AGE-RELATED NEUROIMMUNOLOGY OF DEGENERATION AND REPAIR

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AGE-RELATED NEUROIMMUNOLOGY OF DEGENERATION AND REPAIR

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EDITORIAL
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Editorial: Age-Related Neuroimmunology of Degeneration and Repair

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Keywords: neuroimmunology, aging, neurodegeneration, therapy, peripheral nervous system, central nervous system, immune activation

Editorial on the Research Topic

Age-Related Neuroimmunology of Degeneration and Repair

Our understanding of the cellular, molecular, and physiological components of an aging brain has increased over the last few decades, yet the role of the immune system in governing the repair and/or degeneration of the CNS during aging is less explored. It is critical to understand the functions of different immune cells involved in aged brains, with specific interests in degenerative conditions of both the central nervous system (CNS) as well as the peripheral nervous system (PNS). With this in mind, we launched the topic, "Age-Related Neuroimmunology of Degeneration and Repair," in October 2019 with an aim to enhance our understanding of the role of the immune system in aging brains. Despite COVID-19-related delays, we are glad that the topic was well-received, which is showcased in publication of high-quality papers within the topic as this remains one of the most viewed compilations on Frontiers' platform today. The collection in this Research Topic span a broad variety of age-related conditions, including Parkinson's disease (Zhang et al., Lin C-H. et al., Chen et al.), Alzheimer's disease (AD) (Pollock et al., Yin et al., Han et al.), stroke (Wang et al.), cognitive decline (Jiang et al.), as well as conditions affecting the peripheral nervous system. The articles presented herein extend from basic science inquiries into the immunological properties underlying some of these conditions all the way to studies examining potential novel therapeutic compounds.

This Research Topic benefits from a series of comprehensive review articles, exploring several different facets of neuroinflammation in the context of aging and disease. Of particular note, Mayne et al. provide an extensive overview of the association between aging and several neurodegenerative diseases, including an analysis of the roles potentially played by the adaptive immune system, an arm of age-related neuroinflammation currently understudied. On another note, Han et al., discuss the critical role of astrocyte senescence in mediating AD pathology, a cell type that has garnered attention in recent years in regards to their immunological roles within the CNS. In addition, the role of gut microbiota did not go unnoticed, and dysbiosis in AD is the focus of the review by Zhu et al.. The collection was also enriched by the discussions on relevance of checkpoint inhibition, particularly in tau pathology, in a commentary by Baruch and Yoles and its response by Lin Y et al..

Another highlight of this Research Topic is the inclusion of two articles focusing on the aging peripheral nervous system, another area that has been considerably underappreciated in neuroimmunology. Stratton et al. perform a very nice study examining the immunological

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factors underlying the age-related impairment in peripheral nerve regeneration. In this study, they find that bone marrow transplantation from aged mice into young ones is sufficient to overcome the impairment in functional recovery in the peripheral nerve, suggesting that the young microenvironment plays a significant role in reversing any age-related intrinsic changes in the hematopoietic stem and progenitor cell populations. This study then shows through transcriptional profiling that the age-related impairment in peripheral nerve regeneration may be attributed to defects in monocyte chemoattractant protein-1 signaling. Hagen and Ousman provide an overview of another aspect of neuroimmunology of the peripheral nervous system, focusing on the autoimmune condition known as Guillain-Barré Syndrome. In this review, they summarize the various roles played by many different immune cells in this disease and present a conceptual framework looking into the potential impact an aging immune system has on this disease.

Overall, we hope that this Research Topic adds value to the existing literature and guides future research on the roles of the immune system in the age-related degeneration and repair of the central and peripheral nervous systems.

AUTHOR CONTRIBUTIONS

DKK and KSR contributed equally to the editorial.

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GENERAL COMMENTARY
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Commentary: Chronic PD-1 Checkpoint Blockade Does Not Affect Cognition or Promote Tau Clearance in a Tauopathy Mouse Model

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Keywords: Alzheimer's disease, immune checkpoints, immunotherapy, neurodegeneration, PD-1, PD-L1, chronic treatment

A Commentary on

Chronic PD-1 Checkpoint Blockade Does Not Affect Cognition or Promote Tau Clearance in a Tauopathy Mouse Model
by Lin, Y., Rajamohamedsait, H. B., Sandusky-Beltran, L. A., Gamallo-Lana, B., Mar, A., and Sigurdsson, E. M. (2020). Front. Aging Neurosci. 11:377. doi: 10.3389/fnagi.2019.00377

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In a recent paper in *Frontiers in Aging Neuroscience* (Lin et al., 2020), the authors purported to examine treatment efficacy of Programmed cell death protein (PD)-1 antibody blockade in a tauopathy mouse model in a weekly administration regimen, which the authors refer to as "chronic," supposedly based on the studies by Schwartz's team (Baruch et al., 2016; Rosenzweig et al., 2019). We wish to highlight several conceptual and technical critical issues in both study design and treatment approach, that preclude reaching any conclusion from this work. Accordingly, the title of the article and data interpretation are misleading. Furthermore, we wish to use this commentary and encourage the community to investigate Schwartz's therapeutic approach by using good scientific practice, and based on the suggested mechanism of action.

In the study by Lin et al. (2020), the authors used homozygous female JNPL3 mice, a mouse model of tauopathy, in which the same group has previously shown beneficial effects of active and passive tau immunization (Asuni et al., 2007; Boutajangout et al., 2011). In their previous studies, treatment started at the age of 2 months and outcome measurements (behavior and brain pathology) were tested at the ages of 4 to 8 months. In those studies, the authors emphasized that homozygous JNPL3 mice suffer from progressive sensorimotor abnormalities, but remain relatively healthy in these aspects at least until 8 months of age. Nevertheless, at 12 months of age these mice are severely impaired with hindlimb paralysis that prohibit any ability for behavioral testing (Asuni et al., 2007). The authors also described that the neurofibrillary pathology was much more extensive in females, up to the last time point tested—8 months of age.

Given the above-described previous reports by this team, it is surprising that the current study (Lin et al., 2020) is based upon results from an experiment performed using 22 female JNPL3 mice at the advanced age of 10–11 months, much older than previously used and at which according to the authors—the female mice suffer from severe motor disability. This cohort was divided into two groups, and tested for behavior and brain pathology, at 13–14 and 14–15 months of age, respectively. The authors apparently justified the use of such an aged cohort by claiming that there was a "shift" in their colony, and therefore mice could be tested at a more advanced age (EM Sigurdsson, "personal observation"; Methods section, Lin et al., 2020). Yet, no quantitative

parameters were presented, neither in the present study nor in any of their previous publications to support this claim, and no data were shown using the authors' own tau therapy approach to validate testing of the mice at this old age. Rather, in Sigurdsson's previous work (Boutajangout et al., 2011) locomotor activity of the IgG-treated mice showed "distance traveled" of ∼7,800 cm per mouse on average over 15 min. The same test, in the current paper, showed ∼3,000 cm for IgG-treated (control treatment) mice—less than half of the previously reported value. The authors also reported that 27% of the mice in their current study (4 control mice and 2 treated mice) died during the experiment, which strongly indicates that the animals were at a much more advanced stage of the disease than that previously tested, with a severe motor deficit. Therefore, the current results cannot be interpreted without a positive-control, e.g., using the authors tau immunization approach as in Sigurdsson's previous works (Asuni et al., 2007; Boutajangout et al., 2011) to verify feasibility of detecting any treatment response in this "shifted" colony. In addition, age-matched healthy control mice are missing, as historical controls are meaningless in behavioral measures.

Independently of the above critical issues, the regimen of weekly treatment for "chronic PD-1/PD-L1 immune checkpoint blockade," has not only never been suggested as a therapeutic protocol for achieving long-term effects in Alzheimer's disease, but is in contrast to previous studies using PD-1 or PD-L1 blocking antibodies (Baruch et al., 2016; Rosenzweig et al., 2019). Specifically, it was shown that a single treatment with PD-1/PD-L1 blocking antibody is sufficient to mitigate cognitive decline and reduce brain pathology, and that chronic beneficial effect on cognitive performance over 12 weeks was achieved by 3 monthly injections of anti-PD-1 antibody in 5XFAD mice (Rosenzweig et al., 2019). In line with these results, ImmunoBrain Checkpoint Ltd. tested the effect of anti-PD-L1 antibody administration on cognitive performance in the double mutant tauopathy mouse model (K257T/P301S; double mutant, DM-hTAU), and found that a chronic beneficial effect could be maintained over a period of 4 months by injections every 6 weeks (**Figure 1**). Thus, for a chronic course of treatment, intermittent blockade is needed, where each treatment session includes a period of immune checkpoint blockade followed by a period free of antibody exposure. The issue of intermittent rather than continuous exposure was discussed in the two papers cited above, as well as in an Opinion article by Schwartz (2017).

Critically, the justification by Lin et al. for the selected weekly injections of anti-PD-1 antibody is based on their regimen for tau antibody therapy. Such justification ignores the fact that choice of regimen for any antibody therapy must be based on its mechanism of action. There is no scientific or therapeutic basis to justify any mechanistic linkage between anti-amyloid/tau antibody approaches employed in Alzheimer's disease, and the use of anti-PD-1/PD-L1 antibodies, which represent a completely different mechanism of action of the therapeutic approach.

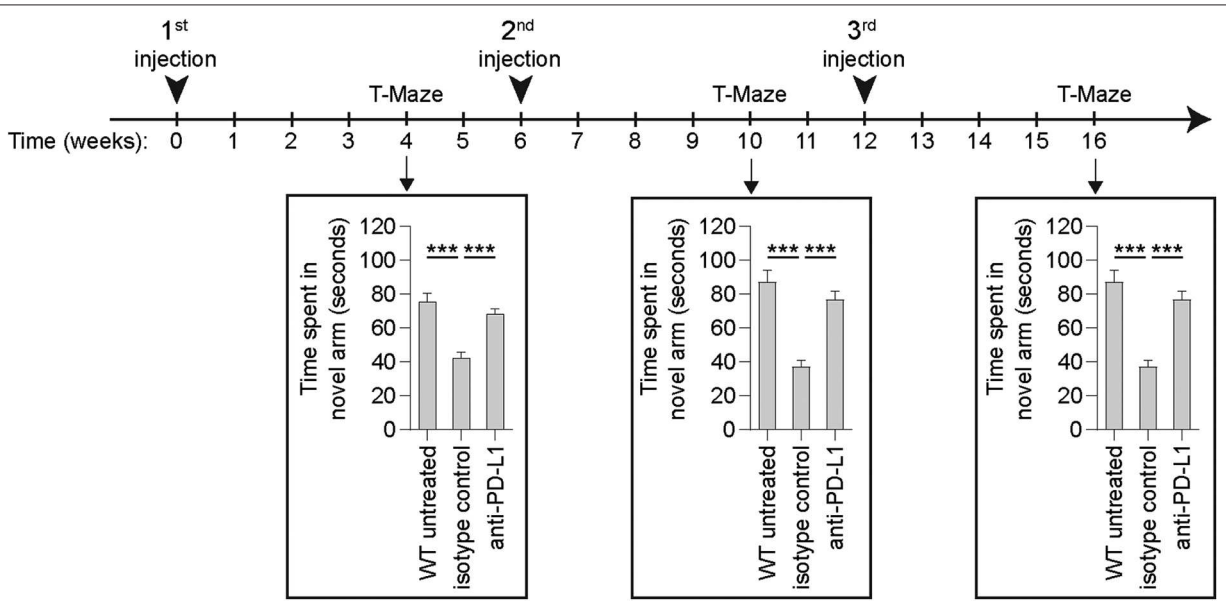

FIGURE 1 | Longitudinal assessment of cognitive performance of DM-hTAU mice following anti-PD-L1 intermittent treatment regimen. Male and female DM-hTAU mice at the age of 6–7 months were treated by intraperitoneal injection of either 1.5 mg/mouse of anti-PD-L1 antibody, or 1.5 mg/mouse isotype control antibody, once every 6 weeks. Untreated age-matched wild-type (WT) mice were used as an additional control group. Using the same protocols described in Rosenzweig et al. (2019), mice were evaluated for the effect on cognitive performance using the T-maze task, 4 weeks after each injection. Preference to spend time in the novel arm of the maze is a measure of short-term spatial memory. $n = 54$ DM-hTAU mice and $n = 13$ WT mice for the T-maze at 4 weeks from treatment initiation; $n = 48$ DM-hTAU mice and $n = 21$ WT mice for the T-maze at 10 weeks from treatment initiation; and $n = 30$ DM-hTAU mice and $n = 15$ WT mice for the T-maze at 16 weeks from treatment initiation. One-way ANOVA followed by Fisher's *post-hoc* test. Error bars represent mean ± s.e.m.; $^{***}P < 0.001$ vs. indicated groups. Mice were sacrificed along study progression for additional measurements, not presented here [ImmunoBrain Checkpoint Ltd.].

While amyloid and tau antibodies are designed to directly dampen the pathology within the brain, PD-1/PD-L1 antibodies are targeting immune cells outside the brain. Thus, PD-1/PD-L1 blockade in mouse models of Alzheimer's disease initiates a chain of immunological events that start in the periphery and culminate within the brain's territory; beginning with the antibody recognizing its cellular targets in the periphery and transiently breaking immune tolerance, and this is followed by migration of specialized immune cell populations from the circulation to the brain (thoroughly described in: Baruch et al., 2016; Schwartz, 2017; Rosenzweig et al., 2019). Immune cells (primarily of myeloid origin) that are recruited to the brain, act by enhancing clearance of toxic elements, improving neuronal function and reducing inflammation. This central effect, within the brain's territory, does not require the presence of the PD-1/PD-L1 antibody, which by that time has been cleared from the circulation. Thus, as opposed to the concept of maintaining continuous exposure with amyloid/tau antibodies for chronic effect on brain pathology, for immune checkpoint blockade, injections should be given intermittently to maintain a chronic beneficial effect. Indeed, in Rosenzweig et al., it was stated that "*…the beneficial effect of the immunotherapy for AD and dementia does not require continuous exposure to the antibody, and that the effect is mechanistically different from that underlying the current anti-PD-L1 treatment used in cancer therapy*" (Rosenzweig et al., 2019).

In summary, Lin et al. performed an experiment missing key appropriate control groups, using a cohort of aged "shifted" transgenic mice, which exhibit a clear motor deficit, and for which no behavioral or pathological data are available. The anti-PD-1-based therapy was used in a regimen that lacks scientific basis, and contradicts the previously available literature describing the dynamics of the therapy. These deficiencies preclude reaching any conclusion from this work, and as such only contribute to the confusion in the field.

ETHICS STATEMENT

Animal experiments detailed herein complied with the regulations formulated by the Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute of Science, Israel.

AUTHOR CONTRIBUTIONS

KB and EY conceived and wrote this commentary.

REFERENCES

Asuni, A. A., Boutajangout, A., Quartermain, D., and Sigurdsson, E. M. (2007). Immunotherapy targeting pathological tau conformers in a tangle mouse model reduces brain pathology with associated functional improvements. *J. Neurosci.* 27, 9115–9129. doi: 10.1523/JNEUROSCI.2361-07.2007

Baruch, K., Deczkowska, A., Rosenzweig, N., Tsitsou-Kampeli, A., Sharif, A. M., Matcovitch-Natan, O., et al. (2016). PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. *Nat. Med.* 22, 135–137. doi: 10.1038/nm.4022

Boutajangout, A., Ingadottir, J., Davies, P., and Sigurdsson, E. M. (2011). Passive immunization targeting pathological phospho-tau protein in a mouse model reduces functional decline and clears tau aggregates from the brain. *J. Neurochem.* 118, 658–667. doi: 10.1111/j.1471-4159.2011.07337.x

Lin, Y., Rajamohamedsait, H. B., Sandusky-Beltran, L. A., Gamallo-Lana, B., Mar, A., and Sigurdsson, E. M. (2020). Chronic PD-1 checkpoint blockade does not affect cognition or promote tau clearance in a tauopathy mouse model. *Front. Aging Neurosci.* 11:377. doi: 10.3389/fnagi.2019.00377

Rosenzweig, N., Dvir-Szternfeld, R., Tsitsou-Kampeli, A., Keren-Shaul, H., Ben-Yehuda, H., Weill-Raynal, P., et al. (2019). PD-1/PD-L1 checkpoint blockade harnesses monocyte-derived macrophages to combat cognitive impairment in a tauopathy mouse model. *Nat. Commun.* 10:465. doi: 10.1038/s41467-019-08352-5

Schwartz, M. (2017). Can immunotherapy treat neurodegeneration? *Science* 357, 254–255. doi: 10.1126/science.aai8231

Conflict of Interest: KB and EY work at ImmunoBrain Checkpoint Ltd., on the development of PD-1/PD-L1 immune checkpoint blockade approach for Alzheimer's disease. KB is an inventor of intellectual property licensed by ImmunoBrain Checkpoint Ltd.

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GENERAL COMMENTARY
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Response: Commentary: Chronic PD-1 Checkpoint Blockade Does Not Affect Cognition or Promote Tau Clearance in a Tauopathy Mouse Model

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Keywords: tau, Alzheimer's disease, tauopathy, PD-1 blockade, antibody, therapy, mouse models, behavior

A Commentary on

Commentary: Chronic PD-1 Checkpoint Blockade Does Not Affect Cognition or Promote Tau Clearance in a Tauopathy Mouse Model
by Baruch, K., and Yoles, E. (2020). Front. Aging Neurosci. 12:135. doi: 10.3389/fnagi.2020.00135

In a recent commentary on our article in Frontiers in Aging Neuroscience (Lin et al., 2020), Baruch and Yoles attempt to highlight perceived critical issues with our study and suggest that our scientific practice and conclusions are poor or misleading (Baruch and Yoles, 2020). They proclaim four main areas of concern—control groups, phenotype "shift," presumptive motor impairment and antibody dosing regimen—which they argue obscure our conclusions. Here we respond to each area in turn in order to clarify the commenters' many misinterpretations and to encourage thoughtful and unbiased criticism of our and others' work.

The main purpose of our study was to assess treatment efficacy of PD-1 antibodies in tauopathy. We thus compared the phenotype of JNPL3 tauopathy mice randomized to anti-PD-1 antibody and IgG control groups. We agree that including age-matched wild-type mice would have provided a useful reference for the magnitude of any deficits or improvements. However, this would not change the clear fact that we observed no significant differences between anti-PD-1 antibody- and IgG control-treated JNPL3 groups in cognition or related tauopathy markers. With regard to a positive control group, as our main objective was not to show superiority of a particular treatment, concurrent testing of other reference compounds was beyond the scope of our study. Nonetheless, we did observe a significant behavioral effect of PD-1 blockade on a control measure—an increase in locomotor activity in an open field. This effect on locomotion may reveal an important caveat regarding therapeutic target specificity of PD-1 blockade (see below). Importantly, it further emphasizes our consistent lack of anti PD-1 antibody effects across a range of cognitive tests.

We indicated in our manuscript that the functional phenotype of our JNPL3 mouse colony currently appears considerably later than we originally described at earlier ages when we first started using this model many years ago (Asuni et al., 2007; Boutajangout et al., 2011). Such gradual shifts in phenotype have been observed in many transgenic Alzheimer's disease related mouse models with Aβ and/or tau pathologies in the over 20 years since their earliest description (Jankowsky and Zheng, 2017; Buck et al., 2018; Gotz et al., 2018; Hyman and Tanzi, 2019). With this in mind, and as we clearly outlined in the Discussion section of our article, we intentionally chose

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middle-aged animals to better relate to the prior work of Rosenzweig et al. (2019), starting the anti-PD-1 antibody treatment when the tauopathy mice have moderate to severe tau pathology. Indeed, the extent of tau pathology in our JNPL3 mice appears roughly comparable to their DM-hTAU model animals which, at their age treated, were described to "show pronounced cognitive deficits" (Rosenzweig et al., 2019). Although these models differ in tau mutations and background strains, experimental therapies should ideally be replicable across models (Latta-Mahieu, 2018). It is also notable that, in an earlier article, the authors emphasized that the therapy works at an advanced stage of pathology in Aβ plaque mice (Baruch et al., 2016). The commenters further pointed out that 6 of 22 mice died during the course of our study to argue that they were severely impaired. Deterioration and/or sudden death are rather common phenomena across transgenic Alzheimer's models, particularly with advancing age, and does not necessarily relate to motor deficits across the entire cohort. For example, although not clearly specified by the authors, a minimum of 12 of 67 animals were excluded from behavioral analysis due to severe motor impairments in the Rosenzweig et al. (2019) study (see page 4, difference in number of mice in Suppl Figure 3, $n = 67$ vs. Figure 2C, $n = 55$). No count of the number of excluded mice were provided in (Baruch et al., 2016).

Consistent with our observed phenotype shift, we categorically did not observe any gross or obvious motor impairments in our JNPL3 mice. We stated this observation explicitly in our article, and further confirmed and quantified locomotor activity using two sensorimotor behavioral tests as well as across five cognitive tests that require varying degrees of locomotion. The commenters highlight the fact that the mean distance traveled in the open field by the JNPL3 mice in our article (Lin et al., 2020) was approximately half of that observed in a prior publication (Boutajangout et al., 2011), and suggest that this indicates gross motor deficits. Later, they conversely argue that historical controls are meaningless in behavioral measures. What they neglect to mention are some of the key factors that likely contribute to this discrepancy, independent of motor impairments, including open field size and the age of the animals. It is well-known that mice tend to explore less both within a smaller arena (e.g., 56 cm diameter in the current vs. 70 cm diameter in the prior study) and at older ages (13–14 months in the current vs. 5–6 months in the prior study). For reference, a large-scale behavioral study of normal C57Bl/6J mice demonstrated an age-dependent reduction in locomotion within a 40 x 40 cm open field—with mean distance measures comparable and lower than we observed for JNPL3 in our testing conditions (Shoji et al., 2016; Lin et al., 2020). We have also previously found that other transgenic mouse models without motor impairments, such as htau mice at 11–12 months of age, travel 1,500–3,000 cm over an initial 15-min open field test (Congdon et al., 2016). Moreover, we transparently report the locomotor distances in our cognitive tests, such as the Barnes maze, where it is evident that JNPL3 mice typically travel further to reach the target and make more commission errors than wild-type reference controls, despite similar average velocities (Shoji et al., 2016). Finally, we also included fear conditioning in our cognitive test battery, a paradigm in which the expression of learning does not depend on locomotion, and found no significant impact of PD-1 blockade, consistent with our other cognitive assays.

