubator (donkey anti-sheep Alexa 488 (Thermo Fisher Scientific, RRID: AB_2534082); 1:400, donkey anti-human Alexa 594 (Jackson ImmunoResearch Laboratories, Inc., RRID: AB_2340572); 1:750, donkey anti-rabbit Alexa 647 (Jackson, RRID: AB_2340572); 1:400 and donkey anti-mouse Alexa 488 (Abcam, RRID: AB_2732856)). Colonic tissues were mounted using fluorescence mounting medium (DAKO Australia Private Ltd; Botany, NSW, Australia). Tissue samples were imaged using a confocal microscope (Nikon Confocal Microscope: A1; Version 4.10). A Z-series of images of myenteric plexus sections (6.5 µm/step with total tissue thickness approximately 60 µm) was captured for each animal and saved in the ND2 file format.

Analysis of nNOS neuron populations in the myenteric plexus

Images of colonic tissue containing the myenteric plexus were analysed using ImageJ (1.52a, NIH, Bethesda, MD, USA). Five intact myenteric ganglia were randomly selected from each wholemount colonic tissue sample (approximately 1 cm^2) for each animal. We then counted the number of Hu- and nNOS-labelled cells from each ganglion. The nNOS neuronal population was estimated as the percentage of nNOS cells in a ganglion co-labelled with Hu.

Intestinal macrophage density and morphology

Z-series images of wholemount tissue were analysed using the Imaris software volume function to assess the cell density and morphology of intestinal macrophages (Imaris 64X 9.1.0; Bitplane AG, UK). Three proximal colon areas of 0.25 mm^2 per tissue per animal were selected as regions of interest (ROI). The presence of macrophages in the muscle layer was established by visualising the z-position of Iba-1-positive cells (macrophages) relative to that of Hu-positive cells (neurons). Macrophages with a z-position outside the location of the neurons were considered to be situated in the muscle layer within the LMMP preparation. Sphericity and cell density data were also recorded and analysed using GraphPad Prism software (Boston, MA, USA; version 9.0.1).

RT-PCR

Proximal colons were snap-frozen and RNA extracted using QIAzol reagents and RNeasy Mini Kits (Qiagen, Valencia, CA, USA). RNA was then transcribed to cDNA using Quantitect Reverse Transcription kits (Qiagen) and analyzed by qRT-PCR with a QuantStudio 7 Flex instrument (Applied Biosystems, Mulgrave, Vic, Australia) using Taqman Gene Expression Assays (Applied Biosystems). We compared the relative quantitative measure of *Cx3cr1* expression (NCBI reference sequence: NM_133534.1, Taqman assay ID: Rn02134446_s1) with the housekeeping gene *Gapdh* (NCBI reference sequence: NM_017008.3, Taqman assay ID: 4352338E) as an endogenous control. We analysed mRNA expression using $2^{-\Delta\Delta C(t)}$, where C(t) is the threshold cycle at which fluorescence is first detected significantly above background.

Ex vivo video imaging of colonic motility

The setup for rat colon has been described in our previous publication (25). Briefly, the proximal to mid colon (5-7 cm measured from the caecum end) was dissected from each animal. Each colon preparation was placed into a beaker containing Krebs solution (118 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM

$MgSO_4$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11 mM D-glucose in mM; bubbled at RT with carbogen gas: 95% O_2 and 5% CO_2) at 4°C. The colon preparation was then placed into an organ bath chamber, which was connected to an in-flow reservoir containing Krebs solution via inlet tubes and was continuously superfused with Krebs solution bubbled with carbogen and maintained between 33-35°C. The oral end of the colon preparation was cannulated to the inlet tube and secured using standard cotton sewing thread. The faecal content was removed by applying gentle positive pressure from the inflow reservoir. The anal end of colon was then cannulated to the outlet tube. An intraluminal pressure was created by using a rubber stopper with a glass tube (5 mm inside diameter) inserted through its centre to seal onto the inflow reservoir. Intraluminal pressure was calculated by measuring the vertical distance from the tissue to the meniscus of Krebs solution within the glass tube of inflow reservoir and maintained at constant level throughout the experiment (i.e., the meniscus was 5.5-6.5 cm above the height of the colon segment). Colonic motility was recorded using a Logitech camera (QuickCam Pro 4000; I-Tech, Ultimo, NSW, Australia) mounted directly above the organ bath at a standard distance of 10 cm. Each colon was given 30 min to equilibrate before we recorded four 15 min videos of spontaneous contractile activity under control conditions. Subsequently, 100 µM Nω-nitro-L-arginine (NOLA) was added to the inflow reservoir, to inhibit nitric oxide, and contractile activity was recorded for another four x 15 min. After NOLA application, a final four x 15 min videos were recorded, considered as the washout period. These final recordings enabled us to assess the restoration of the inhibitory stimulus and to ensure the tissue remained viable for the duration of the experiment.

Pair-feeding motility

Transient ablation of macrophages in the *Cx3cr1-Dtr* causes anorexia-induced weight loss while the macrophages remain depleted (17). Therefore, to verify that any changes in intestinal motility were due to the absence of intestinal macrophages and not to any anorexia or weight loss that accompanies it, we performed a pair-feeding experiment (17). We fed a cohort of macrophage-intact rats the mean voluntary consumption of the DT-treated *Cx3cr1-Dtr* rats to induce a similar weight loss to that associated with the macrophage ablation. Rats were then anaesthetized for tissue collection and assessment of colonic motility as described above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad; version 9.0.1). We assessed nNOS neuronal populations, cell density and sphericity of intestinal macrophages, RT-PCR, percentage changes of resting gut diameter, contraction magnitude and contraction frequency before and after NOLA treatment using Student's unpaired t-tests. We assumed statistical significance when $p < 0.05$. A repeated measures two-way analysis of variance (ANOVA) was used to compare the resting gut diameter, contraction magnitude and contraction frequency of macrophage-intact and *Cx3cr1-Dtr* rat colon under control conditions and with NOLA treatment. Tukey *post hoc* tests were used to identify where significant differences occurred in the case of a significant interaction. Data are presented as mean with maximum and minimum. Sample sizes are included in the individual results sections.

Results

Macrophage ablation reduces Iba-1-positive cell density in the myenteric plexus

To verify that intestinal macrophages are depleted upon DT injection in *Cx3cr1-Dtr* rats, we assessed numbers of Iba-1-positive cells in the myenteric plexus. As expected, at 48 hr after DT injection there was significant loss of intestinal macrophages in the myenteric plexus ($t_{(15)} = 6.09$, $p < 0.0001$, n = 7-10 animals per group; Figures 1A, E, M). Resident intestinal macrophages are particularly important in the bidirectional communication between the nervous and immune systems (29, 30), so we also assessed the CD163-positive (resident) subpopulation of macrophages. Notably, most intestinal macrophages surrounding ganglia expressed CD163 (Figures 1C, D, blue arrows). Similar to Iba-1-expressing cells, CD163-positive cells were ablated upon DT injection ($t_{(7)} = 10.25$, $p < 0.0001$, n = 4-5 animals per group; Figures 1B, F–H, O). Iba-1-positive cells in the smooth muscle layers were also significantly reduced after DT ($t_{(6)} = 3.85$, $p = 0.0085$, n = 4 per group; Figure 1Q). Additionally, *Cx3cr1* mRNA was drastically reduced after DT injection to be almost undetectable ($t_{(16)} = 14.9$, $p < 0.0001$, n = 9 per group; Figure 1R), verifying the efficacy of our model in depleting its target cells. Colocalization analysis indicated that about 70% of Iba-1 positive macrophages in the rat colon also expressed CD163.

As anticipated from our previous work in the brain (18), the morphology of the macrophages remaining after depletion differed from macrophages in intact rats, with both Iba-1-positive cells and CD163-positive cells being significantly more spherical in the *Cx3cr1-Dtr* rats than in those not given DT (Iba-1-positive cells: $t_{(15)} = 3.60$, $p = 0.0026$, n = 7-10 animals per group, Figures 1I, J, N; CD163-positive cells: $t_{(7)} = 7.89$, $p < 0.0001$, n = 7-10 animals per group, Figures 1K, L, P).

Macrophage ablation decreases body weight and shortens small intestine and colon

After verifying the conditional knockout of intestinal macrophages in *Cx3cr1-Dtr* rats, we investigated if the loss of intestinal macrophages affected the overall anatomy of the gastrointestinal tract. Consistent with previous findings from our group (17), the body weights of *Cx3cr1-Dtr* rats were significantly

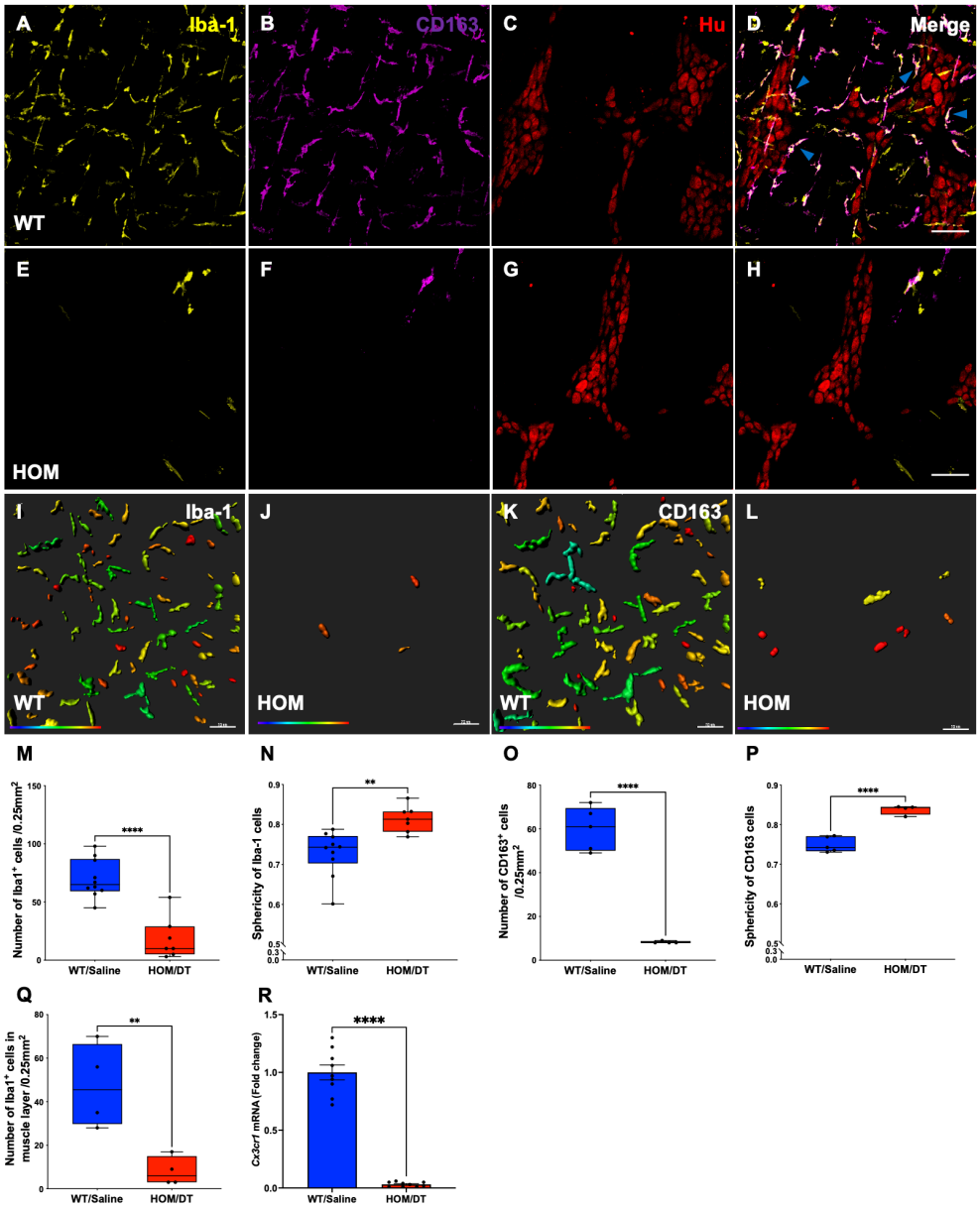


FIGURE 1
Cx3cr1-cell ablation significantly reduced the density and increased the sphericity of Iba-1 cells and CD163 cells in the myenteric plexus of the proximal colon. **(A)** Macrophage-intact proximal colon myenteric plexus ganglia immunolabelled with ionized calcium binding adaptor molecule 1 (Iba-1), **(B)** cluster of differentiation 163 (CD163) and **(C)** Hu. **(D)** Iba-1, CD163 and Hu merged. **(E)** *Cx3cr1-Dtr* rat proximal colon 48 hr after diphtheria toxin (DT) immunolabelled with Iba-1, **(F)** CD163 and **(G)** Hu. **(H)** Iba-1, CD163 and Hu merged. **(I)** Imaris colour-gradient of sphericity of Iba-1 cells in macrophage-intact proximal colon and **(J)** *Cx3cr1-Dtr* rat proximal colon. **(K)** Imaris colour-gradient of sphericity of CD163 cells in macrophage-intact proximal colon and **(L)** *Cx3cr1-Dtr* rat proximal colon. **(M)** Numbers of Iba-1-expressing cells per 0.25 mm^2. **(N)** Cx3cr1-cell ablation leads to a significant increase in the sphericity of Iba-1-positive cells in the myenteric plexus of *Cx3cr1-Dtr* rats given DT compared to WT. **(O)** Numbers of CD163-expressing cells per 0.25 mm^2. **(P)** Cx3cr1-cell ablation leads to a significant increase in the sphericity of CD163-positive cells in the myenteric plexus of *Cx3cr1-Dtr* rats given DT compared to those not given DT. **(Q)** Number of Iba-1-positive cells in the muscle layer per 0.25mm^2. **(R)** *Cx3cr1* gene expression in proximal colon of *Cx3cr1-Dtr* rats given DT compared to those not given DT. Data are mean with maximum and minimum. ** $p \leq 0.01$, **** $p \leq 0.0001$, Scale bar = 100 µm for confocal images, 70 µm for Imaris colour-gradient images. Sphericity colour gradient scale = 0.2-0.9; red indicates more spherical, blue indicates more elongated.

reduced at 48 hr after DT injection ($t_{(24)} = 4.03$, $p = 0.0005$, n = 11-15 animals per group; Figure 2A). We also found that both small intestine ($t_{(31)} = 3.32$, $p = 0.002$, n = 13-20 animals per group; Figure 2B) and colon length ($t_{(44)} = 3.09$, $p = 0.003$, n = 18-28 animals per group; Figure 2C) were shortened by intestinal macrophage ablation.

Effects of macrophage depletion on colonic motility

Next, we examined if the loss of intestinal macrophages in the myenteric plexus had any impact on colonic motility. We measured the resting gut diameter, contraction magnitude and frequency of

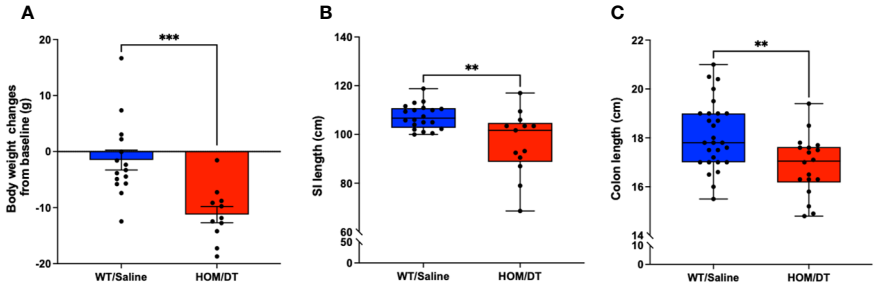


FIGURE 2
Macrophage-ablated *Cx3cr1-Dtr* rats have reduced body weight, small intestinal and colon length. Depletion of microglia and monocytes leads to significantly reduced **(A)** body weight, **(B)** small intestine length and **(C)** colon length in *Cx3cr1-Dtr* rats 48 hr after diphtheria toxin (DT) injection, relative to macrophage-intact rats. Data are mean with maximum and minimum. ** $p \leq 0.01$ and *** $p \leq 0.001$.

contraction in the proximal colon as described previously (25). Our results demonstrated that macrophage-ablated rats have a wider resting colon diameter than macrophage-intact rats under control conditions (i.e., without NOLA; main effect of genotype: $F_{(1,15)} = 7.16$, $p = 0.0017$, n = 8-9 animals per group, Figures 3A, B). The loss of intestinal macrophages also led to a decrease in contraction magnitude (main effect of genotype: $F_{(1,15)} = 45.0$, $p < 0.0001$, n = 8-9 animals per group, Figure 3C), suggesting the importance of intestinal macrophages in colonic motility under control conditions. On the other hand, inhibiting nNOS (via NOLA treatment) stimulated smooth muscle contraction and thus reduced resting gut diameter (main effect of NOLA: $F_{(1,15)} = 4.85$, $p = 0.044$, n = 8-9 animals per group, Figures 3A, B), but caused a decrease in contraction magnitude (main effect of NOLA: $F_{(1,15)} = 5.69$, $p = 0.031$, n = 8-9 animals per group, Figure 3C). As expected, inhibiting nNOS increased the frequency of contractions in the proximal rat colon. However, we observed a significantly higher increase in contraction frequency in macrophage-ablated rats (interaction effect: $F_{(1,16)} = 7.84$, $p = 0.013$, n = 8-9 animals per

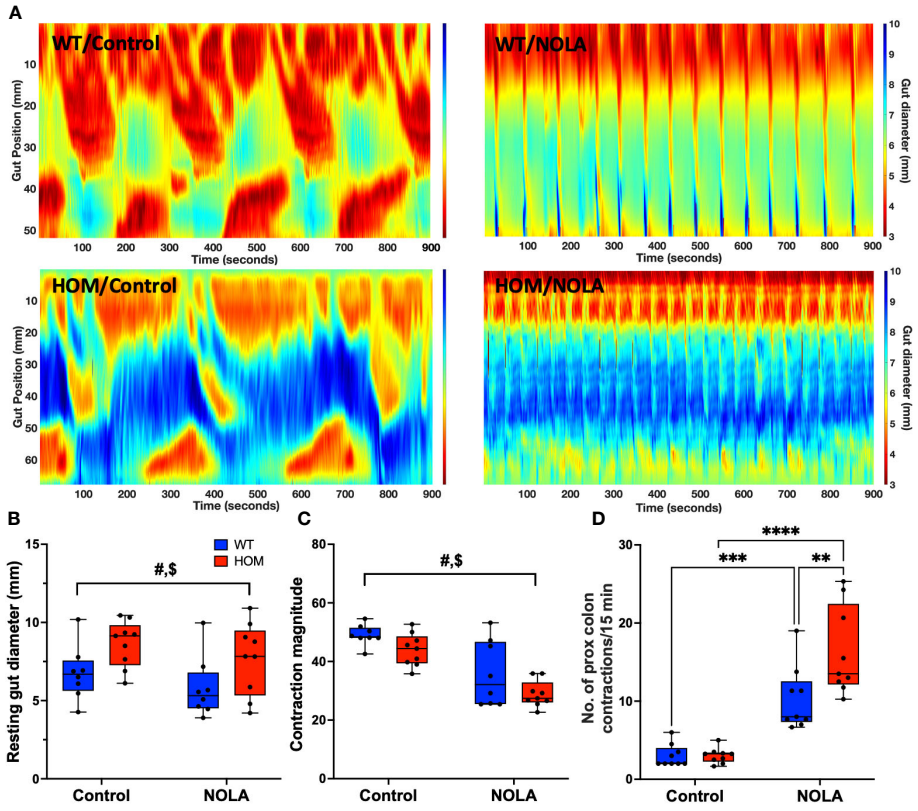


FIGURE 3
Intestinal macrophages regulate, but are not necessary for, colonic motility. **(A)** Representative spatiotemporal heatmaps of macrophage-intact (WT) and *Cx3Cr1-Dtr* rats under control conditions and with Nω-nitro-L-arginine (NOLA) treatment. **(B)** Resting gut diameter of control and macrophage-ablated rats. **(C)** Contraction magnitude of control and *Cx3Cr1-Dtr* rats. **(D)** Contraction frequency per 15 min in the proximal colon of control and macrophage-ablated rats. # two-way ANOVA with main effect of genotype, $ main effect of NOLA treatment, ** Tukey *post-hoc* test $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

group, NOLA effect on macrophage-intact rat colon: p = 0.0008, NOLA effect on *Cx3cr1-Dtr* rat colon: p < 0.0001, Figure 3D). The increase in contraction frequency upon inhibition of nNOS indicates that intestinal macrophages can regulate colonic motility without major inhibitory neuronal input.

Effect of pair-feeding on colonic motility

It has been reported that acute fasting or restricted energy intake may have a direct or indirect effect on gastrointestinal motility through satiety hormones (31–33). In our *Cx3cr1-Dtr* rats, we have consistently reported a decrease in body weight (Figure 2A) as well as food intake (17) upon DT injection. Therefore, we performed a pair-feeding experiment to verify if the changes we saw in intestinal structure and colonic motility were macrophage-related or were instead due to a decrease in food intake. As expected, the pair-fed macrophage-intact rats had a significant decrease in body weight when compared to controls ($t_{(14)} = 2.90$, $p = 0.01$, n = 8 animals per group, Figure 4A). Compared with previous weight changes from *Cx3cr1-Dtr* rats 48 after DT injection (dotted line, Figure 4A), indicating weight loss after DT injection was largely due to reduced food intake. Interestingly, there were no differences in the length of the small intestine or colon between pair-fed rats and *ad libitum*-fed, macrophage-intact, controls (Figures 4B, C). In terms of resting gut diameter, contraction magnitude and contraction frequency, we also did not observe any significant differences between pair-fed and *ad libitum*-fed, macrophage-intact, control rats (Figures 4D–F), together indicating a macrophage-specific effect on colonic motility rather than one related to food intake or changes in digestion.

Macrophage ablation does not affect neuron numbers in the myenteric plexus

Neurons in the myenteric plexus are mainly responsible for regulating colonic motility (3, 15). Based on our findings that the loss of intestinal macrophages led to an increase in colonic motility in the absence of the major inhibitory neuronal input (i.e. with NOLA), we assessed whether the size or proportion of the population of nNOS-expressing neurons in the myenteric plexus was changed in response to the loss of macrophages. The number of myenteric neurons per ganglion remained unchanged upon macrophage ablation (Figures 5B, E, M), consistent with the findings from De Schepper et al., who showed, in an embryonic macrophage-depletion model that apoptosis of neurons caused by the loss of intestinal macrophages does not take place in mice until day 7 (30). There was also no significant change in the number of neurons expressing nNOS per ganglion in the myenteric plexus (Figures 5A, C, F, N). Proportions of acetylcholinergic (ChAT)-expressing neurons within the myenteric plexus were also unaffected (Figures 5G–L, O).

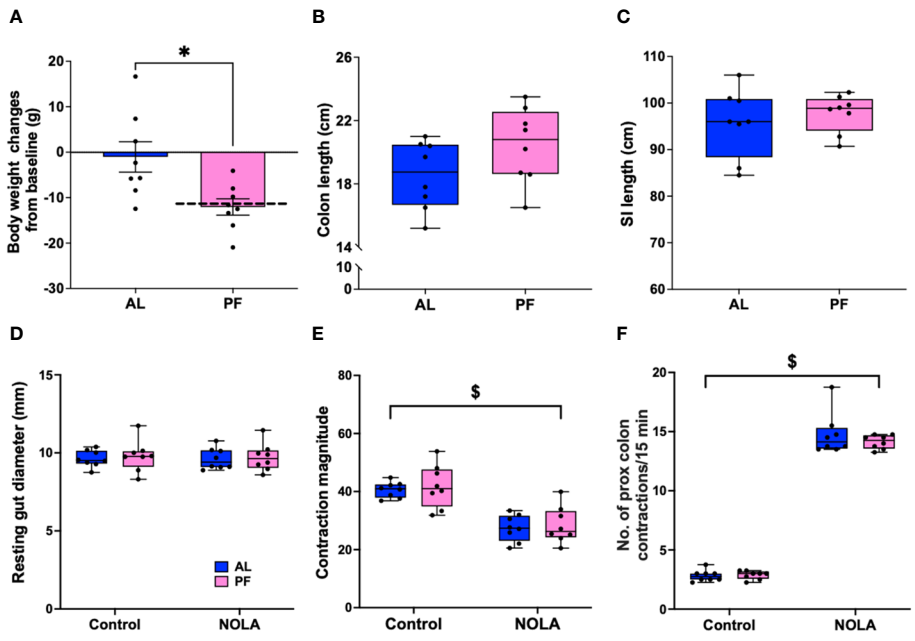


FIGURE 4
Food restriction does not affect colonic motility. **(A)** Body weight changes between *ad libitum*-fed rats (AL) and macrophage-intact (WT) rats pair-fed (PF) to that consumed by the diphtheria toxin (DT)-injected *Cx3cr1-Dtr* rats (PF), dotted line indicated the body weight changes of *Cx3cr1-Dtr* rats 48 hrs after DT injection. **(B)** Colon length of AL and PF rats. **(C)** Small intestine length of AL and PF rats. **(D)** Resting gut diameter of AL and PF rats under control conditions and with NOLA treatment. **(E)** Contraction magnitude of AL and PF rats under control conditions and with NOLA treatment. **(F)** Contraction frequency in 15 min of AL and PF rats under control conditions and with NOLA treatment. $ two-way ANOVA with main effect of NOLA treatment. * p < 0.05.

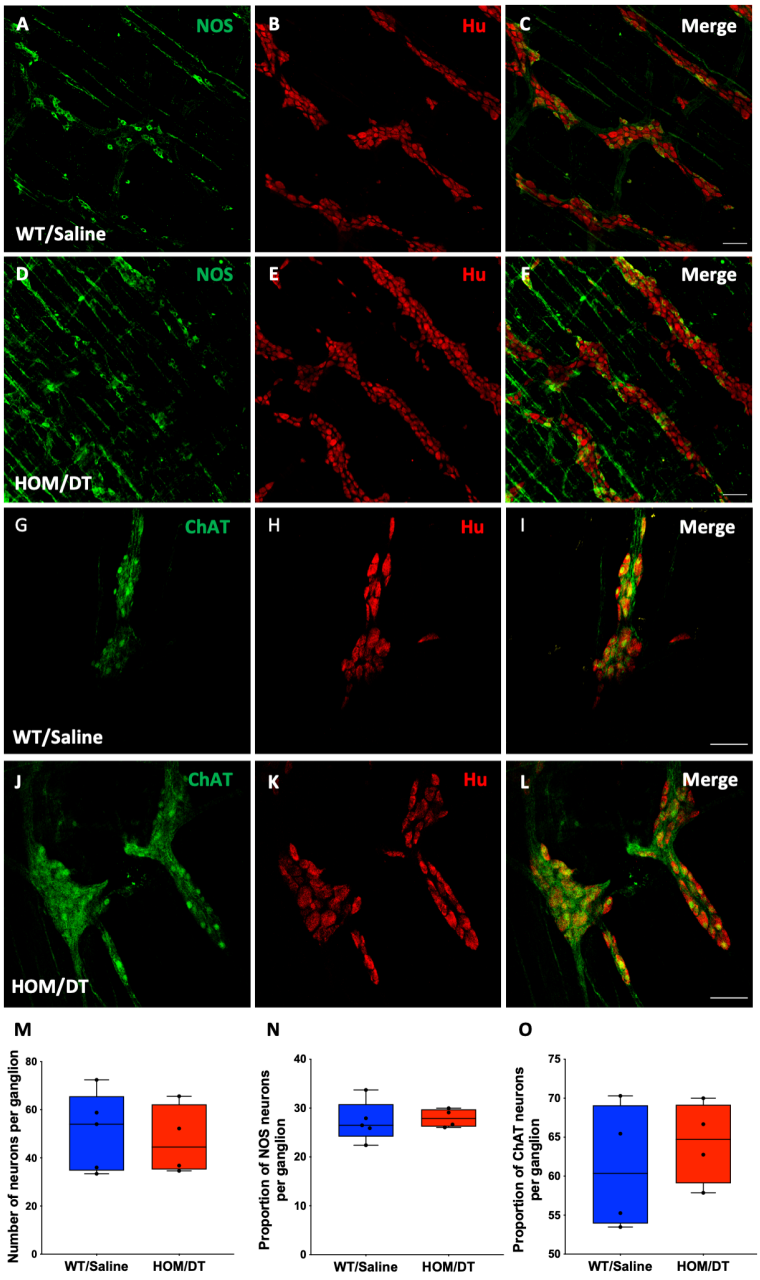


FIGURE 5
Cx3cr1 ablation does not affect the number of neurons in the myenteric plexus of the rat proximal colon. **(A–C)** control proximal colon myenteric plexus ganglia immunolabelled with **(A)** neuronal nitric oxide synthase (nNOS), **(B)** Hu and **(C)** nNOS and Hu merged. **(D–F)** *Cx3cr1-Dtr* rat proximal colon myenteric plexus ganglia immunolabelled with **(D)** nNOS, **(E)** the pan-neuronal marker, Hu, **(F)** nNOS and Hu merged. **(G–I)** control proximal colon myenteric plexus ganglia immunolabelled with **(G)** choline acetyltransferase (ChAT), **(H)** Hu and **(I)** ChAT and Hu merged. **(J–L)** *Cx3cr1-Dtr* rat proximal colon myenteric plexus ganglia immunolabelled with **(J)** ChAT, **(K)** Hu, **(L)** ChAT and Hu merged. **(M)** Macrophage ablation does not affect the number of neurons (Hu-labelled cells) per ganglion, **(N)** the number of nNOS or **(O)** ChAT neurons per ganglion. Data are displayed as mean with maximum and minimum values. Scale bars = 100 µm.