Apart from the substantial evidence described above, it is unclear to us how anyone would argue that an 8 cm mouse traveling 30 m in 15 min (2 m/min) has a gross motor deficit that would preclude interpretation of our results. It is interesting to note that Baruch et al. (2016) and Rosenzweig et al. (2019) do not include any quantitative motor control tests in their studies despite acknowledging "animals that showed motor deficits were excluded from the behavioral analyses." Moreover, cognitive performance on their primary cognitive task—a radial arm water maze—requires extreme motor capacity (e.g., swimming), yet no distance measures or trial omission errors are reported. Without proper motor controls or complete and transparent reporting of test results, their behavioral data are not easily interpretable, as enhancements in motor function might incorrectly be attributed to improvements in cognition. Given that our only significant effect—using the same anti PD-1 antibody as in their studies—was an increase in locomotor activity, a potential parsimonious explanation may be that PD-1 blockade acts primarily, or most effectively, to improve motor rather than cognitive function. We encourage future studies to include the appropriate motor controls and measures to avoid potential confusion in the field.

The commenters suggest that our experimental design, namely a weekly dosing schedule as opposed to the singular or intermittent dosing schedule previously reported (Baruch et al., 2016; Rosenzweig et al., 2019), may have somehow prevented us from observing a protection from cognitive impairment in our tauopathy mouse model. There is no evidence or scientific basis for such a claim. Various doses of anti PD-1 antibody were used in prior studies, with similar benefits shown with high vs. moderate antibody dose (Rosenzweig et al., 2019). The high dose, 1.5 mg/mouse, was also presented in the commenters' new data in Figure 1 (Baruch and Yoles, 2020; administered every 6 weeks). In addition, we noted that in the Baruch et al. (2016) study, the authors emphasized that "repeated treatment sessions are needed to maintain the beneficial effects on cognition and memory" and for "maintaining a long lasting beneficial effect on disease pathology" (Baruch et al., 2016). We thus specifically chose our dose to be 10 mg/kg, or about 1/5 of the aforementioned highest dose [assuming an average mouse weight of 30 g (1.5 mg/30 g = 50 mg/kg)]. Administering the dose per weight instead of the same dose for each animal should reduce variance in antibody levels between animals, whose weight typically varies by up to 20%. We did not find any information in previous articles regarding the half-life of this, or related anti PD-1 antibodies, but we are aware that the half-life of exogenous antibodies is on average about 2 weeks. Therefore, average circulating antibody levels are likely to be comparable in the Baruch et al. (2016) and Rosenzweig et al. (2019) studies and our report. Moreover, considering the typical half-life of exogenous antibodies, the short anti-PD-1 or anti-PD-L1 treatments used in these previous studies are likely to have resulted in continuous antibody exposure. Both higher doses administered at longer intervals and lower doses given at shorter intervals can lead

to similar average circulating antibody levels. It is thus entirely unclear if the former approach has any particular benefits for treating neurodegenerative diseases.

In summary, we highlight the evidence, apparently overlooked or unappreciated by the commenters, showing that our experimental design was appropriate for testing our hypothesis, the age of our model was carefully selected based on tauopathy severity, and the mice had no gross motor deficits across multiple quantitative measures. Our dosing regimen was also chosen rationally, based on all existing information, and with no evidence that it is functionally distinct from earlier studies. There is absolutely no confusion that, in our model and experimental conditions, PD-1 checkpoint blockade does not significantly affect cognition or promote tau clearance. Hence, we stand by the title of the article. We wish to emphasize that we do not interpret our results as discrediting or invalidating any prior findings, but to add to existing scientific knowledge. We only suggest, as we did in our article, that further research in this area is warranted.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Asuni, A. A., Boutajangout, A., Quartermain, D., and Sigurdsson, E. M. (2007). Immunotherapy targeting pathological tau conformers in a tangle mouse model reduces brain pathology with associated functional improvements. *J. Neurosci.* 27, 9115–9129. doi: 10.1523/JNEUROSCI.2361-07.2007

Baruch, K., Deczkowska, A., Rosenzweig, N., Tsitsou-Kampeli, A., Sharif, A. M., Matcovitch-Natan, O., et al. (2016). PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. *Nat. Med.* 22, 135–137. doi: 10.1038/nm.4022

Baruch, K., and Yoles, E. (2020). Commentary: chronic PD-1 checkpoint blockade does not affect cognition or promote tau clearance in a tauopathy mouse model. *Front. Aging Neurosci.* 12:135. doi: 10.3389/fnagi.2020.00135

Boutajangout, A., Ingadottir, J., Davies, P., and Sigurdsson, E. M. (2011). Passive immunization targeting pathological phospho-tau protein in a mouse model reduces functional decline and clears tau aggregates from the brain. *J. Neurochem.* 118, 658–667. doi: 10.1111/j.1471-4159.2011.07337.x

Buck, K., Jahn, T. R., and Gasparini, L. (2018). "Experimental models of tau aggregation," in *Conn's Handbook of Models for Human Aging*, eds J. L. Ram and P. M. Conn (Cambridge, MA: Elsevier), 953–973. doi: 10.1016/B978-0-12-811353-0.00070-1

Congdon, E. E., Lin, Y., Rajamohamedsait, H. B., Shamir, D. B., Krishnaswamy, S., Rajamohamedsait, W. J., et al. (2016). Affinity of Tau antibodies for solubilized pathological Tau species but not their immunogen or insoluble Tau aggregates predicts in vivo and ex vivo efficacy. *Mol. Neurodegener.* 11, 62–85. doi: 10.1186/s13024-016-0126-z

Gotz, J., Bodea, L. G., and Goedert, M. (2018). Rodent models for Alzheimer disease. *Nat. Rev. Neurosci.* 19, 583–598. doi: 10.1038/s41583-018-0054-8

Hyman, B., and Tanzi, R. E. (2019). Effects of species-specific genetics on Alzheimer's mouse models. *Neuron* 101, 351–352. doi: 10.1016/j.neuron.2019.01.021

Jankowsky, J. L., and Zheng, H. (2017). Practical considerations for choosing a mouse model of Alzheimer's disease. *Mol. Neurodegener.* 12:89. doi: 10.1186/s13024-017-0231-7

Latta-Mahieu, M., et al. (2018). Systemic immune-checkpoint blockade with anti-PD1 antibodies does not alter cerebral amyloid-beta burden in several amyloid transgenic mouse models. *Glia* 66:492–504. doi: 10.1002/glia.23260

Lin, Y., Rajamohamedsait, H. B., Sandusky-Beltran, L. A., Gamallo-Lana, B., Mar, A., and Sigurdsson, E. M. (2020). Chronic PD-1 checkpoint blockade does not affect cognition or promote tau clearance in a tauopathy mouse model. *Front. Aging Neurosci.* 11:377. doi: 10.3389/fnagi.2019.00377

Rosenzweig, N., Dvir-Szternfeld, R., Tsitsou-Kampeli, A., Keren-Shaul, H., Ben-Yehuda, H., Weill-Raynal, P., et al. (2019). PD-1/PD-L1 checkpoint blockade harnesses monocyte-derived macrophages to combat cognitive impairment in a tauopathy mouse model. *Nat. Commun.* 10:465. doi: 10.1038/s41467-019-08352-5

Shoji, H., Takao, K., Hattori, S., and Miyakawa, T. (2016). Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. *Mol. Brain* 9:11. doi: 10.1186/s13041-016-0191-9

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REVIEW
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Astrocyte Senescence and Alzheimer's Disease: A Review

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Astrocytes are the largest group of glial cells in the brain and participate in several essential functions of the central nervous system (CNS). Disruption of their normal physiological function can lead to metabolism disequilibrium and the pathology of CNS. As an important mechanism of aging, cellular senescence has been considered as a primary inducing factor of age-associated neurodegenerative disorders. Senescent astrocytes showed decreased normal physiological function and increased secretion of senescence-associated secretory phenotype (SASP) factors, which contribute to Aβ accumulation, tau hyperphosphorylation, and the deposition of neurofibrillary tangles (NFTs) in Alzheimer's disease (AD). Astrocyte senescence also leads to a number of detrimental effects, including induced glutamate excitotoxicity, impaired synaptic plasticity, neural stem cell loss, and blood–brain barrier (BBB) dysfunction. In this review article, we have summarized the growing findings regarding astrocyte senescence and its putative role in the pathologic progress of AD. Additionally, we also focus on the significance of targeting astrocyte senescence as a novel and feasible therapeutic approach for AD.

Keywords: Alzheimer's disease, astrocytes, senescence, senescence-associated secretory phenotype, senolytic drugs, tau aggregate

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INTRODUCTION

Alzheimer's disease (AD) is a chronic degenerative disorder of the brain related to progressive decline of memory and cognition (McGeer and McGeer, 2007; Long and Holtzman, 2019). The disease is characterized by brain atrophy, extracellular accumulation of beta-amyloid peptide (Aβ) (Glenner and Wong, 1984; Hsiao et al., 1996), neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein (Goedert et al., 2006), and loss of synapses and dysfunctions of neurotransmission (DeKosky and Scheff, 1990; Rajmohan and Reddy, 2017), as well as neuroinflammation (Heneka et al., 2015).

Many of the cellular pathologies of AD present on neurons, such as neuronal extracellular deposits of Aβ, intracellular deposition of neurofibrillary tangles (NFTs), and Lewy bodies (Hansen et al., 1990; Hardy and Higgins, 1992; Goedert et al., 2006). These classical pathologies are still central to diagnosing AD. However, although neurons have significant correlations with AD, other cell types and factors in the brain may also contribute to cognitive decline during AD. Additionally, astrocytes are the major glial cells and are vital for the normal physiological functions of the central nervous system (CNS) (Sofroniew and Vinters, 2010; Matias et al., 2019). They perform critical roles in regulation of homeostasis and metabolism of the neurons, mediating uptake and recycling

of neurotransmitters. Astrocytes also play a key role in maintenance of the blood–brain barrier (BBB). They also act as modulators of synaptic plasticity and transmission, supporting the view that astrocytes play an integral role in the initiation and progression of cognitive decline and AD (Acosta et al., 2017; Palmer and Ousman, 2018; Munger et al., 2019).

Aging is considered the most significant risk factor for the occurrence and development of AD (Kritsilis et al., 2018). The incidence of AD has been shown to increase with advancing age and cellular senescence (Baker et al., 2011). Studies regarding to the link and role of senescence in age-related diseases have become increasingly common, and are gradually becoming a new research area (Bhat et al., 2012; Baker and Petersen, 2018). Transcriptome analysis of AD and the aged human brain showed neurons and other non-neuronal CNS cell types including astrocytes, microglia, and oligodendrocytes displayed senescence-associated phenotypes (Salminen et al., 2011; Bhat et al., 2012; Boccardi et al., 2015; Boisvert et al., 2018; Bussian et al., 2018; Zhang P. et al., 2019). Although studies regarding astrocyte senescence during AD and the effects of senescent astrocytes in AD progression have recently increased, an understanding of its precise mechanisms is still lacking. Here, we review our current view on the role of astrocyte senescence in the pathogenesis of AD.

ASTROCYTES

Astrocytes are the most abundant type of glial cells in the brain and are classified into two main groups: fibrous astrocytes and protoplasmic astrocytes (Sofroniew and Vinters, 2010). Fibrous astrocytes are characterized by the presence of numerous fibrils in their cytoplasm and are distributed mainly in the white matter. Protoplasmic astrocytes are typically located in the gray matter and have thick, short, highly branched processes with fewer fibrils (Sofroniew and Vinters, 2010). Despite heterogeneity of astrocytic subtype, these glia are responsible for multifarious complex and essential functions for CNS physiology, including the provision of nutrients to the neuron, regulation of synaptic plasticity, releasing transmitters (called gliatransmitters) in a Ca^{2+}-dependent manner, supporting the blood–brain barrier (BBB), and maintaining the extracellular ion balance (Hussaini and Jang, 2018; Verkhratsky and Nedergaard, 2018).

Astrocytes have been shown to become activated in response to various stimuli and diseases of the CNS. One common and classical feature of reactive astrocytes is the release of a variety of effector molecules including chemokines, cytokines, and proteases (Liddelow et al., 2017; Ahmad et al., 2019). Interestingly, these factors overlap with the secretions of senescent astrocytes (Boisvert et al., 2018). The proteins associated with astrocyte activation, glial fibrillary acidic protein (GFAP) and vimentin, increased during aging (Porchet et al., 2003). Also, senescent astrocytes share many of the similar phenotypes with A1-like reactive astrocytes, including cellular morphological change and proinflammation secretions. It is possible that many previous studies, which focused on reactive astrocytes, may have been focusing on senescent astrocytes. Although Cohen et al. reviewed the different features between reactive and senescent astrocytes, it is necessary to elucidate the characteristics of astrocyte senescence further (Cohen and Torres, 2019). In the next section, we will describe the phenotypes of senescent astrocytes in more detail.

ASTROCYTE SENESCENCE: ASTROSENESCENCE

According to Cohen et al., astrocytes can initiate a senescence program similar to that of other cell types in response to various stressors, termed "astrosenescence" (Cohen and Torres, 2019). Ponten and Macintyre (1968) compared the characteristics of glial cells isolated from malignant and benign tissue to examine the effect of long-term culturing on CNS cells. This study indicated that primary cells from normal tissue had a limited lifetime and stopped dividing after a set number of passages. This might be the first study to examine replicative senescence in glia cells. With time, numerous studies demonstrated that after exhausted replication, oxidative stress, proteasome inhibition, high glucose, or HIV infection, astrocytes show changes in several classical hallmarks of cellular senescence.

Primary astrocytes isolated from the cerebral cortex that underwent replicative senescence displayed a series of established markers of cellular senescence including a growth arrest, increased expression of senescence-associated genes *p53* and $p21^{WAF1}$, and increased senescence-associated β-galactosidase (SA-β-Gal) activity (Evans et al., 2003; Blasko et al., 2004; Pertusa et al., 2007). Astrocytes have shown to undergo stress-induced premature senescence as well. For example, after ionizing radiation or H_2O_2 or proteasome inhibitor treatment, both human and mouse astrocytes displayed classical senescence features, such as decreased proliferation, increased SA-β-Gal activity, and the upexpression of *p53*, $p21^{WAF1}$, and $p16^{INK4A}$ (Bitto et al., 2010; Turnquist et al., 2019). Notably, astrocytes were found to be more sensitive to senescence-inducing stimuli than fibroblasts (Gorg et al., 2015, 2018). Also, Aβ oligomers can induce cellular senescence and promote production of senescence-associated secretory phenotypes (SASPs) in human astrocytes (Mombach et al., 2015). Furthermore, human astrocytes infected with HIV showed signs of DNA damage and premature senescence (Cohen et al., 2017). Therefore, astrocytes have been shown to undergo cellular senescence *in vitro* and *in vivo* due to various stimuli and factors.

CHARACTERISTICS OF ASTROCYTE SENESCENCE

Cellular senescence is a catchall for a set of states in which cells stop dividing and then exhibit a multitude of cellular and molecular changes. Evidence suggests that there is a significant variation in the senescent phenotype that is dependent on both cell type and triggering insults (Gorgoulis et al., 2019). Senescent astrocytes exhibit both classic characteristics as well as other cell types and also demonstrated particular phenotypes (Bhat et al.,

2012; Buhlman, 2017; Baker and Petersen, 2018). Key features of senescent astrocytes include the following: permanent cell cycle arrest, altered morphology, increased GFAP and vimentin, chromatin alterations and formation of senescence-associated heterochromatic foci (SAHFs), upexpression of high-mobility group B (HMGB) proteins, reduced expression of nuclear lamina protein laminB1, downregulation of neurotrophic growth factors, and upregulation of SASP factors as well as SA-β-Gal (Bitto et al., 2010; Boccardi et al., 2015; Chinta et al., 2015; Boisvert et al., 2018; Turnquist et al., 2019; **Figure 1**).

Cell Arrest and GFAP

Typically, senescent astrocytes exhibit permanent cell cycle arrest as well as other cell types, which is thought to be regulated by the p53/p21^{WAF1} and p16^{INK4A}/pRB pathway (Evans et al., 2003; Bitto et al., 2010; Turnquist et al., 2019). p21^{WAF1} is namely the CIP/KIP (CDK interacting protein/kinase inhibitory protein) that is capable of inhibiting CDK2, but paradoxically, it is also necessary for cell cycle progression (Hernandez-Segura et al., 2018). In the context of astrocyte senescence, p53 upregulates the expression of p21^{WAF1}, which inhibits cyclin D–dependent kinase CDK2 activity and the initial cell cycle arrest. Interestingly, this p53-dependent stable proliferative arrest was independent of telomere erosion in human astrocyte *in vitro* study (Evans et al., 2003). p16^{INK4A} is a member of the INK4A family that mediates permanent cell cycle arrest by inhibiting CDK4 and CDK6, which leads to retinoblastoma protein (RB) hypophosphorylation, blocking cell cycle entry to the S phase (Hernandez-Segura et al., 2018). Importantly, as its expression increased in the brain with time, p16^{INK4A} is also a biomarker of natural brain aging (Berchtold et al., 2008; Baker and Petersen, 2018).

Additionally, GFAP is a class III intermediate filament protein, which is the most widely used marker for astrocytes (Eun et al., 2016). In the human brain, the level of GFAP was significantly increased in the hippocampus in people over 65 years of age. GFAP upexpression has been the general change observed in astrocyte senescence *in vitro* and *in vivo* (Nichols et al., 1993; Boisvert et al., 2018; Lye et al., 2019). Larsson et al. have demonstrated that cell proliferation in the granular layer of the dentate gyrus is increased after knockout GFAP/vimentin

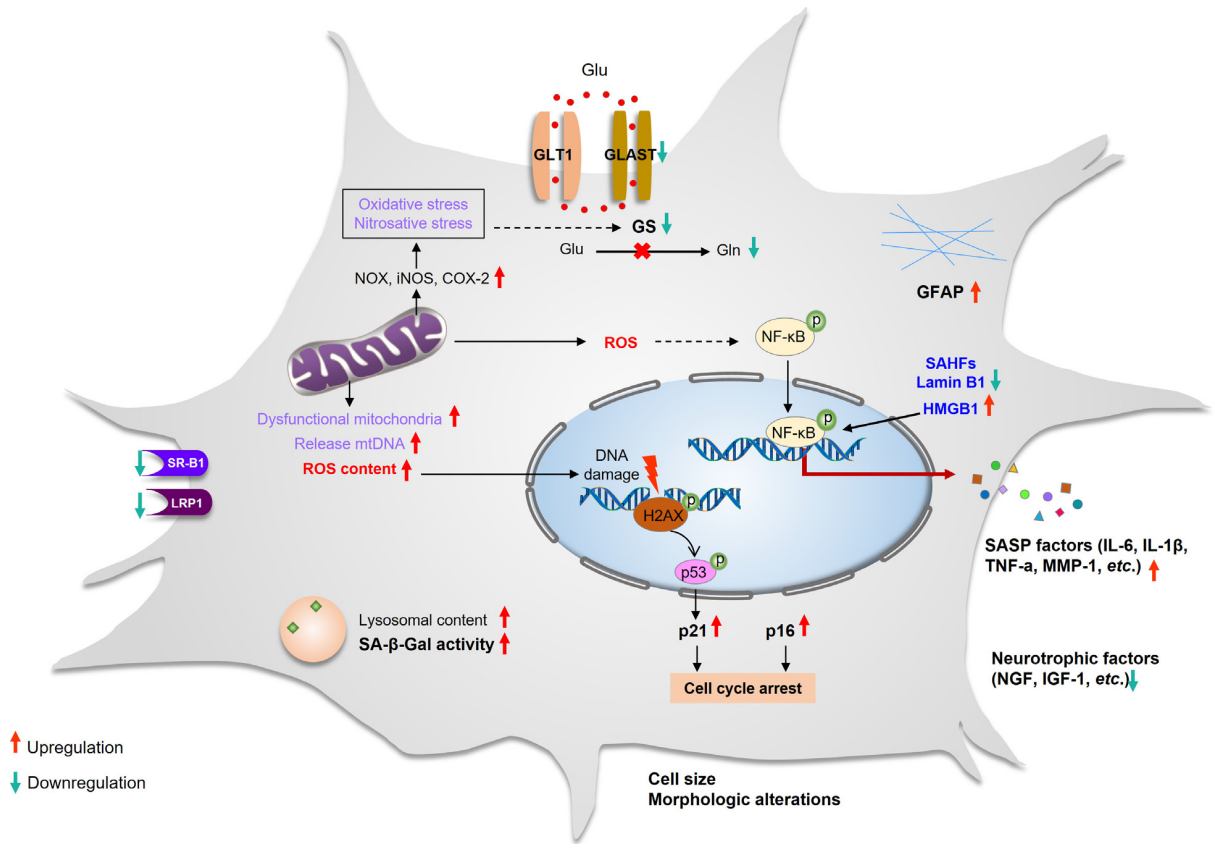

FIGURE 1 | Characteristics of astrocyte senescence. Senescent astrocytes that have undergone cell cycle arrest and have enlarged sharply. The expression of GFAP increased, whereas the expression of glutamate transporters (GLAST and GS) and SR-B1 and LRP1 decreased. The lysosomal content has increased, and the lysosomes have high β-galactosidase activity and dysfunctional mitochondria that produce high levels of ROS and release mtDNA. They have DNA damage and SAHFs, and their nuclear integrity is compromised due to the loss of laminB1. The elevated ROS activates the NF-κB pathway and promotes SASP production. The secretion of neurotrophic factors is decreased. GFAP, glial fibrillary acidic protein; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter-1; GS, glutamine synthetase; iNOS, inducible nitric oxide synthase; LRP1, lipoprotein receptor–related protein 1; NOX, NADPH oxidase; ROS, reactive oxygen species; SAHFs, senescence-associated heterochromatic foci; SASP, senescence-associated secretory phenotype; SR-B1, scavenger receptor B1.

(Larsson et al., 2004). Several studies have demonstrated that the expression of GFAP appears to increase with aging in rodents and human astrocytes (Iram et al., 2016), although activated astrocytes also involve the upregulation expression of GFAP (Liddelow et al., 2017). This implies that astrocyte senescence associated with an increase in GFAP expression, which results in the upregulation of GFAP, may be a new biomarker of astrocyte senescence (**Figure 1**).

Nuclear Changes

In senescent cells, chromatin alterations and remodeling including formation of SAHFs and nuclear DNA enriched for histones modification were stained densely by DAPI (Kosar et al., 2011). These nuclear changes have been associated with attenuated expression of proliferation-promoting genes, which leads to irreversible cell cycle arrest of senescence (Hernandez-Segura et al., 2018; Gorgoulis et al., 2019). Unlike γH2AX, which is a ubiquitously expressed DNA damage response (DDR) marker, the formation of SAHFs in senescent cells varies between cell types (Seoane et al., 2017; Gorgoulis et al., 2019). Studies have shown that cultured human astrocytes displayed both increased levels of γH2AX and formation of SAHFs (Myung et al., 2008; Bitto et al., 2010; Souza et al., 2015; Seoane et al., 2017; **Figure 1**). Senescent astrocytes showed nuclear enlargement, which is generally present in specific cell types such as fibroblasts and CNS cells (Yoon et al., 2016; Bang et al., 2019). When DNA damage and DNA repair occur during the cell cycle, the size of the cell and nucleus is slightly increased (Amodeo and Skotheim, 2016). It seems that the enlargement of the nucleus in senescent cells may be caused by DNA damage and cell cycle arrest.

Senescent astrocytes also display changes in nuclear morphology and integrity of the nuclear envelope owing to the downregulation of nuclear lamina proteins, such as lamin B1 (Freund et al., 2012). Additionally, HMGB1 plays a crucial role in DDR and cellular inflammation (Enokido et al., 2008; Castiglioni et al., 2011), which is increased in astrocytes with aging (Enokido et al., 2008; **Figure 1**).

Senescence-Associated Secretory Phenotype

Oxidative stress-induced and cultured senescent astrocytes cause several transcriptomic changes. More specifically, genes associated with proinflammation cytokines, such as interleukin (IL)-6, IL-8, chemokines, and proteinases were upregulated (Crowe et al., 2016; Hou et al., 2018). These proinflammation factors are termed the SASPs, which are considered to be a downstream consequence of the DDR (Salminen et al., 2011; Seoane et al., 2017). Additionally, Aβ peptides and environmental toxins, such as ammonia and paraquat, have also been shown to induce senescence in cultured astrocytes with increased production of SASP. SASP is highly heterogeneous in senescent astrocytes that are induced by different stimuli (Bhat et al., 2012; Crowe et al., 2016; Clarke et al., 2018; Hou et al., 2018). However, the high expression of IL-6 is a more common feature in astrocyte senescence (Blasko et al., 2004; Hou et al., 2017).

Several studies indicated that SASP-related genes upregulated in senescent astrocytes is mediated by the p38/MAPK and NF-κB pathway (Chien et al., 2011; Salminen et al., 2011; Mombach et al., 2015). Bhat et al. reported that inhibition of p38/MAPK activity both in presenescent and senescent human astrocytes suppress the increased level of SASP factors (Bhat et al., 2012). Following the hypothesis that DNA damage is an essential driver of SASP, HMGB1 may be another important regulator. In the brain, HMGB1 is upexpressed in astrocytes during aging (Enokido et al., 2008). Davalos et al. reported that HMGB1 could increase NF-κB transactivation efficiency through interaction with NF-κB complexes and therefore augment and strengthen the inflammatory reaction of the SASP (Qiu et al., 2010; Davalos et al., 2013). Recently, the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon (IFN) genes (STING) pathway, NOTCH signaling, and mammalian target of rapamycin (mTOR) signaling have also been shown to regulate SASP in fibroblast and other cell types (Herranz et al., 2015; Capell et al., 2016; Hoare et al., 2016; Loo et al., 2019). However, the mechanisms involved in CNS cells have not been extensively studied. SASPs have been shown to operate as autocrine and paracrine signals to reinforce the senescent state and induce senescence or degenerative changes of the surrounding bystander cells (Coppe et al., 2010; Acosta et al., 2013). These observations indicate that SASPs can generate a low-level, chronic inflammation and an age-dependent detrimental cycle to strengthen the senescence state and enhance age-related neurodegenerative disorders.

Lysosomal and Mitochondrial Dysfunction

The upregulation of lysosomal proteins and increased lysosomal content is the main characteristic of cellular senescence (Lee et al., 2006). Enhanced lysosomal content can be detected by measuring the activity of the lysosomal enzyme senescence-associated beta-galactosidase (SA-β-Gal), which is measured at pH 6.0 using in situ staining with the chromogenic substrate X-gal (Kurz et al., 2000; Lee et al., 2006). Therefore, SA-β-Gal is used as the most common hallmark for detecting senescent cells. Senescent astrocytes showed elevated SA-β-Gal activity *in vitro* and *in vivo* (Evans et al., 2003; Bitto et al., 2010; Bhat et al., 2012; Cohen and Torres, 2019). Consistent with others, our previous research also demonstrated the accumulation of dysfunctional lysosomes in stress-induced premature senescence (SIPS) (Han et al., 2016; Tai et al., 2017).

It is known that the number of mitochondria increases during senescence. However, the membrane potential of mitochondria is decreased, leading to intensified ROS production and the release of mitochondrial DNA (mtDNA) (Passos et al., 2007). Meanwhile, elevated mtDNA in the cytoplasm can lead to STING-meditated SASP production (Li and Chen, 2018). Enhanced mitochondria content during senescence could be the result of the accumulation of dysfunctional mitochondria and reduced mitochondrial fission (Tai et al., 2017; Kim et al., 2018). In addition, impaired mitophagy is also likely to contribute to dysfunctional mitochondria accumulation in senescent cells (Fivenson et al., 2017; Vasileiou et al., 2019). Studies using either senescent astrocytes from aged rats or *in vitro* culturing showed that mitochondrial dysfunction and damage was associated with an increase in oxidative/nitrosative stress, RNA oxidation,

upregulation of ROS, and inducible nitric oxide synthase (iNOS) expression levels (**Figure 1**; Pertusa et al., 2007; Bellaver et al., 2017; Bang et al., 2019).

Glutamate Signaling Dysfunction

Due to the high expression of glutamate transporters, GLAST (human homologs, EAAT1) and GLT-1 (human homologs, EAAT2) (Zhang X. et al., 2019), astrocytes can further regulate neuronal function via the efficient uptake of the synaptically released excitatory and inhibitory neurotransmitters glutamate and γ-aminobutyrate (GABA). Glutamate is the primary excitatory neurotransmitter in the mammalian CNS and is critical for learning and memory (Verkhratsky and Kirchhoff, 2007). Nevertheless, excessive extracellular glutamate levels lead to neuronal death as a result of glutamate excitotoxicity (Danbolt, 2001). The released glutamate from neurons is mainly taken up into astrocytes by GLAST and GLT-1 and is then converted to glutamine by glutamine synthase (GS), which is expressed in astrocytes (Pajarillo et al., 2019; Zhang X. et al., 2019). The expression and activity of GS are age dependent. The protein level of GS has been found to decrease dramatically in aged astrocytes (Bellaver et al., 2017; Shi et al., 2017). In addition, the expression of the glutamate transporter GLAST displays an age-dependent decrease. However, there was an increase in the expression of GLT-1 in senescent astrocytes (Bellaver et al., 2017). Other research has shown only reduced expression and activity of GS, with no changes observed in the expression of GLAST andGLT-1 in senescent astrocytes (Boisvert et al., 2018). Additionally, the activity of GS is very sensitive to nitrosative and oxidative stress (Knorpp et al., 2006; Bellaver et al., 2017). Therefore, oxidative stress could decrease the ability of astrocytes to supply metabolic substrates to neurons and aggravate the impairment of GS activity (Crowe et al., 2016; Gonzalez-Reyes et al., 2017). It is likely that senescent astrocytes with reduced capacity for glutamate uptake and clearance contribute to glutamate excitotoxicity in neurodegenerative diseases.

Cholesterol Synthesis

Cholesterol is an essential metabolic substrate for the normal physiological functions of the brain, supporting neuronal homeostasis, synaptic integrity, and receptor function. In the CNS, cholesterol content is largely independent of dietary intake because of the existence of the BBB (Giudetti et al., 2016). Astrocytes are thought to be crucial in brain cholesterol synthesis and transport owing primarily to their expression of sterol regulatory element-binding protein 2 (SREBP2) and apoE (Ferris et al., 2017). SREBP2 is an important transcription factor that regulates the expression of HMG-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol synthesis (Kim et al., 2010). In senescent astrocytes, the expression of HMGCR and genes associated with cholesterol synthesis is significantly decreased, while the mRNA level of cholesterol transport-related genes is increased (Boisvert et al., 2018). It appears that there is an overall dysregulation of cholesterol metabolism in senescent astrocytes, and this may lead to decreased synaptic support.

FUNCTIONAL CONSEQUENCES OF SENESCENT ASTROCYTES IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disease causing cognitive, memory, and behavioral dysfunctions in older age. The pathophysiological mechanism of AD has been extensively studied for many years, which has led to several hypotheses, such as the Aβ, tau, cholinergic, and inflammation hypotheses (Hardy and Higgins, 1992; McGeer and McGeer, 2007; Hampel et al., 2018).

Numerous studies have shown that impaired astrocytes are involved in the initiation and progression of AD (Acosta et al., 2017; Gonzalez-Reyes et al., 2017; Matias et al., 2019). One most likely mechanism is astrocytic neuroinflammation (Benzing et al., 1999; Blasko et al., 2004). Also, a common feature of astrocyte senescence is the production of proinflammatory factors known as the SASP. In addition, studies have shown evidence that astrocytes surrounding Aβ plaques are positive for IL-6, a key SASP component (Bhat et al., 2012; Munger et al., 2019). All these data support the view that astrocyte senescence is a key and novel contributor to AD pathogenesis.

The first study to investigate the role of astrocyte senescence in AD involved human astrocytes treated with the $A\beta_{1-42}$ oligomer *in vitro* (Bhat et al., 2012). After treatment, astrocytes displayed classical phenotypes of cellular senescence including increased SA-β-Gal activity and increased $p16^{INK4A}$ expression. Most notably, not only was there an age-dependent increase in p16-positive astrocytes in the frontal cortex tissue, there was a further increase in senescent astrocytes in brain tissue from AD patients compared to non-diseased tissues from age-matched individuals (Bhat et al., 2012; Turnquist et al., 2016). Also, a recent study suggested that removal of senescent astrocytes and microglia by senolytic agents or genetic ablation prevents or inhibits NFTs formation and neurodegeneration in AD and tauopathy mouse models (Bussian et al., 2018; Mendelsohn and Larrick, 2018), suggesting that astrocyte senescence contributes to the pathogenesis of neurodegeneration in AD. In this part, we summarize the data that demonstrate the effects of senescent astrocytes in AD (**Figure 2**).

Aβ Accumulation

Inefficient Aβ clearance is detrimental in AD. Many studies have shown that astrocytes play an important role in Aβ clearance and degradation (Nicoll and Weller, 2003; Liu et al., 2017). Astrocyte receptors involved in uptake and clearance of Aβ are the low-density lipoprotein receptor-related protein 1 (LRP1) and scavenger receptor B1 (SR-B1) (Basak et al., 2012; Ries and Sastre, 2016). However, the mechanisms governing the receptor-mediated uptake of Aβ are not fully understood. Notably, in aged astrocytes, the expressions of LRP1 and SR-B1 are reduced (Iram et al., 2016), suggesting the ability of senescent astrocytes to uptake and degrade Aβ may be impaired.

In AD, senescent astrocytes are observed in regions surrounding Aβ plaque and the appearance of SA-β-Gal–positive

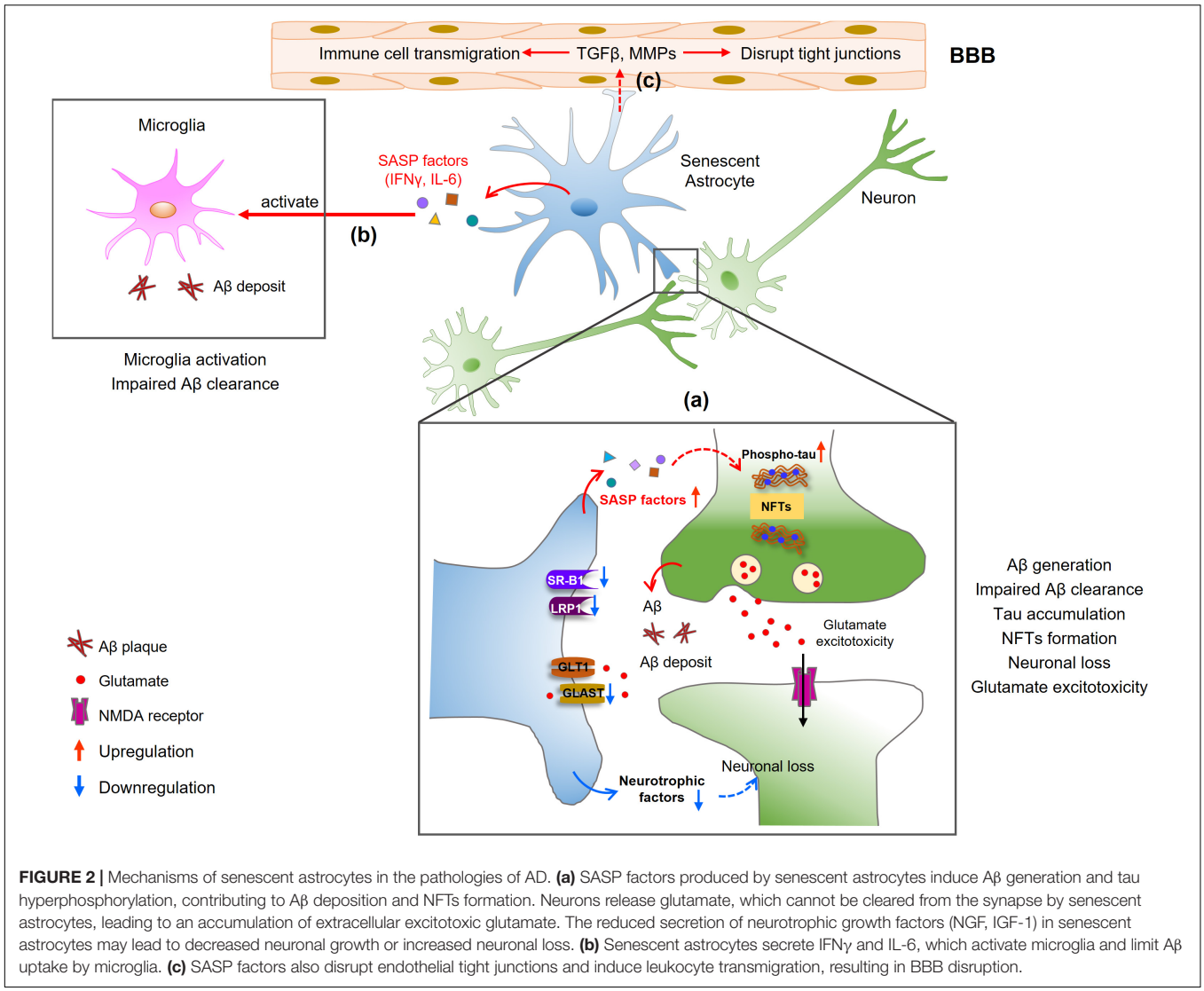

FIGURE 2 | Mechanisms of senescent astrocytes in the pathologies of AD. **(a)** SASP factors produced by senescent astrocytes induce Aβ generation and tau hyperphosphorylation, contributing to Aβ deposition and NFTs formation. Neurons release glutamate, which cannot be cleared from the synapse by senescent astrocytes, leading to an accumulation of extracellular excitotoxic glutamate. The reduced secretion of neurotrophic growth factors (NGF, IGF-1) in senescent astrocytes may lead to decreased neuronal growth or increased neuronal loss. **(b)** Senescent astrocytes secrete IFNγ and IL-6, which activate microglia and limit Aβ uptake by microglia. **(c)** SASP factors also disrupt endothelial tight junctions and induce leukocyte transmigration, resulting in BBB disruption.

astrocytes was earlier than Aβ accumulation (Bhat et al., 2012). The expression of BACE1 and γ-secretase subunits PS1, PS2, and PEN2 is increased by cellular stress in astrocytes (Grolla et al., 2013; Frost and Li, 2017). Subsequent studies have demonstrated that $A\beta_{1-42}$ itself can induce astrocyte senescence and further induce the astrocytic APP and β-secretase processing, resulting in a further increase in oligomeric and fibrillary Aβ (Frost and Li, 2017; Garwood et al., 2017). Additionally, the proinflammatory factors secreted by senescent astrocytes can also increase APP expression and Aβ generation in neurons (Del Bo et al., 1995). This means the higher release of SASP factors reduces the ability of the glia cells to promote Aβ clearance and facilitates its accumulation in the brain.

Tau Accumulation and NFT Formation

Abnormal phosphorylation of the microtubule-associated protein tau and the subsequent accumulation of NFTs are the major pathological mechanisms of AD. Many studies have documented the vital role of senescent astrocytes in tau hyperphosphorylation and NFT formation (Bussian et al., 2018; Mendelsohn and Larrick, 2018). Musi et al. (2018) demonstrated that neurons carrying NFTs themselves become senescent, causing toxicity to nearby neurons. They performed a transcriptomic analysis of NFT-containing human neurons from the postmortem AD brain and revealed increased levels of the cellular senescence hallmark. The expression of senescence-associated genes, such as $p21^{WAF1}$ and SASP proinflammatory genes, was significantly upregulated in the brain of the AD transgenic mouse model with NFTs. The authors treated with senolytics to remove the senescent cells, and found a reduction in total NFT density, neuronal loss, and ventricular enlargement. Although they link the expression of biomarkers of cellular senescence with the appearance of the NFTs in the AD mouse model, a causal relationship is not established, and the specific cell types involved cannot be identified. A recent study by Bussian et al. (2018) found the accumulation of $p16^{INK4A}$-expression senescent astrocytes and microglia in the $MAPT^{P301S}$PS19 mouse model of tau-dependent neurodegenerative disease. They then

created a mouse strain (PS19/ATTAC) by crossing *INK-ATTAC* transgenic mice with the PS19 strain to remove p16-positive senescent cells through the administration of an inducer agent. They found that the clearance and removal of senescent astrocytes and microglial cells almost completely prevented hyperphosphorylated tau protein and NFT deposition in PS19/ATTAC mice, preserving cognitive function. Collectively, these results demonstrate that senescent astrocytes have a crucial role in tau accumulation and thus the etiopathology of tau-associated disease (**Figure 2**). These data also suggest a strong correlation between astrocyte senescence, Aβ formation, and tau accumulation, but the underlying mechanism tying together these characteristics with respect to senescent astrocytes in AD remains unclear and inconclusive.

Synaptic Dysfunction and Neuronal Loss

Cognitive and memory impairment in AD is most likely caused by synapse loss and synapse dysfunction rather than mere neuronal loss or neuronal death (Terry et al., 1991; Hong et al., 2016). As the major glial cell types of the brain, astrocytes play a critical role in supporting neuronal growth and modulating synaptic function and transmission, yet the neuroprotective capacity of astrocytes decreased during aging (Pertusa et al., 2007; Yu et al., 2017). Although the mechanism of synapse dysfunction has not yet been fully elucidated, it is most likely a combination of Aβ plaque deposition, tau accumulation, and lesions (Miller et al., 2014; Piacentini et al., 2017). Astrocytes exhibit a senescence-like phenotype around Aβ plaques and NFTs in the brains of AD patients and AD mouse models (Bhat et al., 2012; Lok et al., 2013; Musi et al., 2018), and synapse dysfunction is also found mainly surrounding dense-core Aβ plaques (Koffie et al., 2009). These studies provide support for a relationship between senescent astrocytes and synaptic dysfunction or synapse loss in AD progression. *In vitro*, hippocampal neurons cocultured with senescent astrocytes showed suppressed synaptic maturation and transmission accompanied by a reduction in the size of synaptic vesicles (Kawano et al., 2012; Turnquist et al., 2016). The neuronal loss observed in AD can be attributed to the release of SASP factors such as IL-6. Furthermore, primary astrocytes from aged 5xFAD mice showed elevated SASP factor expression and neurotoxicity as well as impairment in supporting neuronal homeostasis (Iram et al., 2016). Moreover, senescent astrocytes secrete less neurotrophins such as nerve growth factor (NGF), insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF2), brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) (Bernal and Peterson, 2011; Turnquist et al., 2016; Bellaver et al., 2017), which may be lead to decreased neuronal growth and increased neuronal death in age-related neurological disorders.

Astrocytes also modulate synaptic properties via the abnormal release of the gliotransmitter GABA (Morel et al., 2017; Perez-Nievas and Serrano-Pozo, 2018). Moreover, senescent cortical astrocytes contribute to the impairment of synaptic plasticity and cognitive decline via a decline in the production of ATP, which is an important metabolic factor of neuronal activity (Lalo et al., 2014).