Iba-1- but not CD163-positive macrophages repopulate 7 days after DT injection

We next investigated whether intestinal macrophages repopulate after depletion, and if this could rescue some of the effects on colonic motility. We previously reported that microglia are repopulating the brain by 7 days after DT injection in the *Cx3cr1-Dtr* model (17). In accordance with this, we observed that Iba-1 expressing cells had repopulated the proximal colon at this time (Figures 6A, C–E, G, H, M). Although similar numbers of Iba-1-expressing cells were present in the colon 7 days following DT injection, the morphology of these cells remained more rounded in *Cx3cr1-Dtr* rats, similar to that of 48 hr after ablation ($t_{(20)}$ = 2.35, p = 0.029, n = 11 animals per group; Figures 6I, J, N). We also used CD163 as a marker to assess tissue-resident macrophage repopulation. Interestingly, CD163-positive

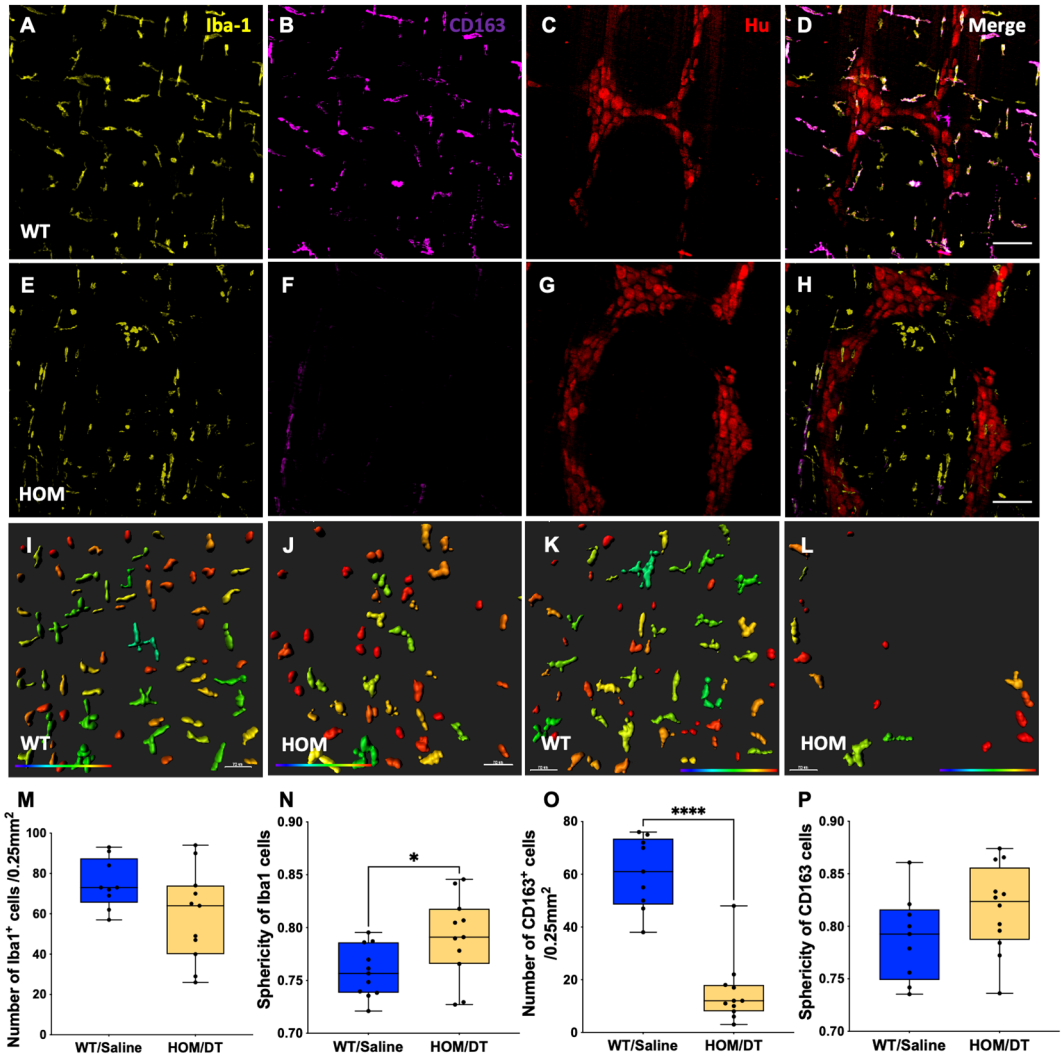


FIGURE 6
Iba-1- but not CD163-positive macrophages repopulate 7 days after DT injection. Proximal colon myenteric plexus was immunolabelled with **(A, E)** Ionized calcium-binding adaptor molecule 1 (Iba-1), **(B, F)** cluster of differentiation 163 (CD163) and **(C, G)** Hu. **(D, H)** Iba-1, CD163 and Hu (merged). **(A–D)** Controls. **(E–H)** Diphtheria toxin (DT)-injected *Cx3cr1-Dtr*. **(I–L)** Imaris colour-gradient of the sphericity of **(I, J)** Iba-1 and **(K, L)** CD163 cells in the **(I, K)** control and **(J, L)** DT-injected *Cx3cr1-Dtr* rat proximal colon. **(M)** Macrophage ablation did not affect the number of Iba-1-positive cells per 0.25 mm^2. **(N)** Sphericity of Iba-1 positive cells remained higher in *Cx3cr1-Dtr* rats than in controls 7 days after macrophage ablation. **(O)** CD163-positive cell numbers remained reduced at 7 days after macrophage ablation in *Cx3cr1-Dtr* rats compared with controls and **(P)** sphericity was unchanged. Data are mean with maximum and minimum, * $p \leq 0.05$, *** $p \leq 0.001$. Scale bars = 100 µm. 70 µm for Imaris colour-gradient images. Cellular sphericity colour gradient scale = 0.2-0.9; red indicates more spherical, blue indicates a more elongated cellular morphology.

macrophages remained depleted at this 7-day time point ($t_{(8)} = 6.94$, $p = 0.0001$, n = 11 animals per group; Figures 6B–D, F–H, O) without a change in sphericity (Figures 6K, L, P).

Macrophage repopulation recovers colon and small intestine length

After confirming that Iba-1-positive macrophages repopulate 7 days after depletion as expected, we next investigated if the gastrointestinal anatomical phenotypes we observed were also reversed at this timepoint. There remained a persistent reduction in body weight in macrophage-ablated rats ($F_{(5,5)} = 1.02$, $p = 0.005$, n = 9-10 animals per group; Figure 7A). However, the difference in colon length was no longer evident ($F_{(5,5)} = 1.04$, $p = 0.28$, n = 6 animals per group; Figure 7C) and the small intestine length was significantly longer in the *Cx3cr1-Dtr* rats 7 days after DT injection than in controls ($F_{(5,5)} = 1.08$, $p = 0.027$, n = 6 animals per group; Figure 7B). Thus, the repopulation of Iba-1-positive intestinal macrophages was sufficient to rescue the shortened small intestine and colon associated with macrophage loss.

Macrophage repopulation effects on colonic motility

As we observed that the shortened colon and small intestinal phenotypes were rescued upon the repopulation of Iba-1-positive-

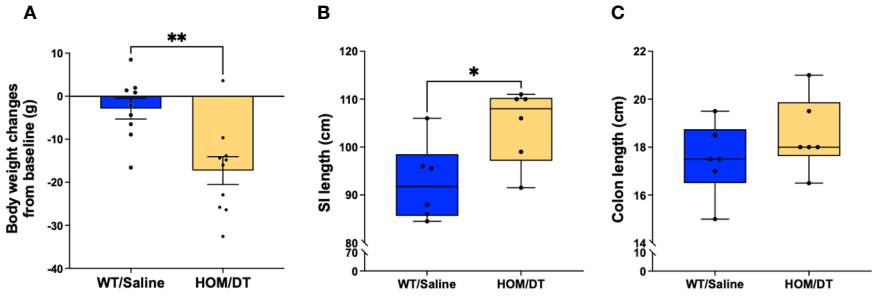


FIGURE 7
Macrophage repopulation leads to increased small intestine length and recovered colon length at 7 days after macrophage loss. **(A)** DT-injected *Cx3cr1-Dtr* rats maintained reduced body weight 7 days after macrophage ablation. **(B)** *Cx3cr1-Dtr* rats had significantly longer small intestines than controls 7 days after macrophage ablation. **(C)** There was no significant difference in colon length between controls and *Cx3cr1-Dtr* rats 7 days after macrophage ablation. Data are depicted as mean with maximum and minimum values. $p \leq 0.05$. * $p \leq 0.05$ and ** $p \leq 0.01$.

only intestinal macrophages, we examined if the exacerbation of the NOLA-induced changes in resting gut diameter and contraction frequency caused by macrophage ablation were similarly restored. There was no significant difference, however, in resting gut diameter and contraction magnitude between the control and macrophage repopulating colon (Figures 8A–C), as opposed to that seen in macrophage-depleted colons. As expected, NOLA treatment led to a decrease in resting gut diameter (main effect of NOLA treatment: $F_{(1,14)} = 6.49$, $p = 0.023$, n = 8 animals per group; Figure 8B) and colonic contraction magnitude (main effect of NOLA treatment: $F_{(1,14)} = 68.6$, $p < 0.0001$, n = 8 animals per group; Figure 8C). Interestingly, the greater increase in contraction frequency upon NOLA treatment we observed in macrophage-ablated rats at 48 hr was also noted at 7 days, despite the repopulation of Iba-1-positive

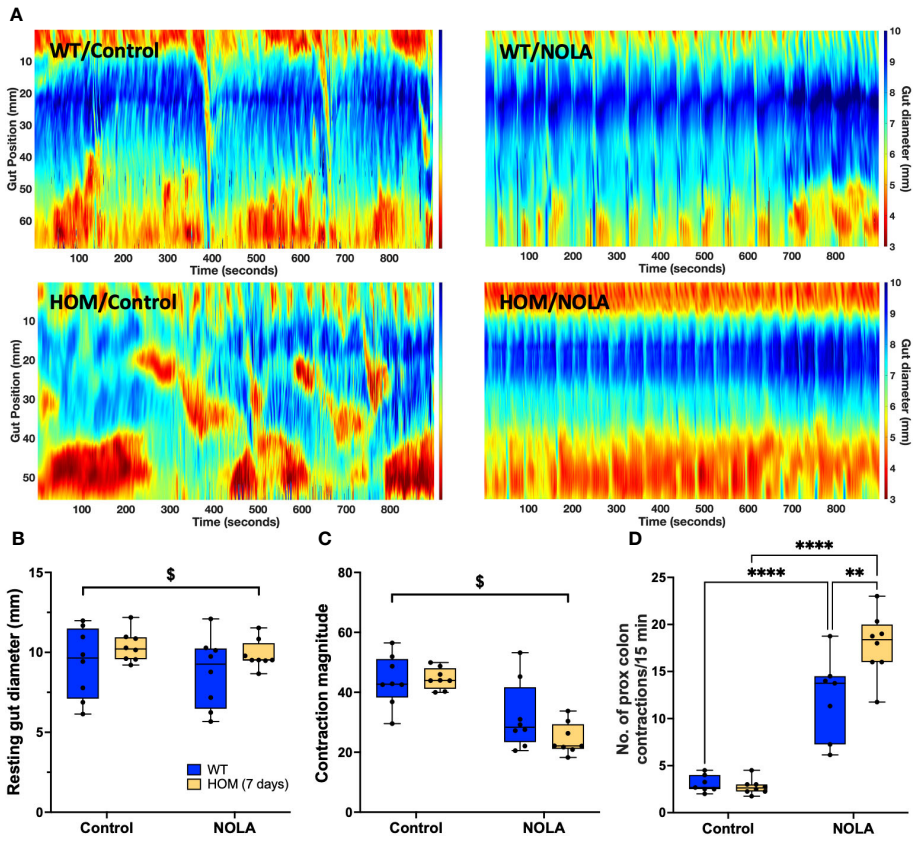


FIGURE 8
Iba-1-positive macrophage repopulation does not rescue motility dysregulation in the absence of inhibitory neuronal input. **(A)** Representative spatiotemporal heatmaps of control (WT) and *Cx3Cr1-Dtr* rats under control conditions and with Nω-nitro-L-arginine (NOLA) treatment. **(B)** Resting gut diameter of control and macrophage-repopulating rats. **(C)** Contraction magnitude of control and *Cx3Cr1-Dtr* rats. **(D)** Contraction frequency per 15 min in the proximal colon of control and macrophage-repopulating rats. # two-way ANOVA with main effect of genotype, $ main effect of NOLA treatment, ** Tukey *post-hoc* test $p < 0.01$, **** $p < 0.0001$.

macrophages (interaction effect: $F_{(1,13)} = 7.72$, $p = 0.016$, n = 8 animals per group, *post hoc* NOLA effect on macrophage-intact rat colon: $p < 0.0001$, *post hoc* NOLA effect on *Cx3cr1-Dtr* rat colon: $p < 0.0001$, n = 8 animals per group; Figure 8D). This finding suggests that Iba-1 positive macrophage repopulation was not sufficient to rescue the dysregulation of motility that occurs without inhibitory neuronal input.

Discussion

Here we show that intestinal macrophages act to restrict intestinal motility. We also identify that this restriction of motility occurs in close interplay with neuronal inputs to the myenteric plexus. Thus, in the absence of intestinal macrophages, colonic motility was normal except when the major inhibitory neuronal input was blocked. In the absence of both this neuronal input and macrophages, colonic contractility was significantly greater than normal. Our findings suggest that this effect is maintained by CD163-positive intestinal-resident macrophages, since the restoration of the non-CD163 population failed to restore this response.

We established here that the *Cx3cr1-Dtr* rat model provides conditional ablation of intestinal macrophages upon DT injection, as for microglia and circulating monocytes in the brain as previously demonstrated (18). The repopulation of intestinal macrophages occurred by 7 days post-DT injection, in line with our previous observations for microglia in brain (18). Although we did not assess additional cell types in this study, our previous work in the ovary has shown that non-immune cell numbers are not affected (34). This rat model is therefore suitable for studying the role of intestinal macrophages in gastrointestinal function. Using this tool, we provide novel evidence that intestinal macrophages are essential in maintaining intestinal structure and that they can also regulate colonic motility in conjunction with the ENS.

This rat model has previously been characterized as a conditional microglia- and circulating monocyte- depletion model and it is striking to observe that the duration of the depletion of intestinal macrophages is very similar to that of microglia, as is the repopulation time frame (17). Cx3cr1, a microglia-associated chemokine, is highly expressed in fully differentiated and mature macrophages in the intestine (35). Notably, most intestinal macrophages in the colon appear to express Cx3cr1 (36). Bain and colleagues characterized colonic cells into those with high expression ($CX3CR1^{high}$), those with intermediate expression ($CX3CR1^{int}$) and those without expression of CX3CR1 ($CX3CR1^{-}$). $CX3CR1^{high}$ cells uniformly express F4/80 and major histocompatibility complex (MHC)II, as well as CD64, a marker that distinguishes macrophages from dendritic cells. The $CX3CR1^{int}$ cells represent a small population of cells that are actively migrating from blood vessels as monocytes before differentiating into mature macrophages. Cells lacking Cx3cr1 expression do not express the relevant macrophage markers outlined above (36), meaning Cx3cr1 is a useful marker for intestinal macrophages and a useful target for depleting this population (17, 37–39). The similarity of microglia and intestinal macrophages extends beyond Cx3cr1 expression, as studies have identified a set of microglia-specific genes and transcription factors that are more highly similar to those of intestinal macrophages than to other macrophage subsets (35, 40). Unlike intestinal macrophages, microglia have been extensively studied for their roles in neuroinflammation and in interactions with other elements of the central nervous system (41). Therefore, the similarities of these two cell types in brain and gut may give us insights into how intestinal macrophages orchestrate gastrointestinal functions in health and disease.

Due to the heterogeneity of immune cells such as dendritic cells, T-cells, and macrophages in the intestine, it is challenging to identify and study intestinal macrophages in isolation. Thus, the ontogeny of intestinal macrophages is of great interest. There is a consensus that intestinal macrophages are continuously replenished by blood monocytes through a series of differentiation events (36, 42). However, recent studies have identified a subset of intestinal macrophages that maintain their own population, named tissue-resident macrophages (30). These self-maintaining macrophages are critical in neuroimmune interactions as they are mainly localised in the submucosal and myenteric plexuses in close proximity to enteric neurons, which in turn regulate intestinal secretion and motility (29). One of the main characteristics of these tissue resident macrophages is the expression of CD163 (30, 43). In general, Iba-1 stains for a broad subset of intestinal macrophages, including recently invaded blood monocytes as well as tissue-resident macrophages (44–46). Our data from the myenteric plexus show that most Iba-1-expressing macrophages also express CD163. This finding aligns with previous work showing that macrophages residing in the muscularis layer are predominantly tissue-resident macrophages (30, 47). A striking finding from our study was that 7 days after DT injection, most repopulating macrophages expressed Iba-1 but not CD163. There is controversy in the literature as to how macrophages are replenished in different tissues in depletion models and during natural turnover. In a lung-resident macrophage ablation *Cd169-Dtr* mouse model, tissue resident macrophages did not repopulate through CCR-2-dependent cells like monocytes, but replenish themselves locally (48). However, in another study evaluating the origin of peritoneal macrophages, Bain et al., proposed that homeostasis of resident peritoneal macrophages is achieved through a combination of self-renewal and monocyte-derived replenishment (49). In our case, we speculate that the rate of monocyte replenishment at the muscularis layer of the colon is faster than the self-renewal of tissue resident macrophages. There is also the possibility that a specific subset of tissue resident macrophages is responsible for macrophage replenishment, as it has been reported that $Tim\text{-}4^{+}$ $CD4^{+}$ macrophages in the intestine are capable of self-renewing (47). Further verification on whether this subtype also expresses CD163 would provide a better understanding of how intestinal macrophages maintain their population. Notably, previous studies on Kupffer cells (liver-resident macrophages) showed that once these cells are depleted in a conditional knockout model, monocytes quickly replenish and repopulate the liver but these repopulating monocytes take at least 15 days to fully express the Kupffer cells transcriptomic profile (50, 51). It is suggested that monocyte

replenishment occurs in two phases, firstly via a quick replenishment phase, and secondly via a slower reprogramming/differentiation stage. We suspect similar mechanisms could also explain our observation that we did not see CD163 macrophages repopulate by 7 days after ablation. Thus, it would be interesting to undertake further immune cell analysis at later timepoints following ablation to identify if CD163-expressing macrophages repopulate and if intestinal motility function is restored as this occurs.

Macrophage sphericity often correlates with cellular activation state (52, 53). Previous studies have demonstrated that activated macrophages, which exhibit an inflammatory phenotype, have higher sphericity (52). In addition to the loss of intestinal macrophages upon DT injection, our cell analysis showed that the remaining macrophages in DT-injected rats had higher sphericity than in controls. One explanation for this observation is that DT injection predominantly ablated macrophages exhibiting lower sphericity, leaving more rounded macrophages behind. When macrophages are in a pro-inflammatory state, their Cx3cr1 expression decreases, meaning that there could be some pro-inflammatory macrophages originally present in the myenteric plexus with lower levels of Cx3cr1 (16). If this was the case, DT may be less effective at removing these pro-inflammatory macrophages leading to the observation of an increase in sphericity. Our model resulted in ablation of approximately 80% of macrophages, however, and about 40% of macrophages in the control groups had similar sphericity to those remaining after ablation. Therefore, a loss of Cx3cr1 expression and therefore a retention of pro-inflammatory macrophages is unlikely to account for the morphological cell differences we see. Another explanation for the observed higher sphericity of remaining intestinal macrophages could be that in response to the initial depletion of macrophages, the remaining macrophages may become pro-inflammatory and act to release cytokines to attract other immune cells to restore homeostasis.

Interestingly, the ablation of intestinal macrophages led to shortening of the small intestine and colon. Such gross anatomical changes are hallmarks of major intestinal disturbances such as colitis (54). However, we did not observe other features from animal models of colitis such as rectal bleeding or an increase in circulating pro-inflammatory cytokines (17). Originally, we suspected that the shortened colon length was due to a reduction in fecal pellet formation in the lumen, leaving the colons less flexible than those with more pellets. However, we did not see any difference in number of pellets inside the colon of rats with ablated macrophages compared to controls ($t_{(31)} = 1.82$, $p = 0.0781$, n = 13 for WT and 20 for HOM/DT; graph not shown), although we did not analyze the size of the pellets, which may also influence the flexibility of the colon. It is also worth noting that macrophages have a protective role in preventing muscle atrophy as well as promoting muscle recovery, suggesting an important interaction between macrophages and skeletal muscle cells (55). The protective role of macrophages could also explain our observation that shortening of the small intestine and colon was rescued upon repopulation of intestinal macrophages. Further histological examination of structures in the gastrointestinal tract such as the mucus lining and muscle thickness in the absence of intestinal macrophages will be important considerations in the future.

Our findings reveal that intestinal macrophages are not crucial for colonic motility under control conditions whereby contraction frequency was not affected by the absence of these cells. While several studies have demonstrated that intestinal macrophages influence gastrointestinal dysmotility in disorders such as inflammatory bowel disease and post-operative ileus (56–58), to our knowledge only two studies have demonstrated an impact on colonic motility under control (homeostatic) conditions (6, 30). In the study by Muller et al., *ex vivo* colonic motility was measured as contraction force generated by a 3 mm colonic ring, followed by a stretch stimulus in adult mice (6). De Schepper et al. measured gastrointestinal motility via ileal muscle strip contractility, gastrointestinal transit and gastric emptying (30). In addition to differences in mechanical measurements, these studies investigated the effects of depleting macrophages at the embryonic phase, not acutely in adulthood as in our work (6, 30).

Another important finding from the present study is that intestinal macrophages are crucial in regulating colonic motility when NOS is inhibited. Blockade of nNOS depletes the major inhibitory signal in the ENS so that smooth muscles are excited at a higher frequency (59). Under these circumstances, intestinal macrophages may act to regulate and even prevent hyper-contraction of the colon. In our study, we observed that macrophage-ablated colons had a much higher increase in contraction frequency upon NOLA treatment when compared to controls. This indicates that intestinal macrophages have an additional inhibitory role in modulating gastrointestinal physiology and reveals their importance specifically in regulating colonic motility. Since the CD163-positive resident intestinal macrophages did not repopulate at the 7-day post DT injection timepoint, we suspect that this subtype of resident intestinal macrophage is crucial in inhibiting colonic motility in addition to the inhibitory neuronal input from the ENS, supporting previous reports that self-maintaining resident macrophages are essential for gastrointestinal transit (30). Although we did not observe changes in neuronal numbers or proportions of nNOS neurons within the myenteric plexus, macrophage depletion could lead to apoptosis of neurons that would not be reflected in Hu/NOS immunostaining alone (60). Notably, our findings suggest that intestinal macrophages can influence contraction frequency but do not affect contraction magnitude. In general, both parameters involve neural-muscular transmission from the myenteric plexus to smooth muscle, under the control of interstitial cells of Cajal (61). The contraction magnitude is chiefly the outcome of the excitation and relaxation of longitudinal muscle and circular muscle, while contraction frequency is mainly determined by the neural input in response to physical tension (14). Therefore, our results imply that intestinal macrophages can directly interact with enteric neurons to exert an inhibitory effect on contraction frequency even when inhibitory neural input is significantly reduced. In terms of a mechanism for this, it has previously been reported that intestinal macrophages can interact with smooth muscle layers

via the transient receptor potential cation channel subfamily V member 4 (TRPV4)- prostaglandin E2 (PGE-2) axis, the IL-17A-iNOS axis or CSF-1/BMP-2 crosstalk with neurons (3). Purinergic neurotransmission may also play a role in the inhibitory regulation of gut motility (62, 63). P2X receptors are expressed in the submucosal plexus, myenteric plexus, as well as the smooth muscle layers (64). In particular, P2X2R receptors localized in intermuscular neurons are involved in the regulation of smooth muscle contraction (65). Therefore, it would be of interest to assess how CD163-expressing macrophages regulate colonic motility through potential downstream effects on these pathways.

In conclusion, this is the first study examining the role of intestinal macrophages in a conditional macrophage ablation rat model and the first such study to utilize *ex vivo* video imaging techniques to assess colonic motility in these rats. Our findings highlight the importance of intestinal macrophages in maintaining gastrointestinal structure and illustrate that tissue resident macrophages are likely to regulate colonic motility in the absence of inhibitory neuronal input. Gastrointestinal disorders where inhibitory neuronal input is suppressed, such as gastroparesis and achalasia, are often caused by bacterial or viral infection with involvement of macrophages (66, 67). Our evidence implicating a role for intestinal macrophages gives insight into how pathophysiology may manifest in these conditions. Future directions should focus on dissecting the precise mechanism of how intestinal macrophages regulate colonic motility and differentiating the subtypes of intestinal macrophages involved in supporting normal intestinal structure. A better understanding of the role of intestinal macrophages will provide macrophage-specific therapeutic targets for various gastrointestinal disorders.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by RMIT University Animal Ethics Committee (AEC #1920). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JY: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. GB: Methodology, Supervision. SX: Investigation. EH-Y: Conceptualization, Funding acquisition, Supervision, Resources, Writing - review & editing. SS: Conceptualization, Funding acquisition, Supervision, Resources, Writing - review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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EDITED BY
Estela Maris Muñoz,
CONICET Dr. Mario H. Burgos Institute of Histology and Embryology (IHEM), Argentina

REVIEWED BY
Alicia Beatriz Penissi,
Universidad Nacional de Cuyo, Argentina
Howard Eliot Gendelman,
University of Nebraska Medical Center, United States

*CORRESPONDENCE
Yasu Zhang
✉ zhangyasu16@163.com

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Bibliometric analysis of global research trends on regulatory T cells in neurological diseases

Qian Gao[1], Xinmin Li[2], Yan Li[2], Junzi Long[1], Mengyang Pan[1], Jing Wang[1], Fangjie Yang[1] and Yasu Zhang[1]*

[1]School of Rehabilitation Medicine, Henan University of Chinese Medicine, Zhengzhou, Henan, China, [2]School of Traditional Chinese Medicine, Henan University of Chinese Medicine, Zhengzhou, Henan, China

This bibliometric study aimed to summarize and visualize the current research status, emerging trends, and research hotspots of regulatory T (Treg) cells in neurological diseases. Relevant documents were retrieved from the Web of Science Core Collection. Tableau Public, VOSviewer, and CiteSpace software were used to perform bibliometric analysis and network visualization. A total of 2,739 documents were included, and research on Treg cells in neurological diseases is still in a prolific period. The documents included in the research were sourced from 85 countries/regions, with the majority of them originating from the United States, and 2,811 organizations, with a significant proportion of them coming from Harvard Medical School. Howard E Gendelman was the most prolific author in this research area. Considering the number of documents and citations, impact factors, and JCR partitions, *Frontiers in Immunology* was the most popular journal in this research area. Keywords "multiple sclerosis," "inflammation," "regulatory T cells," "neuroinflammation," "autoimmunity," "cytokines," and "immunomodulation" were identified as high-frequency keywords. Additionally, "gut microbiota" has recently emerged as a new topic of interest. The study of Treg cells in neurological diseases continues to be a hot topic. Immunomodulation, gut microbiota, and cytokines represent the current research hotspots and frontiers in this field. Treg cell-based immunomodulatory approaches have shown immense potential in the treatment of neurological diseases. Modifying gut microbiota or regulating cytokines to boost the numbers and functions of Treg cells represents a promising therapeutic strategy for neurological diseases.

KEYWORDS

bibliometric analysis, regulatory T cells, neurological diseases, immunomodulation, gut microbiota, cytokines

Introduction

The nervous system serves as the body's command center, and interruptions or impairments of its function can induce neurological diseases, including stroke, spinal cord injury, traumatic brain injury, multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD) (1). Unfortunately, neurological diseases remain highly prevalent, with a scarcity of effective therapeutic strategies (2, 3). As a result, these disorders pose a considerable socioeconomic burden to society, underscoring the urgent need for continued research and development of effective therapies in the field of neurology. While inflammation may not be the direct cause of neurological diseases, accumulating evidence suggests its involvement in their pathogenesis once these diseases have manifested. A

recent research study indicates that disordered innate and adaptive immune responses play a crucial role in the pathological processes of neurological diseases, potentially resulting in autoimmunity, tissue and cellular damage, and subsequent neurological degeneration (4). By addressing the underlying pathological processes that contribute to neurological disease, it is possible to improve the neurological symptoms of individuals affected by these conditions. Therefore, developing effective therapeutic strategies to manage immune-mediated inflammation is crucial in preventing or delaying the onset and progression of neurological diseases.

Regulatory T (Treg) cells are a minor subpopulation of $CD4^+$ T cells defined by constitutive expression of IL-2 receptor alpha (CD25) and forkhead box p3 (Foxp3) (5). In 1995, Sakaguchi et al. formally identified Treg cells as a distinct subset of T cells with the $CD4^+CD25^+$ marker (6). This landmark study elucidated the ability of Treg cells to suppress allogeneic responses and revealed that the depletion of Treg cells could lead to enhanced immune responses and the spontaneous development of autoimmune diseases. Treg cells maintain immune homeostasis by suppressing adaptive immune responses and modulating innate immune responses (7). In addition to promoting self-tolerance, Treg cells also mitigate inflammatory conditions to prevent excessive damage to individual tissues.