Blood-Brain Barrier Dysfunction

The BBB limits the migration of cells and diffusion of molecules freely entering and exiting from the brain. The BBB is crucial for maintaining homeostasis of the brain microenvironment, which consists of perivascular microglia, endothelial cells, pericytes, neurons, and astrocytic end feet (Yamazaki and Kanekiyo, 2017). The impairment function of any of these cells could result in disturbance to the BBB.

In fact, the BBB is seen to leak both in normal aging and AD (Montagne et al., 2015; Yamazaki and Kanekiyo, 2017). However, the underlying mechanism of how aging disrupts the integrity of the BBB remains inconclusive. Astrocytes, although not involved in the formation of the BBB, participate in its maintenance and regulation. Senescent astrocytes produce a variety of SASP factors that influence the permeability of the BBB (Spampinato et al., 2017; Boisvert et al., 2018; Clarke et al., 2018). For instance, matrix metalloproteinases (MMPs), nitric oxide (NO), and VEGFs cause endothelial apoptosis and disrupt endothelial tight junctions (TJs) by downregulation of TJ-related proteins, resulting in BBB disruption (Abbott, 2002; Horng et al., 2017; Spampinato et al., 2017). Some of these molecules include transforming growth factor-β (TGFβ), glial cell–derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), IL-6 and upregulate endothelial cell adhesion molecules (CAMs), which induce leukocyte transmigration (Rochfort and Cummins, 2015). Interestingly, recent research has shown that the accumulation of senescent vascular cells results in compromised BBB integrity and reduced microvessel TJ coverage (Yamazaki et al., 2016). These senescent vascular smooth muscle cells (VSMCs) also contribute to the brain inflammation environment through the upregulation of proinflammatory cytokine IL-6 and chemokines, suggesting that senescent VSMCs have a crucial role in inducing age-dependent BBB breakdown. Yet, it is not clear whether the senescent astrocytes have a positive effect on VSMC senescence, and further studies should continue to explore the role of astrocyte senescence in increased BBB permeability during AD.

Activation of Microglia and Promotion of Chronic Inflammation

Specifically, senescent astrocytes secrete SASP meditators such as IFNγ, CXCL10, IL-6, and TGFβ, which are capable of inducing inflammation (Bhat et al., 2012; Stamouli and Politis, 2016). For example, IFNγ is a potent regulatory cytokine that activates microglia and promotes inflammation in the AD brain (Blasko et al., 2004; Taylor et al., 2018). IL-6 is another typical SASP factor whose expression is upregulated in the aged brain and in those with AD. Its overexpression has been shown to drive neurodegeneration *in vitro* (Bhat et al., 2012). Several SASP factors, including IL-6, IL-1β, TNF-α, MMP-1, MMP-3, and MMP-10, have also been found to be elevated in the cerebrospinal fluid (CSF) and serum of AD patients (Wood et al., 1993; Blum-Degena et al., 1995; Leake et al., 2000; Horstmann et al., 2010; Gezen-Ak et al., 2013). This suggests that senescent astrocytes may sustain a proinflammatory status via the production of SASP factors and SASP-mediated microglia activation and

inflammation may contribute to the pathogenesis of AD and aggravate the course of the disease.

ASTROCYTE SENESCENCE AND OTHER NEURODEGENERATIVE DISEASES

Senescent astrocytes are also more prominent in brain tissues from patients with other neurodegenerative diseases, such as Parkinson's disease (PD) (Chinta et al., 2018; Scott and Williams-Gray, 2018) and amyotrophic lateral sclerosis (ALS) (Turnquist et al., 2016; Vazquez-Villasenor et al., 2019). PD is the second most common age-related neurodegenerative disease, characterized by loss of neurons in the substantia nigra pars compacta (SNpc), accumulation of α-synuclein, and presence of intraneuronal proteinaceous cytoplasmic inclusions (Lewy bodies) (Dauer and Przedborski, 2003). Increased levels of SASP-related factors, IL-1β, IL-6, and TNF-α, have been reported in the CSF, serum, and striatal dopaminergic regions of the patients with PD compared to controls (Mogi et al., 1994; Blum-Degena et al., 1995). Chinta et al. (2018) found that lamin B1 levels are decreased, while the expression of senescence-associated genes, $p16^{INK4A}$ and $p21^{WAF1}$, is increased in astrocytes of SNpc tissues in PD patients. Additionally, conditioned media from senescent astrocytes significantly reduced the viability of dopaminergic neurons and the proliferation and migration of neural progenitor cells (NPCs). Most importantly, they found that selective elimination of senescent astrocytes could repress the development of paraquat-induced neurodegenerative phenotypes associated with sporadic PD (Chinta et al., 2018), demonstrating that astrocyte senescence might be a critical mechanism for PD neurodegeneration. ALS is a fatal neurodegenerative disease characterized by the loss of upper and lower motor neurons (MNs). Postmortem brain tissue from ALS patients exhibits increased numbers of senescent astrocytes (Turnquist et al., 2016). This is further supported by animal models of ALS that show increased expression of the senescence marker $p16^{INK4A}$ in GFAP-positive astrocytes within lumbar spinal cord sections that typically surround damaged motor neurons (Trias et al., 2019). Moreover, SASP cytokine *IL-6* as well as $p16^{INK4A}$ and $p21^{WAF1}$ were more remarkably upregulated in ALS compared to AD (Turnquist et al., 2016). Aged astrocytes are less supportive to motor neuron function (Das and Svendsen, 2015; Turnquist et al., 2016). Senescent astrocytes might exert their neurotoxic effect on neurons through the release of SASP factors, exacerbating neuroinflammation. However, it is not clear whether prevention or clearance of senescent cells in ALS could delay disease progression as is seen in PD models.

THERAPEUTIC APPLICATIONS

Currently, most of the therapeutic approaches of AD focus mainly on the modulation of Aβ production by inhibiting Aβ generation and enhancing Aβ degradation or reducing tau protein deposits and NFT accumulation (Citron, 2010). Others attempt at targeting apoE, neuroinflammation, metabolic dysfunction, and epigenetic changes (Long and Holtzman, 2019). Despite the fact that there are more than 100 different compounds in various stages of clinical trials being tested for use in early-, mid- or late-stage AD, there are few efficacious therapeutic options available (Hara et al., 2019; Long and Holtzman, 2019). The failure of these trials emphasizes a great need for different pharmaceutical therapies to prevent or delay the progression of AD.

Aging is a critical risk factor for most age-related neurodegenerative disease, including AD (Baker and Petersen, 2018; Kritsilis et al., 2018; Hara et al., 2019). A hallmark of aging is senescent cell accumulation. In recent years, a significant finding in aging and age-related diseases research is that selective elimination of senescent cells can extend lifespan and slow the progression of diseases *in vivo* without triggering negative side effects (Baker et al., 2011, 2016; Jeon et al., 2017). This strategy is referred to as senolysis. As mentioned earlier, a great number of studies indicated that astrocyte senescence plays a crucial role in the pathogenesis of AD. Senolytic therapeutic strategies that safely and effectively reduce the detrimental effects of senescent astrocytes, such as the neutralization of SASP or targeted clearance of senescent astrocytes, are gaining considerable attention in AD.

Alleviate Astrocyte Senescence and Decrease SASP Level

Several lines of research suggest that astrocyte senescence occurs in the early stage of disease progression and may induce or aggravate other neurodegenerative pathologies. Thus, therapies to alleviate astrocyte senescence could prevent the onset of AD or delay its progress. In AD, senescent astrocytes exert deleterious effects on neurons via both reduced secretion of neurotrophic growth factors (NGF, IGF-1) and elevated production of SASP factors (Turnquist et al., 2016). Turnquist et al. (2016, 2019) demonstrated that overexpression of Δ133p53 or downregulation of *p53β* can alleviate astrocyte senescence, repress SASP production, and consequently prevent neuronal apoptosis and loss. Additionally, in astrocytes, Δ133p53-mediated upregulation of neurotrophic factors induced a neuroprotection effect. This shift secretion profile demonstrated that SASP may be a promising therapeutic method for slowing the progression of AD. There are two more commonly used SASP neutralization therapies: inhibiting SASP-initiated signaling and blocking the activity of particular components of the SASP.

As mentioned earlier, astrocytic SASP genes are upregulated in a p38/MAPK and NF-κB signaling-dependent manner. Thus, targeting the NF-κB pathway decreased the production of SASP. Similarly, pharmacological inhibition of p38/MAPK abolished SASP secretion by senescent astrocytes. Yet, not all SASP factors are altered in senescent astrocytes. Because several SASP factors are essential to maintaining cell senescence, inhibition of pro-SASP signaling can increase the risk of cancer development (Coppe et al., 2010; Hoare et al., 2016). Alternatively, blockade of specific SASP factors, such as IL-6, is also a viable strategy. Specifically, using IL-6 monoclonal neutralizing antibodies,

which are already approved for clinical applications, i.e., siltuximab (anti-human IL-6) or tocilizumab (anti-human IL-6R), could show some promise.

Clearance of Senescent Astrocytes (Senotherapy)

In many age-related disorders such as osteoarthritis, atherosclerosis, and diabetes mellitus type 2, the removal of senescent cells of transgenic mice models has shown an impaired associated pathology and extended the healthy lifespan (Baker et al., 2011, 2016; Jeon et al., 2017). Success has also been observed in a mouse model of tau-associated pathogenesis. This study was the first to demonstrate a causal relationship between glial senescence and neurodegeneration (Bussian et al., 2018). In this study, Bussian et al. found accumulations of senescent astrocytes and microglia in tau-associated neurodegenerative disease model mice. Elimination of these senescent cells via a genetic approach can reduce tau deposition and prevent the degeneration of cortical and hippocampal neurons. Besides, a new set of pharmacological drugs has been proven to show a similar effect, termed senolytics, which include ABT263, quercetin, and dasatinib (Zhu et al., 2014). Indeed, these pharmacological agents have already been successful in preclinical and clinical trials in several age-associated diseases (reviewed by Childs et al., 2017). Most recently, Zhang X. et al. (2019) showed that clearance of senescent oligodendrocyte progenitor cells in AD model mice with senolytic agents could lessen the Aβ plaque load, reduce neuroinflammation, and ameliorate cognitive deficits. This seno-therapeutic approach is currently being tested in neurodegenerative diseases and despite expected challenges and difficulties, more detailed investigation is warranted.

CONCLUSION AND OUTLOOK

Astrocytes undergo degeneration and senescence in the early stages of AD progression, which may alter the microenvironment of the brain and contribute to early cognitive deficits. However, how senescent astrocytes actively and exactly contribute to the progression of AD is still to be fully characterized. Fortunately, targeting astrocyte senescence using a senolytics approach, among others, is beginning to emerge in AD treatments, with evidence in AD animal models already showing promise. Additionally, other cell types in the brain, including microglia (Flanary, 2005), oligodendrocytes (Zhang P. et al., 2019), neural stem cells (He et al., 2013), and neurons (Jurk et al., 2012), showed senescent phenotypes that are also involved in development of AD. Therefore, the link between cellular senescence of other CNS cell types and AD needs to be further explored.

Furthermore, extensive astrocyte senescence has also been found in other age-related neurodegenerative diseases such as PD and ALS. This indicates that astrocyte senescence seems to be a common characteristic of neurodegeneration, and future research needs to examine this phenomenon in more detail. Additionally, the pathologies of neurodegenerative diseases induced by senescent astrocytes need to be further understood. Such future studies could increase our knowledge of cellular senescence and neurodegeneration medicine.

AUTHOR CONTRIBUTIONS

XH conceived and wrote the manuscript. TZ and HL contributed to manuscript preparation. YM and XG designed the concept of this study and discussed the results with all authors.

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REFERENCES

Abbott, N. J. (2002). Astrocyte-endothelial interactions and blood-brain barrier permeability. *J. Anat.* 200, 629–638. doi: 10.1046/j.1469-7580.2002.00064.x

Acosta, C., Anderson, H. D., and Anderson, C. M. (2017). Astrocyte dysfunction in Alzheimer disease. *J. Neurosci. Res.* 95, 2430–2447. doi: 10.1002/jnr.24075

Acosta, J. C., Banito, A., Wuestefeld, T., Georgilis, A., Janich, P., Morton, J. P., et al. (2013). A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat. Cell Biol.* 15, 978–990. doi: 10.1038/ncb2784

Ahmad, M. H., Fatima, M., and Mondal, A. C. (2019). Influence of microglia and astrocyte activation in the neuroinflammatory pathogenesis of Alzheimer's disease: rational insights for the therapeutic approaches. *J. Clin. Neurosci.* 59, 6–11. doi: 10.1016/j.jocn.2018.10.034

Amodeo, A. A., and Skotheim, J. M. (2016). Cell-Size Control. *Cold Spring Harb. Perspect. Biol.* 8:a019083. doi: 10.1101/cshperspect.a019083

Baker, D. J., Childs, B. G., Durik, M., Wijers, M. E., Sieben, C. J., Zhong, J., et al. (2016). Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* 530, 184–189. doi: 10.1038/nature16932

Baker, D. J., and Petersen, R. C. (2018). Cellular senescence in brain aging and neurodegenerative diseases: evidence and perspectives. *J. Clin. Invest.* 128, 1208–1216. doi: 10.1172/JCI95145

Baker, D. J., Wijshake, T., Tchkonia, T., LeBrasseur, N. K., Childs, B. G., van de Sluis, B., et al. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 479, 232–236. doi: 10.1038/nature10600

Bang, M., Kim, D. G., Gonzales, E. L., Kwon, K. J., and Shin, C. Y. (2019). Etoposide induces mitochondrial dysfunction and cellular senescence in primary cultured rat astrocytes. *Biomol. Ther.* 27, 530–539. doi: 10.4062/biomolther.2019.151

Basak, J. M., Verghese, P. B., Yoon, H., Kim, J., and Holtzman, D. M. (2012). Low-density lipoprotein receptor represents an apolipoprotein E-independent pathway of Abeta uptake and degradation by astrocytes. *J. Biol. Chem.* 287, 13959–13971. doi: 10.1074/jbc.M111.288746

Bellaver, B., Souza, D. G., Souza, D. O., and Quincozes-Santos, A. (2017). Hippocampal astrocyte cultures from adult and aged rats reproduce changes in glial functionality observed in the aging brain. *Mol. Neurobiol.* 54, 2969–2985. doi: 10.1007/s12035-016-9880-8

Benzing, W. C., Wujek, J. R., Ward, E. K., Shaffer, D., Ashe, K. H., Younkin, S. G., et al. (1999). Evidence for glial-mediated inflammation in aged APPSW

transgenic mice. *Neurobiol. Aging* 20, 581–589. doi: 10.1016/s0197-4580(99)00065-2

Berchtold, N. C., Cribbs, D. H., Coleman, P. D., Rogers, J., Head, E., Kim, R., et al. (2008). Gene expression changes in the course of normal brain aging are sexually dimorphic. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15605–15610. doi: 10.1073/pnas.0806883105

Bernal, G. M., and Peterson, D. A. (2011). Phenotypic and gene expression modification with normal brain aging in GFAP-positive astrocytes and neural stem cells. *Aging Cell* 10, 466–482. doi: 10.1111/j.1474-9726.2011.00694.x

Bhat, R., Crowe, E. P., Bitto, A., Moh, M., Katsetos, C. D., Garcia, F. U., et al. (2012). Astrocyte senescence as a component of Alzheimer's disease. *PLoS One* 7:e45069. doi: 10.1371/journal.pone.0045069

Bitto, A., Sell, C., Crowe, E., Lorenzini, A., Malaguti, M., Hrelia, S., et al. (2010). Stress-induced senescence in human and rodent astrocytes. *Exp. Cell Res.* 316, 2961–2968. doi: 10.1016/j.yexcr.2010.06.021

Blasko, I., Stampfer-Kountchev, M., Robatscher, P., Veerhuis, R., Eikelenboom, P., and Grubeck-Loebenstein, B. (2004). How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. *Aging Cell* 3, 169–176. doi: 10.1111/j.1474-9728.2004.00101.x

Blum-Degena, D., Müller, T., Kuhn, W., Gerlach, M., Przuntek, H., and Riederer, P. (1995). Interleukin-1β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. *Neurosci. Lett.* 202, 17–20. doi: 10.1016/0304-3940(95)12192-7

Boccardi, V., Pelini, L., Ercolani, S., Ruggiero, C., and Mecocci, P. (2015). From cellular senescence to Alzheimer's disease: the role of telomere shortening. *Ageing Res. Rev.* 22, 1–8. doi: 10.1016/j.arr.2015.04.003

Boisvert, M. M., Erikson, G. A., Shokhirev, M. N., and Allen, N. J. (2018). The aging astrocyte transcriptome from multiple regions of the mouse brain. *Cell Rep.* 22, 269–285. doi: 10.1016/j.celrep.2017.12.039

Buhlman, L. M. (2017). Parkin loss-of-function pathology: Premature neuronal senescence induced by high levels of reactive oxygen species? *Mech. Ageing Dev.* 161, 112–120. doi: 10.1016/j.mad.2016.06.008

Bussian, T. J., Aziz, A., Meyer, C. F., Swenson, B. L., van Deursen, J. M., and Baker, D. J. (2018). Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. *Nature* 562, 578–582. doi: 10.1038/s41586-018-0543-y

Capell, B. C., Drake, A. M., Zhu, J., Shah, P. P., Dou, Z., Dorsey, J., et al. (2016). MLL1 is essential for the senescence-associated secretory phenotype. *Genes Dev.* 30, 321–336. doi: 10.1101/gad.271882.115

Castiglioni, A., Canti, V., Rovere-Querini, P., and Manfredi, A. A. (2011). High-mobility group box 1 (HMGB1) as a master regulator of innate immunity. *Cell Tissue Res.* 343, 189–199. doi: 10.1007/s00441-010-1033-1

Chien, Y., Scuoppo, C., Wang, X., Fang, X., Balgley, B., Bolden, J. E., et al. (2011). Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes Dev.* 25, 2125–2136. doi: 10.1101/gad.17276711

Childs, B. G., Gluscevic, M., Baker, D. J., Laberge, R. M., Marquess, D., Dananberg, J., et al. (2017). Senescent cells: an emerging target for diseases of ageing. *Nat. Rev. Drug Discov.* 16, 718–735. doi: 10.1038/nrd.2017.116

Chinta, S. J., Woods, G., Demaria, M., Rane, A., Zou, Y., McQuade, A., et al. (2018). Cellular senescence is induced by the environmental neurotoxin paraquat and contributes to neuropathology linked to Parkinson's Disease. *Cell Rep.* 22, 930–940. doi: 10.1016/j.celrep.2017.12.092

Chinta, S. J., Woods, G., Rane, A., Demaria, M., Campisi, J., and Andersen, J. K. (2015). Cellular senescence and the aging brain. *Exp. Gerontol.* 68, 3–7.

Citron, M. (2010). Alzheimer's disease: strategies for disease modification. *Nat. Rev. Drug Discov.* 9, 387–398. doi: 10.1038/nrd2896

Clarke, L. E., Liddelow, S. A., Chakraborty, C., Munch, A. E., Heiman, M., and Barres, B. A. (2018). Normal aging induces A1-like astrocyte reactivity. *Proc. Natl. Acad. Sci. U.S.A.* 115, E1896–E1905. doi: 10.1073/pnas.1800165115

Cohen, J., D'Agostino, L., Wilson, J., Tuzer, F., and Torres, C. (2017). Astrocyte senescence and metabolic changes in response to HIV antiretroviral therapy drugs. *Front. Aging Neurosci.* 9:281. doi: 10.3389/fnagi.2017.00281

Cohen, J., and Torres, C. (2019). Astrocyte senescence: evidence and significance. *Aging Cell* 18:e12937. doi: 10.1111/acel.12937

Coppe, J. P., Desprez, P. Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu. Rev. Pathol.,* 5, 99–118. doi: 10.1146/annurev-pathol-121808-102144

Crowe, E. P., Tuzer, F., Gregory, B. D., Donahue, G., Gosai, S. J., Cohen, J., et al. (2016). Changes in the transcriptome of human astrocytes accompanying oxidative stress-induced senescence. *Front. Aging Neurosci.* 8:208. doi: 10.3389/fnagi.2016.00208

Danbolt, N. C. (2001). Glutamate uptake. *Prog. Neurobiol.* 65, 1–105.

Das, M. M., and Svendsen, C. N. (2015). Astrocytes show reduced support of motor neurons with aging that is accelerated in a rodent model of ALS. *Neurobiol. Aging* 36, 1130–1139. doi: 10.1016/j.neurobiolaging.2014.09.020

Dauer, W., and Przedborski, S. (2003). Parkinson's Disease. *Neuron* 39, 889–909.

Davalos, A. R., Kawahara, M., Malhotra, G. K., Schaum, N., Huang, J., Ved, U., et al. (2013). p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes. *J. Cell Biol.* 201, 613–629. doi: 10.1083/jcb.201206006

DeKosky, S. T., and Scheff, S. W. (1990). Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann. Neurol.* 27, 457–464. doi: 10.1002/ana.410270502

Del Bo, R., Angeretti, N., Lucca, E., De Simoni, M. G., and Forloni, G. (1995). Reciprocal control of inflammatory cytokines, IL-1 and IL-6, and β-amyloid production in cultures. *Neurosci. Lett.* 188, 70–74. doi: 10.1016/0304-3940(95)11384-9

Enokido, Y., Yoshitake, A., Ito, H., and Okazawa, H. (2008). Age-dependent change of HMGB1 and DNA double-strand break accumulation in mouse brain. *Biochem. Biophys. Res. Commun.* 376, 128–133. doi: 10.1016/j.bbrc.2008.08.108

Eun, K., Hwang, S. U., Jeon, H. M., Hyun, S. H., and Kim, H. (2016). Comparative analysis of human, mouse, and pig glial fibrillary acidic protein gene structures. *Anim. Biotechnol.* 27, 126–132. doi: 10.1080/10495398.2015.1126719

Evans, R. J., Wyllie, F. S., Wynford-Thomas, D., Kipling, D., and Jones, C. J. (2003). A P53-dependent, telomere-independent proliferative life span barrier in human astrocytes consistent with the molecular genetics of glioma development. *Cancer Res.* 63, 4854–4861.