Early findings suggested that Treg cells could modulate neuroinflammation and attenuate disease progression (6, 8). However, due to the complexity of the immune system and the heterogeneity of neurological diseases, there were discrepancies and conflicting results among individual studies. For example, a study reported that Treg cells, while often beneficial, could act as a double-edged sword in central nervous system injury, attenuating both protective and inflammatory post-injury immune responses and thus either exacerbating or ameliorating neuronal degeneration (9). Several studies identified that Treg cells acted as a negative player in neurological diseases (10, 11). However, numerous studies found that Treg cells play a beneficial role in neurological diseases, and this viewpoint occupies an important position (12–15). In 1996, a study first reported that the oral administration of antigen induces oral tolerance in animal models of experimental autoimmune disease mainly through the induction of Treg cells that actively suppress immune responses by secreting the TGF-β1 cytokine (8). The findings of another study on experimental autoimmune encephalomyelitis indicated that Treg cells play a facilitative role in the remyelination process and exert suppressive effects on neuroinflammatory responses during the chronic stages of MS (12). The expansion of Treg cells has been shown to effectively suppress immune responses and mitigate dopaminergic neurodegeneration in A53T-α-synuclein PD mice (14). Numerous studies have indicated that Treg cells play a beneficial role in delaying the onset and progression of neurological diseases, while dysfunction in Treg cells may lead to the development of autoimmune disease and the progression of neuroinflammation (15). In short, promoting the production and activity of Treg cells represents promising therapeutic strategies for managing immune-mediated inflammation in neurological diseases. Therefore, it is extremely important to understand the current research status and development trends concerning Treg cells in neurological diseases. Gaining such knowledge can help to further explore immunologic mechanisms and therapeutic strategies of neurological diseases and address relevant clinical problems.

Bibliometric analysis can analyze and visualize scientific outputs, research hotspots, and trending topics of a certain field in public literature databases (16). Bibliometric tools, including VOSviewer and CiteSpace, are commonly applied to visualize results of document analysis, which have been widely used in medical fields (17–20). VOSviewer, a free Java-based software, can be used to analyze a large number of document data in an easy-to-interpret way and display it in the form of a map (17). By using CiteSpace, a Java-based software, research results in a certain field can be visualized to help researchers and experts understand the knowledge domain, research frontiers, and development trends (21). Although bibliometric studies on neurological diseases have been conducted, there has yet to be a bibliometric analysis of Treg cells in neurological diseases (22, 23). This study aimed to bridge this knowledge gap by conducting a bibliometric analysis of documents on Treg cells in neurological diseases. Specifically, this analysis identified major contributors and current research status and evaluated future development prospects and research trends in this field.

Methods

Data sources and search strategy

All data were downloaded from the Web of Science Core Collection online database, and the search strategy was as follows: TS = ("regulatory T cell*" OR "regulatory T-cell*" OR "Treg*" OR "T-reg*") AND TS = ("neuro*") (23). Subsequently, we limited the document types to articles and review articles, and selected documents written in English. Finally, relevant data were exported in a plain text file with full records and cited references.

Bibliometric analysis

We analyzed relevant data in the following aspects: the annual number of documents, countries/regions, organizations, authors, journals, keywords, and references. Online platform (http://www.bioinformatics.com.cn) was used to plot the annual document output. Software Tableau Public was applied to draw the geographic distribution of documents. VOSviewer v.1.6.1 and CiteSpace v.6.1.R6 were applied to perform the bibliometric analysis and network visualization, including co-authorship analysis of countries/regions, organizations and authors, co-occurrence analysis of keywords, citation analysis of journals and documents, and co-citation analysis of references.

Results

The trend of document outputs

A total of 2,739 documents, including 1,737 articles and 1,002 review articles, were collected from the Web of Science Core

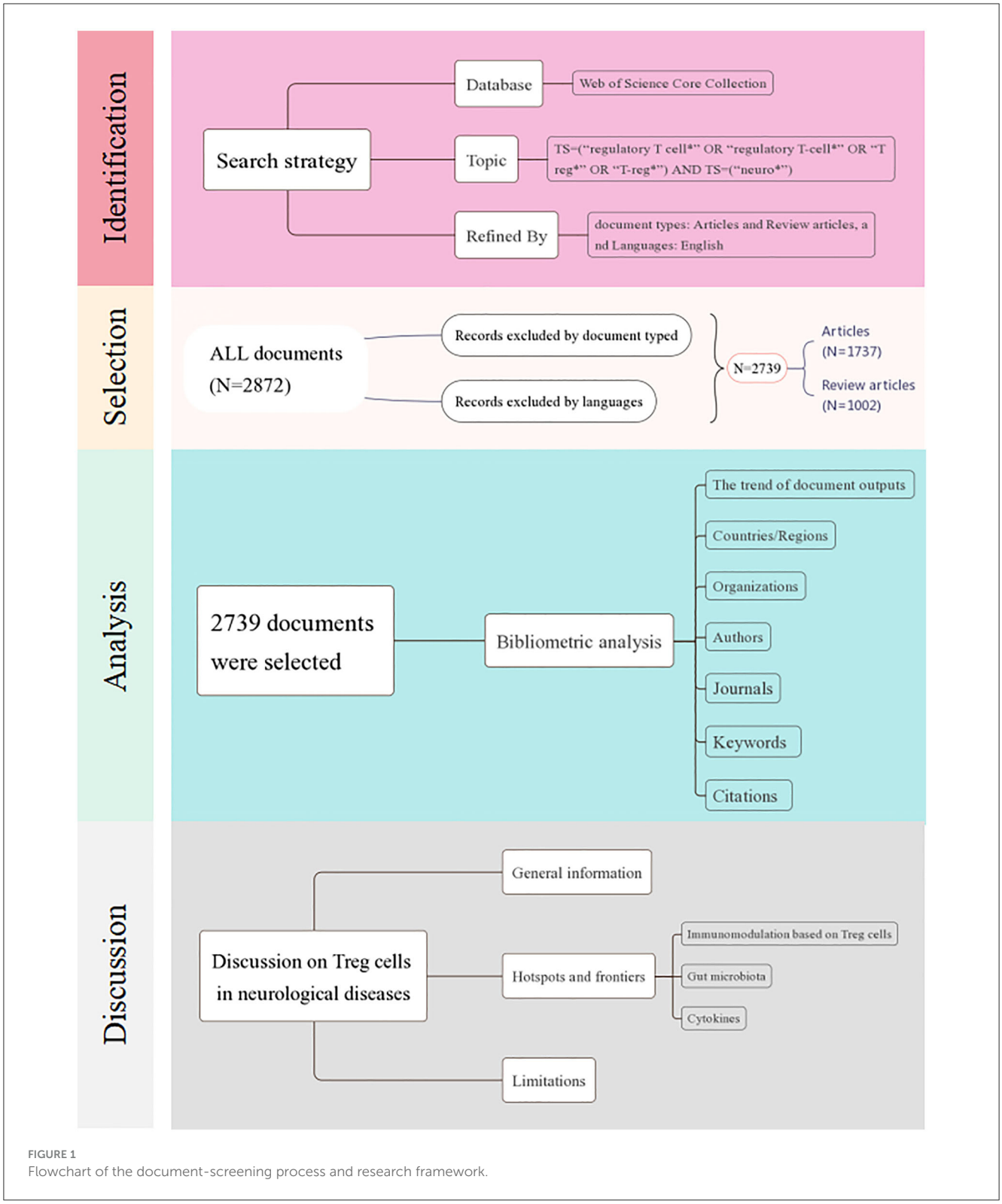


FIGURE 1
Flowchart of the document-screening process and research framework.

Collection on 2 August 2023. The research selection process and research framework are shown in Figure 1. As shown in Figure 2, the research trend can be divided into three stages. During the first stage, spanning from 1991 to 2003 and comprising 20 documents, the output of published research on Treg cells in neurological diseases gradually increased from 1 to 6. This suggests that the field was still in a nascent period, with relatively few studies conducted on the topic at the time. The second stage was from 2004 to 2019, during which the annual output climbed rapidly with a slight fluctuation in 2011. Starting from 2020, the third stage has been a volatile but prolific period in which the annual output of research on Treg cells in neurological diseases has consistently exceeded

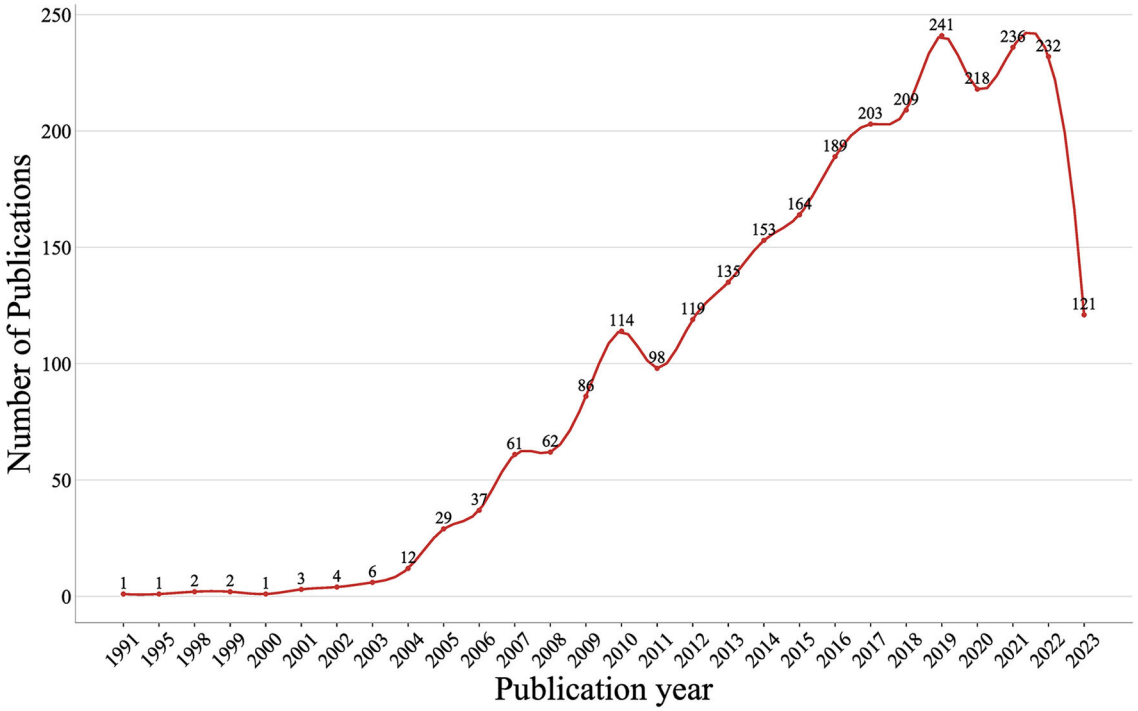


FIGURE 2
Trends of annual documents related to Treg cells in neurological diseases.

210 documents despite fluctuations. Only 7 months of documents were counted in 2023, but the overall trend in published research was stable. It suggested that the role of Treg cells in neurological diseases has attracted extensive attention worldwide since 2004 and remains a continuing hotspot.

Countries/regions

A total of 85 countries/regions dabbled in the role of Treg cells in neurological diseases (Figure 3A). The United States, China, and Germany were the top three countries/regions with the most documents on Treg cells in neurological diseases (Table 1). The United States not only had the largest number of documents and citations but also had the highest total link strength and centrality, making it a leading contributor to research on Treg cells in neurological diseases. The annual output of the top 10 countries/regions is shown in Figure 3B. The United States posted a significantly higher annual output than any other country/region until 2021, after which the United States decreased, while China overtook other countries/regions to rank first. In addition, the United States was the earliest country to focus on this research area, while China did not begin to bloom until 2015. The United States, Germany, France, and England had the highest centrality, indicating that they had a strong bridge role in this field (Figure 3C). Some of the documents were completed in cooperation with multiple countries/regions. The United States had collaborated with 57 countries/regions, and Germany had collaborated with 48 countries/regions.

Organizations

A total of 2,739 documents were published by 2,811 different organizations, and 61 met the threshold (minimum number of documents of an organization: 15). After excluding disjointed organizations, the remaining 59 organizations were visualized (Figure 4A). The top 10 organizations with the most documents are listed in Table 2, and 7 of the top 10 organizations were affiliated with the United States. Harvard Medical School ranked first in terms of the number of documents and citations, total link strength, and centrality, indicating that it was the most prolific organization and had the most cooperation with other organizations. In addition, the University of California system, including the University of California San Francisco (UCSF) and the University of California Los Angeles (UCLA), was another important organization in this research area. As shown in Figure 4B, Harvard Medical School, University of Pittsburgh, and Fudan University (nodes with yellow color) were the most recent organizations to publish more documents. The top three organizations with the strongest citation bursts were Consejo Superior de Investigaciones Cientificas (CSIC) from 2006 to 2013, Harvard University from 2000 to 2009, and Weizmann Institute of Science from 2001 to 2005 (Figure 4C). The citation bursts in many organizations have continued until 2023, suggesting that Treg cells

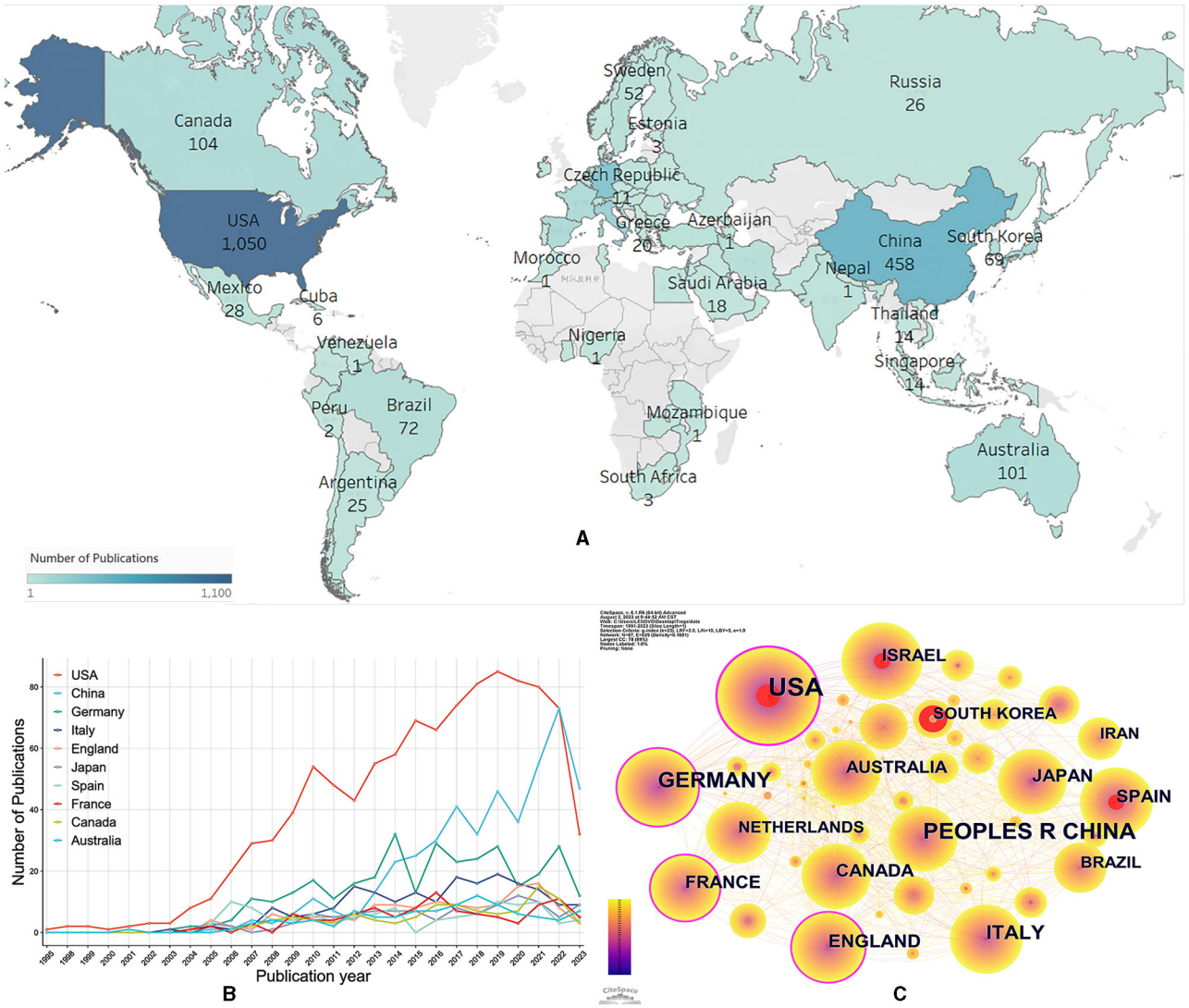


FIGURE 3
Co-authorship analysis of countries/regions. **(A)** Geographic distribution of documents. The darker the color, the more documents in this country/region. **(B)** Trends of annual documents of the top 10 countries/regions. **(C)** Visualization map of countries/regions collaboration analysis. Each node represents a country/region, and the node size is positively correlated with the number of documents. The connection between nodes represents collaboration. Countries/regions with citation bursts are presented with red nodes, and nodes with purple rings have high centrality values.

TABLE 1 **Top 10 countries/regions with the most documents.**

Rank	Country/region	Documents	Citations	Total link strength	Centrality
1	USA	1,050	63,033	554	0.38
2	China	458	12,210	186	0.07
3	Germany	328	20,735	308	0.18
4	Italy	195	10,065	145	0.04
5	England	135	7,240	178	0.1
6	Japan	111	6,519	59	0
7	Spain	108	4,743	100	0.03
8	France	106	4,287	98	0.15
9	Canada	104	6,127	120	0.06
10	Australia	101	5,131	107	0.02

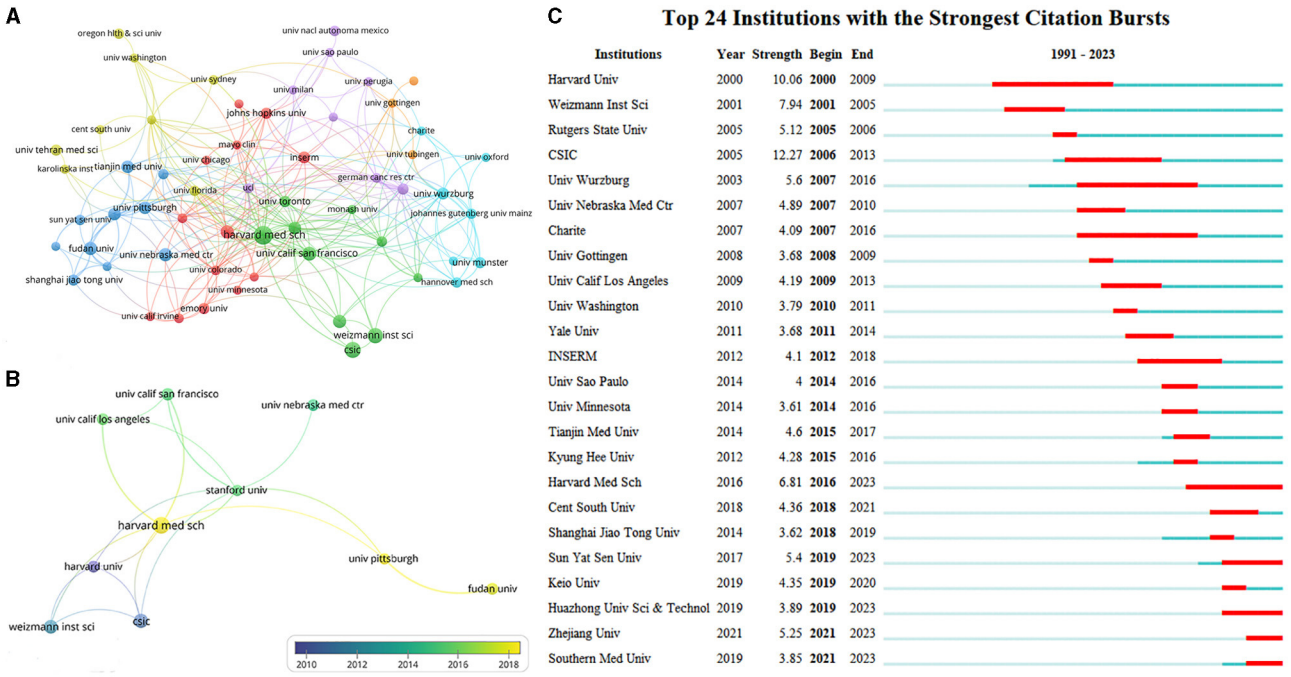


FIGURE 4
Co-authorship analysis of organizations. **(A)** Visualization map of organizations collaboration analysis. Each node represents an organization, and the node size is positively correlated with the number of documents. The connection between nodes represents collaboration, and the distance and thickness of the connection represent the relative strength of the relationship. **(B)** Visualization map of the top 10 organizations' collaboration. The color means the average published year. **(C)** Top 24 organizations with the strongest citation bursts. Minimum duration: 2.

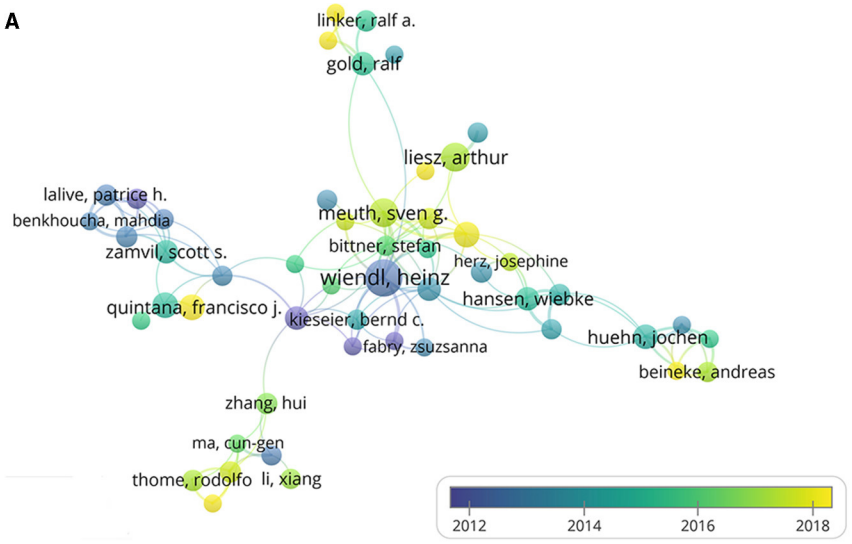
TABLE 2 Top 10 organizations with the most documents.

Rank	Organization	Country/Region	Documents	Citations	Total link strength	Centrality
1	Harvard Medical School	USA	64	4,019	43	0.17
2	Consejo Superior de Investigaciones Cientificas (CSIC)	Spain	47	1,814	5	0.04
3	Weizmann Institute of Science	Israel	45	2,586	6	0.03
4	University of California San Francisco	USA	36	3,426	23	0.05
5	Fudan University	China	35	1,482	16	0.04
6	University of Nebraska Medical Center	USA	35	1,801	3	0.01
7	University of California Los Angeles	USA	34	1,459	26	0.03
8	Harvard University	USA	33	3,543	10	0.09
9	Stanford University	USA	31	2,717	39	0.13
10	University of Pittsburgh	USA	31	2,394	21	0.09

in neurological diseases remain the hotspot for future research by many organizations.

Authors

A total of 13,859 authors were involved in Treg cells in neurological diseases, and 185 met the threshold (minimum number of documents of an author: 5). The largest set of connected items consisted of 51 authors (Figure 5A). The top 15 core authors in this field are listed in Table 3, of which 6 authors came from the United States. The top 15 authors published 271 documents, accounting for 9.89% of the total number. Howard E Gendelman and R Lee Mosley were the top authors with the largest number of documents and citations. Both are affiliated with the University of Nebraska Medical Center in the United States, which ranked sixth in terms of the number of documents about the role of Treg cells in neurological diseases. As shown in the visualization map of authors (Figure 5A), Heinz Wiendl was the center of authors' co-authorship



C

Top 14 Authors with the Strongest Citation Bursts

Authors	Year	Strength	Begin	End	1991 - 2023
Schwartz, M	2001	6.76	2001	2005	
Kipnis, J	2001	6.14	2001	2005	
Gonzalez-rey, E	2005	3.76	2005	2006	
Delgado, M	2005	3.76	2005	2006	
Delgado, Mario	2006	10.97	2006	2010	
Gonzalez-rey, Elena	2006	8.5	2006	2010	
Chorny, Alejo	2006	4.29	2006	2007	
Wiendl, Heinz	2007	3.62	2007	2017	
Gendelman, Howard E	2009	6.44	2009	2011	
Reynolds, Ashley D	2009	5.01	2009	2010	
Stone, David K	2009	4.38	2009	2010	
Mosley, R Lee	2009	4.25	2009	2010	
Offner, Halina	2015	3.8	2015	2018	
Olson, Katherine E	2020	3.97	2020	2023	

FIGURE 5
Co-authorship analysis of authors. **(A)** Visualization map of authors collaboration analysis. Each node represents an author, and the node size is positively correlated with the number of documents. The connection between nodes represents collaboration, and the distance and thickness of the connection represent the relative strength of the relationship. The color means the average published year. **(B)** Cooperative network of Gendelman HE. **(C)** Top 14 authors with the strongest citation bursts. Minimum duration: 2.

TABLE 3 Top 15 authors with the most documents.

Rank	Author	Organization	Country/ Region	Documents	Citations	Total link strength
1	Gendelman HE	University of Nebraska Medical Center	USA	34	1,935	80
2	Mosley RL	University of Nebraska Medical Center	USA	27	1,655	76
3	Delgado M	Consejo Superior de Investigaciones Cientificas (CSIC)	Spain	26	859	48
4	Gonzalez-rey E	Consejo Superior de Investigaciones Cientificas (CSIC)	Spain	24	806	51
5	Bae H	Kyung Hee University	Korea	20	529	33
6	Wiendl, H	University of Münster	Germany	20	1,217	38
7	Offner H	VA Portland Health Care System, Oregon Health & Science University	USA	17	687	23
8	Schwartz M	The Weizmann Institute of Science	Israel	17	1,242	4
9	Olson KE	University of Nebraska Medical Center	USA	14	402	44
10	Appel SH	Houston Methodist Research Institute	USA	12	1,078	25
11	Fragoso G	Univ Nacl Autonoma Mexico	Mexico	12	173	40
12	Hu X	University of Pittsburgh School of Medicine	USA	12	749	27
13	Kipnis J	The Weizmann Institute of Science	Israel	12	569	2
14	Liesz A	Heidelberg University	Germany	12	1,251	10
15	Meuth SG	Heinrich-Heine University of Düsseldorf	Germany	12	606	25

relations and had the longest citation bursts. However, Howard E Gendelman was not involved in the largest connected cooperative network. A total of eight authors cooperated with Howard E Gendelman, and they were all affiliated with the University of Nebraska Medical Center (Figure 5B). Several emerging scholars (nodes with yellow color) have also begun to dabble in this field, suggesting that Treg cells in neurological diseases are still a hotspot for future research. The top three authors with the strongest citation bursts were Marina Delgado from 2006 to 2010, Michal Schwartz from 2001 to 2005, and Howard E Gendelman from 2009 to 2011, indicating that they were leaders in this field in a certain period (Figure 5C).

Journals

A total of 859 journals published 2,739 documents concerning Treg cells in neurological diseases. The top 11 journals are listed in Table 4, they published 618 documents, accounting for approximately 22.56% of the total. *Frontiers in Immunology* with 134 documents, *Journal of Immunology* with 90 documents, and *Journal of Neuroinflammation* with 75 documents were the most prolific journals. Impact factors of the top 11 journals ranged from 3.3 to 15.1, of which *Brain Behavior and Immunity* was the highest, and *Journal of Neuroimmunology* was the lowest. Of the top 11 journals, 6 journals belonged to Q1, 4 journals belonged to Q2, and the remaining 1 journal belonged to Q3. Notably, the *Journal of Experimental Medicine*, with the most citations (4,352 times), was not among the top 11 journals. The document "HIF1 alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of T(H)17 and T-reg cells" published in this journal in July 2011 was cited 1215 times, which ranked second in terms of the number of citations. Although the number of documents published in the *Journal of Experimental Medicine* was relatively small, the quality of documents was relatively high, which had conspicuously pushed forward the progress in this field. In short, both the number and the quality of documents need to be considered in the evaluation of prolific journals. Considering the number of documents and citations, impact factors, and JCR partitions, *Frontiers in Immunology* was the most popular journal in this research area.

Keywords

A total of 4,868 author keywords were involved in 2,558 documents, and 354 met the threshold (minimum number of documents of a keyword: 5). The overlay visualization map showed the co-occurrence relations of keywords (Figure 6A), in which "multiple sclerosis," "inflammation," "regulatory T cells," "neuroinflammation," "autoimmunity," "microglia," "cytokines," "experimental autoimmune encephalomyelitis," "immunotherapy," and "immunomodulation" were identified as high-frequency keywords. Moreover, these keywords were mostly associated with neuroprotection, neuroimmunology, and immunoregulation in 2014; interconnected with myasthenia gravis, MS, and neurodegeneration in 2016; related to PD, AD, and spinal cord injury in 2018; and currently linked to ischemic stroke, gut microbiota, and the gut–brain axis. Recently, the role of gut

TABLE 4 Top 11 journals with the most documents.

Rank	Journal	Documents	Citations	Total link strength	Impact factor (2022)	JCR partition
1	Frontiers in Immunology	134	4,351	603	7.3	Q1
2	Journal of Immunology	90	4,323	463	4.4	Q2
3	Journal of Neuroinflammation	75	3,057	405	9.3	Q1
4	Journal of Neuroimmunology	72	2,117	335	3.3	Q3
5	International Journal of Molecular Sciences	47	1,273	212	5.6	Q1
6	PLoS One	46	1,925	199	3.7	Q2
7	Brain Behavior and Immunity	38	1,440	188	15.1	Q1
8	Scientific Reports	33	955	116	4.6	Q2
9	Journal of Neuroimmune Pharmacology	29	1,144	161	6.2	Q1
10	Immunology	27	1,365	148	6.4	Q2
11	Proceedings of the National Academy of Sciences of the United States of America	27	3,238	299	11.1	Q1

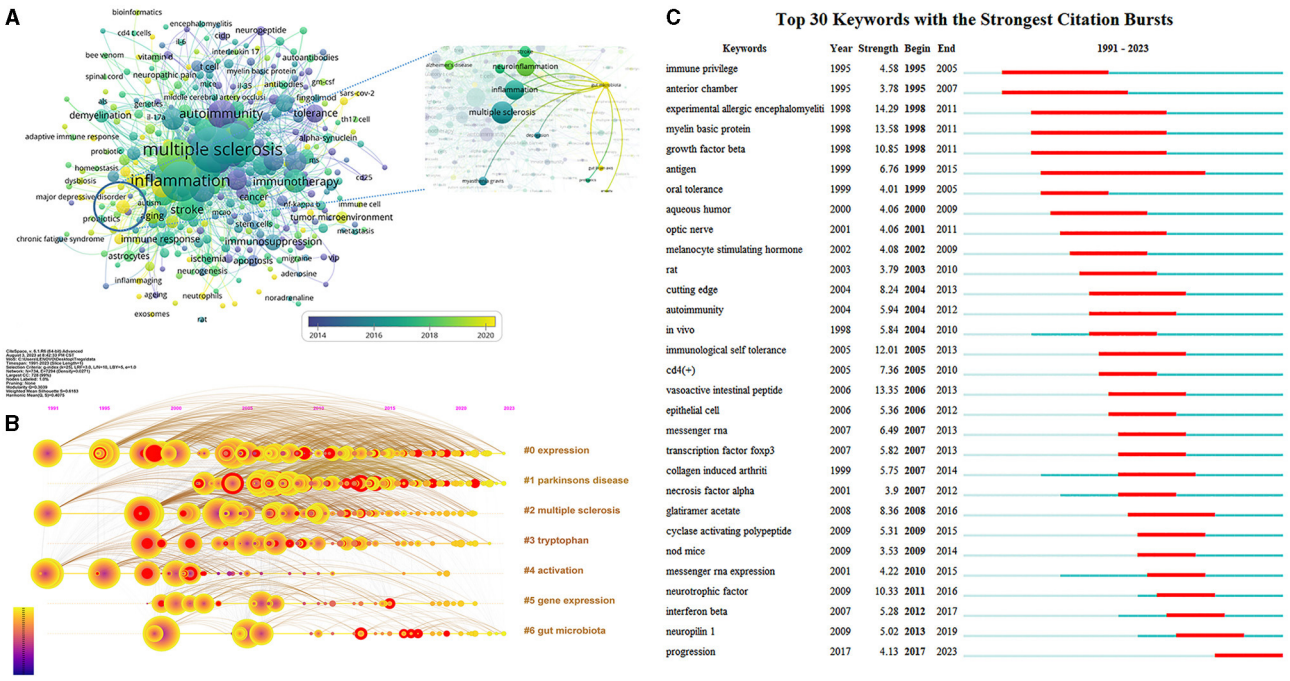


FIGURE 6
Co-occurrence analysis of keywords. **(A)** Visualization map of author keywords analysis. Each node represents an author keyword, and the node size is positively correlated with the number of documents containing the author keyword. The connection between nodes represents co-occurrence. The color means the average published year. **(B)** Timeline view of keywords clustering analysis. The different colored horizontal lines on the right represent the clusters formed by the keywords, nodes on the horizontal lines represent keywords, and the position of nodes on the horizontal lines represents the year in which the document containing the keywords first appeared. **(C)** Top 30 keywords with the strongest citation bursts. Minimum duration: 6.

microbiota in neurological diseases has gained significant attention, with substantial evidence linking it to neuroinflammation.

As shown in Figure 6B, the timeline view of keywords clustering analysis was displayed to show the basic knowledge structure and the evolution over time of Treg cells in neurological diseases. The modularity Q was 0.4075, indicating that the network structure was consequential, and the mean silhouette S was 0.6183, implying that clustering was credible. Keywords with close relationship were automatically grouped into a cluster, which was named by the keyword with the largest log-likelihood rate. Cluster "#0 expression," "#2 multiple sclerosis," and "#4 activation" appeared the earliest, and cluster "#1 Parkinsons disease" appeared the latest. Cluster "#0 expression," "#1 Parkinson's disease," and "#6 gut microbiota" related studies were available in 2023, which

TABLE 5 Top 10 documents with the most citations.

Rank	Title	References	Journal	Citations
1	The pro- and anti-inflammatory properties of the cytokine interleukin-6	Scheller et al. (24)	Biochimica Et Biophysica Acta-Molecular Cell Research	2,058
2	HIF1 alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of T(H)17 and T-reg cells	Shi et al. (25)	Journal of Experimental Medicine	1,215
3	Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization	Setoguchi et al. (26)	Journal of Experimental Medicine	941
4	Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis	Lee et al. (27)	Proceedings of the National Academy of Sciences of the United States of America	910
5	Role of tumor microenvironment in tumorigenesis	Wang et al. (28)	Journal of Cancer	798
6	Inflammatory mechanisms in ischemic stroke: therapeutic approaches	Lakhan et al. (29)	Journal of Translational Medicine	705
7	Safety and Immunologic Effects of Mesenchymal Stem Cell Transplantation in Patients with Multiple Sclerosis and Amyotrophic Lateral Sclerosis	Karussis et al. (30)	Archives of Neurology	679
8	The Immunomodulatory and Anti-Inflammatory Role of Polyphenols	Yahfoufi et al. (31)	Nutrients	676
9	Psychoneuroimmunology Meets Neuropsychopharmacology: translational Implications of the Impact of Inflammation on Behavior	Haroon et al. (32)	Neuropsychopharmacology	626
10	Effects of stress on immune function: the good, the bad, and the beautiful	Dhabhar (33)	Immunologic Research	597

may become frontiers of Treg cells in neurological diseases in future, while cluster "#2 multiple sclerosis," "#3 tryptophan," "#4 activation," and "#5 gene expression" gradually decreased or even disappeared.

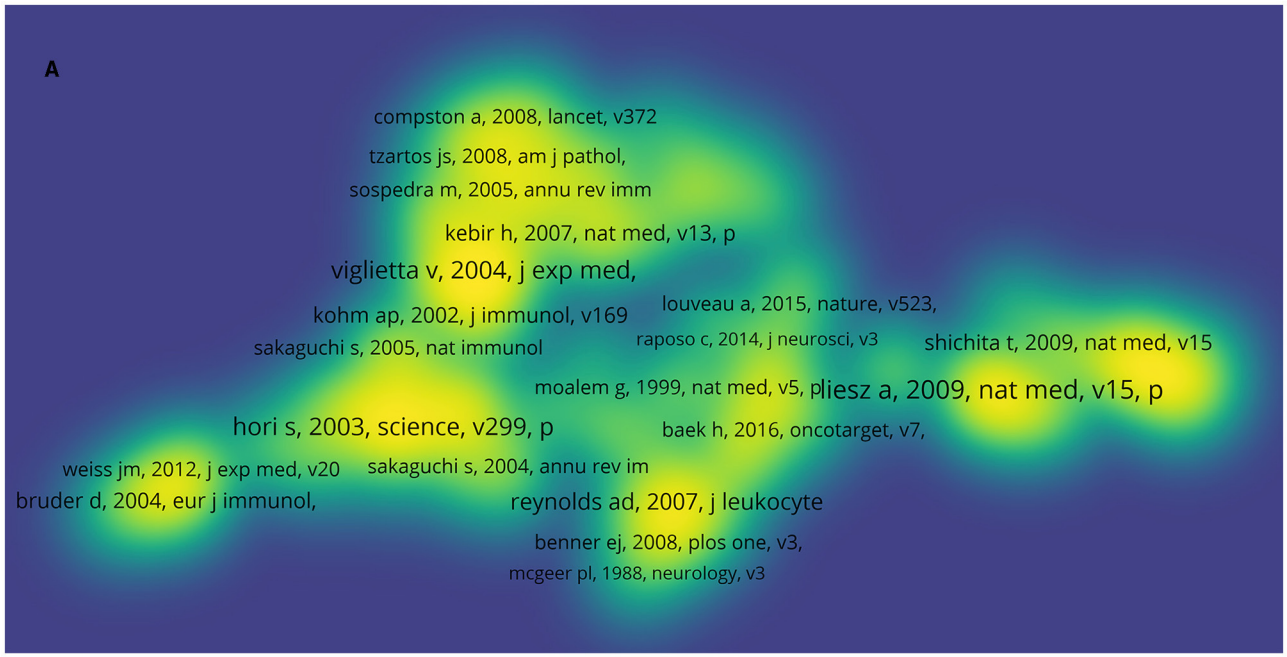
The top 30 keywords with the strongest citation bursts are shown in Figure 6C, which were considered consequential milestones for the science mapping research. "Immune privilege" and "anterior chamber" were important contents of the earliest research, suggesting that the immune privilege of the anterior chamber was an early research hotspot and had occupied a major position in this field. Keywords "antigen" had the longest 16 years of duration burst. In addition, "experimental allergic encephalomyelitis" had the highest burst strength from 1998 to 2011, which implied that scholars can never ignore its equally important existence when conducting research in this field, followed by "myelin basic protein" and "vasoactive intestinal peptide".

Citations

The top 10 documents with the most citations are listed in Table 5, and the range of citations was from 597 to 2,058. The top three documents with the most citations were documents written by Scheller J in 2011 (24), Shi LZ in 2011 (25), and Setoguchi R in 2005 (26), whereby all introduced the role of cytokines in autoimmune neurological diseases. The document written by Lee YK in 2011 (27), pointed out that gut microbiota impacts the balance between pro-and anti-inflammatory immune responses during experimental autoimmune encephalomyelitis. The document written by Wang MN in 2017 (28) focused on the role of tumor microenvironment in tumorigenesis of glioma, glioblastoma, and other cancers. The documents written by Lakhan SE in 2009 (29), Karussis D in 2010 (30), and Haroon E in 2012 (32) introduced the immunologic mechanisms underlying several therapeutic approaches for neurological diseases, such as ischemic stroke, MS, amyotrophic lateral sclerosis, and depression.

The co-citation analysis of cited references was performed by VOSviewer. A total of 162,113 cited references were involved in 2,739 documents, and 131 met the threshold (minimum number of citations of a cited reference: 40). The density visualization map of cited references based on citations is shown in Figure 7A, and the top 10 cited references with the most citations are shown in Table 6. The reference with the most citations was an article written by Liesz A in 2009, which indicated that research in this article may be a research hotspot, followed by references written by Viglietta V in 2004 and Hori S in 2003. Among the top 10 cited references, five references (34, 35, 39, 42, 43) focused on the neuroprotective role of Treg cells in neurological diseases, including stroke, MS, and PD. In total, five references (34, 36, 37, 40, 41) highlighted the important role of cytokines, including Foxp3, IL-10, IL-2, and TGF-β, in the generation, development, and function of Treg cells, suggesting that cytokines have always been the research focus in this field.

Reference burst detection can help find the most influential cited references and discover research frontiers and trends. In total, seven references with the strongest citation bursts were obtained (Figure 7B). The reference written by Liesz A in 2009 had the highest burst and highest number of citations, indicating that the research discussed in this article is authoritative and has been a hotspot in this field. Judging from the past 6 years, a reference



B

Top 7 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	1991 - 2023
Balandina A, 2005, BLOOD, V105, P735, DOI 10.1182/blood-2003-11-3900, DOI	2005	4.34	2005	2010	
Gonzalez-Rey E, 2006, BLOOD, V107, P3632, DOI 10.1182/blood-2005-11-4497, DOI	2006	10.18	2006	2011	
Liesz A, 2009, NAT MED, V15, P192, DOI 10.1038/nm.1927, DOI	2009	25.12	2009	2014	
Brochard V, 2009, J CLIN INVEST, V119, P182, DOI 10.1172/JCI36470, DOI	2009	11.47	2009	2014	
Reynolds AD, 2010, J IMMUNOL, V184, P2261, DOI 10.4049/jimmunol.0901852, DOI	2010	17.86	2010	2015	
Henkel JS, 2013, EMBO MOL MED, V5, P64, DOI 10.1002/emmm.201201544, DOI	2013	11.75	2013	2018	
Liddelow SA, 2017, NATURE, V541, P481, DOI 10.1038/nature21029, DOI	2017	11.83	2017	2023	

FIGURE 7
Co-citation analysis of cited references. **(A)** The density map of cited references based on citations. The opacity of yellow is positively related to citations. **(B)** Top 7 references with the strongest citation bursts. Minimum duration: 6.

published by Liddelow SA in 2017 (44) has become the latest research frontier so far and may continue in the next decade. This reference titled "Neurotoxic reactive astrocytes are induced by activated microglia," suggested that inflammatory cells contribute to the death of neurons in AD, PD, amyotrophic lateral sclerosis, and MS and provided opportunities for the development of cell-based immunotherapies for these diseases.

Discussion

General information

Annual documents on Treg cells in neurological diseases showed an overall upward trend, suggesting that this research field remains an active hotspot. Among 85 countries/regions publishing documents on this topic, the United States was the largest contributor, with double the number of documents and citations compared to China and far ahead of other countries/regions. Additionally, among the top 10 most productive organizations, seven were based in the United States, and among the top 15 most prolific authors, six were also from the United States, underscoring their substantial contributions to this research field. However, China has emerged as a potential contributor in this field, with its annual output overtaking other countries/regions and ranking first in 2022. Harvard Medical School was identified as the most important organization and a major driver of research on the role of Treg cells in neurological diseases. Nearly 25% of relevant research results were published in the top 11 journals, demonstrating their high quality and authoritative role as communication platforms for research related to Treg cells in neurological diseases. Notably, *Frontier in Immunology* was the most popular journal, playing an active role in promoting the development of Treg cells in neurological diseases. Howard E Gendelman, currently affiliated with the University of Nebraska Medical Center, has published the most documents on Treg cells in neurological diseases. These documents primarily focused on neuroimmunity, neuromodulatory, immunomodulation, and neuroprotection. Among these documents, the document "Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease" has achieved the most citations. This study highlighted

TABLE 6 Top 10 references with the most citations.

Rank	Title	References	Journal	Citations
1	Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke	Liesz et al. (34)	Nature Medicine	250
2	Loss of functional suppression by CD4(+)CD25(+) regulatory T cells in patients with multiple sclerosis	Viglietta et al. (35)	Journal of Experimental Medicine	199
3	Control of regulatory T cell development by the transcription factor Foxp3	Hori et al. (36)	Science	174
4	Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells	Fontenot et al. (37)	Nature Immunology	155
5	Regulatory T cells and immune tolerance	Sakaguchi et al. (38)	Cell	143
6	Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease	Reynolds et al. (39)	Journal of Leukocyte Biology	138
7	Reciprocal developmental pathways for the generation of pathogenic effector T(H)17 and regulatory T cells	Bettelli et al. (40)	Nature	125
8	Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases	Sakaguchi et al. (41)	Journal of Immunology	120
9	Regulatory T Cells Attenuate Th17 Cell-Mediated Nigrostriatal Dopaminergic Neurodegeneration in a Model of Parkinson's disease	Reynolds et al. (42)	Journal of Immunology	111
10	The immunology of stroke: from mechanisms to translation	Iadecola et al. (43)	Nature Medicine	103

the potential of Treg cells in regulating neurodestructive immunity and laid the foundation for immunization strategies for PD (42).

There has been a remarkable historical progression in the field of Treg cells in neurological diseases. Initially, research focused on characterizing Treg cells and their role in maintaining immune homeostasis (6, 8). Over time, studies began to explore their involvement in neurological diseases, such as MS, AD, and PD (8, 12–14). Furthermore, this research area has undergone a significant transformation, shifting from the concept of neuroprotective autoimmunity to neuroprotection through neuroimmune transformation. A previous research study focused on the detrimental role of Th17/Th1 cells and the protective role of Treg cells, leading to strategies targeting Th17/Th1 suppression and Treg activation (45, 46). However, our deepening understanding emphasizes the critical balance between Th17/Th1 and Treg cells for neuroprotection. Under specific conditions, these cell subpopulations can convert into each other, playing a pivotal role in balancing immune effects and suppression (47, 48). Correcting Th17/Treg cell imbalance is now a novel approach for disease prevention and treatment (49, 50). As the field evolved, researchers made significant contributions by refining experimental models, developing more precise methodologies to identify and track Treg cells in the central nervous system, and investigating their specific mechanisms of action. However, this research area is still in the development stage and has enormous development potential. To gradually advance the field of Treg cells in neurological diseases and form a consensus, independent investigators, organizations, and countries should prioritize standardizing experimental conditions, including cell sources, assay protocols, and animal models to ensure the replicability and extensibility of their studies. Second, while striving for progress, researchers should actively incorporate diverse perspectives, such as conducting systematic reviews, collaboratively evaluating existing literature, and using data-driven approaches to resolve discrepancies. Importantly, researchers can explore whether Treg cells from different sites and sources exhibit distinct roles or whether there are different phenotypes of Treg cells with specialized functions (51–53).

Hotspots and frontiers

Keywords are powerful tools for understanding the theme and research focus of scientific documents, and they can help identify hotspots and trends of Treg cells in neurological diseases. The most cited documents often signify important research directions and breakthroughs in the field. Co-cited references reflect the historical development and roots of the field, while references with citation bursts reveal the emerging hotspots within it. By combining keyword and citation analyses, we have identified the following aspects as current research hotspots and trends of Treg cells in neurological diseases:

Immunomodulation based on Treg cells

Given their immunosuppressive properties, Treg cells are considered excellent candidates for immunomodulation. Treg cell-based therapeutic strategies have been actively developing in transplantation and autoimmune diseases (46). The absence of Treg cells in the lymphoid aggregates of MS patients' brains indicates that the reduction of Treg cells may play a role in the progression of the disease (54). Thus, therapies based on Treg cells have the potential to ameliorate MS. A phase I clinical trial evaluating the adoptive transfer of Treg cells into patients with relapsing-remitting MS found it to be safe and well-tolerated,

without adverse events (55). Nonetheless, additional research is necessary to assess the efficacy and safety of Treg cell-based therapeutic strategies for patients with MS, given the limited knowledge about how Treg cells influence immune homeostasis and inflammation resolution. The early depletion of Treg cells by anti-CD25 antibody hastened cognitive deficits in APP/PS1 mice and reduced microglial recruitment to amyloid deposits (56). The adaptive transfer of *ex vivo* expanded human Treg cells to immunodeficient 5xFAD-Rag2KO mice resulted in the suppression of neuroinflammation and significant alleviation of amyloid pathogenesis (13). In disagreement, a study suggested that transient Treg cell depletion was followed by amyloid-β plaque clearance, mitigation of the neuroinflammatory response, and reversal of cognitive decline in the AD mouse model (10). Although there are disagreements regarding the role of Treg cells in the progression of AD, it is undeniable that Treg cell modulation is a new treatment option. PD is a neurodegenerative disorder characterized by neuroinflammation that may be caused by an imbalance between Treg cells and Th17 cells. Treg cells have been shown to attenuate Th17 cell-mediated death of nigrostriatal dopaminergic neurons (57). An *in vitro* study revealed that human adipose tissue-derived mesenchymal stem cells could inhibit the differentiation of $CD4^+$ T cells isolated from patients with PD into Th17 cells. This inhibitory effect was mainly mediated by an increase in Treg cells and secretion of IL-10, indicating that Treg cells play an anti-inflammatory and neuroprotection role in PD (49). Immunomodulation through Treg cell expansion was found to be an effective treatment for PD mice in a recent study, providing evidence that immunotherapy may offer a disease-modifying option for patients with PD (14). While a study demonstrated that depleting $Foxp3^+$ Treg cells in transgenic DEREG mice significantly reduced lesion volume and improved neurological function during the early phase of middle cerebral artery occlusion (11), many studies have shown that an increase in Treg cells could potentially improve long-term stroke recovery (46, 58, 59). Researchers found that Treg cell-derived osteopontin contributed to a tissue-reparative microglial response. This response led to improved oligodendrocyte regeneration and remyelination during the chronic stages of stroke (58). The use of a CD28 superagonist to expand and amplify Treg cells attenuated the inflammatory response, reduced infarct volume, and improved outcomes in experimental stroke (59).

Recently, engineered Treg cells have been used for adoptive immunotherapy. First, human Treg cells are isolated from human peripheral blood, umbilical cord blood, or thymus. These Treg cells are then cultured *in vitro* to generate polyclonal Treg cells or antigen-specific Treg cells. Finally, qualified Treg cells are infused into patients to treat related diseases (60). Therefore, immunomodulatory strategies based on Treg cells are novel and promising therapies for neurological diseases and deserve continued research by scholars.

Gut microbiota

Substantial evidence has indicated that the gut–brain axis likely plays a crucial role in neurological diseases, with an altered gut microbiota potentially having significant implications on immune responses in both the gut and distal effector immune sites such as the central nervous system (61). A study involving experimental autoimmune encephalomyelitis mice found that the gut microbiota greatly influenced the balance between pro- and anti-inflammatory immune responses. This discovery suggested that modulating gut microbiota could provide new targets for treating extraintestinal inflammatory diseases such as MS (27). Specific metabolites of gut microbiota, such as the tryptophan metabolite FICZ [6-formylindolo (3-2b) carbazole], are associated with the production of pro-inflammatory cytokines and the generation of Th17 cells. Conversely, commensal bacteria and their metabolites, including *Lactobacilli* and *Bacillus*-derived poly-gamma-glutamic acid (gamma-PGA), can stimulate Treg cell generation to promote immune suppression. Therefore, the immunomodulatory effects of gut microbiota may be mediated primarily *via* the Th17/Treg axis (62). Exposure to MS microbiota or MS-associated *Acinetobacter calcoaceticus* extract was shown to alter lymphocyte differentiation in healthy individuals, resulting in an increase in Th1 cells and a decrease in $CD25^+Foxp3^+$ Treg cells, while exposure to the *Parabacteroides distasonis* extract increased Treg cell differentiation (63). Patients with MS display a reduction in commensal microbiota levels compared to healthy individuals, and therapies targeting the microbiota have been demonstrated to increase the microbiota and improve MS by decreasing Th1- and Th17-cell levels and increasing Treg cell levels (64). Myasthenia gravis is an autoantibody-mediated neurological disease, and Th17/Treg imbalance contributes to the pathogenesis of myasthenia gravis. Studies have reported that correcting Th17/Treg imbalances may be a novel therapeutic approach to myasthenia gravis by modifying the gut microbiota (50). Butyrate promotes the expression of Foxp3 and differentiates naive T cells into Treg cells by inhibiting histone deacetylase. Therefore, the reduction of short-chain fatty acid-producing bacteria in patients with PD reduces the number of Treg cells, thereby exacerbating the neuroinflammatory response (65). Patients with neurological diseases often exhibit gut microbial dysbiosis and altered microbial metabolites, highlighting the potential of microbial components or commensal bacteria as immunomodulatory agents to correct Th17/Treg imbalances and then treat neurological diseases (50). Therefore, developing therapeutic interventions targeting the gut microbiome could represent a promising strategy for managing neurological diseases.

Cytokines

Cytokines are under active investigation as immune modulators to boost the numbers and functions of Treg cells in neurological diseases. The development and function of $CD4^+CD25^+$ Treg cells are regulated by Foxp3, while peripheral $CD4^+CD25^-$ T cells can acquire suppressor function through ectopic Foxp3 expression. This discovery opens up a new way for cell-based therapies for autoimmunity (37). IL-2 is an essential factor for the development, survival, and function of $Foxp3^+$ natural Treg cells, playing a critical role in maintaining Treg cell homeostasis (26, 38). Studies have revealed that low-dose IL-2 therapy can selectively promote the persistence and survival of Treg cells while limiting effects on other T-cell subsets. The

therapeutic efficacy of this approach has been demonstrated in both animal models and clinical trials, highlighting its potential as a promising treatment option (66, 67). The aberrant TGF-β signaling observed in individuals with MS is strongly associated with Treg cell dysfunction (68). Consequently, targeting and modulating TGF-β signaling may hold promise for addressing this defect and potentially alleviating the symptoms of MS. IL-6 plays a pivotal role in regulating the balance between Th17 and Treg cells. Specifically, IL-6 supports the differentiation of Th17 cells from naive T cells together with TGF-β and inhibits TGF-β-induced Treg differentiation (69). Tocilizumab, an anti-IL-6 receptor monoclonal antibody, has been approved for treating inflammatory diseases (24). Therefore, the utilization of cytokines as immune modulators to regulate the differentiation and function of Treg cells represents a significant therapeutic approach in the treatment of neurological diseases. Furthermore, relevant immunomodulatory agents have transformed recent clinical practice to prevent and reverse the pathology of neurological diseases. However, a delivery system that can cross the blood–brain barrier to carry immunomodulatory agents is still the direction of scholars' unremitting exploration.

Limitations

This study is the first bibliometric analysis to systematically analyze documents related to Treg cells in neurological diseases. Nevertheless, there are still some deficiencies here. First, only English language articles and reviews published in the Web of Science Core Collection were collected, which may lead to language and publication bias. As bibliometric analysis is closely linked to timeliness, it is essential to continuously update the results and trends of research on Treg cells in neurological diseases to keep pace with ongoing scientific exploration. This will enable a more comprehensive understanding of the topic as well as provide more precise predictions of future trends. Finally, this review discusses from the perspective of the neuroprotective role of Treg cells, however, varying perspectives exist in certain studies. Therefore, before reaching a consensus, it is important to consider multiple aspects when targeting Treg cells for the treatment of neurological diseases and exercise caution in their use. However, given the large enough number of documents in this analysis, we believe that this study provides an instructive perspective for the research of Treg cells in neurological diseases and guides future research in this field.

Conclusion

Through VOSviewer, CiteSpace, and Tableau Public software, we have carried out a bibliometric analysis on Treg cells in neurological diseases. The study of Treg cells in neurological diseases continues to be a hot topic. The United States was the largest contributor among 85 countries/regions, and China was the most potential country. More than half of the top 10 most prolific organizations were located in the United States, and Harvard Medical School was the most important organization in this field. Nearly half of authors who make major contributions belonged to the United States organizations when publishing documents. *Frontiers in Immunology* was the most popular journal in this research area. Immunomodulation, gut microbiota, and cytokines represent the current research hotspots and frontiers in this field. Treg cell-based immunomodulatory approaches have shown immense potential in the treatment of neurological diseases. Modifying gut microbiota or regulating cytokines to boost the numbers and functions of Treg cells represents a promising therapeutic strategy for neurological diseases. Hence, we can conclude from these documents that future therapeutic strategies for neurological diseases should leverage the therapeutic potential of Treg cells, with an emphasis on modulating their activity to promote neuroprotection.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

QG: Conceptualization, Data curation, Writing—original draft, Writing—review and editing. XL: Conceptualization, Validation, Writing—review and editing. YL: Funding acquisition, Project administration, Validation, Writing—review and editing. JL: Methodology, Writing—review and editing. MP: Formal analysis, Writing—review and editing. JW: Visualization, Writing—review and editing. FY: Software, Writing—review and editing. YZ: Funding acquisition, Project administration, Supervision, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Rajnikant Mishra,
Banaras Hindu University, India

REVIEWED BY
Christian Schachtrup,
University of Freiburg, Germany
Nicholas Dopkins,
NewYork-Presbyterian, United States

*CORRESPONDENCE
Veit Rothhammer
veit.rothhammer@uk-erlangen.de

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The role of midkine in health and disease

Emely Elisa Neumaier, Veit Rothhammer* and Mathias Linnerbauer

Department of Neurology, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany

Midkine (MDK) is a neurotrophic growth factor highly expressed during embryogenesis with important functions related to growth, proliferation, survival, migration, angiogenesis, reproduction, and repair. Recent research has indicated that MDK functions as a key player in autoimmune disorders of the central nervous system (CNS), such as Multiple Sclerosis (MS) and is a promising therapeutic target for the treatment of brain tumors, acute injuries, and other CNS disorders. This review summarizes the modes of action and immunological functions of MDK both in the peripheral immune compartment and in the CNS, particularly in the context of traumatic brain injury, brain tumors, neuroinflammation, and neurodegeneration. Moreover, we discuss the role of MDK as a central mediator of neuro-immune crosstalk, focusing on the interactions between CNS-infiltrating and -resident cells such as astrocytes, microglia, and oligodendrocytes. Finally, we highlight the therapeutic potential of MDK and discuss potential therapeutic approaches for the treatment of neurological disorders.