Ferris, H. A., Perry, R. J., Moreira, G. V., Shulman, G. I., Horton, J. D., and Kahn, C. R. (2017). Loss of astrocyte cholesterol synthesis disrupts neuronal function and alters whole-body metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 114, 1189–1194. doi: 10.1073/pnas.1620506114

Fivenson, E. M., Lautrup, S., Sun, N., Scheibye-Knudsen, M., Stevnsner, T., Nilsen, H., et al. (2017). Mitophagy in neurodegeneration and aging. *Neurochem. Int.* 109, 202–209. doi: 10.1016/j.neuint.2017.02.007

Flanary, B. (2005). The role of microglial cellular senescence in the aging and Alzheimer diseased brain. *Rejuvenation Res.* 8, 82–85. doi: 10.1089/rej.2005.8.82

Freund, A., Laberge, R. M., Demaria, M., and Campisi, J. (2012). Lamin B1 loss is a senescence-associated biomarker. *Mol. Biol. Cell* 23, 2066–2075. doi: 10.1091/mbc.E11-10-0884

Frost, G. R., and Li, Y. M. (2017). The role of astrocytes in amyloid production and Alzheimer's disease. *Open Biol.* 7:170228. doi: 10.1098/rsob.170228

Garwood, C. J., Ratcliffe, L. E., Simpson, J. E., Heath, P. R., Ince, P. G., and Wharton, S. B. (2017). Review: astrocytes in Alzheimer's disease and other age-associated dementias: a supporting player with a central role. *Neuropathol. Appl. Neurobiol.* 43, 281–298. doi: 10.1111/nan.12338

Gezen-Ak, D., Dursun, E., Hanagasi, H., Bilgic, B., Lohman, E., Araz, O. S., et al. (2013). BDNF, TNFalpha, HSP90, CFH, and IL-10 serum levels in patients with early or late onset Alzheimer's disease or mild cognitive impairment. *J. Alzheimers Dis.* 37, 185–195. doi: 10.3233/JAD-130497

Giudetti, A. M., Romano, A., Lavecchia, A. M., and Gaetani, S. (2016). The role of brain cholesterol and its oxidized products in Alzheimer's Disease. *Curr. Alzheimer Res.* 13, 198–205. doi: 10.2174/1567205012666150921103426

Glenner, G. G., and Wong, C. W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120, 885–890. doi: 10.1016/s0006-291x(84)80190-4

Goedert, M., Klug, A., and Crowther, R. A. (2006). Tau protein, the paired helical filament and Alzheimer's disease. *J. Alzheimers Dis.* 9, 195–207.

Gonzalez-Reyes, R. E., Nava-Mesa, M. O., Vargas-Sanchez, K., Ariza-Salamanca, D., and Mora-Munoz, L. (2017). Involvement of astrocytes in Alzheimer's Disease from a neuroinflammatory and oxidative stress perspective. *Front. Mol. Neurosci.* 10:427. doi: 10.3389/fnmol.2017.00427

Gorg, B., Karababa, A., and Haussinger, D. (2018). Hepatic encephalopathy and astrocyte senescence. *J. Clin. Exp. Hepatol.* 8, 294–300. doi: 10.1016/j.jceh.2018.05.003

Gorg, B., Karababa, A., Shafigullina, A., Bidmon, H. J., and Haussinger, D. (2015). Ammonia-induced senescence in cultured rat astrocytes and in human cerebral cortex in hepatic encephalopathy. *Glia* 63, 37–50. doi: 10.1002/glia.22731

Gorgoulis, V., Adams, P. D., Alimonti, A., Bennett, D. C., Bischof, O., Bishop, C., et al. (2019). Cellular senescence: defining a path forward. *Cell* 179, 813–827. doi: 10.1016/j.cell.2019.10.005

Grolla, A. A., Fakhfouri, G., Balzaretti, G., Marcello, E., Gardoni, F., Canonico, P. L., et al. (2013). Abeta leads to Ca(2)(+) signaling alterations and transcriptional changes in glial cells. *Neurobiol. Aging* 34, 511–522. doi: 10.1016/j.neurobiolaging.2012.05.005

Hampel, H., Mesulam, M. M., Cuello, A. C., Farlow, M. R., Giacobini, E., Grossberg, G. T., et al. (2018). The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain* 141, 1917–1933.

Han, X., Tai, H., Wang, X., Wang, Z., Zhou, J., Wei, X., et al. (2016). AMPK activation protects cells from oxidative stress-induced senescence via autophagic flux restoration and intracellular NAD(+) elevation. *Aging Cell* 15, 416–427. doi: 10.1111/acel.12446

Hansen, L., Salmon, D., Galasko, D., Masliah, E., Katzman, R., DeTeresa, R., et al. (1990). The Lewy body variant of Alzheimer's disease: a clinical and pathologic entity. *Neurology* 40, 1–8.

Hara, Y., McKeehan, N., and Fillit, H. M. (2019). Translating the biology of aging into novel therapeutics for Alzheimer disease. *Neurology* 92, 84–93. doi: 10.1212/WNL.0000000000006745

Hardy, J. A., and Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256, 184–185.

He, N., Jin, W. L., Lok, K. H., Wang, Y., Yin, M., and Wang, Z. J. (2013). Amyloid-beta(1-42) oligomer accelerates senescence in adult hippocampal neural stem/progenitor cells via formylpeptide receptor 2. *Cell Death Dis.* 4:e924. doi: 10.1038/cddis.2013.437

Heneka, M. T., Carson, M. J., Khoury, J. E., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405.

Hernandez-Segura, A., Nehme, J., and Demaria, M. (2018). Hallmarks of cellular senescence. *Trends Cell Biol.* 28, 436–453. doi: 10.1016/j.tcb.2018.02.001

Herranz, N., Gallage, S., Mellone, M., Wuestefeld, T., Klotz, S., Hanley, C. J., et al. (2015). mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nat. Cell Biol.* 17, 1205–1217. doi: 10.1038/ncb3225

Hoare, M., Ito, Y., Kang, T. W., Weekes, M. P., Matheson, N. J., Patten, D. A., et al. (2016). NOTCH1 mediates a switch between two distinct secretomes during senescence. *Nat. Cell Biol.* 18, 979–992. doi: 10.1038/ncb3397

Hong, S., Beja-Glasser, V. F., Nfonoyim, B. M., Frouin, A., Li, S., Ramakrishnan, S., et al. (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science* 352, 712–716. doi: 10.1126/science.aad8373

Horng, S., Therattil, A., Moyon, S., Gordon, A., Kim, K., Argaw, A. T., et al. (2017). Astrocytic tight junctions control inflammatory CNS lesion pathogenesis. *J. Clin. Invest.* 127, 3136–3151. doi: 10.1172/JCI91301

Horstmann, S., Budig, L., Gardner, H., Koziol, J., Deuschle, M., Schilling, C., et al. (2010). Matrix metalloproteinases in peripheral blood and cerebrospinal fluid in patients with Alzheimer's disease. *Int. Psychogeriatr.* 22, 966–972. doi: 10.1017/S1041610210000827

Hou, J., Cui, C., Kim, S., Sung, C., and Choi, C. (2018). Ginsenoside F1 suppresses astrocytic senescence-associated secretory phenotype. *Chem. Biol. Interact.* 283, 75–83. doi: 10.1016/j.cbi.2018.02.002

Hou, J., Kim, S., Sung, C., and Choi, C. (2017). Ginsenoside Rg3 prevents oxidative stress-induced astrocytic senescence and ameliorates senescence paracrine effects on glioblastoma. *Molecules* 22:E1516. doi: 10.3390/molecules22091516

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., et al. (1996). Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274, 99–102.

Hussaini, S. M. Q., and Jang, M. H. (2018). New roles for old glue: astrocyte function in synaptic plasticity and neurological disorders. *Int. Neurourol. J.* 22, S106–S114. doi: 10.5213/inj.1836214.107

Iram, T., Trudler, D., Kain, D., Kanner, S., Galron, R., Vassar, R., et al. (2016). Astrocytes from old Alzheimer's disease mice are impaired in abeta uptake and in neuroprotection. *Neurobiol. Dis.* 96, 84–94. doi: 10.1016/j.nbd.2016.08.001

Jeon, O. H., Kim, C., Laberge, R. M., Demaria, M., Rathod, S., Vasserot, A. P., et al. (2017). Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nat. Med.* 23, 775–781. doi: 10.1038/nm.4324

Jurk, D., Wang, C., Miwa, S., Maddick, M., Korolchuk, V., Tsolou, A., et al. (2012). Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. *Aging Cell* 11, 996–1004. doi: 10.1111/j.1474-9726.2012.00870.x

Kawano, H., Katsurabayashi, S., Kakazu, Y., Yamashita, Y., Kubo, N., Kubo, M., et al. (2012). Long-term culture of astrocytes attenuates the readily releasable pool of synaptic vesicles. *PLoS One* 7:e48034. doi: 10.1371/journal.pone.0048034

Kim, J. H., Jittiwat, J., Ong, W. Y., Farooqui, A. A., and Jenner, A. M. (2010). Changes in cholesterol biosynthetic and transport pathways after excitotoxicity. *J. Neurochem.* 112, 34–41. doi: 10.1111/j.1471-4159.2009.06449.x

Kim, Y. M., Youn, S. W., Sudhahar, V., Das, A., Chandhri, R., Cuervo Grajal, H., et al. (2018). Redox regulation of mitochondrial fission protein drp1 by protein disulfide isomerase limits endothelial senescence. *Cell Rep.* 23, 3565–3578. doi: 10.1016/j.celrep.2018.05.054

Knorpp, T., Robinson, S. R., Crack, P. J., and Dringen, R. (2006). Glutathione peroxidase-1 contributes to the protection of glutamine synthetase in astrocytes during oxidative stress. *J. Neural. Transm.* 113, 1145–1155. doi: 10.1007/s00702-005-0389-y

Koffie, R. M., Meyer-Luehmann, M., Hashimoto, T., Adams, K. W., Mielke, M. L., Garcia-Alloza, M., et al. (2009). Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. *Proc. Natl .Acad. Sci. U.S.A.* 106, 4012–4017. doi: 10.1073/pnas.0811698106

Kosar, M., Bartkova, J., Hubackova, S., Hodny, Z., Lukas, J., and Bartek, J. (2011). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16(ink4a). *Cell Cycle* 10, 457–468. doi: 10.4161/cc.10.3.14707

Kritsilis, M., Rizou, V. S., Koutsoudaki, P. N., Evangelou, K., Gorgoulis, V. G., Papadopoulos, D., et al. (2018). Ageing, cellular senescence and neurodegenerative disease. *Int. J. Mol. Sci.* 19:E2937. doi: 10.3390/ijms19102937

Kurz, D. J., Decary, S., Hong, Y., and Erusalimsky, J. D. (2000). Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* 113(Pt 20), 3613–3622.

Lalo, U., Rasooli-Nejad, S., and Pankratov, Y. (2014). Exocytosis of gliotransmitters from cortical astrocytes: implications for synaptic plasticity and aging. *Biochem. Soc. Trans.* 42, 1275–1281. doi: 10.1042/BST20140163

Larsson, A., Wilhelmsson, U., Pekna, M., and Pekny, M. (2004). Increased cell proliferation and neurogenesis in the hippocampal dentate gyrus of old GFAP(-/-)Vim(-/-) mice. *Neurochem. Res.* 29, 2069–2073. doi: 10.1007/s11064-004-6880-2

Leake, A., Morris, C. M., and Whateley, J. (2000). Brain matrix metalloproteinase 1 levels are elevated in Alzheimer's disease. *Neurosci. Lett.* 291, 201–203. doi: 10.1016/s0304-3940(00)01418-x

Lee, B. Y., Han, J. A., Im, J. S., Morrone, A., Johung, K., Goodwin, E. C., et al. (2006). Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 5, 187–195.

Li, T., and Chen, Z. J. (2018). The cGAS-cGAMP-STING pathway connects DNA damage to inflammation, senescence, and cancer. *J. Exp. Med.* 215, 1287–1299. doi: 10.1084/jem.20180139

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., et al. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541, 481–487. doi: 10.1038/nature21029

Liu, C. C., Hu, J., Zhao, N., Wang, J., Wang, N., Cirrito, J. R., et al. (2017). Astrocytic LRP1 mediates brain abeta clearance and impacts amyloid deposition. *J. Neurosci.* 37, 4023–4031. doi: 10.1523/JNEUROSCI.3442-16.2017

Lok, K., Zhao, H., Zhang, C., He, N., Shen, H., Wang, Z., et al. (2013). Effects of accelerated senescence on learning and memory, locomotion and anxiety-like behavior in APP/PS1 mouse model of Alzheimer's disease. *J. Neurol. Sci.* 335, 145–154. doi: 10.1016/j.jns.2013.09.018

Long, J. M., and Holtzman, D. M. (2019). Alzheimer Disease: an update on pathobiology and treatment strategies. *Cell* 179, 312–339. doi: 10.1016/j.cell.2019.09.001

Loo, T. M., Miyata, K., Tanaka, Y., and Takahashi, A. (2019). Cellular senescence and senescence-associated secretory phenotype via the cGAS-STING

signaling pathway in cancer. *Cancer Sci.* 111, 304–311. doi: 10.1111/cas.14266

Lye, J. J., Latorre, E., Lee, B. P., Bandinelli, S., Holley, J. E., Gutowski, N. J., et al. (2019). Astrocyte senescence may drive alterations in GFAPalpha, CDKN2A p14(ARF), and TAU3 transcript expression and contribute to cognitive decline. *Geroscience* 41, 561–573. doi: 10.1007/s11357-019-00100-3

Matias, I., Morgado, J., and Gomes, F. C. A. (2019). Astrocyte heterogeneity: impact to brain aging and disease. *Front. Aging Neurosci* 11:59. doi: 10.3389/fnagi.2019.00059

McGeer, P. L., and McGeer, E. G. (2007). NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. *Neurobiol. Aging* 28, 639–647. doi: 10.1016/j.neurobiolaging.2006.03.013

Mendelsohn, A. R., and Larrick, J. W. (2018). Cellular senescence as the key intermediate in tau-mediated neurodegeneration. *Rejuvenation Res.* 21, 572–579. doi: 10.1089/rej.2018.2155

Miller, E. C., Teravskis, P. J., Dummer, B. W., Zhao, X., Huganir, R. L., and Liao, D. (2014). Tau phosphorylation and tau mislocalization mediate soluble abeta oligomer-induced AMPA glutamate receptor signaling deficits. *Eur. J. Neurosci.* 39, 1214–1224. doi: 10.1111/ejn.12507

Mogi, M., Harada, M., Kondo, T., Riederer, P., Inagaki, H., Minami, M., et al. (1994). Interleukin-1β, interleukin-6, epidermal growth factor and transforming growth factor-α are elevated in the brain from parkinsonian patients. *Neurosci. Lett.* 180, 147–150. doi: 10.1016/0304-3940(94)90508-8

Mombach, J. C., Vendrusculo, B., and Bugs, C. A. (2015). A model for p38MAPK-induced astrocyte senescence. *PLoS One* 10:e0125217. doi: 10.1371/journal.pone.0125217

Montagne, A., Barnes, S. R., Sweeney, M. D., Halliday, M. R., Sagare, A. P., Zhao, Z., et al. (2015). Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* 85, 296–302. doi: 10.1016/j.neuron.2014.12.032

Morel, L., Chiang, M. S. R., Higashimori, H., Shoneye, T., Iyer, L. K., Yelick, J., et al. (2017). Molecular and functional properties of regional astrocytes in the adult brain. *J. Neurosci.* 37, 8706–8717. doi: 10.1523/JNEUROSCI.3956-16.2017

Munger, E. L., Edler, M. K., Hopkins, W. D., Ely, J. J., Erwin, J. M., Perl, D. P., et al. (2019). Astrocytic changes with aging and Alzheimer's disease-type pathology in chimpanzees. *J. Comp. Neurol.* 527, 1179–1195. doi: 10.1002/cne.24610

Musi, N., Valentine, J. M., Sickora, K. R., Baeuerle, E., Thompson, C. S., Shen, Q., et al. (2018). Tau protein aggregation is associated with cellular senescence in the brain. *Aging Cell* 17, e12840. doi: 10.1111/acel.12840

Myung, N. H., Zhu, X., Kruman, I. I., Castellani, R. J., Petersen, R. B., Siedlak, S. L., et al. (2008). Evidence of DNA damage in Alzheimer disease: phosphorylation of histone H2AX in astrocytes. *Age* 30, 209–215. doi: 10.1007/s11357-008-9050-7

Nichols, N. R., Day, J. R., Laping, N. J., Johnson, S. A., and Finch, C. E. (1993). GFAP mRNA increases with age in rat and human brain. *Neurobiol. Aging* 14, 421–429. doi: 10.1016/0197-4580(93)90100-p

Nicoll, J. A., and Weller, R. O. (2003). A new role for astrocytes: beta-amyloid homeostasis and degradation. *Trends Mol. Med.* 9, 281–282. doi: 10.1016/s1471-4914(03)00109-6

Pajarillo, E., Rizor, A., Lee, J., Aschner, M., and Lee, E. (2019). The role of astrocytic glutamate transporters GLT-1 and GLAST in neurological disorders: potential targets for neurotherapeutics. *Neuropharmacology* 161:107559. doi: 10.1016/j.neuropharm.2019.03.002

Palmer, A. L., and Ousman, S. S. (2018). Astrocytes and Aging. *Front. Aging Neurosci.* 10:337. doi: 10.3389/fnagi.2018.00337

Passos, J. F., Saretzki, G., Ahmed, S., Nelson, G., Richter, T., Peters, H., et al. (2007). Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence. *PLoS Biol.* 5:e110. doi: 10.1371/journal.pbio.0050110

Perez-Nievas, B. G., and Serrano-Pozo, A. (2018). Deciphering the astrocyte reaction in Alzheimer's Disease. *Front. Aging Neurosci.* 10:114. doi: 10.3389/fnagi.2018.00114

Pertusa, M., Garcia-Matas, S., Rodriguez-Farre, E., Sanfeliu, C., and Cristofol, R. (2007). Astrocytes aged in vitro show a decreased neuroprotective capacity. *J. Neurochem.* 101, 794–805. doi: 10.1111/j.1471-4159.2006.04369.x

Piacentini, R., Li Puma, D. D., Mainardi, M., Lazzarino, G., Tavazzi, B., Arancio, O., et al. (2017). Reduced gliotransmitter release from astrocytes mediates tau-induced synaptic dysfunction in cultured hippocampal neurons. *Glia* 65, 1302–1316. doi: 10.1002/glia.23163

Ponten, J., and Macintyre, E. H. (1968). Long term culture of normal and neoplastic human glia. *Acta Pathol. Microbiol. Scand.* 74, 465–486. doi: 10.1111/j.1699-0463.1968.tb03502.x

Porchet, R., Probst, A., Bouras, C., Draberova, E., Draber, P., and Riederer, B. M. (2003). Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer's disease. *Proteomics* 3, 1476–1485. doi: 10.1002/pmic.200300456

Qiu, J., Xu, J., Zheng, Y., Wei, Y., Zhu, X., Lo, E. H., et al. (2010). High-mobility group box 1 promotes metalloproteinase-9 upregulation through Toll-like receptor 4 after cerebral ischemia. *Stroke* 41, 2077–2082. doi: 10.1161/STROKEAHA.110.590463

Rajmohan, R., and Reddy, P. H. (2017). Amyloid-beta and phosphorylated tau accumulations cause abnormalities at synapses of Alzheimer's disease Neurons. *J. Alzheimers Dis* 57, 975–999. doi: 10.3233/JAD-160612

Ries, M., and Sastre, M. (2016). Mechanisms of abeta clearance and degradation by glial cells. *Front. Aging Neurosci.* 8:160. doi: 10.3389/fnagi.2016.00160

Rochfort, K. D., and Cummins, P. M. (2015). The blood-brain barrier endothelium: a target for pro-inflammatory cytokines. *Biochem. Soc. Trans.* 43, 702–706. doi: 10.1042/BST20140319

Salminen, A., Ojala, J., Kaarniranta, K., Haapasalo, A., Hiltunen, M., and Soininen, H. (2011). Astrocytes in the aging brain express characteristics of senescence-associated secretory phenotype. *Eur. .J Neurosci.* 34, 3–11. doi: 10.1111/j.1460-9568.2011.07738.x

Scott, K. M., and Williams-Gray, C. H. (2018). Targeting aged astrocytes may be a new therapeutic strategy in Parkinson's Disease. *Mov. Disord.* 33, 758–759. doi: 10.1002/mds.27387

Seoane, M., Costoya, J. A., and Arce, V. M. (2017). Uncoupling oncogene-induced senescence (OIS) and DNA damage response (DDR) triggered by DNA hyper-replication: lessons from primary mouse embryo astrocytes (MEA). *Sci. Rep.* 7:12991. doi: 10.1038/s41598-017-13408-x

Shi, X., Wang, B., Liu, Y., Zhang, J., Huang, Y., Cao, P., et al. (2017). Carnosine modulates glutamine synthetase expression in senescent astrocytes exposed to oxygen-glucose deprivation/recovery. *Brain Res. Bull.* 130, 138–145. doi: 10.1016/j.brainresbull.2017.01.014

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol.* 119, 7–35. doi: 10.1007/s00401-009-0619-8

Souza, D. G., Bellaver, B., Raupp, G. S., Souza, D. O., and Quincozes-Santos, A. (2015). Astrocytes from adult Wistar rats aged in vitro show changes in glial functions. *Neurochem. Int.* 90, 93–97. doi: 10.1016/j.neuint.2015.07.016

Spampinato, S. F., Merlo, S., Sano, Y., Kanda, T., and Sortino, M. A. (2017). Astrocytes contribute to Abeta-induced blood-brain barrier damage through activation of endothelial MMP9. *J. Neurochem.* 142, 464–477. doi: 10.1111/jnc.14068

Stamouli, E. C., and Politis, A. M. (2016). [Pro-inflammatory cytokines in Alzheimer's disease]. *Psychiatriki* 27, 264–275.