KEYWORDS

midkine, CNS, inflammation, malignancy, injury

Introduction

Growth factors are essential for the development and functioning of the central nervous system (CNS). As soluble molecules, they play vital roles in cell-to-cell communication and regulate a multitude of functions, including cell proliferation and differentiation. One of these growth factors is the heparin-binding growth factor midkine (MDK). MDK, together with the structurally related growth factor pleiotrophin (PTN), belongs to the family of neurite promoting growth factors and has originally been identified in embryonal carcinoma (EC) cells in 1988 (1).

MDK is expressed in a small number of embryonic tissues, including the CNS. The expression pattern of MDK during mouse gestation indicates that the growth factor is required for the generation of epithelial tissue, remodeling of the mesoderm (2), and neurogenesis (3). Early studies by Kadomatsu et al. (2) describe an upregulation of MDK during midgestation in mouse embryos, while its expression in adult mice has initially only been described in the kidney. The polypeptide MDK with a molecular weight of about 13 kDa (4) consists of a N-terminal domain, held together by three disulfide bridges and a C-terminal domain, stabilized by two disulfide bridges (5). Notably, early studies have

suggested that the neurite outgrowth promoting functions of MDK are highly dependent on both the C-terminally located heparin-binding domain and the sulfide bonds (6–8).

Recent research has indicated that MDK is highly upregulated in response to various pathological conditions, both in the CNS and the periphery (9, 10) (Figure 1, Table 1), and can be exploited as a biomarker and therapeutic target (35, 36), highlighting the pivotal role of the growth factor in the context of disease. In this review, we will summarize the involvement and function of MDK in the context of peripheral disorders and CNS pathologies, including brain injuries, brain tumors, as well as neuroinflammatory and neurodegenerative diseases. Furthermore, we will review existing therapeutic strategies targeting MDK in neoplastic diseases and discuss the therapeutic value of MDK for the treatment of CNS disorders.

Cellular sources of MDK

In the periphery, numerous cell types have been identified to produce MDK under basal and pathological conditions (9). In addition to monocytes, macrophages, and monocyte-derived dendritic cells (mDCs), also non-hematopoietic cell types such as endothelial cells are capable of producing MDK (4).

Within the CNS MDK is mainly expressed during development until midgestation, while its mRNA levels decrease in postnatal life (4, 9). In mice MDK is mainly expressed by oligodendrocyte precursor cells (OPCs), followed by fetal astrocytes, neurons, and newly formed oligodendrocytes, while in humans fetal astrocytes represent the major source of MDK in the CNS (9). Moreover, *in vitro* studies show MDK expression by cultured neurons and activated astrocytes, but not microglia (37).

Inducers of MDK expression

In monocytes, polymorphonuclear neutrophils (PMNs), and endothelial cells, MDK expression is induced during hypoxia (38). Binding of hypoxia-inducible factor-1α (HIF-1α) to hypoxia response elements (HREs) in the *MDK* promotor activates expression of the gene (Figure 1), while MDK in turn increases HIF-1α expression in a positive feedback loop (39). In addition to hypoxia, MDK expression is driven by the master regulator of pro-inflammatory pathways, nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) (Figure 1), which can be activated by reactive oxygen species (ROS), pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1β, as well as

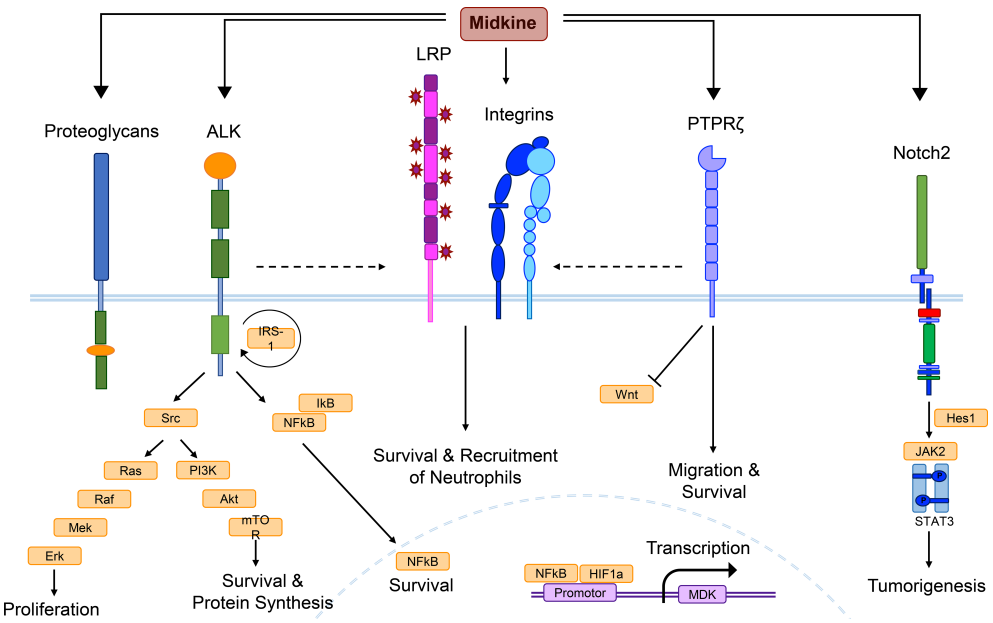

FIGURE 1
MDK receptor candidates and signaling pathways. Midkine (MDK) is a multifunctional molecule, whose effects are probably regulated via different receptor-ligand interactions, as well as complex formation of receptor candidates, and cross-talk between the receptors. Low density lipoprotein receptor-related proteins (LRPs) and integrins are thought to build the core of the MDK receptor complex, while other candidates such as the anaplastic lymphoma kinase (ALK) or the protein tyrosine phosphatase ζ (PTPζ) might be recruited. This figure combines signaling pathways discovered in different cell types under several pathological conditions and does not show the determined signaling of MDK in a specific cell type. Binding of MDK to the ALK receptor induces the phosphorylation of the insulin receptor substrate-1 (IRS-1) and its interaction with the ALK receptor, followed by the activation of several signaling pathways. Src kinase phosphorylation results in mitogen-activated protein (MAP)-kinase signaling, which includes a phosphorylation cascade of the proteins Ras, Raf, Mek, and Erk, supporting cell proliferation. Another downstream effect of Src is the phosphoinositide (PI)-3-kinase signaling, including Akt and mTOR activation, promoting survival and protein synthesis. MDK/ALK signaling also induces the expression of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), a growth factor inhibited by IκB proteins until it reaches the nucleus, where it stimulates cell survival. The core complex of LRP and integrins contributes to cellular survival and the recruitment of neutrophils, while MDK binding to PTPζ additionally promotes survival, as well as cell migration and negatively regulates Wnt signaling. Neurogenic locus notch homolog protein 2 (Notch2) activation mediates the interaction between Hes1 and the Janus kinase 2 (Jak2)/STAT3 complex, inducing tumorigenesis. The promotor of the MDK gene entails binding sites for NF-κB and hypoxia-inducible factor-1α (HIF-1α).

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TABLE 1 The major roles of MDK in pathological conditions.

Disease	Effects	Models	References
Alzheimer's disease	Inhibits Aβ fibril formation and Aβ-induced cytotoxicity	*in vitro*	(11, 12)
Cardiac ischemia-reperfusion injury	Prevents myocardial apoptosis	*in vivo*	(13)
Cerebral infarct	Reparative neurotrophic functions during early phase	*in vivo*	(14)
Gastric cancer	Confers chemoresistance	*in vitro*	(15–17)
Glioblastoma	Induces stem-like properties of glioma initiating cells; induces cannabinoid resistance; modulates immunosuppressive tumor microenvironment	*in vitro* *in vivo*	(17–20)
Inflammatory breast cancer	Recruits monocytes	*in vitro*	(21)
Leukemia	Promotes B cell survival	*in vivo*	(22)
Melanoma	Modulates tumor microenvironment towards tolerogenic and immune-resistant states	*in vitro* *in vivo*	(23)
Multiple Sclerosis	Suppresses expansion of T_{reg} cells, deteriorating disease course	*in vivo*	(24, 25)
Neuroblastoma	Promotes tumorigenesis	*in vitro* *in vivo*	(26, 27)
Neuromyelitis optica	Correlates with IL-23 levels	*in vivo*	(28)
Pancreatic cancer	Contributes to chemoresistance, proliferation and migration of cancer cells	*in vitro*	(15, 16, 26, 27)
Renal ischemia-reperfusion injury	Promotes migration of neutrophils and macrophages	*in vivo*	(29)
Rheumatoid arthritis	Leads to activation and migration of neutrophils and inflammatory leukocytes; induces release of pro-inflammatory cytokines	*in vivo*	(18–20, 30, 31)
Systemic lupus erythematosus	Correlates with increased IL-17 levels	*in vivo*	(32)
Transient forebrain injury	Promotes tissue repair	*in vivo*	(33)
Traumatic brain injury	Polarizes microglia towards an anti-inflammatory state; recruits neutrophils and macrophages; increases apoptotic neurons around lesions; potentiates secondary injury	*in vivo*	(34)

(Continued)

TABLE 1 Continued

Disease	Effects	Models	References

bacterial components, such as lipopolysaccharide (LPS) (9), among others. Together, both its regulation by hypoxia and pro-inflammatory NF-κB signaling indicate the relevance of MDK signaling in response to inflammatory stimuli.

While numerous studies have shed light on the role of MDK in non-CNS diseases, the regulation of MDK in CNS pathologies is less defined (Table 1). During development of experimental autoimmune encephalomyelitis (EAE), a preclinical animal model of Multiple Sclerosis (MS), T helper (T_H) cells have been identified as MDK producer cells (24). However, in glial cells it can be speculated that similar processes drive the expression of MDK. In these lines, hypoxic conditions following ischemia, or the presence of pro-inflammatory cytokines such as TNFα and IL-1β under neuroinflammatory conditions (40, 41) may induce NF-κB-dependent upregulation of MDK by astrocytes, microglia, or oligodendrocytes. Whether this upregulation in fact occurs and whether it is part of a protective or inflammatory activation state must be addressed by future studies.

MDK receptors and signaling

So far, several plasma membrane molecules have been identified as MDK receptors, including integrins, proteoglycans, neurogenic locus notch homolog protein 2 (Notch2) (42), ALK (43), low-density lipoprotein receptor-related protein (LRP) (44), and protein tyrosine phosphatase ζ (PTPζ) (45) (Figure 1). Integrins with MDK-binding properties include the heterodimers $\alpha_6\beta_1$ and $\alpha_4\beta_1$, while the family of MDK-binding proteoglycans can be subdivided into syndecans (46), glypican-2 (47), PG-M/versican (48), and neuroglycan C (49).

Instead of binding to a single one of these receptors, MDK exerts its multifaceted functions through binding to a multimolecular receptor complex (Figure 1), with PTPζ as the most established component (4, 50). The formation of the receptor complex, the arrangement of MDK-binding molecules, and the crosstalk between receptor subunits coordinate the signal transduction in response to MDK binding via several signaling pathways, depending on the cellular context, thereby facilitating the diverse functions of the growth factor (51) (Figure 1).

Protein tyrosine phosphatase ζ

The signaling cascade elicited through binding of MDK to the receptor component PTPζ has been associated to various functions and cell types. Binding of macrophage migration inhibitory factor (MIF) to its receptor CD74 on mature and malignant B cells leads to an increased expression of MDK, which in turn increases B cell

survival by autocrine MDK-signaling through PTPζ (22). Furthermore, the receptor PTPζ mediates MDK signals that suppress osteoblast proliferation via negative regulation of Wnt signaling (Figure 1) by dephosphorylation of β-catenin (52). However, it is not known whether MDK itself is able to induce the phosphatase activity of the receptor, or if further components of the PTPζ complex are needed to initiate dephosphorylation (51). Not only in the periphery, but also within the CNS, PTPζ has been shown to be involved in several MDK-dependent signaling pathways, including the promotion of neuronal survival (53) and the migration of neurons (45) (Figure 1), which is especially important during neurogenesis (54, 55).

Anaplastic lymphoma kinase

The MDK/ALK signaling pathway is well established in diverse tissues and has been elucidated in numerous studies (43, 56–59). MDK binding to ALK results in phosphorylation of the insulin receptor substrate-1 (IRS-1), leading to enhanced activation of Src kinases (43), mitogen-activated protein (MAP)-kinase and phosphoinositide (PI)-3-kinase signaling (60), as well as the induction of the transcriptional activation of NF-κB (56) (Figure 1). As mentioned above, NF-κB acts as central mediator of inflammatory responses (61) and regulates fundamental cellular processes including differentiation, proliferation, and survival (57). In these lines, MDK/ALK signaling is especially involved in neoplastic diseases (18, 58, 62, 63), as it initiates, for example, an autocrine growth and survival signal via the suppression of caspases (58), as well as the enhancement of B-cell lymphoma-2 (Bcl-2) (62), an anti-apoptotic protein and oncogene. Both pathways counteract anti-tumor immunity (64, 65), thereby implicating MDK signaling in tumor resistance. In melanoma, MDK activates mTOR via a similar signaling pathway (Figure 1), leading to an increased expression of vascular endothelial growth factor receptor 3 (VEGFR3) and the stimulation of lymphangiogenic signals, resulting in metastatic growth in lymph nodes and the lungs (63).

In the CNS, MDK-dependent ALK signal transmission in glioma cells results in the activation of the Akt/mTOR1 axis (Figure 1), preventing autophagy-mediated cell death by tetrahydrocannabinol (THC), thereby contributing to the cannabinoid-resistance of gliomas (18). While MDK negatively contributes to cancer progression and metastasis formation via its anti-apoptotic and growth-promoting effects in the peripheral compartment as well as in the CNS, these functions may also have beneficial roles in the context of injuries and tissue regeneration. It is conceivable that the anti-apoptotic and proliferative effects of MDK possess the capacity to mediate tissue-protection and ameliorate inflammatory and demyelinating processes in the CNS (9, 66).

Low-density lipoprotein receptor-related protein

In inflammatory diseases such as myocarditis, the interaction of MDK with the receptor LRP1 and members of the β_2 integrin family is critical for MDK-induced PMN recruitment (Figure 1) and neutrophil extracellular trap (NET) formation (67). Here, MDK contributes to a process called NETosis (68), which results in inflammation and tissue injury through direct damage (67). In squamous cell carcinoma, MDK triggers phosphorylation and thereby activation of paxillin and signal transducer and activator of transcription (STAT) 1α pathways in an integrin-dependent manner, resulting in the overexpression of genes implicated in cell migration and tissue invasion (59).

In the CNS, binding of MDK to LRP1 has been shown to induce cell survival in embryonic neurons (44) (Figure 1), while MDK-signaling through the integrins α_4 and α_6 promotes neurite outgrowth (69). Upon binding of MDK to the receptor component LRP, MDK is internalized and transported to the nucleus (70). The nuclear translocation of the growth factor is enabled by the shuttle proteins nucleolin (71) and laminin binding protein precursor (LBP) (72), and necessary for the promotion of cell survival via the MDK/LRP signaling pathway (70) (Figure 1). Similar to the anti-apoptotic effects of MDK/ALK signaling in the CNS, MDK binding to LRP1 may thereby support regeneration and re-myelination in response to CNS insult.

Neuroglycan C

In the CNS, the receptor neuroglycan C has been identified as important MDK signal transducer involved in process elongation of OPCs (49). These cells are not only important during synapse formation, but also for the re-myelination of axons in demyelinating diseases such as MS (73). Emerging literature on OPCs furthermore describes their potential for the establishment and remodeling of neural circuits (74), which supports the function of MDK in the developing brain. The receptor neuroglycan C might function in complex with LRP1 and integrins, which strongly bind to one another and may form the core of the MDK receptor complex in the CNS (69) (Figure 1).

In summary, the complex interplay of MDK receptor signaling facilitates the intricate and context-dependent functions of the growth factor in a broad variety of cell types, which regulate numerous inflammatory and non-inflammatory functions (Figure 1). Both, in the periphery and the CNS, MDK elicits pro-inflammatory and anti-apoptotic effects, driving inflammation, tumor progression, and metastasis. Nevertheless, upon injury or trauma, increased MDK expression has the potential to positively influence disease outcomes by promoting differentiation, reducing cell death, and increasing regeneration.

MDK and its roles in injury, cancer, inflammation, and autoimmunity

Besides its important role during development and differentiation of various cell types (2), MDK has been shown to be upregulated in various pathological conditions in the periphery and the CNS, reaching from neoplastic diseases to inflammatory diseases and injuries (Figure 2, Table 1).

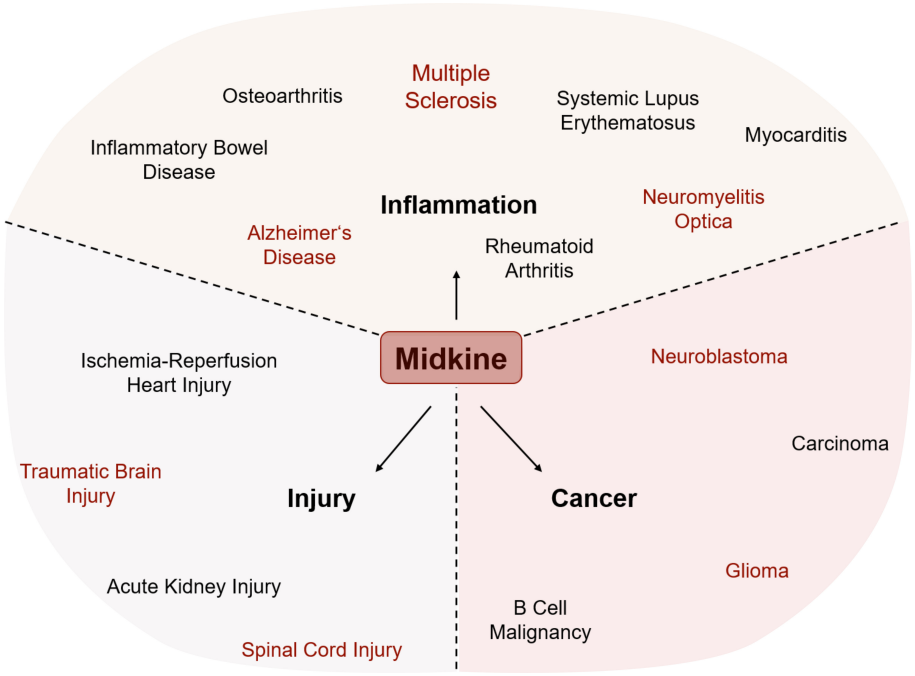

FIGURE 2
Involvement of MDK in various pathological conditions. The expression of Midkine (MDK) is increased in all shown conditions, while it has opposing functions within injury, inflammation, and cancer. MDK influences the outcome by either promoting recovery or deteriorating the course of disease and/or pathogenesis. Conditions written in red are central nervous system (CNS)-related injuries, brain tumors, or neuroinflammatory diseases.

MDK in the context of neoplastic diseases

In the context of neoplastic diseases, increased MDK levels have been demonstrated more than 20 years ago by several studies covering various types of cancer (Figure 2). In human tissue samples of prostatic (75) and hepatocellular (76) carcinomas, increased protein levels of MDK have been detected via immunohistochemical staining, while mRNA levels of MDK were increased in human gastric carcinoma specimens (77). *In vitro* studies suggest that MDK promotes proliferation and migration of pancreatic cancer cells (15) and is furthermore contributing to chemoresistance in ductal adenocarcinomas (16). These observations are supported by more recent studies in the context of breast cancer and melanoma, where MDK shapes the tumor microenvironment and promotes tumor-resistance (21, 23).

Aside from its numerous roles in tumor-formation and -resistance in peripheral tissues, MDK has also been implicated to play a role in the development and tumorigenicity of brain tumors (Figure 2). Among these, neuroblastomas belong to the most prevalent malignant pediatric solid tumors (78), while gliomas are the most frequent primary tumors of the CNS in adults (79).

In vitro studies using primary neuroblastomas and neuroblastoma cell lines suggest that MDK not only promotes peripheral neoplasms but is also involved in tumor growth and differentiation in the CNS (80). This hypothesis has been supported by reduced tumor growth in several MDK-depleted neuroblastoma cell lines (26). Moreover, elevated MDK blood levels can be linked to poor prognostic factors in neuroblastoma patients (81, 82), supporting the relevance of MDK in neuroblastoma tumorigenesis. Similarly, increased MDK levels in the CNS correlate with a poor prognosis and lower survival of glioblastoma patients (83), indicating an involvement of the growth factor in disease progression. A hallmark of glioblastomas is their ability to relapse in patients within a certain cell population, called glioma initiating cells (GICs), which exhibit stem-like characteristics (19). Because MDK has been implicated to promote the growth of neural stem cells and progenitor cells *in vitro* (84), it is likely that MDK is also involved in glioblastoma initiation. Studies with GIC cultures show increased MDK mRNA and protein levels, while inhibition of MDK reduces the ability of neurosphere generation by GICs, as well as the number of stemness biomarkers in culture (19).

Overall, these and other observations (16, 17) underscore the role of MDK signaling for chemoresistance and tumorigenesis in the context of solid brain tumors. These findings not only emphasize the relevance of MDK as a therapeutic target, but also illustrate its potential as early diagnostic and independent prognostic marker.

Although the functions of MDK in the context of solid tumors inside and outside the CNS exhibit remarkable similarities, it still remains unclear if the tumor promoting effects of MDK on peripheral and central neoplasms underlie a common mechanism. One conceivable common mechanism is mediated through the proto-oncogene p53. *MDK* is known to harbor p53 binding sites, where binding of the appropriate protein activates the transcription of MDK in gliomas, while knockdown of p53 downregulates the

expression of mRNA and protein levels of MDK (20). In these lines, it has been suggested that the p53-induced overexpression of MDK in gliomas drives the anti-inflammatory polarization of microglia, thereby remodeling the tumor immunosuppressive microenvironment (20). Studies of low-grade gliomas (LGGs) using neurofibromatosis type 1 (NF1) as a genetic model system describe MDK as an upstream mediator regulating the activation of T cells, the release of cytokines, and thereby tumor growth in *NF1*-mutant murine and human neurons (85). Further reports describe the tumorigenic role of MDK in the context of NF1 (86, 87), indicating that MDK activation of T cells is a crucial mechanism in NF1-LGG pathogenesis. Additional commonalities include the control of MDK expression by NF-κB signaling and hypoxia. Activation of both pathways is a defining feature of the tumor microenvironment, irrespective of the tissue and cancer type (88). It is therefore conceivable that MDK is part of a common response mechanism to malignant tumors, and therefore potentially represents a central target for therapeutic intervention.

Overall, these findings demonstrate the importance of MDK as a central mediator of tumorigenesis, irrespective of tissue and cell type. Uncovering the exact signals that drive MDK expression and its transduction through its various binding partners in the tumor microenvironment is therefore of highest interest to identify novel therapeutic strategies that overcome tumor resistance.

MDK in the context of autoimmune and inflammatory diseases

Besides its various functions in cancer, MDK has been described as an important regulator of autoimmune and inflammatory diseases (Figure 2). One of them is rheumatoid arthritis (RA), the most common inflammatory arthritis affecting joints as well as potentially other organs. The disease is characterized by synovial inflammation, hyperplasia, and the production of autoantibodies followed by cartilage and bone destruction (89). Main drivers of synovitis are leukocyte accumulation and the production of pro-inflammatory cytokines such as TNFα and IL-6 (89). MDK has been detected in inflamed synovial tissue of RA patients but not in healthy controls (90). Here, MDK leads to the activation and migration of neutrophils into inflamed tissue by either acting as chemoattractant or by inducing the release of pro-inflammatory cytokines including IL-8, IL-6, and CCL2 (30). Notably, the migration of inflammatory leukocytes into RA synovial tissue is suppressed in MDK knock-out mice (31), where disease activity is diminished. Similar observations have been made in the context of the autoimmune disease systemic lupus erythematosus (SLE). Its pathogenesis is characterized by the production of autoantibodies against nuclear and cytoplasmic antigens affecting several organs. Patients undergo periods of remission and relapse showing organ-specific symptoms (91). In SLE patients, elevated MDK plasma levels correlate with rash and increased levels of IL-17, a pro-inflammatory cytokine produced by T_H17 cells (32). Increased levels of circulating MDK have not only been described in peripheral autoimmune diseases but also in other inflammatory conditions like ulcerative colitis (UC) (92) and Crohn's disease (CD) (93), two main forms of inflammatory bowel diseases (IBDs) (Figure 2).

Additionally, MDK has been implicated in the regulation of primary degenerative and inflammatory diseases of the CNS (28, 94) (Figure 2). Alzheimer's disease (AD), for instance, is a complex neurodegenerative disorder and one of the major causative factors for cognitive impairment. Molecular hallmarks of its pathogenesis include plaque formation by extracellular aggregates of β-amyloid (Aβ) peptides and intracellular neurofibrillary tangles made of hyperphosphorylated tau (τ) protein (95). While AD is not considered a primary inflammatory disorder, it has become increasingly clear that secondary inflammation is a key driver of disease progression (96, 97). In these lines, increased MDK levels have been found in serum and plaques of AD patients (94). These observations match the increase in inflammatory markers in AD patients and support the idea of a close interaction between amyloid pathology and inflammation. In this context, MDK has been shown to inhibit Aβ fibril formation and Aβ-induced cytotoxicity (11, 12), highlighting the tissue-protective potential of the growth factor in AD.

In autoimmune CNS disorders like neuromyelitis optica (NMO) and MS, increased levels of MDK have been associated to a poor prognosis (28). This is in line with reports of a direct correlation between MDK serum levels and IL-23 levels (98), a pro-inflammatory cytokine that drives pathological functions of T_H17 cells (99). Moreover, MDK mRNA expression in mice is highly upregulated upon EAE induction and correlates with disease progression and clinical symptoms (100). *In vivo* studies using MDK-deficient mice describe an expansion of regulatory T (T_{reg}) cell populations upon EAE induction, which in turn reduces the numbers of autoreactive T_H1 and T_H17 cells (Figure 3), resulting in disease amelioration compared to control mice (25). T_{reg} cell development is regulated through the transcription factors STAT3 (24) and STAT5 (25) and based on a MDK-dependent suppression of tolerogenic dendritic (DC_{reg}) cells, which usually promote T_{reg} cell differentiation (24) (Figure 3). The functional relevance of MDK in the context of EAE is further supported by observations of decreased inflammatory infiltration in spinal cords of MDK-deficient mice, concomitant with reduced disease severity compared to controls (25). The beneficial outcomes of MDK deficiency in EAE can be reversed by exogenous application of recombinant MDK, which exacerbates disease severity and indicates an overall detrimental function of the growth factor in autoimmune neuroinflammation (25).

In conclusion, MDK plays important roles in the onset and progression of autoimmune and inflammatory diseases in the periphery and the CNS. MDK serum levels are elevated in patients with inflammatory, autoimmune, and neurodegenerative diseases, while *in vivo* studies reveal that MDK contributes to inflammation via the induction of pro-inflammatory cytokines, the recruitment and activation of inflammatory immune cells, as well as the suppression of regulatory mechanisms (Figure 3). While these data support the notion that MDK is an important mediator of inflammation not only in peripheral pathologies but also following CNS insult, future studies are needed to delineate mechanisms and target cells in the CNS (Figure 3).

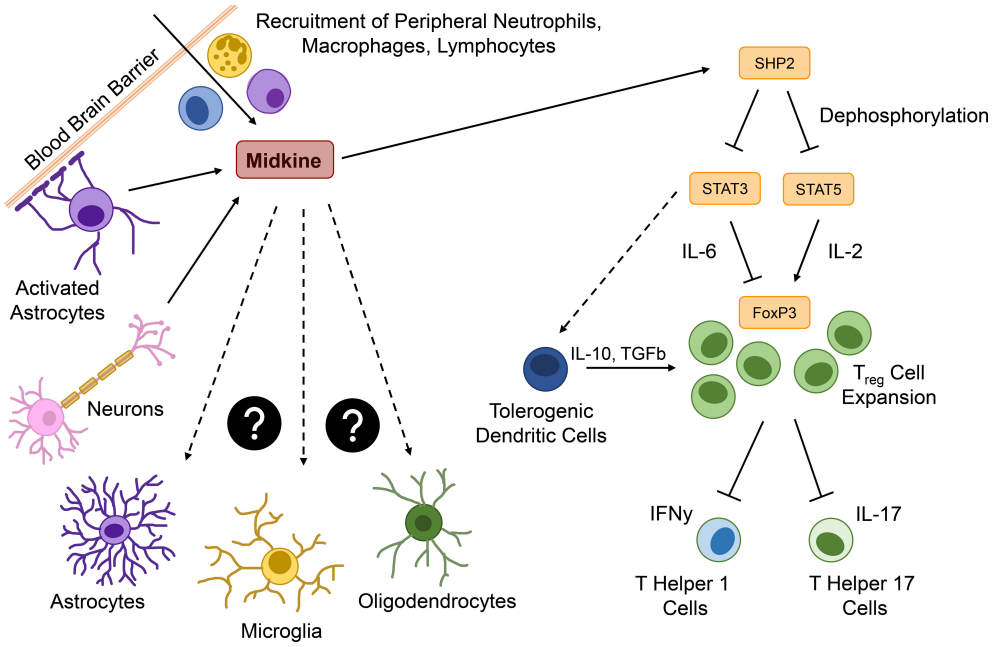

FIGURE 3
Major functions of MDK in the context neuroinflammation. Within the brain, Midkine (MDK) is expressed by activated astrocytes or neurons and acts as a chemoattractant for peripheral immune cells, such as neutrophils, macrophages, and lymphocytes. MDK promotes the expression of the tyrosine phosphatase SHP2, which dephosphorylates signal transducer and activator of transcription (STAT) 3 and 5. STAT5 usually induces the expression of the transcription factor forkhead-box-protein p3 (FOXP3) in an interleukin (IL)-2-dependent way, promoting the expansion of regulatory T (T_{reg}) cells. STAT3 is required for the IL-6-dependend inhibition of Foxp3 expression and might be involved in the induction of tolerogenic dendritic cells (DC_{reg}), which additionally promote a T_{reg} cell expansion. T_{reg} cells downregulate the interferon γ (IFNγ) expressing T helper (T_H) 1 and the IL-17 expressing T_H17 cells. The pro-inflammatory cytokine IL-23 triggers pathological features in IL-17 producing T cells and might be in correlation with MDK expression. All MDK-induced events lead to continuous neuroinflammation, while effects of MDK on central nervous system (CNS) resident cells such as astrocytes, microglia, and oligodendrocytes is still unknown.

MDK in the context of acute injury

Aside from neoplastic diseases and primary inflammatory disorders, upregulation of MDK can also be observed in injuries of peripheral organs such as the heart or the kidney (66, 101) (Figure 2). Upon renal ischemia-reperfusion, a process frequently leading to excessive tissue injury and destructive inflammatory responses (102), MDK promotes the migration of neutrophils and macrophages to the site of injury (29) while in cardiac ischemia-reperfusion injury MDK prevents myocardial apoptosis (13).

Similarly, in the injured CNS, numerous functions of MDK have been proposed. Traumatic brain injury (TBI) starts with primary tissue damage directly caused by the insult, followed by secondary tissue damage, which is induced by pathological processes after the primary insult and leads to necrosis and apoptosis of cells in the CNS (103). Major consequences of traumatic insults are blood brain barrier (BBB) breakdown, subsequent infiltration of immune cells into the brain (104), and neuroinflammation. *In vivo* studies with wild type and MDK-deficient mice demonstrated that MDK-deficiency does not affect astrogliosis following TBI (34), confirming earlier results of *in vitro* experiments, where MDK treatment of purified astrocyte and microglia cell cultures did neither induce astrogliosis nor microgliosis (37). Astrogliosis is a process in which astrocytes respond to CNS damage or disease by transcriptional remodeling and an altered activation state (105). Depending on the severity and permanence of this state, astrogliosis is associated to beneficial and necessary functions, but can also lead to harmful effects by cellular hypertrophy, proliferation, and the secretion of pro-inflammatory cytokines (106, 107). The same is true for microglia, the tissue-specific macrophages of the CNS (108). Following their activation, microglia can exert neurotrophic as well as neurotoxic functions (108). However, while treatment of microglia with MDK *in vitro* resulted in no major alterations, Takada et al. (34) observed a shift to an anti-inflammatory microglia polarization state during the acute phase of TBI in MDK-deficient mice. In addition, the authors observed that MDK-deficiency leads to a decrease in apoptotic neurons around lesions, thereby reducing cerebral atrophy and neurological deficits after TBI (34). As the growth factor also features chemoattractant properties, especially the recruitment of neutrophils and macrophages (34, 109) (Figure 3), it is conceivable that increased BBB permeability upon primary traumatic insult allows MDK to amplify the recruitment of peripheral immune cells and thereby potentiates secondary injury (34). Indeed, a reduction in the transgression of immune cells into the CNS was observed in MDK-deficient mice, supporting the notion that MDK regulates immune cell infiltration in the context of TBI.

Aside from TBI, MDK is expressed in early stages of cerebral infarct, a condition where the blood supply to the brain is disrupted,

leading to ischemia and hypoxia, and finally to necrotic tissue in the brain. MDK has been detected at the sites of nerve damage, where it seems to act as a reparative neurotrophic factor (14). These findings align with the transcriptional regulation of MDK by HIF-1α (Figure 1) and highlight the reparative potential of MDK in hypoxia-driven disorders. *In vivo* studies in rat showed an upregulation of MDK mRNA, as well as protein levels following transient forebrain injury (33) and increased expression of MDK in damaged areas of traumatic spinal cord injury in regards to tissue repair (110).

These data collectively suggest that MDK is part of a central inflammatory response mechanism that governs injury responses, as well as numerous autoimmune-, inflammatory-, and cancer pathologies (Figure 2, Table 1). Depending on the inflammatory state and the microenvironment at the site of injury MDK exerts opposing functions and either promotes the amplification or suppression of pathological processes. Due to increased MDK levels in several diseases, the growth factor may be of high relevance as disease marker and target for drug development. Especially in the CNS, where MDK may drive the infiltration of peripheral immune cells and the pro-inflammatory activation of glial cells during acute insult, its protective functions on microglia, oligodendrocytes, and neurons underscore its therapeutic potential for regenerative processes in response to acute CNS insult. However, further studies are needed to clarify MDK signaling pathways involved in CNS pathologies, but also cancer progression, metastasis, inflammation, and other peripheral pathological conditions.

MDK as a mediator of neuro-immune crosstalk

The regulatory functions of MDK are indispensable during development, where the growth factor mediates embryogenesis, organogenesis, as well as neurogenesis (111). While in healthy adults, MDK is only expressed in the kidney, several pathological conditions are accompanied by an increase in MDK levels in the periphery, as well as the CNS (10, 36) (Figure 2). In these lines, it is becoming increasing clear that MDK is not only an important mediator of disease processes within a specific compartment, but also functions as mediator of neuro-immune cell-to-cell crosstalk.

While under homeostatic conditions, the CNS is shielded from the periphery by the BBB, inflammation induced barrier dysfunction may foster the MDK-dependent interaction between CNS-resident and peripheral cell types. In these lines, the infiltration of MDK-expressing immune cells through a leaky BBB may stimulate context-specific MDK-signaling events in CNS-resident cells, or *vice versa* (Figure 3). While the effects of MDK on glial cells are not fully understood, MDK has been shown to induce an anti-inflammatory polarization state in microglia *in vivo* (34). Even though there are no direct effects on astrocytes revealed so far (34), MDK-induced polarization of microglia might regulate crosstalk between glial cells (112) and thereby indirectly modulate the functions of astrocytes, as well as other CNS-resident cells, such as neurons and oligodendrocytes, which may exert the described neuroprotective effects of MDK (84, 113). On the other hand, MDK expressed by CNS-resident cells upon insult or inflammation may act as a mediator of neuro-immune crosstalk by promoting the recruitment of peripheral immune cells through a leaky BBB into the CNS (Figure 3), thereby fueling inflammatory processes within the CNS and ultimately leading to disease deterioration and additional activation of glial cells. The importance of MDK as a mediator of neuro-immune crosstalk is furthermore exemplified by its role in the suppression of regulatory functions within the CNS. Here, the secretion of MDK by CNS-resident cells induces tyrosine phosphatase SHP2 expression, which dephosphorylates and thereby inactivates STAT3 and STAT5. This cascade results in the suppression of DC_{reg} cells, and consequently T_{reg} cells, leading to increased numbers of effector T cells (Figure 3), and the exacerbation of inflammatory processes *in vivo* (24, 25).

Similar mechanisms of MDK may contribute to the development and pathogenesis of neoplastic diseases, where neurons, microglia, macrophages, and T cells in the tumor microenvironment control formation, growth, and progression of malignant solid tumors (85, 114–118). Here, MDK not only recruits peripheral immune cells, but also activates $CD8^+$ T cells, establishing a neuro-immune-cancer axis that promotes tumor growth (85). The exact routes and mechanisms of crosstalk, and how MDK derived from the periphery versus CNS-derived MDK regulates inflammatory reactions still need to be addressed in future studies.

MDK as putative biomarker

Due to the distinct expression of MDK in various pathological conditions, especially malignancies and inflammatory diseases (Figure 2, Table 1) the growth factor has been considered as a putative biomarker (36, 119–121). While the potential of MDK as a biomarker has been proposed for several cancer types and inflammatory diseases, further studies are required to delineate its specificity as a biomarker. In hepatocellular carcinoma (HCC), MDK enables a discrete discrimination of patients with early HCC from those with cirrhosis (122). The assessment of serum and urinary MDK levels furthermore facilitates the early detection of non-small cell lung cancer (NSCLC) (123) and aids clinical decision making, as high MDK levels correlate with poor prognosis in NSCLC patients (124). Recently, the growth factor MDK has additionally been described as candidate biomarker in lung adenocarcinoma, one of the most common types of lung cancer (125). As MDK is a systemic lymphangiogenesis-inducing factor, its detection might function as a prognostic marker for melanoma patients (63). In brain tumors both MDK and PTN might be useful as early diagnostic and independent prognostic markers, as MDK overexpression correlates with the rapid progression of astrocytomas (126) and a poor survival outcome in high-grade gliomas (127). Studies in the context of autoinflammatory diseases such as RA, SLE, UC, and CD have proposed MDK as a marker for the detection of inflammatory disease activity (92, 93, 128, 129), with a performance comparable to, and potentially superior to

established disease activity markers like C-reactive protein (CRP) (92, 93). Finally, a recent has described MDK levels in the cerebrospinal fluid of Parkinson's disease (PD) patients as a supportive diagnostic biomarker (130), highlighting its potential for other neurodegenerative disease such as MS or AD.

MDK as therapeutic target

Beyond its significance as a biomarker, the involvement of MDK in numerous diseases, including injuries, malignancies, and inflammatory disorders of the periphery and CNS (Table 1), harbors significant potential as a therapeutic target. Depending on the type of disease and the function of the growth factor within a pathological condition, therapeutic approaches could consist of MDK blockage or the exogenous supplementation of MDK.

In neoplastic diseases, MDK promotes tumor growth, differentiation, and therapy-resistance. In these lines, MDK-targeted strategies may have great therapeutic potential, particularly in refractory cancer settings. Recent studies demonstrate that blockage of MDK signaling by various approaches rescues tumor resistance. For instance, the use of the small molecule inhibitor iMDK (131), small interfering RNAs (siRNAs) (132), or MDK blockage using anti-MDK monoclonal antibodies (133) restores tumor apoptosis and inhibits tumor growth in mice. A promising human MDK blockade system has already been established *in vitro* using prostate cancer xenografts, where synthetic siRNA in combination with the chemotherapeutic paclitaxel (PTX) affects tumor cell proliferation, apoptosis, and angiogenesis (132). Another way to target MDK is via an antisense oligodeoxynucleotide molecule based on the secondary structure of MDK mRNA, referred to as antisense oligoDNA, or morpholino antisense oligomers. Treatment with antisense MDK suppresses tumorigenicity in mouse rectal carcinoma cells and other xenograft models *in vitro* and reduces tumor growth in nude mice *in vivo* (134). A recent study precisely looked into the effects of MDK within the HCC microenvironment and postulated MDK inhibition as valuable therapeutic addition to anti-PD-1 immunotherapy in HCC patients, as the standard treatment, sorafenib, leads to an immunosuppressive tumor microenvironment due to increased MDK expression (135). MDK-TRAP, a MDK-binding peptide derived from the MDK receptor LRP1, inhibits, similar to anti-MDK antibodies, the binding between MDK and LRP1, thereby decreasing cell growth and colony formation in G401 cells and CMT-93 cells (136). As MDK/LRP1 signaling contributes to anchorage-independent tumor cell growth, its disruption might be a promising cancer treatment approach, along with MDK-TRAP and polyclonal antibodies. The blockage or inhibition of MDK-mediated effects prior to or during chemotherapy might increase treatment effectiveness and benefits patients who are not responding to conventional treatments.

Therapies with siRNA, oligoDNA, and other drugs inhibiting MDK have not yet been tested for CNS-related neoplastic diseases but might represent enormous therapeutic potential for the treatment of glioblastomas. While in neuroblastomas PTN expression is linked to good prognosis, high MDK mRNA levels are detected in tumors with poor prognosis (80). This is of particular interest, as knockout of PTN and its receptor ALK exerts antitumorigenic effects in glioblastoma animal models (137). Monoclonal antibodies directed against MDK may allow targeting these tumorigenic effects in the CNS, however, current candidates still lack the necessary efficacy (138, 139). Moreover, RNA aptamers against MDK hold great potential for therapeutic treatment of neuroblastomas (27). Aptamers are biochemical agents that specifically recognize a particular target, usually a protein (140). They bind their target with high affinity and function similarly to antibodies, which is why they have been considered as highly effective therapeutics. *In vitro* and *in vivo* studies with tumor xenografts depict a suppressed growth of neuroblastoma cells upon intratumoral administration of RNA aptamers specific for MDK (27). The clinical efficacy of anti-MDK aptamers has additionally been shown for autoimmune disorders of the CNS, such as MS. Anti-MDK aptamers induce T_{reg} cell expansion *in vitro*, while treatment of EAE mice with MDK-specific RNA aptamers results in a delayed disease onset and lower clinical scores (25). This attenuation of autoinflammatory processes has also been observed when anti-MDK RNA aptamers were administered post EAE onset, once the disease is established, demonstrating its therapeutic potential in a clinically relevant setting (25).

Collectively, the blockade of MDK harbors great potential as therapeutic strategy in neoplastic and autoimmune diseases of both the periphery and the CNS. Nonetheless, it is important to better understand the upstream and downstream regulators of MDK signaling in order to develop novel therapeutic strategies.

In contrast to MDK-targeting strategies that aim to reduce MDK levels in target tissues, several approaches have been proposed that incorporate distinct features of the growth factor or focus on an exogenous or endogenous increase of MDK levels. In these lines, MDK might be a candidate for cancer vaccine development, as it has been shown that MDK-primed cytotoxic T cells are able to lyse tumor cells (36). Another novel therapeutic strategy for peripheral tumors expressing MDK is the promotor-based conditionally replicative adenovirus therapy, which has been tested in pancreatic cancer cell lines *in vitro* (141). This gene therapy involves an oncolytic virus containing part of the *MDK* promotor, named Ad-MDK. The virus is capable of killing tumor cells and even though the growth factor MDK itself is not involved in this kind of therapy, its solely expression in cancer tissues allows a tumor-selective replication of the virus containing the *MDK* promotor and might be a promising new cancer therapy (141). As shown for pancreatic carcinomas, Ad-MDK gene therapy enables glioblastoma-specific expression of oncolytic viruses, highlighting the use of MDK for the treatment of malignant glioblastomas (142). Gene therapy might also be a useful tool in non-neoplastic CNS-affecting diseases as MDK is thought to be involved in neural repair upon brain injuries. Studies in mice show that the injection *MDK* encoding adenovirus after ischemic injury decreases the infarct volume and protects against ischemic damage (143, 144). Similarly, intrathecal administration of MDK promotes functional recovery upon spinal cord injury in rats (37), supporting the beneficial effects of elevated MDK levels following CNS insult (113). Altogether, the endogenous or exogenous elevation of MDK

levels in the CNS represents a promising treatment option for various injuries of the nervous system. So far, multiple non-invasive approaches for drug delivery into the CNS have been tested, including intranasal administration (145–147), focused ultrasound (148) or nanobiotechnology-based delivery techniques (149). These approaches may harbor great potential for the exogenous elevation or reduction of MDK levels in the CNS. Additionally, gene and cellular therapies may represent useful long-term strategies for numerous CNS-affecting disorders (150).

Conclusion

The multifunctional growth factor MDK is a central factor in numerous pathologies (Table 1, Figure 2) and harbors great potential as biomarker and therapeutic target (121). Depending on disease, MDK exerts diverse functions that drive or suppress disease progression. As we have discussed in this review, MDK exerts tumorigenic functions by promoting tumor growth, differentiation, and chemoresistance in neoplastic diseases (16, 26, 27, 78, 82). Additionally, MDK contributes to the onset and progression of inflammatory and autoimmune diseases through its chemoattractant properties (29–31, 90) and the suppression of regulatory mechanisms (24, 25). While these mechanisms collectively contribute to disease progression, it has become clear that MDK can also exert tissue-protective functions (11, 12) that attenuate neurodegeneration and support repair in the periphery (13, 29) and CNS (34).

Altogether, these diverse functions allow a wide range of MDK-centered therapeutic strategies. Numerous studies have already demonstrated beneficial outcomes following MDK blockade in inflammatory disorders and malignancies (25, 131–134).

The next step is now to evaluate these strategies in combination with established therapies in order to increase treatment efficacy and to overcome tumor-resistance. Moreover, as central mediator of neuro-immune crosstalk, MDK has great potential as therapeutic target in CNS disorders. While inhibition or blockade of MDK signaling may be a promising option for neoplastic, inflammatory, or autoimmune diseases affecting the CNS, endogenous or exogenous increase of MDK levels could improve the outcome in the context of acute CNS injuries and ischemia. In these lines, particularly recently emerging opportunities of non-invasive drug delivery into the CNS further support the therapeutic potential of MDK-centered therapies in the treatment of CNS disorders (145–149). Finally, beyond its functions as therapeutic target and a critical modulator of disease processes, MDK offers great potential as putative biomarker in the context of various malignancies and disorders (92, 93, 122–124, 127–129). Future studies will be necessary to evaluate each individual benefit of MDK as a biomarker and compare them to well established markers. In summary, MDK unveils new therapeutic avenues that necessitate further validation in future studies.

Author contributions

EN: Writing – original draft, Writing – review & editing. VR: Writing – review & editing. ML: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Rajnikant Mishra,
Banaras Hindu University, India

REVIEWED BY
Hermelinda Salgado-Ceballos,
Mexican Social Security Institute (IMSS), Mexico
Faezeh Sahebdel,
University of Minnesota Twin Cities, United States

*CORRESPONDENCE
Jianjun Li
13718331416@163.com

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Impact of inflammation and Treg cell regulation on neuropathic pain in spinal cord injury: mechanisms and therapeutic prospects

Chunjia Zhang[1,2], Yan Li[3], Yan Yu[3], Zehui Li[1,2], Xin Xu[1,2], Zuliyaer Talifu[4], Wubo Liu[1,2,5], Degang Yang[1,2], Feng Gao[1,2], Song Wei[1,2], Liang Zhang[1,2], Han Gong[1,2], Run Peng[1,2], Liangjie Du[1,2] and Jianjun Li[1,2,3,5]*

[1]School of Rehabilitation, Capital Medical University, Beijing, China, [2]Department of Spinal and Neural Functional Reconstruction, China Rehabilitation Research Center, Beijing, China, [3]Institute of Rehabilitation medicine, China Rehabilitation Research Center, Beijing, China, [4]School of Population Medicine and Public Health, Chinese Academy of Medical Sciences/Peking Union Medical College, Beijing, China, [5]Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China

Spinal cord injury is a severe neurological trauma that can frequently lead to neuropathic pain. During the initial stages following spinal cord injury, inflammation plays a critical role; however, excessive inflammation can exacerbate pain. Regulatory T cells (Treg cells) have a crucial function in regulating inflammation and alleviating neuropathic pain. Treg cells release suppressor cytokines and modulate the function of other immune cells to suppress the inflammatory response. Simultaneously, inflammation impedes Treg cell activity, further intensifying neuropathic pain. Therefore, suppressing the inflammatory response while enhancing Treg cell regulatory function may provide novel therapeutic avenues for treating neuropathic pain resulting from spinal cord injury. This review comprehensively describes the mechanisms underlying the inflammatory response and Treg cell regulation subsequent to spinal cord injury, with a specific focus on exploring the potential mechanisms through which Treg cells regulate neuropathic pain following spinal cord injury. The insights gained from this review aim to provide new concepts and a rationale for the therapeutic prospects and direction of cell therapy in spinal cord injury-related conditions.

KEYWORDS

spinal cord injury, neuropathic pain, Treg cells, inflammation, cell therapy

1 Introduction

Spinal cord injury (SCI) occurs when there is injury or damage to the spinal cord due to an external force that may result in neurological dysfunction. This injury can affect, to varying degrees, the sensory, motor, and autonomic functions of the body (1). Such injuries result in severe deterioration in the quality of life of patients and increase disability and mortality rates for spinal cord injuries (2, 3). Trauma accounts for approximately 90% of spinal cord injuries (4). Neuropathic pain is a complex disorder caused by neurological lesion or disease and has become a major prognostic challenge for clinical patients due to its difficult-to-treat and often ineffective treatment options (5). A new definition of neuropathic pain was proposed by a panel of experts in 2008: 'pain that occurs as a direct consequence of an injury or disease affecting the somatosensory system' (6), which has since been endorsed by the International Association for the Study of Pain (IASP) (7). Peripheral or central neurological lesions can result in loss of sensation in the innervated areas of the damaged nerves or in areas of the body that correspond to areas of the spinal cord or brain that have been directly or indirectly damaged as a result of the lesion or disease. Therefore, sensory hypersensitivity in the affected area is often accompanied by sensory loss when most neuropathic pain occurs (8). Pain resulting from SCI can affect the patients' quality of life and severely impact the prognosis, which can result in lifelong consequences. Neuropathic pain (NP), a complex and heterogeneous disorder, affects approximately 8% of the adult population and has significant implications for both patients and healthcare systems (9). The International Association for the Study of Pain (IASP) defines NP as pain that arises directly from a lesion or disease affecting the somatosensory system (10). The etiology of NP can be attributed to damage to either the peripheral nerves, resulting in peripheral neuropathic pain (PNP), or the central nerves, resulting in central neuropathic pain (CNP). PNP is commonly associated with conditions such as Complex Regional Pain Syndrome (CRPS) and Failed Back Surgery Syndrome (FBSS), including cancer and diabetes. CNP typically arises following a stroke, spinal cord injury, or multiple sclerosis (11). Around 8% of cases of central pain syndromes manifest in post-stroke patients (12), while spinal cord injury patients constitute approximately 30-50% of cases (13), and those suffering from multiple sclerosis comprise around 20-25% (14). NP is characterized by spontaneous pain (pain that occurs without provocation, such as burning sensations and tingling), allodynia (pain resulting from non-harmful stimuli), and hyperalgesia (an increased response to painful stimuli). Pain after SCI can manifest itself in a variety of ways, and as scar tissue recedes, chronic pain emerges, limiting the prognosis of SCI and affecting neuroplasticity (15, 16). The damage to nerve fibers and neurons following SCI can also cause chronic symptoms of neuropathic pain, which may be closely related to the neuroinflammatory response (17). Neuropathic pain is pain due to nerve fiber damage or chronic compression, which can manifest as a tingling, burning, or electric shock-like pain (8). It is an abnormal pain response due to damage to the nervous system, which may manifest as hypersensitivity or spreading of pain (18). Nerve fiber degeneration or sustained compression on nerve fibers can give rise to neuropathic pain, attributed to aberrant firing or heightened release of neurotransmitters. The propagation of nerve impulses becomes irregular, and these abnormal transmissions can lead to distortion or amplification of pain sensations (19). Subsequent to a spinal cord injury, certain neurons may exhibit augmented excitability, resulting in an excessive amplification of pain signaling. This abnormal excitability might involve neurons beyond the injury site, causing pain sensations to radiate into unaffected areas. The heightened neuronal activity engenders impaired nerve conduction, thereby disrupting the transmission of pain information through the central nervous system (20). A neuroinflammatory response occurs, characterized by tissue swelling and increased pressure in the vicinity of the nerves. Consequently, inflammatory mediators like tumor necrosis factor, prostaglandins, and cytokines are released, thereby leading to aberrant pain perception (8, 21, 22). Alterations and remodeling of neural circuits in the central nervous system manifest following spinal cord injury. This, in turn, can elicit a painful response to otherwise innocuous stimuli, consequently inducing neuropathic pain characterized by sensations of tingling, burning, and electric shock-like pain (8, 23).

Following SCI, the inflammatory response is complex and is driven by a variety of cellular and signaling molecules, including inflammatory factors and injury-associated molecules (e.g., high mobility group protein (HMGB1), heat shock protein (HSP), etc.). These inflammatory mediators subsequently recruit and activate immune cells to further exacerbate the inflammatory response (24). Intervention of the inflammatory response leads to inflammatory cell infiltration, neuronal degeneration, and abnormal neurotransmitter release, which in turn exacerbates the perception and development of neuropathic pain (25). Microenvironmental imbalance and parenchymal cell infiltration are key to secondary SCI (26, 27). The immune response is involved in post-injury microenvironmental regulation; its regulatory role is achieved through interactions with other immune cells, which can regulate the activation state of immune cells and control the intensity of the inflammatory and immune response through cell contact and cytokine secretion (28, 29).

Regulatory T (Treg) cells, a specialized subpopulation of immunosuppressive T cells, are essential for maintaining immune homeostasis (30). There is mounting evidence indicating the involvement of the adaptive T-cell immune response, within the immune system, in the development of neuropathic pain. Previous investigations have demonstrated the infiltration of T cells into the spinal cord (31, 32), the site of injury (33), and the dorsal root ganglion (DRG) subsequent to peripheral nerve injury (34). These studies highlight the indispensable role of T cell regulation in neuropathic pain. Although the precise contributions of distinct T cell subpopulations to neuropathic pain remain unclear, it has been observed that helper T (Th)1 cells are capable of augmenting pain sensitivity through the production of inflammatory cytokines, namely interferon gamma (33). Conversely, helper T (Th)2 cells have shown the ability to diminish pain sensitivity in animal models of nerve injury by generating anti-inflammatory cytokines, such as IL-10 (33). Simultaneous investigations have also suggested a potential link between microglia-mediated gender dimorphism in pain and Treg-mediated regulation of microglia activation and attenuation of pain hypersensitivity (35). Treg cells exert a localized immunosuppressive effect by targeting immune cells to reduce the inflammatory response and decrease self-attack (36). Regulation of Treg cell number and

function is an important part of the pathogenesis of various immune diseases. Treg cells act as key negative regulators of inflammation in various pathological states, including autoimmunity, injury, and neurodegeneration (37–43). Regulatory T cells (Tregs) play a crucial role in maintaining self-tolerance and the dynamic equilibrium of the immune microenvironment (44). Given their involvement in immunoregulation during the inflammatory response to neuropathic pain, it is imperative to examine the functions of inflammation and Treg cell regulation in the context of neuropathic pain following spinal cord injury.

This review describes the relationship between the inflammatory response and Treg cell regulation following SCI (Figure 1), as well as the critical role of Treg cells in the development of neuropathic pain after SCI. The overarching objective of this review is to gain profound insights into the underlying mechanisms of neuropathic pain following SCI and to provide novel avenues for cellular therapeutic interventions.

2 The inflammatory response is associated with neuropathic pain after SCI

2.1 Release of inflammatory mediators and inflammation-mediated pain transmission

The release of inflammatory mediators and inflammation-mediated pain transmission play an important role in the progression of disease after SCI (45). During the inflammatory response, immune cells and nerve cells interact to trigger the release of inflammatory mediators, including cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β) and chemokines (e.g., CXCL1, CXCL2). These inflammatory mediators are involved in the inflammatory response and regulate inflammation-related signaling pathways (46, 47).

In addition, the release of inflammatory mediators participates in the inflammation-mediated pain transduction process. Following SCI, inflammatory mediators stimulate sensory neurons and dorsal root ganglion cells, leading to increased neuronal excitability (48). This abnormal state of excitability induces pain perception, which is mediated through signaling pathways, during which inflammatory mediators act as signaling molecules that interact with their corresponding receptors and ion channels to regulate neuronal excitability (49, 50). Such receptors and channels include TRPV1 channels, ATP receptors, and acid-sensing ion channels. Inflammatory mediators alter neuronal excitatory thresholds and enhance neuropathic pain by modifying the activity and expression of these channels (51–54). Therefore, inflammatory mediators are involved in pain transmission following SCI.

2.2 Effect of the infiltration and activation of immune cells on pain after SCI

SCI elicits an immune response, resulting in the accumulation of inflammatory and immune cells. Immune cell infiltration occurs at the site of SCI where immune cells interact with neurons (55, 56). These infiltrating immune cells mainly include monocytes,

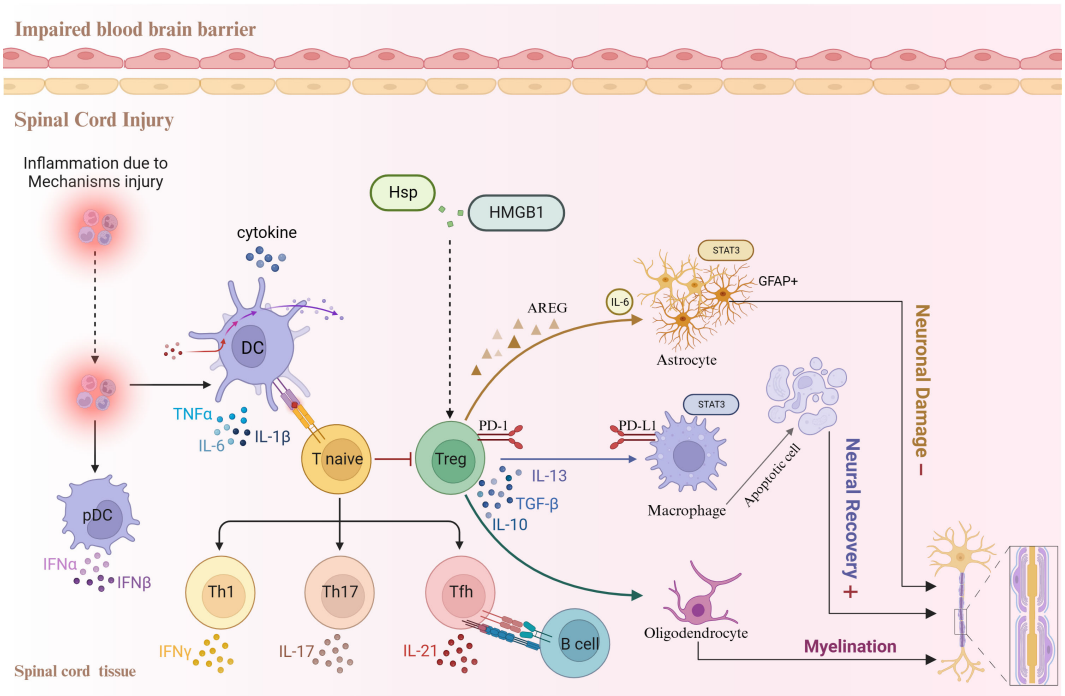

FIGURE 1
Relationships between the inflammatory response, Treg cells, and other cell types following spinal cord injury.

macrophages, T cells, and B cells (57). Different cell types release different immune molecules at the site of injury after SCI, and the peak period of cellular infiltration varies (Figure 2). Immune cells are activated to release a series of cytokines and chemicals, including TNF-α, IL-1β, and IL-6, which an act directly on neurons to increase excitability and decrease inhibition (58). The interaction between immune cells and nerve cells leads to increased neuronal excitability, triggering or enhancing the nociceptive transmission of pain through direct stimulation of neurons or via effects on synaptic transmission (59). Related studies have shown that inhibiting the infiltration and activation of immune cells, or targeting and modulating cytokines and chemicals released by immune cells, can attenuate pain sensation following SCI (60–63). Therefore, modulation of the infiltration and activation of immune cells may be a novel approach to treat pain after SCI.