Tai, H., Wang, Z., Gong, H., Han, X., Zhou, J., Wang, X., et al. (2017). Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence. *Autophagy* 13, 99–113. doi: 10.1080/15548627.2016.1247143

Taylor, J. M., Moore, Z., Minter, M. R., and Crack, P. J. (2018). Type-I interferon pathway in neuroinflammation and neurodegeneration: focus on Alzheimer's disease. *J. Neural. Transm.* 125, 797–807. doi: 10.1007/s00702-017-1745-4

Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., et al. (1991). Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* 30, 572–580. doi: 10.1002/ana.410300410

Trias, E., Beilby, P. R., Kovacs, M., Ibarburu, S., Varela, V., Barreto-Nunez, R., et al. (2019). Emergence of microglia bearing senescence markers during paralysis progression in a rat model of inherited ALS. *Front. Aging Neurosci.* 11:42. doi: 10.3389/fnagi.2019.00042

Turnquist, C., Beck, J. A., Horikawa, I., Obiorah, I. E., Von Muhlinen, N., Vojtesek, B., et al. (2019). Radiation-induced astrocyte senescence is rescued by Delta133p53. *Neuro Oncol.* 21, 474–485. doi: 10.1093/neuonc/noz001

Turnquist, C., Horikawa, I., Foran, E., Major, E. O., Vojtesek, B., Lane, D. P., et al. (2016). p53 isoforms regulate astrocyte-mediated neuroprotection and neurodegeneration. *Cell Death Differ.* 23, 1515–1528. doi: 10.1038/cdd.2016.37

Vasileiou, P. V. S., Evangelou, K., Vlasis, K., Fildisis, G., Panayiotidis, M. I., Chronopoulos, E., et al. (2019). Mitochondrial homeostasis and cellular senescence. *Cells* 8:686. doi: 10.3390/cells8070686

Vazquez-Villasenor, I., Garwood, C. J., Heath, P. R., Simpson, J. E., Ince, P. G., and Wharton, S. B. (2019). Expression of p16 and p21 in the frontal association cortex of ALS/MND brains suggests neuronal cell cycle dysregulation and astrocyte senescence in early stages of the disease. *Neuropathol. Appl. Neurobiol.* 46, 171–185. doi: 10.1111/nan.12559

Verkhratsky, A., and Kirchhoff, F. (2007). Glutamate-mediated neuronal-glial transmission. *J. Anat.* 210, 651–660. doi: 10.1111/j.1469-7580.2007.00734.x

Verkhratsky, A., and Nedergaard, M. (2018). Physiology of astroglia. *Physiol. Rev.* 98, 239–389. doi: 10.1152/physrev.00042.2016

Wood, J. A., Wood, P. L., Ryan, R., Graff-Radford, N. R., Pilapil, C., Robitaille, Y., et al. (1993). Cytokine indices in Alzheimer's temporal cortex: no changes in mature IL-1β or IL-1RA but increases in the associated acute phase proteins IL-6, α2-macroglobulin and C-reactive protein. *Brain Res.* 629, 245–252. doi: 10.1016/0006-8993(93)91327-o

Yamazaki, Y., Baker, D. J., Tachibana, M., Liu, C. C., van Deursen, J. M., Brott, T. G., et al. (2016). Vascular cell senescence contributes to blood-brain barrier breakdown. *Stroke* 47, 1068–1077. doi: 10.1161/STROKEAHA.115.010835

Yamazaki, Y., and Kanekiyo, T. (2017). Blood-brain barrier dysfunction and the pathogenesis of Alzheimer's Disease. *Int. J. Mol. Sci.* 18:E1965.

Yoon, K. B., Park, K. R., Kim, S. Y., and Han, S. Y. (2016). Induction of nuclear enlargement and senescence by sirtuin inhibitors in glioblastoma Cells. *Immune Netw.* 16, 183–188. doi: 10.4110/in.2016.16.3.183

Yu, C., Narasipura, S. D., Richards, M. H., Hu, X. T., Yamamoto, B., and Al-Harthi, L. (2017). HIV and drug abuse mediate astrocyte senescence in a beta-catenin-dependent manner leading to neuronal toxicity. *Aging Cell* 16, 956–965. doi: 10.1111/acel.12593

Zhang, P., Kishimoto, Y., Grammatikakis, I., Gottimukkala, K., Cutler, R. G., Zhang, S., et al. (2019). Senolytic therapy alleviates Abeta-associated oligodendrocyte progenitor cell senescence and cognitive deficits in an Alzheimer's disease model. *Nat. Neurosci.* 22, 719–728. doi: 10.1038/s41593-019-0372-9

Zhang, X., Lao, K., Qiu, Z., Rahman, M. S., Zhang, Y., and Gou, X. (2019). Potential astrocytic receptors and transporters in the pathogenesis of Alzheimer's Disease. *J. Alzheimers Dis.* 67, 1109–1122. doi: 10.3233/jad-181084

Zhu, Y., Armstrong, J. L., Tchkonia, T., and Kirkland, J. L. (2014). Cellular senescence and the senescent secretory phenotype in age-related chronic diseases. *Curr. Opin. Clin. Nutr. Metab. Care* 17, 324–328. doi: 10.1097/MCO.0000000000000065

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Macrophages and Associated Ligands in the Aged Injured Nerve: A Defective Dynamic That Contributes to Reduced Axonal Regrowth

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The regenerative capacity of injured peripheral nerves is diminished with aging. To identify factors that contribute to this impairment, we compared the immune cell response in young vs. aged animals following nerve injury. First, we confirmed that macrophage accumulation is delayed in aged injured nerves which is due to defects in monocyte migration as a result of defects in site-specific recruitment signals in the aged nerve. Interestingly, impairment in both macrophage accumulation and functional recovery could be overcome by transplanting bone marrow from aged animals into young mice. That is, upon exposure to a youthful environment, monocytes/macrophages originating from the aged bone marrow behaved similarly to young cells. Transcriptional profiling of aged macrophages following nerve injury revealed that both pro- and anti-inflammatory genes were largely downregulated in aged compared to young macrophages. One ligand of particular interest was macrophage-associated secreted protein (MCP1), which exhibited a potent role in regulating aged axonal regrowth *in vitro*. Given that macrophage-derived MCP1 is significantly diminished in the aged injured nerve, our data suggest that age-associated defects in MCP1 signaling could contribute to the regenerative deficits that occur in the aged nervous system.

Keywords: aging, nerve injury, macrophages, microenvironment, MCP1, axonal regeneration

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INTRODUCTION

The mammalian PNS can regenerate following nerve injury, but the extent of recovery depends on several factors, including age; where age is negatively correlated with regeneration (Huebner and Strittmatter, 2009). Indeed, both axon regrowth and behavioral recovery are diminished in aged animals relative to young animals following an equivalent nervous injury (Pestronk et al., 1980; Tanaka and Webster, 1991; Vaughan, 1992; Verdú et al., 2000). The mechanisms

underlying this age-related neuro-regenerative deficiency remains unknown. Effective treatments that target the aging population to enhance recovery following nerve injury do not exist.

Previous work has demonstrated that aged axons retain their capacity for regrowth, equivalent to young axons, given the appropriate environment (Painter et al., 2014; Scheib and Höke, 2016). This suggests that the surrounding injury environment within the aged nerve contributes to the suppression of regeneration. Secreted factors from macrophages—located in regions distal to the injury site—can directly affect axonal regrowth (Kigerl et al., 2009). Depending on macrophage phenotypes, axonal regrowth can be either enhanced or suppressed. Others have provided a thorough characterization of nerve macrophages as well as demonstrated the importance of macrophages by preventing their accumulation following nerve injury (Tanaka and Webster, 1991; Barrette et al., 2008; Stratton et al., 2018; Ydens et al., 2020). This revealed that the number of regenerating nerve fibers and the extent of functional recovery is significantly reduced in mice where macrophage accumulation is inhibited.

Here, we sought to explore macrophage dynamics that are associated with defective axonal regeneration in aged mice. Specifically, we investigated changes in macrophage dynamics as a potential contributor to the loss of regenerative competence within the aged nerve. We discovered that the injured microenvironment within the aged nerve plays a critical role in shaping macrophage dynamics. Also, we demonstrated that the number of macrophages expressing Chemokine (C-C motif) ligand 2 (CCL2/MCP1) was reduced following nerve injury in aged mice and that exposing aged axons to MCP1 was able to significantly enhance their regrowth (Niemi et al., 2016). This data could suggest that reduced MCP1 signaling might contribute to the regenerative deficiency observed in the aged nervous system.

MATERIALS AND METHODS

Rodent Nerve Injuries

All experiments using animals were carried out following the Canadian Council of Animal Care Guidelines, and approved by the University of Calgary's Health Sciences Animal Care Committee. All surgeries and electrophysiological readings were carried out under isoflurane (2%–5%) inhalation anesthetic with postoperative pain control provided by intraperitoneal injections of buprenorphine (0.05 mg/kg) on the day of surgery, followed by buprenorphine on the days following. All surgical and electrophysiological procedures were carried out following the shaving of the hind limb area in an aseptic fashion using 70% ethanol. Under operating microscopes (M651, Leica), one sciatic nerve per animal was exposed at the sciatic notch and crushed for 10–20 s using beveled Dumont No. 5 forceps (L4$\frac{1}{4}$inch, Inox alloy, Sigma).

Mice

Mice were maintained at the University of Calgary's Animal Facility, under standard housing conditions. This included housing with 4–5 mice under a 12-h light-dark cycle, at 20–24°C, with unlimited food and water. *Cx3cr1-GFP/Ccr2-RFP* mice on a *C57BL/6* background (stock no.: 017586; stock No.: 005582, Jackson Laboratories), were kindly donated by Dr. Kubes (University of Calgary) in 2014. Mice were maintained *via* crossing *Cx3cr1-GFP*$^{+/+}$ *Ccr2-RFP*$^{+/+}$ male and *C57BL/6* female mice to obtain *Cx3cr1-GFP*$^{+/-}$ *Ccr2-RFP*$^{+/-}$ heterozygous offspring where both monocytes and macrophages were confirmed to have GFP and RFP reporters (Dal-Secco et al., 2015). *Sox2*^{tm2HochGFP} mice were maintained on a mixed background (The Jackson Laboratory). Young mice were aged 3–6 months, and aged mice were 16–24 months old.

CMAPs

Compound muscle action potential (CMAP) amplitudes were measured at 4, 6, and 8-weeks post-injury from uninjured and injured nerves, with the average value recorded from three measurements at the same stimulation intensity. The body temperature of animals was kept constant at 37°C ± 0.5°C throughout the experiment on a heating pad. The sciatic nerve was stimulated just above the sciatic notch using bipolar hook electrodes and the electromyogram activity was recorded (100×; 100 Hz to 1 kHz) using bipolar recording electrodes inserted into the gastrocnemius muscle of the corresponding hind limb. The experimenter was blinded to the post-injury time during recording.

Dorsal Root Ganglion (DRG) Neuronal Cell Culture

Dorsal root ganglions (DRGs) were dissected out, trimmed of nerve roots and connective tissue, and washed in L15 medium. The cleaned DRGs were incubated in 0.1% collagenase IV/L15 at 37°C for 60 min, then dissociated by pipette trituration. The resulting cell suspension was centrifuged at 100 *g* for 6 min, and the pellet resuspended in L15 medium for density gradient filtration: the suspension was carefully laid over a 15% BSA/L15 solution and centrifuged as above, followed by a final L15 resuspension and centrifugation. The final pellet of purified DRG neurons was resuspended in DMEM/F12 media and plated on PDL/laminin-coated 96-well plate at a density of 2,500 cells/mL. Growth media consisted of DMEM/F12 enriched with 1× N2 supplement, 50 U/ml penicillin-streptomycin, 0.1% BSA, and 0.1 ng/ml NGF. In each well, either MCP1 (500 ng/ml), NGF (100 ng/ml), Il6 (500 ng/ml), or Il1β (50 ng/ml) were added as per experimental conditions, with PBS used as a control. The plated neurons were allowed to grow for 18–24 h at 37°C before being fixed in 4% PFA/1× PHEM and washed in 1× PBS. Cell culture experiments were performed in triplicates and were repeated at least three times on independent days.

Immunocytochemistry

Fixed DRG neurons were incubated in blocking solution (5% donkey serum, 0.3% Triton X-100/1× PBS) for 30 min, primary antibodies in diluent (0.1% Triton X-100, 0.1% BSA, 0.04% EDTA/1× PBS) for 1 h, washed twice in PBS, secondary antibodies in diluent for 1 h, Hoechst-33258 (14530, Sigma, 1:500 in PBS) for 10 min, and finally washed twice in PBS.

All steps were performed at RT. Primary antibodies used were a cocktail of anti-Neurofilament200 (mouse, N5389, Sigma-Aldrich, 1:800) and anti-Tubulin β3 (mouse, 801213, Bio Legend, 1:1,000) to facilitate automated neurite analysis. The secondary antibody used was Alexa Fluor 488 donkey anti-mouse (A21202, Invitrogen, 1:200). Each well was imaged at 10× using the ImageXpress system (Molecular Devices) and a composite was stitched using Microsoft Image Composite Editor (Microsoft). The composite images were each divided into nine images of equal size and all analyzed using the MetaXpress (Molecular Devices) automated neurite outgrowth function.

Immunohistochemistry

Before tissue collection, mice were euthanized using CO_2 asphyxiation. Dissected nerves were post-fixed in 4% PFA at 4°C overnight and subsequently allowed to equilibrate in 30% sucrose at 4°C overnight, before embedding in cryoprotectant (VWR Clear Frozen Section Compound) for storage at −80°C. Sciatic nerve segments stretching from 2 mm proximal to 1 cm distal from the crash site were collected. Twelve micron thick longitudinal sections were cut and stored at −80°C. Two to three high-quality sections per nerve per condition were selected for immunohistochemistry. Briefly, sections were permeabilized and blocked in 0.3% Triton X-100 and 5% BSA (1+ hour at room temperature) before primary antibody incubation overnight at room temperature. Immunostains were done using anti-ionized calcium-binding adaptor molecule 1 (rabbit, Wako, 1:500), Anti-Murine JE/MCP-1 (rabbit, Peprotech, 1:500), anti-Stathmin-2 (rabbit, NBP1-49461, Novus Biologicals, 1:1,000), Purified anti-Tubulin β3 (mouse, 801213, BioLegend, 1:1,000). After washing, cells were incubated at room temperature for 1–2 h with secondary antibodies conjugated to either Alexa Fluor 488, 555 or 647 (donkey anti-mouse, rabbit, rat, goat, 1:200, Invitrogen), and Hoechst-33258 (1:1,000, 14530, Sigma), then washed and cover-slipped. All immunohistochemical (IHC) stains were confirmed positive with appropriate no primary (secondary alone) controls. Image collection and quantification was done using either Nikon A1 or a Leica SP8 confocal microscope. Whole nerve montages were collected using a 63× objective lens, Tile-Scan and Z-stack features, and maximum projection images. For all other images, sections were imaged at 63× objective, 10–20 serial z-stacks in 0.5–1.0 μm steps, then maximum projected before quantifying. Three images per animal were collected from the crash site. All imaging of a given stain was performed using the same laser settings. We used ImageJ and designated markers to count cells or cellular components of interest, as well as nuclear stains. All quantification was done while blinded.

Monocyte Migration Assay

Blood was collected from young and aged mice (n = 3 each) and centrifuged for 15 min at 300 *g*. The buffy coat was collected and the red blood cells were lysed. Using HBSS with 0.1% BSA, the cells were resuspended (1×10^6 blood cells) and plated in the bottom of migration assay wells with 0.6 ml of HBSS with 0.1% BSA and 1/3 of the nerve section dissected into 1 mm pieces. The control wells contained 5% FBS. All wells were topped with 100 μl of cell suspension (1×10^5 cells/well). Four hours later, the cells were collected from the bottom of the wells and analyzed using FACS sorting. The membranes were collected, fixed, and coverslipped with permount. The nerve tissue and supernatant from the bottom of the wells were collected and stored at −80°C.

Bone Marrow Transplants

The recipient mice were lethally irradiated with a dosing schedule of 2 × 5.5 Gy, 4 h apart using a gamma irradiator with a cesium-137 source (Gammacell 40; Best Theratronics). Donors were euthanized and the femurs isolated on ice. The bone marrow was flushed from the femurs with 10 ml of HBSS and then centrifuged for 15 min at 300 *g*. The red blood cells were lysed and bone marrow cells resuspended at 5×10^7 bone marrow cells/ml in HBSS. Two-hundred microliter (10×10^6 bone marrow cells) were injected *via* the tail vein of prepared recipients. Recipient mice were used for experimentation at 8 weeks post-transplant.

Macrophage FACs Isolations and RNAseq

At Day 3 and 8 post-nerve injury, *Cx3cr1*-GFP$^+$ *Ccr2*-RFP$^+$ double-positive cells were FACS collected from 5 months (young) and 18 months (aged) *Cx3cr1*^{GFP}/*Ccr2*^{RFP} mice. Following euthanasia, sciatic nerves were isolated, sterilized, and cleared of hair, membranes, and connective tissue. Nerves were finely chopped using a sterile blade, then put in collagenase (2 mg/ml, Worthington, Lakewood, NJ, USA) at 37°C for 30 min, triturating every 10 min until solutions appeared cloudy, then filtered with a 70 μm filter. Samples were centrifuged for 5 min at 200 *g*, the supernatant discarded, then re-suspended in 0.5% BSA in HBSS before FACS purification using a BD FACS Aria machine. A stringent initial gate was used to exclude debris and cell doublets. Wild-type cells were used as negative controls to determine gates for detecting GFP and RFP, and then macrophages were purified according to these gates. The number of cells collected from each animal is listed: Day 3 young (n = 7)—19,041, 13,548, 23,989, 8,621, 16,751, 8,621, 16,751; Day 3 aged (n = 7)—8,472, 11,194, 5,044, 2,338, 2,928, 2,338, 2,928. Due to low levels of RNA, samples were pooled so two samples per condition were analyzed. Total RNA ranged from 153 to 261 ng per pooled sample. All cells were sorted directly into lysis buffer (Ambion), then triturated and vortexed before storage at −80°C. For RNA extractions, all samples were processed together following manufacturers' recommendations (AM1561, Ambion). Total RNA extracts were quantitated using a Qubit Fluorimeter and the Qubit HS assay kit (Invitrogen). All samples were subjected to reverse transcription and Multiple Displacement Amplification (MDA) with REPLI-g SensiPhi DNA Polymerase and oligo-dT primers as per the Qiagen REPLI-g Single Cell RNA Library preparation kit (150073) and the manufacturer's protocol. The amplified cDNA product was then sheared to an average of 300 bp using a Covaris S220 sonicator and Covaris microtubes with AFA fibers (520045). Illumina compatible libraries were then prepared using the Qiagen GeneRead Adapter I Set A 12-plex (180985) index adapters as per the REPLI-g single-cell RNA

library prep kit's protocol. Products were quantitated and sized using both a Qubit fluorimeter with QuantiFluor dsDNA System dye (Promega E2670) and an Agilent 2200 TapeStation with D1000 ScreenTape and reagents (5067–5583). Five hundred to six hundred nanogram of the final library was obtained from each sample. Each library was quantitated using the Kapa qPCR Library Quantitation Kit for Illumina (KK4835) on a StepOne Plus qPCR instrument before preparing a single pool containing equal amounts of each library. This pool was then subjected to on-board cluster formation and sequencing on an Illumina NextSeq 500 sequencer with a high-output v2 75 cycle sequencing kit (FC-404-2005) as per the standard Illumina protocols. After sequencing, the bcl data was converted to fastq data files using the Illumina BCL2FASTQ utility. The sequencing run produced 475 million clusters passing filter (density = 206 K/mm^2) and 34.8 gigabases of sequencing with quality scores of ≥Q30 (91.4%). The number of assigned reads for each sample was between 39.1 million and 43.9 million. Reads were pseudoaligned to the mouse NCBI RefSeq transcript database (O'Leary et al., 2016) dated January 2017, using Kallisto 0.42.4 (Bray et al., 2016). Sleuth (Pimentel et al., 2017) was used for differential gene-level expression using a linear model containing one term: the nominal age factor. Genes passing the Wald Test with Benjamini-Hochberg corrected *p*-values (a.k.a. false discovery rate, FDR) less than 0.05 were considered differentially expressed. Differentially expressed genes were annotated and analyzed for enrichment using Ingenuity Pathway Analysis (Qiagen N.V., Redwood City, CA, USA).

Quantification and Statistical Analysis

For comparisons of groups across multiple groups, a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* comparison was used (GraphPad Prism 5). For comparisons of groups across two groups, an unpaired Student *t*-test was used. For comparisons of multiple factors across two groups, multiple unpaired Student *t*-tests with a false discovery rate of 5% were used. *P*-values < 0.05 was considered significant. All other statistical details can be found in figure legends and "Materials and Methods" section.

RESULTS

Diminished Axonal Regeneration in Aged Rodents Is Associated With the Aged Microenvironment

To confirm that axonal regeneration is defective in aged mice, we compared CMAP amplitudes in muscles innervated by previously injured nerves (**Supplementary Figure S1A**). This revealed that following a nerve crush injury, CMAP amplitudes were reduced to 9.06 ± 6.48% in the young group and 8.96 ± 4.71% (of uninjured) in the aged group at 4 weeks post-injury (**Supplementary Figure S1B**). Consistent with the literature (Verdú et al., 1995), by 8 weeks this had recovered to 52.34 ± 13.36% in young mice and only 33.84 ± 8.49% in aged mice (**Supplementary Figure S1B**). This confirms that axonal reinnervation is compromised in aged mice.

To understand whether this was due to intrinsic defects in the capacity of PNS neurons to regenerate axons, we used an *in vitro* approach to compare young vs. aged axon growth. We cultured DRG neurons from 2-month and 24-month old mice in parallel then assessed the percent of neurons with outgrowth, mean outgrowth per neuron, and branches per neuron after 16–24 h in culture. Interestingly, there was no significant difference in any metrics we assessed in young axons as compared to aged axons (**Supplementary Figures S1C–F**), consistent with other findings (Painter et al., 2014; Scheib and Höke, 2016). Together, this suggests that the aged microenvironment plays a role in dysregulated neuro-regenerative responses in aged mice.

Fewer Macrophages Are Found at the Injury Site of Aged Nerves

There are several potential factors in the aged microenvironment that might account for defects in axonal regeneration, including; defects in fibrosis (Martinod et al., 2017); deficient Schwann cell function (Painter et al., 2014); and shortcomings in the immune system (Montecino-Rodriguez et al., 2013). Given the widespread presence of macrophages at nerve injury sites and the prominent role macrophages play in PNS regeneration (Barrette et al., 2008; Stratton et al., 2018), it is highly likely that these cells are involved. Thus, we first assessed macrophage populations over time in the injured nerves of aged vs. young mice, to establish macrophage dynamics during nerve healing in aging. This revealed that at 3 days following injury, there were 3.6 times fewer macrophages in the aged nerves as compared to the young nerves (**Supplementary Figures 2A,B**; Day 3 Aged, 37.45 ± 12.56 vs. Day 3 Young, 135.5 ± 28.82) consistent with the findings of other studies (Painter et al., 2014). Intriguingly, macrophage numbers in aged nerves had already returned to values comparable to young nerves by 8 days (Day 8 Aged, 278.1 ± 18.86; Day 8 Young, 222.8 ± 44.56). Given that others have shown that macrophages enhance axonal regeneration (Kigerl et al., 2009), this could suggest that defective regeneration in aging is partially attributed to the absence of macrophages during the critical early-stage post-injury in the aged microenvironment.

Monocyte Recruitment Is Reduced With Age Due to Defects in PNS Recruitment Signals

To further our understanding of why there were reduced macrophage numbers in the aged injured nerve, we compared the motility and PNS-specific recruitment signals for young and aged monocytes *in vitro* (**Figures 1A–C**). We placed young or aged monocytes in a permeable vessel subjected to either young or aged nerve conditioned media (CM). We found that young monocytes were able to migrate similarly towards both young and aged nerve CM suggesting that young monocytes can overcome any age-related defects in recruitment signals from aged nerves (29.60 ± 2.14 and 26.00 ± 1.75, respectively). Aged monocytes however migrated significantly less to aged CM than to young CM (16.00 ± 0.71 and 25.80 ± 3.40, respectively). This suggests that the aged environment, rather than defects in aged

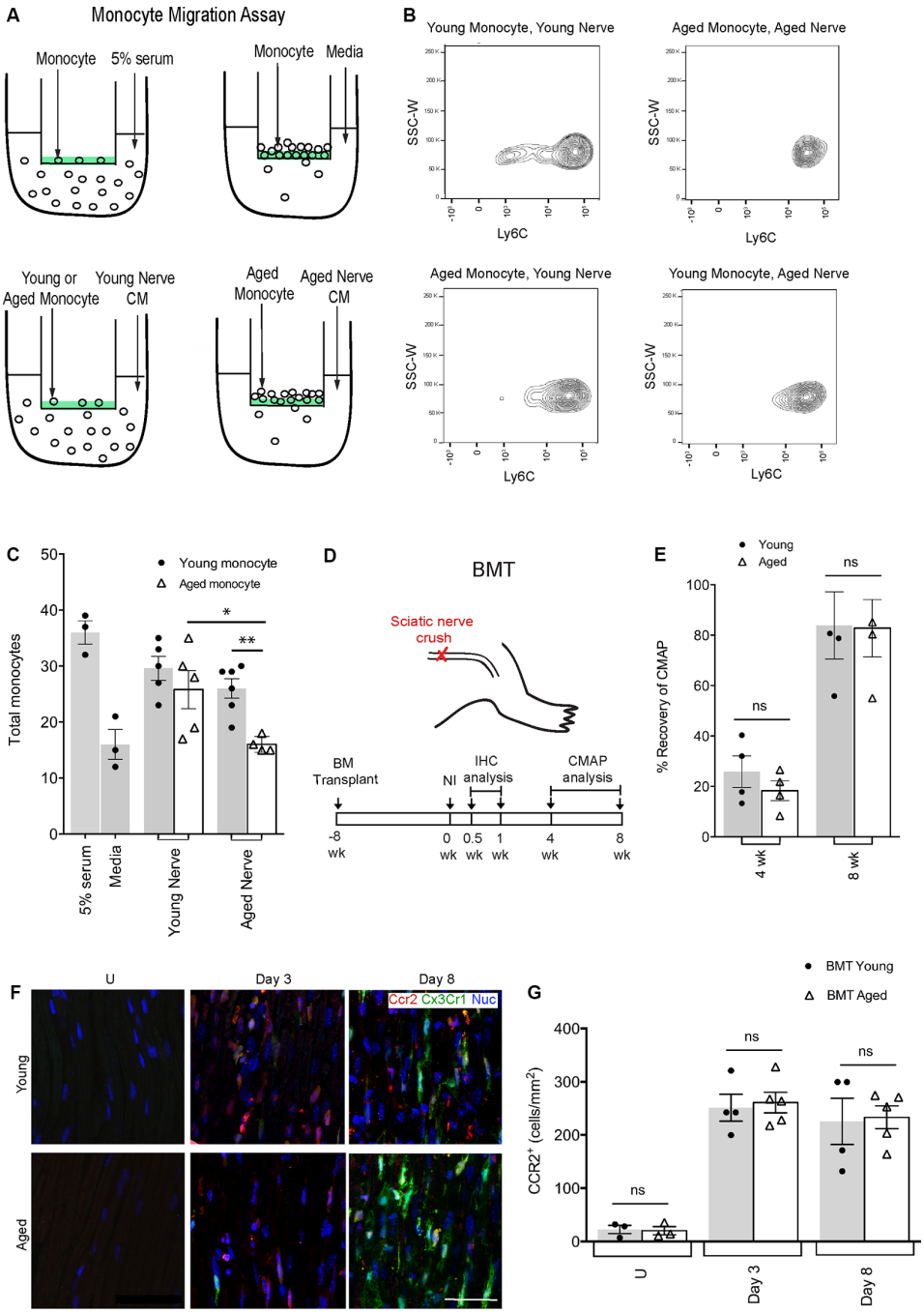

FIGURE 1 | Defects in aged macrophage dynamics can be rescued by providing a young environment. **(A)** Experimental design for monocyte migration assay. Monocytes were placed on a vessel containing a semipermeable membrane, inside a well containing either 5% serum (positive control), media (negative control), young nerve conditioned media (CM) or aged nerve CM. **(B)** Representative flow cytometry plots demonstrating a decrease in LyC6+ monocytes in the condition where aged monocytes were subject to aged CM (Aged Monocyte, Aged Nerve) compared to other groups. This effect was particularly evident in the $LyC6^{low}$ monocyte population. **(C)** Quantification revealed aged monocytes subjected to the aged nerve CM had similar migration as the negative control (media), while young monocytes subjected to young nerve CM had similar values to the positive control (5% serum). Aged monocytes in aged CM showed significantly lower migration than young monocytes in the aged CM. Aged monocytes in the aged CM also showed significantly lower migration than aged monocytes in young CM. $^{*}p < 0.05$ and $^{**}p < 0.02$. Error bars indicate ± SEM. **(D)** Experimental design for bone marrow transplant (BMT) experiments. The timeline shows BMT was performed at 8 weeks, sciatic nerve crush injury at 0 weeks, immunohistochemical (IHC) analysis at 0.5 and 1-week post-injury, and compound muscle action potential (CMAP) analysis at 4 and 8 weeks post-injury. **(E)** Quantification of CMAP analysis showing no significant difference in the percent recovery of CMAP amplitude between the young and aged animals at 4 and 8 weeks post-injury, thus illustrating similar functional recovery. $n = 4$ per group; ns, no significance. Error bars indicate ± SEM. **(F,G)** Representative IHC images and supporting quantification showed that at day 3 and 8 post-injury, there was no significant difference in macrophage numbers between young and aged animals. $n = 4$–5 per group; ns, no significance. Error bars indicate ± SEM. Scale bar, 50 μm.

monocyte motility/chemoattractant sensing, is necessary for defective recruitment.

A Young Environment Can Rescue Defects in Aged Macrophage Accumulation and Functional Recovery

To further clarify the environmental influence in regulating aged macrophage dynamics, we performed bone marrow transplants (BMT; **Figure 1D**). Bone marrow from *Cx3cr1-GFP*$^+$/*Ccr2-RFP*$^+$ donor mice (either 18–20-month-old or 2-month-old) was transplanted into young recipient C57BL/6 mice. By 8 weeks post-BMT, over 90% of circulating monocytes were *Ccr2*-RFP$^+$ and *Cx3cr1*-GFP$^+$ and thus derived from aged or young transplanted bone marrow (not shown). We then performed peripheral nerve crush injuries and assessed *Ccr2*-RFP$^+$/*Cx3cr1*-GFP$^+$ macrophages at 3 and 8-days post-injury (**Figures 1F,G**). We found no difference in nerve macrophage numbers between the aged or young BMT mice at either time point, suggesting that any defects in macrophage accumulation in aging (**Supplementary Figures S2A,B**) could be rescued given a more permissive (young animal) environment. Importantly, the percent recovery of CMAPs was also the same for recipients of young or aged bone marrow (**Figure 1E**). Together these data suggest that aged macrophages are inherently capable of supporting nerve repair if provided appropriate instructions.

MCP1, a Chemokine Primarily Expressed by Macrophages, Is Downregulated With Advanced Age and Can Significantly Enhance Axonal Growth *in vitro*

A previous transcriptional profiling study using bulk-RNAseq of injured nerves from 2 and 24-month old mice highlighted age-associated changes in gene expression (Painter et al., 2014). Genes associated with immune signaling were especially intriguing (Stratton et al., 2018). While some genes such as *Tnf, Lif*, and *Igf1* showed no difference with regards to age, *Il1*β, *Il6*, and *Ccl2* (MCP1) were of particular interest as they were found to be downregulated in aging (**Figure 2A**). To better understand the direct role that Il1β, Il6, and MCP1 may play in regulating axonal regeneration under-aged conditions, we assessed axonal regrowth in aged DRG neurons following their stimulation with these factors *in vitro* (**Figures 2B,C**). While l1β had no effect, we found that MCP1 consistently increased neurite regrowth; it increased the percentage of neurons with neurites from 39.93 ± 1.13% to 53.91 ± 0.83%, the mean outgrowth per neuron from 264.6 ± 51.94 μm to 655.6 ± 49.99 μm and the number of branches per neuron from 7.46 ± 1.66 to 21.48 ± 1.44 (**Figure 2C**). Il6 also showed a statistically significant increase in the mean outgrowth per neuron (**Figure 2C**). Il6 is a known pro-regenerative cytokine, and the modest increase may be explained by its action on initiating regenerative cascades or overriding inhibitory signals (Cao et al., 2006; Pieraut et al., 2011; Cox et al., 2017).

We next assessed the cell types expressing MCP1. As expected from prior genetic analyses (Stratton et al., 2018), our immunofluorescence analysis showed significant colocalization between MCP1+ and CCR2+ macrophages (78.58 ± 6.79% of MCP1 positive cells were macrophages; **Figures 2D,E**). Quantification also showed MCP1 was expressed by Schwann cells (10.81 ± 3.83%), and other nucleated endoneurial cells (12.26 ± 2.74%), but not axons (0%; **Figures 2D,E**). Next, to determine if *Ccl2* (MCP1) was differentially expressed between aged vs. young macrophages, we assessed the transcriptional profile of macrophages post-nerve injury from Cx3cr1-GFP/Ccr2-RFP transgenic reporter mice and found no significant difference between groups (**Figures 2F,G**). However, IHC analysis revealed a marked decrease in the number of MCP1+ CCR2+ macrophages in aged animals as compared to young (**Figures 2H,I**). This suggests that while *Ccl2 (MCP1)* transcription in macrophages is not dysregulated with aging, the overall numbers of recruited MCP1+ macrophages are diminished with advanced age, likely contributing to a net reduction of *Ccl2 (*MCP1*)* in the aged nerve after injury (Painter et al., 2014).

Both Pro- and Anti-inflammatory Cytokines Expressed by Macrophages Following Nerve Injury Are Downregulated in Aged Mice Compared to Young Mice

To identify other transcriptional differences between aged and young macrophages following nerve injury, we compared the transcriptomes of macrophages from aged and young nerves at day 3 post-injury (**Figure 3**; young data previously analyzed in (Stratton et al., 2018). Analysis of the top 50 most differentially regulated transcripts revealed a separation in the pattern of gene expression between aged and young macrophages (**Figure 3C**). The expression of some well-characterized macrophage-associated factors *(Itgam, Mrc1*) but not others (*Ccr2, Cx3cr1*) were downregulated with aging (**Figure 3D**). In aged macrophages, we also observed suppression of factors typically associated with both a pro-inflammatory macrophage phenotype (*Il6, Cxcl10, Il12*β*, Il1*β*, Tnf*) and an anti-inflammatory state (*Trem2, Stat6, Il10, Hbegf, and Retnla*; **Figure 3E**).

When we assessed the top regulated canonical pathways in aging, we found the three most highly downregulated pathways were; Retinoic acid receptor (RAR) Activation, Estrogen receptor signalling and Peroxisome proliferator-activated receptors (PPAR) Signaling; while the top regulated activation z-scores for cellular functions were; "Organismal death" (Activation Z-score: 2.2; *P*-value: 6.4 × 10–03), "Organization of cytoskeleton" (Activation Z-score: −2.1; *P*-value: 6.7 × 10–04), "Microtubule dynamics" (Activation Z-score: −2.1; *P*-value: 1.6 × 10–03) and "Coordination" (Activation Z-score: −2.2; *P*-value: 8.3 × 10–03). Others have also described age-associated dysregulation of RAR and PPAR signaling in myeloid cells and found that phagocytosis impairment due to a dysregulation in this pathway delayed nervous system regeneration (Natrajan et al., 2015).

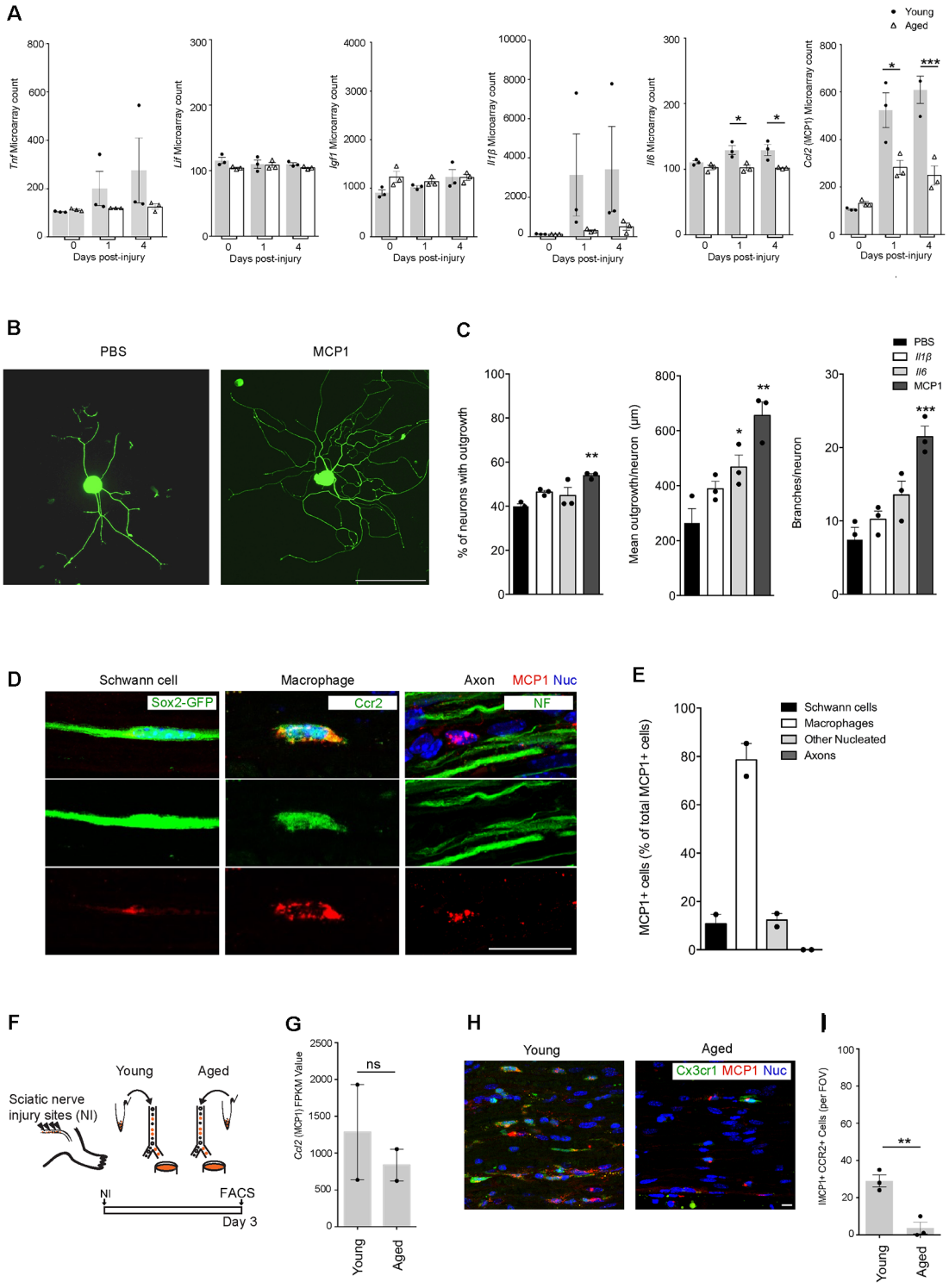

FIGURE 2 | MCP1 positive macrophages are reduced in aging, and MCP1 is a potent promoter of axonal outgrowth. **(A)** Selection of ligands expressed in nerves before and after the injury as per microarray dataset (12). Note the decrease in aged nerves of some (*Il6*, *Ccl2*) but not other (*Igf-1*, *Lif*) cytokines and growth factors known to be expressed by macrophages (19). $*p < 0.03$ for the *Il6* graph (vs. PBS), $*p < 0.05$; $***p < 0.0001$ for the *MCP1* graph (vs. PBS). **(B,C)** Representative IHC images **(B)** and supporting quantification **(C)** demonstrating that neurite outgrowth was increased in dorsal root ganglion (DRG) neurons cultured *in vitro* with MCP1 vs. PBS, IL1β, and IL6. IL6 showed significant improvement in the mean outgrowth per neuron compared to PBS **(C)**. The percent of neurons with outgrowth, mean outgrowth per neuron, and branches per neuron were all shown to be enhanced with MCP1. $**p < 0.007$, $**p < 0.002$ and $***p < 0.0001$ (vs. PBS) for

(*Continued*)

FIGURE 2 | Continued
graphs 1–3, respectively. Scale bar, 200 μm. **(D,E)** Representative IHC images **(D)** and quantification **(E)** demonstrated that MCP1 is primarily expressed by macrophages (Ccr2, green) in the injured nerve, and not Schwann cells (Sox2-GFP, green), axons (NF, green) and other nucleated cells. Scale bar, 5 μm. **(F)** Experimental design for macrophage RNAseq analysis. Macrophages were FACS collected from young and aged *Cx3cr1*^{GFP}/*Ccr2*^{RFP} mice 3 days after injury. Cells were lysed and sequenced as per the methods section. **(G)** Macrophage RNAseq analysis revealed no significant difference between the *Mcp1* FPKM values in young vs. aged groups. FPKM, Fragments Per Kilobase of transcript per Million mapped reads. ns = no significance. **(H,I)** Representative IHC images **(H)** and supporting quantification **(I)** demonstrated less MCP1+ macrophages per field of view (FOV) between aged and young injured nerves. $^{**}p < 0.005$. Scale bar, 10 μm. All error bars indicate ± SEM.

DISCUSSION

To date, numerous studies have outlined the phenomenon of impaired axonal regeneration in aged animals, however, the causes of this from a macrophage perspective have yet to be fully explored. Through observing similar growth rates between young and aged DRG neurons *in vitro*, it was found that the declining regenerative capacity with age could be attributed to the aged microenvironment, which aligns with previously published literature (Kang and Lichtman, 2013; Painter et al., 2014; Scheib and Höke, 2016). In previous experiments, age-mismatch sciatic nerve grafts demonstrated that aged animals could achieve robust functional recovery upon receiving a young nerve graft (Painter et al., 2014; Scheib and Höke, 2016). Conversely, young animals receiving aged nerve grafts displayed similar regeneration to the aged phenotype. We found that aged macrophage numbers can be rescued in young nerve environments and achieve functional recovery similar to young conditions. This provides compelling evidence that macrophage dysregulation is likely one, of several surrounding dysregulated mechanisms, in the aged nerve injury environment. Several factors likely contribute to dysregulated macrophage dynamics. Blood vessel function is impaired, including dysfunction of endothelial, smooth muscle cells, and extracellular matrix vasculature, which could impair infiltration (Rubio-Ruiz et al., 2014). Reduced expression of chemoattractants at the injury site, such as Il6, important for initiating recruitment could also contribute (Tofaris et al., 2002; Painter et al., 2014) as our data suggests. An alteration in macrophage phenotype (Chen et al., 2015), or reduced phagocytic capacity (Natrajan et al., 2015), would also modify macrophage function in a manner which is detrimental to regeneration. And finally, as we have suggested here, a reduction in pro-regenerative macrophage secreted factors, not limited to MCP1, may also contribute.