2.3 The role of glial cells in the inflammatory response and the maintenance of neuropathic pain

Glial cells, including astrocytes and microglia, are involved in the inflammatory response following SCI (64–66). These glial cells become activated after SCI and release multiple inflammatory mediators (67, 68) (Figure 2). Primary injury is initiated by an initial insult to the spinal cord, leading to mechanical damage and subsequent opening of the blood-brain barrier (BBB). This process is characterized by oxidative damage, edema, ischemia, and heightened glutamate excitability (25, 69). Within the initial few hours, these mechanisms contribute to the onset of secondary damage, whereby immune cells infiltrate the damaged region via the vascular system, resulting in cell death and exacerbated injury. Various cell types are involved in this secondary phase, exerting distinct temporal influences on disease progression (25, 70). Spinal cord injury induces the activation and recruitment of multiple glial cells, leading to intricate downstream effects on neuronal function (71). The formation of the glial scar after SCI involves the participation of various cell types. Astrocyte activation begins on day 1 post-injury and reaches its peak at day 14 (72–75). Schwann cell recruitment starts 21 days after SCI (76, 77). Meningeal cells become involved 3 days after SCI and reach their peak at day 14 (78–80). Fibroblast activation initiates 3 days after SCI and peaks at day 7-14 (81–84). Finally, a limited degree of structural tissue regeneration and repair takes place in the weeks to months following spinal cord injury (25). Astrocytes, the most common glial cell type in the spinal cord, play an important role in maintaining normal neuronal function, regulating the blood–brain barrier, and removing intercellular metabolites (85, 86). The deleterious effects of astrocytes during SCI are produced through reactive astrocytes. Two types of reactive astrocytes have been identified: the A1 astrocyte and the A2 astrocyte (87, 88). The former plays a destructive role and promotes the inflammatory response, whereas the latter plays a restorative role in ischemia-induced inflammation and inhibits the inflammatory response (89). The imbalance between the A1 and A2 responses is an important mechanism in the development of neuropathic pain after SCI.

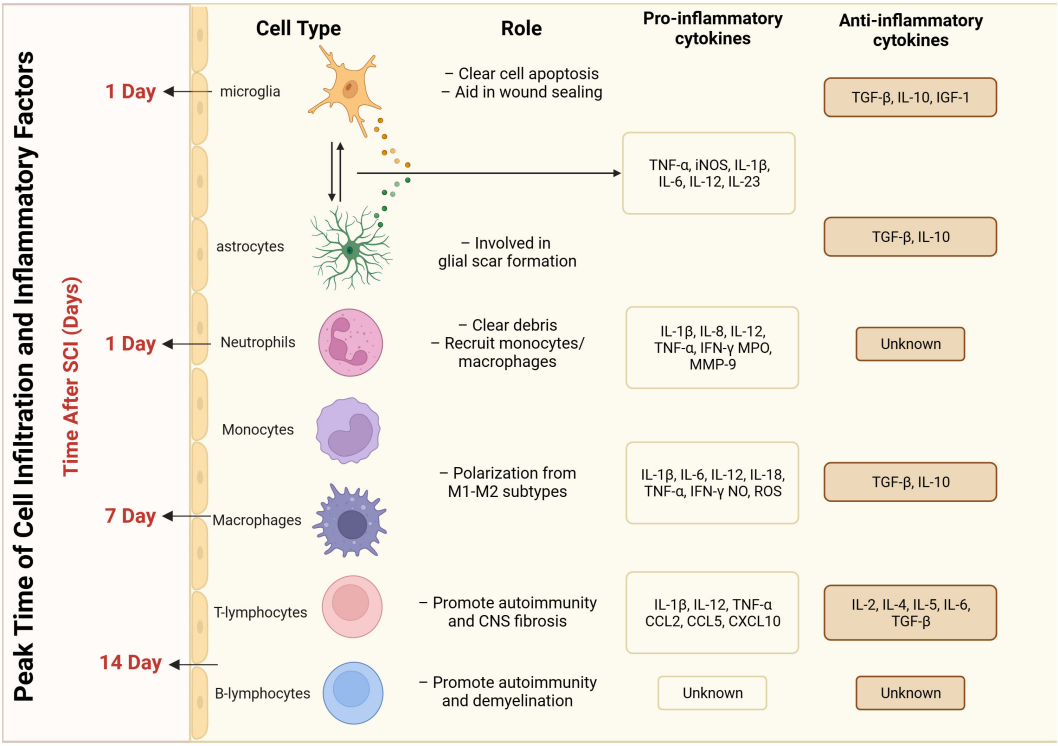

FIGURE 2
Peak time of infiltration and inflammatory factors released by different cell types after spinal cord injury.

Excessive A1 astrocyte responses and excessive release of inflammatory mediators may lead to neuronal activation and abnormal nociceptive transmission, resulting in the development of neuropathic pain (90). The A2 astrocyte response is a major contributor to the development of neuropathic pain. In addition, deficiencies in the A2 astrocyte response may affect tissue repair and anti-inflammatory mechanisms, perpetuating the inflammatory response and exacerbating the degree and duration of pain (91).

Microglia are mainly found in the gray matter regions of the central nervous system. Following SCI, microglia are also activated and participate in the inflammatory response, promoting neuronal excitability and inflammation-mediated nociceptive transmission (92). Microglia act as powerful neuromodulators to regulate salience transmission and pain transmission through multiple inflammatory mediators (e.g., pro-inflammatory and anti-inflammatory factors) acting on neurons and other glial cells (93) (Figure 2). Microglia have multiple cell surface receptors that dynamically and multifacetedly regulate the inflammatory response after SCI by interacting with neurons, astrocytes, immune cells, and others (94). Studies have reported that microglia are involved in inflammatory responses, pain signaling, and synaptic remodeling after SCI. Microglia maintain the inflammatory response and the enhancement of pain afferent signaling through the recruitment of immune cells after injury, forming synaptic structures with neurons. Vesicles released through these structures enhance neuronal excitability and strengthen pain signaling (95–98). Furthermore, it has been reported that the HMGB1–RAGE axis contributes to the major macrophage/microglia-mediated pro-inflammatory response, and that inhibition of this pathway exerts neuroprotective functions after SCI. This cascade modulation of the immune microenvironment has emerged as a prospective therapeutic approach for the treatment of SCI (99). In addition to the well-studied microglia and astrocytes, oligodendrocytes, as the main myelin-producing glial cells, are critical in maintaining myelin for fast and efficient conduction of electrical impulses along the axon and for maintaining axon integrity (100). Some studies have reported that Treg cells are involved in oligodendrocyte differentiation and myelination, which has a positive effect on SCI recovery (101).

3 Regulatory role of Treg cells in SCI

3.1 Function and characterization of Treg cells

Treg cells are an immunosuppressive subpopulation of CD4+ T helper cells, which have important immunomodulatory functions and unique characteristics that help to maintain immune homeostasis and inhibit overactivation of the immune response (102, 103). The functions of Treg cells are characterized by immunosuppression, immune tolerance, and immune homeostasis.

Recent advances in Treg cell biology have identified Treg cells residing in specific tissues for the maintenance of tissue homeostasis and repair (104), such as in the secondary prevention of ischemic stroke where they suppress immune responses by directly inhibiting the activation and function of other immune cells (105). Treg cells play an important role in the immune system as self-tolerance regulators, preventing damage to tissues from the immune response and reducing the occurrence of autoimmune diseases. Furthermore, Treg cells are involved in tumor development and progression by suppressing tumor immunity; Treg cells can be activated by chemokines (e.g., CCR4-CCL17/22, CCR8-CCL1, CCR10-CCL28, and CXCR3-CCL9/10/11), are chemotactically attracted to the tumor microenvironment, and participate in microenvironment regulation (106–108).

The dysregulation of the Th17 and Treg cell balance in neurological disorders can significantly impact disease progression (39). Excessive activation of Th17 cells and insufficient regulation by Treg cells can contribute to immune-mediated neuroinflammation and injury, thereby promoting disease progression (109, 110). Additionally, emerging evidence highlights the interconnectedness of the gut, spinal cord, and immune cells in spinal cord injury disorders, which establishes a “gut-spinal cord-immune” axis. Treg cell regulation in the intestinal environment, along with the promotion of IL-10 secretion, can modulate the dynamic equilibrium between Treg and IL-17γδ T cells, suppress inflammatory responses, and enhance motor function recovery in rats. Collectively, these studies underscore the crucial regulatory function of Treg cells in the pathogenesis of spinal cord injury (111).

CD25 is one of the hallmark features of Treg cells. Increased expression of CD25 by Treg cells is closely associated with immunosuppressive functions (112). In addition, Treg cells express the transcription factor FOXP3 in the nucleus, which is a major marker of Treg cell identity and a key molecule in the regulation of their function (113, 114). Transforming growth factor β (TGF-β) and IL-10 are examples of the multiple inflammatory suppressive cytokines produced by Treg cells; these factors inhibit the activation and immune response of other cells, leading to immunosuppression and immunomodulation (115, 116). Short-chain fatty acids (SCFAs) modulate Treg cells in the gut and affect the balance of Treg cells and IL-17+γδ T cells in the spinal cord, which has been reported to suppress inflammatory responses and promote locomotor function in SCI rats (111). Treg cells interact with amphiregulin (AREG) through the AREG/epidermal growth factor receptor (EGFR) signaling pathway to participate in immune regulation while controlling skeletal muscle function and regeneration (117). In summary, Treg cells play an important role in immune regulation by suppressing the activation and function of other immune cells, maintaining immune homeostasis, and preventing damage to host tissues from the immune response.

3.2 Treg cell regulation of the inflammatory response after SCI

A reduction in the number of Treg cells is closely associated with the inflammatory response following SCI. Treg cells regulate the degree of inflammation through a variety of mechanisms; they can inhibit the activity of other immune cells (e.g., Th1, Th2, and Th17 cells) and reduce the production and release of inflammatory mediators (118, 119). The number of Treg cells was reported to be

significantly reduced in the spinal cord and its surrounding tissues, which resulted in a decrease in the immunomodulatory function of Treg cells, allowing an over-activated inflammatory response to develop at the site of injury (103). Treg cells reduce the extent of the inflammatory response by inhibiting the activation of other immune cells and the production of inflammatory factors. The inflammatory response in SCI is directly suppressed by inhibiting the activation signaling of other immune cells through the binding of the surface molecules cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD-1) to their ligands (120, 121). Treg cells act as regulatory antigen presenters through specific ligand–receptor interactions, inhibiting antigen activation and inflammatory responses by immune cells (120). Treg cells also interfere with inflammatory signaling pathways, and can reduce the production of inflammatory factors and inflammatory responses by inhibiting the activation of the nuclear transcription factor NF-κB (122–124). In addition, some studies have reported that by regulating the TUG1/miR-214/HSP27 signaling pathway, the proportion of Treg cells can be reduced, thereby alleviating acute SCI (125). Treg cells can also regulate neuroendocrine circuits by inhibiting cytokine secretion and release; therefore, regulating neuronal activity Janyga et al., 2023[1]. Treg cells downregulate TNF-α and IL-6 expression in microglia by inhibiting STAT3 pathway activation, which ultimately improves the damaged spinal cord microenvironment and promotes the recovery of neurological function after SCI (126). MBP-Th2 cell transplantation after SCI changes the state dominated by Th1 and M1 cells to a state dominated by Th2, Treg, and M2 cells. This changes the local immune microenvironment by increasing the number of Th2 cells, thus producing beneficial effects on the spinal cord and promoting the repair of SCI.

3.3 Inhibition of neuropathic pain by Treg cells

Neuroimmune communication has emerged as a key neuropathic pain mechanism in previous studies, which reported that both the innate and adaptive immune systems are associated with neuroinflammatory changes in neuropathic pain (127). Local infiltration of macrophages, T cells, astrocytes, and activated microglia following SCI results in the release of multiple inflammatory mediators (pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-17, and IFN-g) to maintain nociceptive hypersensitivity (128–131) (Figure 2). In mice, mechanical pain after nerve injury can be alleviated by intrathecal injection of Treg cells (132). Furthermore, CD28 agonists can alleviate mechanical pain hypersensitivity due to injury in rats with chronic compression injury of the sciatic nerve by modulating the number of T cells, macrophages, and other immune cells in the sciatic nerve and dorsal root ganglion (44, 133). The elimination of Treg cells using a CD25 antibody leads to prolonged mechanical abnormalities in sciatica mice (44). Depletion of FoxP31 Treg cells in transgenic DEREG mice leads to a transient increase in mechanical pain hypersensitivity (134).

Treg treatment also modulates the amount of reactive astrocytes of different phenotypes to reduce neurotoxicity by attenuating astrocyte GFAP expression. Interestingly this therapeutic effect of Treg cells manifests itself differently in female and male mice, with a reduction in the number of neurotoxic astrocytes in peripherally injured male mice, and conversely, in peripherally injured females, the number of protective astrocytes was increased with peripheral nerve injury (135). Brain Treg cells inhibit neurotoxic astrocyte proliferation and protect neurons from damage by producing the low-affinity EGFR ligand AREG (136). Male mouse meningeal Treg cell administration may induce an anti-inflammatory shift in microglia phenotype via Treg-associated effector cytokines IL-10 and TGF-β (137–139).

The inhibitory role of Treg cells in neuropathic pain has been confirmed by a series of animal studies, as described above. The results of clinical trials similarly suggest that the number of T helper cells producing IL-17 is reduced, while the number of Treg cells is increased in patients with chronic lower back pain. Correspondingly, mRNA expression levels of FOXP3 and TGF-β were elevated in peripheral blood mononuclear cells according to cytokine profiling assays (140, 141). This phenomenon has been speculated to reduce pain levels in patients through the suppression of inflammatory responses (142). In summary, Treg cells reduce inflammatory damage to neurons by modulating neuroimmune interactions, and reducing neuronal hyperexcitability and abnormal alterations in synaptic plasticity, which in turn attenuates the onset and progression of neuropathic pain.

4 Clinical application strategies for Treg cell enhancement and synergy

4.1 Therapeutic strategies for Treg cell increase and functional improvement

The number of Treg cells can be increased by exogenous donor acquisition or endogenous proliferation and expansion, for example, with the use of growth factors, immunomodulators, or cell therapy, among others (143–145). Targeting Treg cell ligand–receptor interactions using specific cytokines or drugs (e.g., IL-10, TGF-β, and IL-2, etc.) that enhance Treg cell immunosuppression, enhancing immunomodulation, improves the function of Treg cells (146, 147). Improving Treg cell migration and accurately localizing damaged sites by altering chemical gradients, enhancing cytokine adhesion, and modulating inflammatory factor expression is one of the important therapeutic strategies (148–150).

4.2 Synergistic effects of Treg cells with immunosuppressants and other treatments

Treg cells, as natural immunomodulatory cells, can inhibit immune cell activity, and can function synergistically with immunosuppressive

1 Janyga S, Kajdaniuk D, Czuba Z, Ogrodowczyk-Bobik M, Urbanek A, Kos-Kudła B, et al. Interleukin (IL)-23, IL-31, and IL-33 play a role in the course of autoimmune endocrine diseases. *Endocr Metab Immune Disord Drug Targets*. (Ahead of Print) (2023). doi: 10.2174/1871530323666230908143521

agents (e.g., immunosuppressant drugs or cytokine inhibitors) to reduce inflammatory and autoimmune responses (151–153). Cell therapy for SCI is emerging as a new research hotspot (154). Currently common cell therapies, such as stem cell therapy or gene therapy, can repair and regenerate tissues. Treg cell combined with stem cell therapy is a potentially more desirable therapeutic tool, and the synergistic combination of drugs with Treg cells enhances the immune-suppressive effect of the drugs, reduces the side effects, and lowers the risk of immune tolerance (155). Treg deficiency affects the gut microbiota and bile acid metabolism, induces IL-6 expression, and triggers a lethal inflammatory response. Antibiotics can modulate the gut microbiota and bile acid metabolism by inhibiting IL-6 levels, thus preventing the lethal inflammation caused by Treg deficiency (156). In studies on immune-related diseases associated with tumors, Treg cells are implicated in tumor development and progression by suppressing anti-tumor immune responses. Therefore, there is a critical need in the field of cancer immunotherapy to deplete Treg cells and modulate their function to enhance anti-tumor immune responses (106). Research has demonstrated that dendrobine significantly reduces Foxp3 expression, increases serum IL-17 levels, and enhances Th17 cell function while suppressing Treg cell function. Additionally, *in vivo*, dendrobine and cisplatin may synergistically regulate the Treg/Th17 cell balance rather than induce apoptosis (157). All-trans retinoic acid is involved in regulating the differentiation of helper T cells (Th) and Treg cells. Furthermore, all-trans retinoic acid maintains the stability of thymus-derived Treg cells under inflammatory conditions (158). Severe asthma development is particularly associated with Th17 cell and neutrophil activation, and studies have shown that asthma patients can effectively suppress airway inflammation by increasing the Treg/Th17 cell ratio using statins in combination with corticosteroids (159, 160). Tregs are key target cells involved in asthma relief, and it has been suggested that glucocorticoid application reduces the number and activity of Tregs in various asthma mouse models, potentially through thymic T-cell production inhibition (161). Immunomodulators (e.g., anti-CD3 antibody, anti-CD25 antibody, etc.) can directly influence the number and activity of Treg cells, and targeted depletion of Treg cells can activate tumor-specific effector T cells and enhance the efficacy of tumor immunotherapy (162). Cancer immunotherapy primarily focuses on immune checkpoint molecules, and blocking CTLA-4 primarily activates T cells and suppresses Treg cells. PD-L1 plays a dominant role in Th1 and Th17 immunity, while PD-L2 primarily impacts Th2 immunity (163). The use of Treg cells as a cell-based therapeutic approach was initially demonstrated in mouse models; Treg cells were found to have a beneficial role in pathogenesis (36). However, the immune rejection faced by this therapeutic approach is considered to be one of the important challenges.

5 Prospects and challenges in the application of Treg cell therapy

From the current degree of clinical application, firstly, there still exists a certain technical difficulty in large-scale preparation of high-purity Treg cells. Second, further clinical research and validation are still needed to determine the therapeutic mechanism and safety of Treg cells. In addition, the survival time and functional stability of Treg cells also requires further improvement. Overall, although there are still some challenges and limitations in the clinical application of Treg cells, they have great potential in regulating inflammatory responses and treating immune-related diseases. Therefore, Treg cells are expected to be a potential target for regulating inflammatory responses and treating neuropathic pain after SCI, which will bring better therapeutic effects and treatment strategies for SCI patients.

6 Conclusions

Spinal cord injury (SCI) is a severe neurotraumatic condition that frequently results in the development of neuropathic pain. Neuropathic pain following spinal cord injury (SCI) is a complex spectrum of disorders characterized by a multitude of pathophysiologic mechanisms and associations with psychosocial factors, posing significant challenges in its management (164). While research in recent decades has shed light on the pathophysiology of neuropathic pain after SCI, therapeutic advancements have been limited. Given the high prevalence of chronic neuropathic pain, future research will prioritize the investigation of targeted therapies, identification of reliable biomarkers, and evaluation of combination therapies targeting multiple mechanisms to enhance treatment efficacy. Inflammation is known to play a critical role in the early stages following SCI, but excessive inflammation can exacerbate painful symptoms. Treg cells have a pivotal function in regulating inflammation and reducing neuropathic pain. Treg cells regulate inflammatory responses by influencing cytokine expression and other immune cell functions. However, inflammation also hinders the activity of Treg cells, thus exacerbating neuropathic pain. Therefore, besides suppression of the inflammatory response, enhancing the regulatory function of Treg cells may also offer new therapeutic avenues for the treatment of neuropathic pain caused by SCI. It is very valuable and meaningful to study the potential regulatory function of Treg cells in neuropathic pain after spinal cord injury or even central nervous system injury. Future research on neuropathic pain after spinal cord injury may focus on the development of new immunomodulatory drugs, assessment of the number and function of patients' Tregs cells to form a personalized treatment plan, the development of vaccines to regulate the immune system, and novel cell therapies based on the *in vitro* expansion of Tregs technology and the transfer of Tregs cells into the patient's body. However, it is important to note that the clinical application of Tregs for the treatment of neuropathic pain requires a careful consideration of human Treg cell purity, stability, and functional role in neuropathic pain disorders.

Author contributions

CZ: Data curation, Formal Analysis, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft. YL:

Formal Analysis, Investigation, Writing – review & editing. YY: Formal Analysis, Investigation, Resources, Writing – review & editing. ZL: Software, Writing – review & editing. XX: Data curation, Formal Analysis, Writing – review & editing. ZT: Resources, Writing – review & editing. WL: Supervision, Visualization, Writing – review & editing. DY: Conceptualization, Writing – review & editing. FG: Project administration, Writing – review & editing. SW: Software, Writing – review & editing. LZ: Data curation, Writing – review & editing. HG: Methodology, Writing – review & editing. RP: Validation, Writing – review & editing. LD: Visualization, Writing – review & editing. LJ: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Estela Maris Muñoz,
Institute of Histology and Embryology of Mendoza Dr. Mario H. Burgos (IHEM)-UNCuyo-CONICET, Argentina

REVIEWED BY
Song Hu,
Chinese Academy of Medical Sciences and Peking Union Medical College, China
Alicia Mabel Seltzer,
National Scientific and Technical Research Council (CONICET), Argentina
Maria Silvia Di Genaro,
National University of San Luis, Argentina

*CORRESPONDENCE
Weiming Sun
sunweiming@email.ncu.edu.cn
Lang Shuai
shuailangnc@126.com

†These authors have contributed equally to this work and share the first authorship

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Causal effects of immune cell surface antigens and functional outcome after ischemic stroke: a Mendelian randomization study

Weiming Sun[1,2,3*†], Jiawei Gui[4†], Keqi Wan[1,2†], Yize Cai[5], Xiangli Dong[6], Guohua Yu[1,2], Chafeng Zheng[1,2], Zhen Feng[1,2,3] and Lang Shuai[1,2*]

[1]Department of Rehabilitation Medicine, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, China, [2]The First Clinical Medical College, Jiangxi Medical College, Nanchang University, Nanchang, China, [3]Postdoctoral Innovation Practice Base, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, China, [4]HuanKui Academy, Jiangxi Medical College, Nanchang University, Nanchang, China, [5]School of Public Policy, Nanchang University, Nanchang, China, [6]Department of Psychosomatic Medicine, The Second Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, China

Objective: While observational studies link immune cells with post-stroke functional outcome, the underlying immune mechanisms are not well understood. Immune cell surface antigens are actively involved in the biological behavior of immune cells, investigating immune cell surface antigens could deepen our comprehension of their role and biological processes in stroke recovery. Therefore, we aimed to investigate the immunological basis of stroke outcome by exploring the causal relationship between immune cell surface antigens and functional outcome after ischemic stroke in a Mendelian randomization study.

Methods: Genetic variants related to immune cell surface antigens and post-stroke functional outcome were selected for two-sample Mendelian randomization (MR) analysis. 389 fluorescence intensities (MFIs) with surface antigens were included. Inverse variance weighted (IVW) modeling was used as the primary MR method to estimate the causal effect of exposure on the outcome, followed by several alternative methods and sensitivity analyses. Additional analysis of the association between immune cell surface antigens and risk of ischemic stroke for assessment of collider bias.

Results: We found that suggestive associations between CD20 on switched memory B cell (OR = 1.16, 95% CI: 1.01-1.34, p = 0.036) and PDL-1 on monocyte (OR = 1.32, 95% CI: 1.04-1.66, p = 0.022) and poor post-stroke functional outcome, whereas CD25 on CD39+ resting Treg (OR = 0.77, 95% CI: 0.62-0.96, p = 0.017) was suggestively associated with good post-stroke functional outcome.

Conclusion: The elevated CD20 on switched memory B cell, PDL-1 on monocyte, and CD25 on CD39+ resting Treg may be novel biomarkers and potential causal factors influencing post-stroke functional outcome.

KEYWORDS

immune cell, surface antigens, ischemic stroke, prognosis, Mendelian randomization

1 Introduction

Ischemic stroke, accounting for 62.4% of stroke events in 2019, is the predominant stroke type with significant long-term neurological impairment and high mortality (1). The complex and poorly understood pathogenesis of ischemic stroke leads to uncertain treatment strategies. Despite available treatments like thrombus removal, their limited effectiveness and narrow therapeutic window often result in an unfavourable outcome for many patients (2). Hence, there is an urgent need to identify novel biomarkers and therapeutic targets for ischemic stroke treatment.

Recent studies indicate that ischemic stroke triggers neuroinflammation, characterized by lymphopenia and dysfunction of immune cells, highlighting the critical role of the immune response in stroke outcome (3, 4). Understanding how immunity influences neurological recovery is thus essential. The characteristics of immune cells in stroke patients mirror the body's immune status and are strongly linked to prognosis (5, 6). For instance, regulatory T cells (Tregs), a crucial subset of immunosuppressive T cells, are believed to modulate immune responses in ischemic strokes, impacting prognosis (7, 8). CD4+ Treg levels at admission predict the modified Rankin Scale (mRS) score three months post-stroke, correlating positively with outcome (9). Immune cell surface antigens, key in immune cell differentiation, activation, and signaling, determine immune cell properties, indicating changes in function and status and reflecting their phenotype. Targeting specific surface antigens on immune cells could improve ischemic stroke therapy outcomes (10). Yet, the exact relationship between these antigens and the post-stroke functional outcome remains to be elucidated with existing studies potentially affected by reverse causation and confounding factors. Given that specific immune cell surface antigens might impact post-stroke functional outcome, further research is essential to deepen our understanding.

Mendelian randomization (MR), utilizing germline genetic variants to investigate the causal effects of exposures on outcome, is a pivotal methodology in epidemiological etiological inference (11–13). The general independence of genetic variations from environmental influences and outcomes provides us with a favorable tool to study the causality of several complex exposures and outcomes. Therefore, we performed a two-sample MR framework using genome-wide association studies (GWAS) data to explore the potential causal associations between immune cell surface antigens and post-stroke functional outcome.

2 Methods

2.1 Study design

In this study, we utilized GWAS summary statistics for a two-sample MR analysis to determine the causal effect of immune cell surface antigens on post-stroke functional outcome (Figure 1). Instrumental variables (IVs) in MR must meet three core assumptions: (1) association with the exposure; (2) independence from confounders; (3) influence on the outcome exclusively through the exposure. Our analysis relied on publicly available GWAS summary statistics from cohorts primarily of European ancestry. We carefully reviewed the original studies, and found that sample overlap was negligible. An overview of the GWAS summary data sources is presented in Table 1.

2.2 Genetic instruments for immune cell surface antigens

In this MR study, we sourced genetic variants linked to immune cell surface antigens (measured by median fluorescence intensities, MFIs) from the publicly available GWAS Catalog (https://www.ebi.ac.u/gwas/home). The initial genome-wide GWAS analysis utilized data from 3,757 individuals of European ancestry (14).

The MFI represents the median expression level of a fluorescent-conjugated antibody bound to a cell, directly proportional to the median quantity of antigen expressed in that cell. The distribution was normalized for overall and daily fluctuations to control batch effects in MFIs. A total of 389 MFIs with surface antigens were included in seven panels (maturation stages of T cell, Treg, TBNK, DC, B cell, monocyte, and myeloid cell, respectively). All immune cells used to measure MFIs were collected from the participant's peripheral blood. Details of the 389 MFIs are listed in Supplementary Table S1. Genetic variants were screened based on the following conditions: (1) Single nucleotide polymorphisms (SNPs, refer to DNA sequence polymorphisms caused by variation in a single nucleotide at the genomic level) associated with MFIs of immune cell surface antigens ($P< 1\times10\text{-}5$) and not in linkage disequilibrium (LD) with other SNPs ($r^2< 0.001$ within a clumping window of 10000 kb); (2) a phenotypic variance explained (PVE, evaluated using the R^2) > 0.5% and a F statistic >10; the F statistic was calculated as follows: $\frac{R2(N-K-1)}{K(1-R2)}$ (R^2, phenotypic variance explained; N, effective sample size; K, the quantity of genetic variants); (3) a minor allele frequency (MAF) > 0.05; (4) exclusion of SNPs associated with the outcome ($P< 1\times10\text{-}5$). The remaining SNPs were utilized as IVs. Subsequently, we harmonized the alleles and effects between the exposure and outcome. When the SNPs were not identified in the outcome data, the proxy SNPs ($r^2 >$ 0.8) from 1000 genomes European reference data were used to replace them. The SNPs that have palindromic alleles with intermediate allele frequencies (MAF > 0.42) were removed. Furthermore, we applied Steiger filtering to exclude SNPs that explained more of the variance in the outcome than the exposure. In the reverse MR analysis, the screening criteria for IVs were the same as above.

2.3 Outcome data sources

We derived GWAS summary statistics for post-ischemic stroke functional outcome from the Genetics of Ischemic Stroke Functional Outcome (GISCOME) network (15), comprising 6,021 patients across 12 studies from Europe, Australia, and the United States (16). Participants were European ancestry and aged 18 or

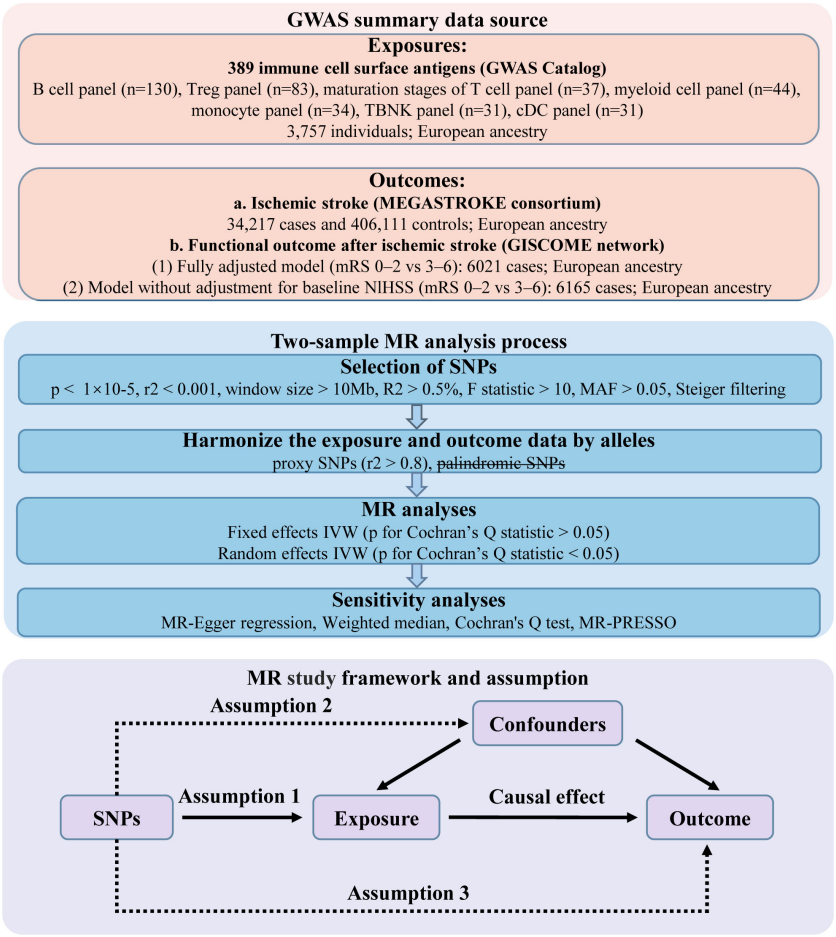

FIGURE 1
Design of the present Mendelian randomization study of the associations between immune cell surface antigens and post-stroke functional outcome.

above. Post-stroke functional outcome refers to a person's level of physical, mental and cognitive ability after a stroke, which includes a range of factors such as mobility, strength, coordination, speech, language, memory (15, 17). The main focus of research has been on unfavorable functional outcomes for stroke patients, including cognitive impairment or dementia, dependency, disability, motor impairment, psychological impairment (depression or anxiety) and death (18, 19). The mRS approximately 3 months post-stroke was selected to assess functional outcome. mRS assesses dependency of stroke patients and ranges from 0 (no symptoms) to 5 (completely dependent and bed ridden), and death was included in scale (mRS score = 6), which is a commonly used scale for measuring the degree of disability of people who have suffered a stroke or other causes of neurological disability. We classified a 'poor' outcome as an mRS score > 3 (2,280 cases) and a 'good' outcome as a score< 2 (3,741 cases). In our analyses, the mRS was analyzed as 2 dichotomous variables (score of 0–2 vs 3–6), and the results were adjusted for age, sex, ancestry, and baseline stroke severity as evaluated by the NIH Stroke Scale (NIHSS).

TABLE 1 An overview of the GWAS summary data sources in this study.

Traits	Data source	Sample size or cases/controls	Number of SNPs	Ancestry	Publication year	PMID
389 immune cell surface antigens	GWAS Catalog	3,757 individuals	~22 million	European	2020	32929287
Ischemic stroke	MEGASTROKE consortium	34,217 cases; 406,111 controls	~8.3 million	European	2018	29531354
Functional outcome after ischemic stroke	GISCOME network	6021 cases	~8.5 million	European	2019	30796134

2.4 Assessment of collider bias

To assess whether the causal association between MFIs of immune cell surface antigens and functional outcome after ischemic stroke is attributable to collider bias, we also performed an MR analysis between immune cell surface antigens and the risk of ischemic stroke. The summary statistics were obtained from the MEGASTROKE consortium, which included 406,111 controls and 34,217 patients with ischemic stroke (20). Participants were drawn from 17 studies and were restricted to Europeans only. SNPs that met the MEGASTROKE criteria (n_cases > 50% and oevar_imp > 0.5) were selected for the MR analysis.

2.5 Statistical analysis

The inverse-variance weighted (IVW) method was adopted as the main MR analysis. To account for multiple hypothesis testing, we applied Bonferroni correction with a significance threshold of $P< 1.285 \times 10^{-4}$ (0.05/389), indicating statistical significance. We also considered results with p-values of 1.285 × 10-4 to 0.05 nominally significant. Sensitivity analyses were performed using the weighted median, MR-Egger regression, and MR-Pleiotropy Residual Sum and Outlier (MR-PRESSO). The weighted median method yields consistent estimates when over 50% of the weights originate from valid instrumental variables (21). MR-Egger regression, both for the intercept and slope, assessed directional pleiotropy and provided robust estimates adjusted for its presence (22). Specifically, MR Egger regression tests for the presence of directional pleiotropy by examining the intercept term and provides an approximately unbiased estimate of the causal effect of exposure on outcome by incorporating the intercept into the regression model (22). MR-PRESSO was utilized to detect and account for potential horizontal pleiotropy and to identify and exclude any outliers with such effects (23). In cases where pleiotropy and heterogeneity were absent, a significant result ($P< 0.05$) obtained via the IVW method was considered positive, provided that the effect estimates from other methods were consistent with those of the IVW method. Cochran's Q statistic assessed heterogeneity among instrumental variables. If heterogeneity was present ($P< 0.05$), a random-effects IVW model was applied. For comparison, we conducted an MR analysis using GISCOME GWAS data without adjusting for baseline NIHSS. All statistical analyses were carried out using the MR-PRESSO (version 1.0) (23) and TwoSampleMR (version 0.5.7) (24) packages in the R software environment.

3 Results

3.1 Thirteen immune cell surface antigens as potential causal mediators of functional outcome after stroke in the main MR analysis

The number of SNPs as IVs generated by 389 MFIs of immune cell surface antigens for MR analysis ranged from 7 to 30, and all IVs passed Steiger filtering. Notably, all IVs exhibited F statistics exceeding 10, with a minimum F statistic of 19.54, indicating the significant effectiveness of these IVs (Supplementary Table S2).

In the IVW MR analysis of the expression levels of immune cell surface antigens and post-stroke functional outcome, 13 suggestive MFIs of surface antigens were identified, of which 4 were in the B cell panel, 1 in maturation stages of the T cell panel, 6 in the Treg panel, 1 in myeloid cell panel, and 1 in the monocyte panel (Figure 2).

The forest plot in Figure 3 presents the IVW estimates of the associations between the levels of these immune cell surface antigens and post-stroke functional outcome.

Genetically elevated levels of surface antigens in the DC and TBNK panel were not strongly associated with post-stroke functional outcome (all $p > 0.05$). We also conducted a reverse MR analysis (IVW method), which did not reveal a causal effect of post-stroke functional outcome on these immune cell surface antigens, suggesting no reverse causal effect (Supplementary Table S3).

3.2 Sensitivity analyses

To ensure the robustness of our findings, we conducted multiple sensitivity analysis methods to assess the presence of potential pleiotropy in the results obtained from the MR analysis described above.

Our sensitivity analyses yielded consistent and reassuring results (Table 2). Specifically, we found no evidence of heterogeneity, as indicated by all p-values for Cochran's Q test exceeding 0.05. This suggests a lack of substantial variability among the instrumental variables used in the MR analysis. Furthermore, our assessment of directional pleiotropy using the MR-PRESSO global test and MR-Egger intercept revealed no significant deviations from the IVW method. All p-values exceeded 0.05, indicating that the potential for pleiotropy did not substantially influence our findings. Additionally, the weighted median and MR-PRESSO methods produced effect estimates that were concordant with those obtained from the IVW method. This consistency reinforces the reliability of our results. However, it is worth noting that in the MR-Egger (slope) analysis, we observed that the effects of CD24 on switch memory B cell and CD3 on EM CD4+ maturation stages of T cell were estimated in the opposite direction compared to the results obtained from other MR analysis methods. This discrepancy suggests the need for a cautious interpretation of these particular associations.

As a comparative analysis, we performed MR analysis based on GISCOME GWAS data without adjustment for baseline NIHSS. This analysis revealed that four immune cell surface antigens maintained suggestive causal associations with post-stroke functional outcome based on IVW estimates (Figure 4). Importantly, this comparison analysis showed no significant evidence of directional pleiotropy or global heterogeneity (Supplementary Table S4).

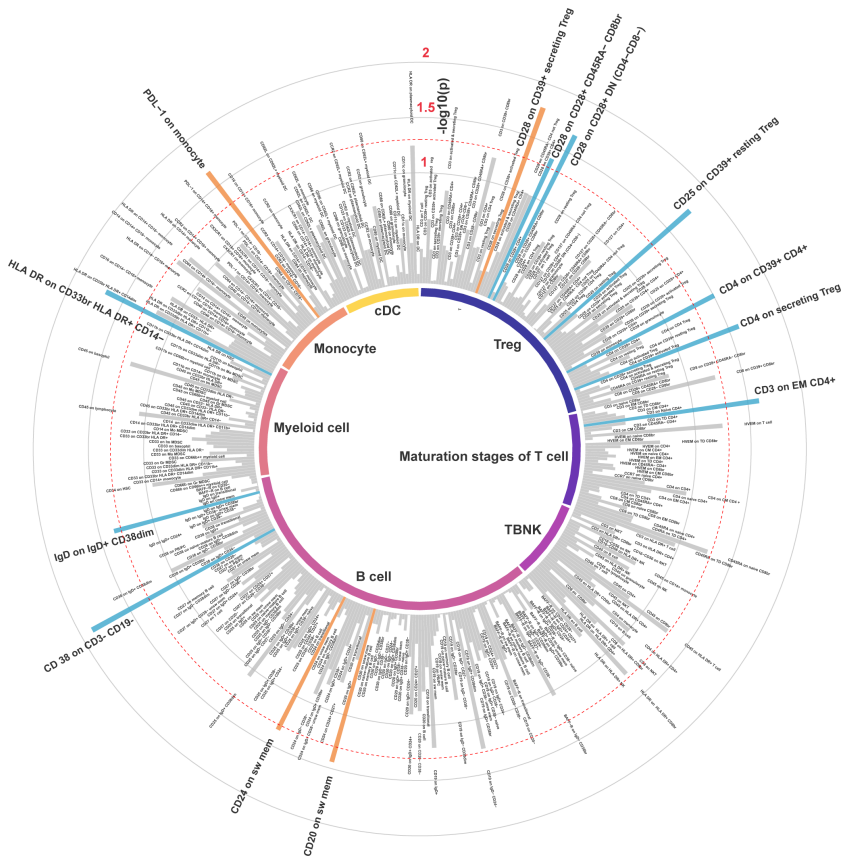

FIGURE 2
Inverse variance weighted estimates of the causal association between 389 immune cell surface antigens and post-stroke functional outcome. The red dashed line indicates the threshold of significance ($P< 0.05$). Orange bars represent deleterious mediators of post-stroke functional outcome, whereas blue bars represent protective mediators of post-stroke functional outcome.

Immune cell surface antigens	SNPs	OR(95% CI)	p
B cell panel			
CD20 on sw mem	25	1.16 (1.01−1.34)	0.036
CD24 on sw mem	24	1.17 (1.00−1.36)	0.045
CD38 on CD3− CD19−	15	0.82 (0.71−0.96)	0.013
IgD on IgD+ CD38dim	16	0.82 (0.68−0.99)	0.043
Maturation stages of T cell panel			
CD3 on EM CD4+	14	0.84 (0.72−0.98)	0.025
Treg panel			
CD28 on CD39+ secreting Treg	18	1.20 (1.03−1.40)	0.016
CD28 on CD28+ CD45RA− CD8br	17	0.84 (0.72−0.99)	0.041
CD28 on CD28+ DN (CD4−CD8−)	19	0.84 (0.72−0.97)	0.022
CD25 on CD39+ resting Treg	11	0.77 (0.62−0.95)	0.017
CD4 on CD39+ CD4+	25	1.15 (1.01−1.30)	0.031
CD4 on secreting Treg	17	0.85 (0.73−0.98)	0.026
Myeloid cell panel			
HLA DR on CD33br HLA DR+ CD14−	11	0.82 (0.69−0.97)	0.021
Monocyte panel			
PDL−1 on monocyte	12	1.32 (1.04−1.66)	0.022

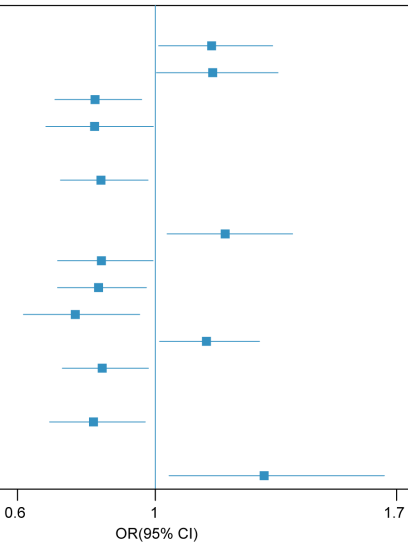

FIGURE 3
The forest plot of 13 immune cell surface antigens with functional outcome after ischemic stroke adjustment for baseline stroke severity (p for Inverse variance weighted method< 0.05).

TABLE 2 Sensitive analyses between 13 immune cell surface antigens and functional outcome after ischemic stroke (adjustment for baseline stroke severity).

MFIs of immune cell surface antigens	MR-Egger (slope)		weighted median		MR-PRESSO		MR-Egger (intercept)	MR-PRESSO global test	Cochran's Q test
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	p	p	p
B cell panel									
CD20 on sw mem	1.11 (0.77-1.59)	0.59	1.28 (1.04-1.56)	0.02	1.16 (1.01-1.34)	0.046	0.77	0.49	0.47
CD24 on sw mem	0.96 (0.64-1.43)	0.84	1.08 (0.86-1.35)	0.53	1.17 (1.01-1.34)	0.042	0.31	0.64	0.65
CD38 on CD3- CD19-	0.90 (0.66-1.23)	0.52	0.79 (0.65-0.98)	0.03	0.82 (0.72-0.95)	0.015	0.55	0.68	0.66
IgD on IgD+ CD38dim	0.75 (0.44-1.28)	0.31	0.89 (0.70-1.13)	0.34	0.82 (0.68-0.99)	0.061	0.72	0.30	0.25
Maturation stages of T cell panel									
CD3 on EM CD4+	1.03 (0.76-1.40)	0.87	0.93 (0.76-1.13)	0.46	0.84 (0.73-0.97)	0.030	0.17	0.46	0.62
Treg panel									
CD28 on CD39+ secreting Treg	1.43 (1.05-1.96)	0.04	1.16 (0.94-1.43)	0.18	1.20 (1.06-1.37)	0.012	0.22	0.78	0.78
CD28 on CD28+ CD45RA- CD8br	0.88 (0.63-1.22)	0.44	0.81 (0.64-1.02)	0.08	0.84 (0.76-0.93)	0.004	0.80	0.99	0.99
CD28 on CD28+ DN (CD4- CD8-)	0.82 (0.60-1.12)	0.22	0.80 (0.63-1.00)	0.05	0.84 (0.75-0.94)	0.006	0.87	0.95	0.94
CD25 on CD39+ resting Treg	0.69 (0.44-1.09)	0.14	0.67 (0.49-0.91)	0.01	0.77 (0.62-0.95)	0.037	0.61	0.49	0.45
CD4 on CD39+ CD4+	1.26 (0.98-1.63)	0.09	1.14 (0.95-1.36)	0.15	1.15 (1.01-1.30)	0.042	0.42	0.35	0.32
CD4 on secreting Treg	0.82 (0.59-1.14)	0.25	0.89 (0.73-1.09)	0.26	0.85 (0.76-0.94)	0.009	0.84	0.92	0.91
Myeloid cell panel									
HLA DR on CD33br HLA DR + CD14-	0.59 (0.33-1.04)	0.10	0.80 (0.64-1.01)	0.06	0.82 (0.71-0.94)	0.019	0.44	0.71	0.74
Monocyte panel									
PDL-1 on monocyte	1.60 (0.94-2.72)	0.12	1.32 (0.96-1.80)	0.09	1.32 (1.04-1.66)	0.043	0.26	0.35	0.31

3.3 Two immune cell surface antigens were associated with the risk of stroke

To comprehensively evaluate our analyses, particularly in the context of prognostic implications, we investigated the potential presence of collider bias by examining the association of immune cell surface antigen levels with ischemic stroke risk using data from the MEGASTROKE dataset.

The MR analysis outcome revealed that CD24 on switch memory B cell (OR = 1.03, 95% CI: 1.001-1.067, p = 0.041) and CD4 on CD39+ CD4+ Treg (OR = 1.03, 95% CI: 1.002-1.054, p = 0.031) were weakly associated with an increased risk of ischemic stroke (Figure 5). These associations raise the possibility that the observed relationships between these specific immune cell surface antigens and poor post-stroke functional outcome may, in part, be influenced by collider bias. However, it is essential to note that our assessment of collider bias suggests that any such bias, if present, is likely to be minimal. This indicates that while these immune cell surface antigens may have some impact on both ischemic stroke risk and post-stroke functional outcome, collider bias is unlikely to be a major driver of the observed associations.

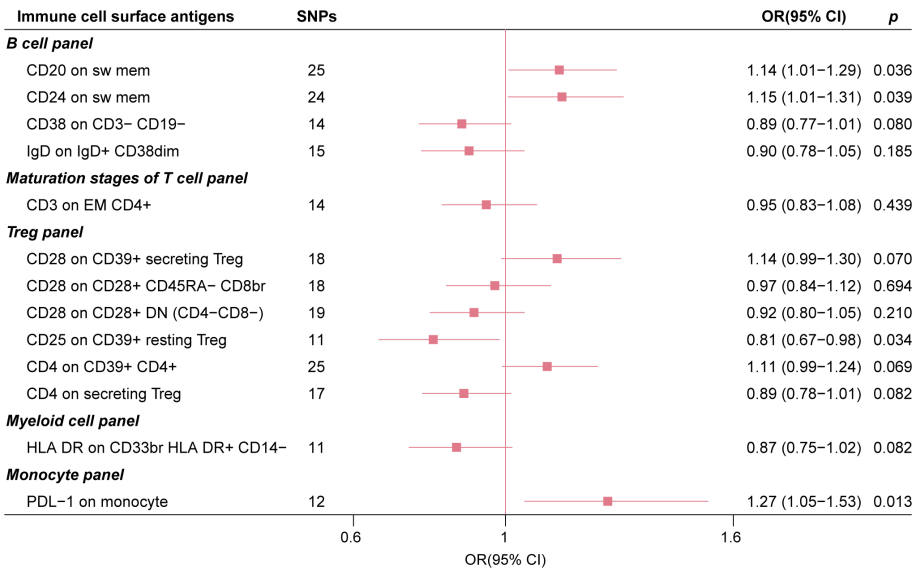

FIGURE 4
The forest plot of 13 immune cell surface antigens with functional outcome after ischemic stroke without adjustment for baseline stroke severity.

4 Discussion

The clinical significance of post-stroke functional outcome lies in its impact on the overall quality of life and long-term prognosis of stroke survivors, which is critical in determining appropriate care and rehabilitation efforts (17). In addition, it can help predict the risk of future health complications such as falls, infections, and depression (2). Actively exploring immunological factors that affect stroke recovery and intervening can help patients maximize their recovery and regain independence after stroke. While numerous observational studies have provided substantial evidence of immune cell involvement in stroke onset and outcome, they suffer from inherent limitations, including uncontrollable biases and the heterogeneity of study metrics. Immune cell surface antigens can potentially unravel the precise roles of these immune cells following ischemic stroke. However, previous investigations have primarily overlooked the contribution of cell surface antigen biological functions in the context of stroke. This study marks the inaugural effort to explore the causal relationship between immune cell surface antigens and post-stroke functional outcome using MR analysis.

In the initial primary MR analysis, we identified 13 immune cell surface antigens associated with post-stroke functional outcome. Following rigorous sensitivity analyses and an assessment of

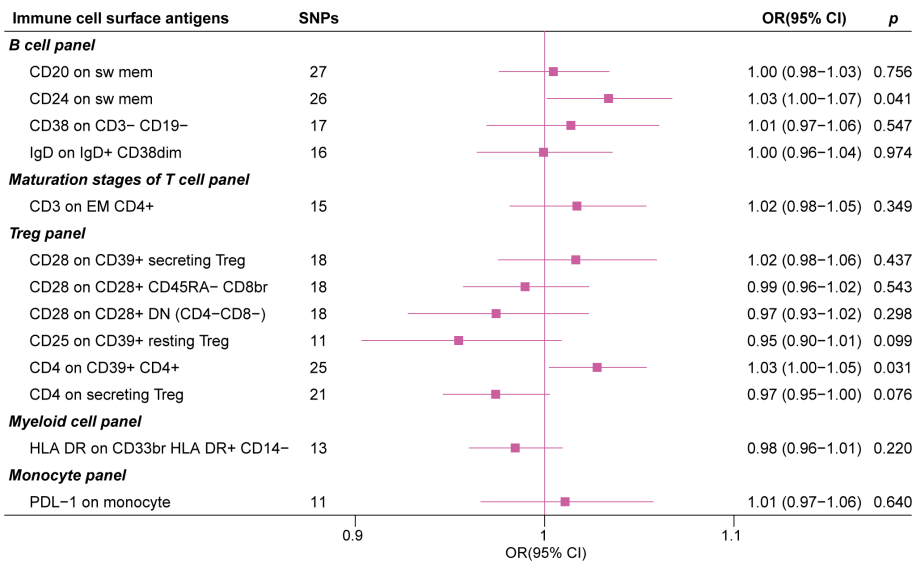

FIGURE 5
The forest plot of 13 immune cell surface antigens with risk of ischemic stroke.

collider bias, we ultimately identified three robust immune cell surface antigens with suggestive causal associations linked to post-stroke functional outcome. Specifically, elevated levels of CD20 on switched memory B cell and PDL-1 on monocyte were associated with poorer post-stroke functional outcome. In contrast, increased expression of CD25 on CD39+ resting Treg was linked to a more favorable post-stroke functional outcome (19).

CD20, encoded by MS4A1, is a non-glycosylated protein belonging to the membrane-spanning 4-domain family A (MS4A) protein family (25). Beginning with late pre-B lymphocytes, most B cells express CD20, and its expression is diminishing in terminally differentiated plasma cells. Therefore, CD20 can be used as a marker for developing B cells, and CD20-specific inhibitors are commonly used to treat B cell malignancies and autoimmune diseases (26). However, the precise biological function of CD20 and regulatory mechanisms remain elusive. Tedder TF et al. have suggested that CD20 might influence B cell proliferation and activation by modulating Ca2+ transmembrane transport (27). A case report demonstrated that CD20 deficiency reduced circulating memory B cell counts, impaired Ig isotype switching, and diminished IgG antibody levels (28). Upon activation, memory B cells undergo isotypic transformation from IgD/IgM to IgG/IgA/IgE (29). Thus, CD20 may be involved in the isotypic transformation of memory B cells. Our findings indicate that higher CD20 expression on switched memory B cells is associated with poor long-term outcome in patients with ischemic stroke. This suggests that CD20 expression levels may trigger the conversion of B cells from a specific state to pathogenic entities following ischemic stroke. Notably, the concept that B cells can contribute to central nervous system (CNS) pathology independently of antibody production has been discussed in the context of multiple sclerosis (30). B cells can release factors that disrupt the CNS, leading to oligodendrocyte and neuronal death. Targeting CD20 has proven effective in multiple sclerosis treatment (31).

Similarly, the expression level of PDL-1 on monocytes exhibited a similar effect. Accumulating evidence from animal models and patient studies suggests that ischemic stroke prompts the recruitment of circulating monocytes into the brain, where they differentiate into macrophages or dendritic cells, influencing ischemic injury progression (32, 33). Elevated monocyte counts have been associated with worse stroke outcome and greater stroke severity, making them potential predictive biomarkers for post-stroke functional outcome (34, 35). Our findings suggest that PDL-1 on monocytes may play a role in mediating unfavorable post-stroke functional outcome. PDL-1, the primary ligand for PD-1, is widely expressed in B cells, T cells, DCs, and monocytes, regulating immune function in these cell types (36). Bodhankar S. et al. have shown that homozygous knock-out (PDL-1-/-) mice had reduced monocyte infiltration, smaller infarct sizes in the ischemic hemisphere, and reduced activation status of splenic monocytes compared to wild-type (WT) mice, implying PDL-1's involvement in exacerbating experimental stroke outcome (37). Combining these results with our analysis, it can be inferred that monocytes with high PDL-1 expression may be pivotal in controlling the adverse effects of ischemia.

On the other hand, our results suggest a beneficial role for increased CD25 expression on CD39+ resting Tregs in post-stroke functional outcome. CD25, the alpha-chain of the heterotrimer IL-2 receptor, also known as IL2Rα, is constitutively expressed at high levels in most Tregs. CD25 (α-chain), together with CD122 (β-chain) and CD132 (γ-chain), forms the functional IL-2 receptor (IL-2R), and of these three receptor chains, the binding affinity of CD25 for IL-2 is the highest (38). IL-2 signalling can affect Treg peripheral induction, lineage commitment, stability sustainability, and homeostasis, and CD25 expression is critical for IL-2 signalling to Treg (39, 40). Thus, the expression status of CD25 influences, to some extent, the immunomodulatory functions of Treg involved in re-establishing immune homeostasis and regulating inflammatory response after ischemic stroke. High CD25 expression influences the immunomodulatory functions of Tregs, contributing to immune homeostasis restoration and regulation of inflammatory reactions post-stroke. However, it is essential to note that these effects may be specific to certain Treg subpopulations. Elevated CD25 expression may support the survival and maintenance of resting Tregs, while CD39 surface expression is involved in the hydrolysis of extracellular ATP, essential for immunosuppressive function. These mechanisms likely play a crucial role in long-term immune homeostasis after stroke (41, 42).

Nonetheless, several limitations of this study should be acknowledged. Firstly, measuring immune cell surface antigen levels (MFIs) involves flow cytometry on peripheral blood samples, which can introduce time-dependent artifacts. These time-dependent effects were not considered in the current MR analysis. Additionally, MR estimates might introduce bias when comparing brain and blood, necessitating careful consideration of the tissue specificity of ischemic stroke. Secondly, the GISCOME database lacked outcome data for specific stroke subtypes, preventing an assessment of immune cell surface antigen relationships with functional outcome in different stroke subtypes. Moreover, a lack of available data for replication analysis may have reduced the persuasiveness of our results. Lastly, our MR analysis was limited to subjects of European ancestry, potentially limiting the generalizability of our findings to other populations.

5 Conclusion

This MR study offers compelling evidence that specific immune cell surface antigen levels are associated with adverse post-stroke functional outcome. CD20 on switched memory B cell, PDL-1 on monocyte, and CD25 on CD39+ resting Treg emerge as potential biomarkers and causal factors linked to post-stroke functional outcome. However, the underlying biological mechanisms require further exploration, and the potential of targeting these immune cell surface antigens as a therapeutic strategy to enhance post-stroke recovery warrants further investigation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The author stated that no human studies are presented in the manuscript.

Author contributions

WS: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. JG: Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. KW: Data curation, Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. YC: Formal analysis, Investigation, Methodology, Software, Writing – review & editing. XD: Conceptualization, Formal analysis, Supervision, Visualization, Writing – review & editing. GY: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. CZ: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. LS: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – review & editing. ZF: Methodology, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1353034/full#supplementary-material

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