Previous studies have used high-throughput whole-tissue profiling of immune factors following nerve injury in aged vs. young nerves at acute time points (Painter et al., 2014; Büttner et al., 2018). Given the delay in the accumulation of macrophages with aging, differential expression between groups is difficult to interpret in these studies. Are reductions in immune-associated factors simply due to reductions in infiltrated cell numbers? Or does this reflect a difference in the endogenous capacity of a given macrophage to express genes or proteins of interest? For MCP1, we found that the profile of each individual cell is no different between aged and young macrophages, but there are far fewer macrophages recruited to the injury site in aging which accounts for the reduced overall MCP1 levels in aging conditions (Painter et al., 2014). Employing our macrophage-specific reporter system followed by FACS to prospectively isolate mobilized cells directly from injured nerves enabled us to more directly identify macrophage transcripts, in an unbiased fashion *in vivo*. In support of many conclusions made using bulk tissue analysis approaches, we also observed a decrease in several immune-related genes in macrophages with aging follow acute injury (including *Il*β*, Il6*, and *Cxcl1*). But in contrast to others (Painter et al., 2014), we found that several genes were unchanged in macrophages, including *Ccl11*, *Il16* and *Ccl2*; while others that were upregulated in our dataset were not altered in other datasets (*Cxcl10*, *Il10*, Painter et al., 2014; Büttner et al., 2018). Perhaps this discrepancy reflects changes due to regulation in other cell types, such as the mesenchymal population, or other immune cells which also express several immune-associated genes (Carr et al., 2019) and are sequenced when using whole-tissue techniques. In any case, our data identifies several candidate immunomodulatory factors in macrophages that are regulated with aging within the injured peripheral nerve. This highlights the importance of including population-specific gene expression studies or single-cell studies to better elucidate cell-type-specific transcriptional changes within injured tissues (Stratton et al., 2018; Ydens et al., 2020). Two other studies have used this type of approach to study nerve injury but only in young mice. Together these studies similarly report that *Trem2, Chil3, Arg1*, and several other pro and anti-inflammatory genes are dynamically regulated following injury (Stratton et al., 2018; Ydens et al., 2020). Ydens et al. (2020) also described two steady-state resident macrophage phenotypes and confirmed that the major macrophage type present following injury is the infiltrating macrophage, consistent with the findings reported by fate mapping strategies (Plemel et al., 2020).

We were surprised to find that genes associated with either a classic inflammatory (M1) or anti-inflammatory (M2) macrophage phenotype did not have opposing patterns of expression correlating with aging (Lee et al., 2013; Jablonski et al., 2016). Instead, both gene sets were largely suppressed acutely in aging following nerve injury. There are suggestions that a pro-inflammatory environment is detrimental to neuro-regeneration and an anti-inflammatory environment is beneficial (Mokarram et al., 2012). As an extension, it is reasonable to suggest that a pro-inflammatory environment in aging might be a contributing factor that impairs efficient regeneration, whereas an anti-inflammatory environment would benefit the aging environment (Büttner et al., 2018). Interestingly, both our results and others (Büttner et al., 2018) do not support this suggestion. Rather, both pro- and anti-inflammatory genes are suppressed acutely following nerve injury and largely continue to be dysregulated as time passes, or with macrophage modulation therapies (Büttner et al., 2018). Like us, others have also described

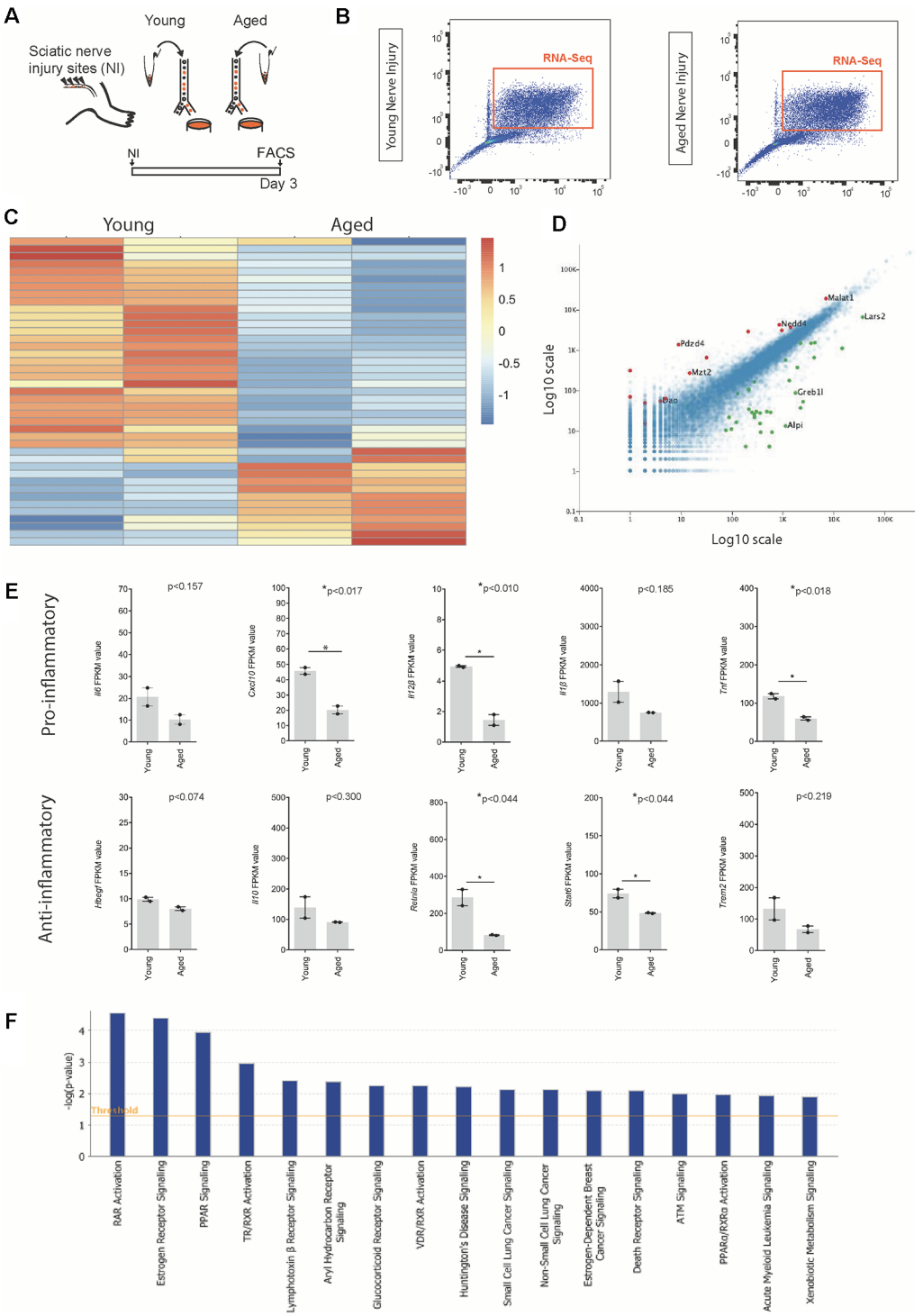

FIGURE 3 | Identification of ligands expressed by macrophages following nerve injury. **(A)** Experimental design for macrophage RNAseq analysis. *Cx3cr1*-GFP^{+} *Ccr2*-RFP^{+} double-positive macrophages were FACS collected from young and aged *Cx3cr1*^{GFP}/*Ccr2*^{RFP} mice animals 3 days after injury. Cells were lysed and sequenced as per the methods section. **(B)** FACS plots depicting cell sorting of *Ccr2*-RFP and *Cx3cr1*-GFP positive macrophage cells from the injured nerve. Cells boxed in orange were collected for RNA sequencing. **(C)** Heat map showing the unique expression profile of the top 50 most differentially regulated transcripts between young and aged mice at 3 days post-injury. Red indicates a fold increase while blue indicates a fold decrease. **(D)** Volcano plot demonstrating significantly upregulated (red, +1) and downregulated (green, −1) genes in aged vs. young macrophages. **(E)** FPKM values of common pro-inflammatory (M1) and anti-inflammatory (M2) macrophage identifiers. Error bars indicate ±SEM. **(F)** Top canonical pathways regulated with aging macrophages at day 3 post-injury (threshold cut off 0.05).

age-associated dysregulation of RAR and PPAR signaling, specifically concerning macrophage phagocytosis in the CNS (Natrajan et al., 2015). In aging, it is thought that a reduction in phagocytosis contributes to delayed nervous system regeneration, where defects in retinoic acid signaling underlie this dysfunction (Natrajan et al., 2015). Now that we have identified dysregulated signaling pathways in aging macrophages *in vivo*, future experiments endeavor to manipulate these signatures in nerve injury environments with the hopes of identifying pathways that can be therapeutically modulated to improve regeneration.

Both acute and chronic dysregulation in macrophage dynamics likely contribute to reduced axonal regrowth (Painter et al., 2014; Scheib and Höke, 2016) but we believe the immediate delay in macrophage numbers may play a crucial role in impaired regeneration. This is because, during acute time points, regeneration is usually extremely efficient and axonal regrowth is widespread (McQuarrie et al., 1977; Danielsson et al., 1996; Wang et al., 2015) thus depriving the aged nerve of pro-regenerative macrophages at this critical stage could interfere with this process. Given macrophages express MCP1 at high levels, and MCP1 promotes axonal regrowth, this could be one possible mechanism underlying why axonal regeneration in aging is reduced. MCP1/CCL2 has an affinity for CCR2 and CCR4 receptors, which are both expressed by neurons (Coughlan et al., 2000; Banisadr et al., 2005; Cédile et al., 2017). Kwon et al. (2015) previously showed that an intraganglionic injection of recombinant MCP1 into the L5 DRG of young rats caused significantly greater macrophage accumulation as well as significantly larger neurite outgrowth while injecting other chemokines (CX3CL1 and CCL3) only resulted in macrophage accumulation (Kwon et al., 2015). Also, Niemi et al. (2016) described the effects of MCP1 signaling on axonal growth in young rodents. By infecting neurons with AAV5-CCL2 they found that increased axonal outgrowth was dependent on CCR2 and STAT3 signaling. These findings agree with our data in aged axons, albeit we demonstrate that exogenous application of MCP1 is sufficient to support enhanced axon growth *ex vivo*. We used semi-automated neurite analysis and found an increase in several parameters: mean outgrowth per cell, an increase in the branching of neurites as well as an increase in the percentage of neurons with outgrowth. This was unique to MCP1 given other chemokines such as CCL3 and CX3CL1 had no effect (not shown).

Interestingly, we found the major cell type expressing MCP1 at the site of injury was macrophages, with no detectable levels in axons and substantially less in Schwann cells or other cells within the endoneurial microenvironment. When Kwon and colleagues assessed the ganglion, where the neuronal cell bodies lie, they attributed the major source of MCP1 to be neurons (Kwon et al., 2015). This illustrates the clear regional differences in protein expression apparent within neurons (Matus et al., 1981; Drake and Lasek, 1984). Others have suggested that Schwann cell-derived MCP1 is essential for glia-derived recruitment signals (Subang and Richardson, 2001; Taskinen and Röyttä, 2001; Tofaris et al., 2002). Our characterization of MCP1 expression across various cell types after nerve injury, reveals for the first time that macrophages are the overwhelming source of MCP1 within the endoneurial environment after injury.

MCP1 is a particularly difficult immune-factor to study *in vivo*, partly because it plays a role in both attracting immune cells (Tofaris et al., 2002) and is a growth factor for peripheral axons. Therefore, manipulation of this protein in macrophages *in vivo* would affect its role as a macrophage chemoattractant and as a neurotrophic factor. Dissecting the relative contribution of each role to overall regeneration would be complex. One could imagine the over-expression of MCP1 could result in over-recruitment of macrophages *in vivo*, which may result in neurotoxic effects that negate the neurotrophic benefit (Gensel et al., 2009; Kigerl et al., 2009). This supports our use of an *in vitro* approach, demonstrating directly that MCP1 does have the capacity to enhance axonal regeneration of aged injured nerves.

While our findings indicate that the addition of MCP1 allows for greater axonal regrowth, which provides support for the use of this chemokine/growth factor as a therapeutic agent for neural repair in aged patients, it is important to note that this chemokine/growth factor is also linked to many deleterious health conditions. MCP1 has been shown to have pro-tumorigenic roles that aid in metastasis and has been linked to cardiovascular disease, type 1 diabetes, and obesity (Deshmane et al., 2009; Panee, 2012). For this reason, any future therapeutic strategies must take caution to provide targeted approaches. Further studies assessing how MCP1 can be administered to promote neural repair without causing unintended negative consequences are of great interest.

DATA AVAILABILITY STATEMENT

The RNAseq data reported in this study has been deposited under GEO submission GSE132882, and portions of this dataset are also found at GSE106927.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Calgary Health Sciences Animal Care Committee, in accordance with the Canadian Council of Animal Care Guidelines.

AUTHOR CONTRIBUTIONS

SJ, JS, and JB wrote the manuscript. JS and JB designed the experiments. JS, SE, NR, AH, SJ, and GY performed the experiments and analyzed the data. RM, SE, NR, SJ, JB, and JS read and edited the article. RM and JB supervised all experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.00174/full#supplementary-material.

FIGURE S1 | Aged rodents display reduced recovery post-nerve injury, while aged axons regrow efficiently *in vitro*. **(A)** Experimental design. CMAP analysis was performed in rodents 4 weeks, 6 weeks, and 8 weeks after a sciatic nerve crush injury. **(B)** Quantification of CMAP data showed that aged animals had significantly less % recovery of CMAP amplitude as compared to the young group. n = 5–6 per group; $^*p < 0.05$. Error bars indicate ± SEM. **(C–F)** Representative images and quantification of *in vitro* DRG growth assay demonstrated that neurites from mice aged 2 months and 24 months showed no significant difference in the percent of neurons with outgrowth **(D)** mean outgrowth per neuron **(E)** and branches per neuron **(F)**. n = 3 per group. Error bars indicate ± SEM. Scale bar, 500 μm. NI, Nerve injured; CMAP, Compound muscle action potential; DRG, Dorsal root ganglion; ns, no significance.

FIGURE S2 | Macrophage response following nerve injury is delayed in aged rodents. **(A,B)** Representative immunohistochemical images demonstrated that following nerve injury, there are reduced macrophage densities at day 3 post-injury in aged rodents compared to young **(A)**. This observation was also supported by quantification **(B)**. n = 3–4 per time point; $^*p < 0.05$. Error bars indicate ±SEM. Scale bar, 50 μm. U, Uninjured.

REFERENCES

Banisadr, G., Gosselin, R., Mechighel, P., Rostène, W., Kitabgi, P., and Parsadaniantz, S. (2005). Constitutive neuronal expression of CCR2 chemokine receptor and its colocalization with neurotransmitters in normal rat brain: functional effect of MCP-1/CCL2 on calcium mobilization in primary cultured neurons. *J. Comp. Neurol.* 192, 178–192. doi: 10.1002/cne.20729

Barrette, B., Hébert, M.-A., Filali, M., Lafortune, K., Vallières, N., Gowing, G., et al. (2008). Requirement of myeloid cells for axon regeneration. *J. Neurosci.* 28, 9363–9376. doi: 10.1523/JNEUROSCI.1447-08.2008

Bray, N. L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal RNA-Seq quantification. *Nat. Biotechnol.* 34, 525–527. doi: 10.1038/nbt.3519

Büttner, R., Schulz, A., Reuter, M., Akula, A. K., Mindos, T., Carlstedt, A., et al. (2018). Inflammaging impairs peripheral nerve maintenance and regeneration. *Aging Cell* 17, 1–15. doi: 10.1111/acel.12833

Cao, Z., Gao, Y., Bryson, J. B., Hou, J., Chaudhry, N., Siddiq, M., et al. (2006). The cytokine interleukin-6 is sufficient but not necessary to mimic the peripheral conditioning lesion effect on axonal growth. *J. Neurosci.* 26, 5565–5573. doi: 10.1523/JNEUROSCI.0815-06.2006

Carr, M. J., Toma, J. S., Johnston, A. P. W., Frankland, P. W., Kaplan, D. R., and Miller, F. D. (2019). Mesenchymal precursor cells in adult nerves contribute to mammalian tissue repair and regeneration. *Cell Stem Cell* 24, 240–256. doi: 10.1016/j.stem.2018.10.024

Cédile, O., Wlodarczyk, A., and Owens, T. (2017). CCL2 recruits T cells into the brain in a CCR2- independent manner. *Acta Pathol. Microbiol. Immunol. Scand.* 125, 945–956. doi: 10.1111/apm.12740

Chen, P., Piao, X., and Bonaldo, P. (2015). Role of macrophages in Wallerian degeneration and axonal regeneration after peripheral nerve injury. *Acta Neuropathol.* 130, 605–618. doi: 10.1007/s00401-015-1482-4

Coughlan, C. M., McManus, C. M., Sharron, M., Murphy, D., Jaffer, S., Chen, W., et al. (2000). Expression of multiple functional chemokine receptors and monocyte chemoattractant protein-1 in human neurons. *Neuroscience* 97, 591–600. doi: 10.1016/s0306-4522(00)00024-5

Cox, A. A., Sagot, Y., Hedou, G., Grek, C., Wilkes, T., Vinik, A. I., et al. (2017). Low-dose pulsatile interleukin-6 as a treatment option for diabetic peripheral neuropathy. *Front. Endrocrinol.* 8:89. doi: 10.3389/fendo.2017.00089

Dal-Secco, D., Wang, J., Zeng, Z., Kolaczkowska, E., Wong, C. H. Y., Petri, B., et al. (2015). A dynamic spectrum of monocytes arising from the in situ reprogramming of $CCR2^+$ monocytes at a site of sterile injury. *J. Exp. Med.* 212, 447–456. doi: 10.1084/jem.20141539

Danielsson, P., Dahlin, L., and Povlsen, B. (1996). Tubulization increases axonal outgrowth of rat sciatic nerve after crush injury. *Exp. Neurol.* 243, 238–243. doi: 10.1006/exnr.1996.0097

Deshmane, S. L., Kremlev, S., Amini, S., and Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interf. Cytokine Res.* 29, 313–326. doi: 10.1089/jir.2008.0027

Drake, P. F., and Lasek, R. J. (1984). Regional differences in the neuronal cytoskeleton. *J. Neurosci.* 4, 1173–1186. doi: 10.1523/JNEUROSCI.04-05-01173.1984

Gensel, J. C., Nakamura, S., Guan, Z., Van Rooijen, N., Ankeny, D. P., and Popovich, P. G. (2009). Macrophages promote axon regeneration with concurrent neurotoxicity. *J. Neurosci.* 29, 3956–3968. doi: 10.1523/JNEUROSCI.3992-08.2009

Huebner, E. A., and Strittmatter, S. M. (2009). Axon regeneration in the peripheral and central nervous systems. *Results Probl. Cell Differ.* 48, 339–351. doi: 10.1007/400_2009_19

Jablonski, K. A., Gaudet, A. D., Amici, S. A., Popovich, P. G., and Guerau-de-Arellano, M. (2016). Control of the inflammatory macrophage transcriptional signature by miR-155. *Public Libr. Sci. One* 11:e0159724. doi: 10.1371/journal.pone.0159724

Kang, H., and Lichtman, J. W. (2013). Motor axon regeneration and muscle reinnervation in young adult and aged animals. *J. Neurosci.* 33, 19480–19491. doi: 10.1523/JNEUROSCI.4067-13.2013

Kigerl, K. A., Gensel, J. C., Ankeny, D. P., Alexander, J. K., Donnelly, D. J., and Popovich, P. G. (2009). Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J. Neurosci.* 29, 13435–13444. doi: 10.1523/JNEUROSCI.3257-09.2009

Kwon, M. J., Shin, H. Y., Cui, Y., Kim, H., Thi, A. H. L., Choi, J. Y., et al. (2015). CCL2 mediates neuron-macrophage interactions to drive proregenerative macrophage activation following preconditioning injury. *J. Neurosci.* 35, 15934–15947. doi: 10.1523/JNEUROSCI.1924-15.2015

Lee, D. C., Ruiz, C. R., Lebson, L., Selenica, M.-L. B., Rizer, J., Rojiani, R., et al. (2013). Aging enhances classical activation but mitigates alternative activation in the CNS. *Neurobiol. Aging* 34:130. doi: 10.1016/j.neurobiolaging.2012.12.014

Martinod, K., Witsch, T., Erpenbeck, L., Savchenko, A., Hayashi, H., Cherpokova, D., et al. (2017). Peptidylarginine deiminase 4 promotes age-related organ fibrosis. *J. Exp. Med.* 214, 439–458. doi: 10.1084/jem.20160530

Matus, A., Bernhardt, R., and Hugh-Jones, T. (1981). High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Cell Biol.* 78, 3010–3014. doi: 10.1073/pnas.78.5.3010

McQuarrie, I. G., Grafstein, B., and Gershon, M. D. (1977). Axonal regeneration in the rat sciatic nerve: effect of a conditioning lesion and of dbcAMP. *Brain Res.* 132, 443–453. doi: 10.1016/0006-8993(77)90193-7

Mokarram, N., Merchant, A., Mukhatyar, V., Patel, G., and Bellamkonda, R. (2012). Effect of modulating macrophage phenotype on peripheral nerve

repair. *Biomaterials* 33, 8793–8801. doi: 10.1016/j.biomaterials.2012.08.050

Montecino-Rodriguez, E., Berent-Maoz, B., and Dorshkind, K. (2013). Causes, consequences, and reversal of immune system aging. *J. Clin. Invest.* 123, 958–965. doi: 10.1172/jci64096

Natrajan, M. S., de la Fuente, A. G., Crawford, A. H., Linehan, E., Nuñez, V., Johnson, K. R., et al. (2015). Retinoid X receptor activation reverses age-related deficiencies in myelin debris phagocytosis and remyelination. *Brain* 138, 3581–3597. doi: 10.1093/brain/awv289

Niemi, J. P., Defrancesco-Lisowitz, A., Cregg, J. M., Howarth, M., and Zigmond, R. E. (2016). Overexpression of the monocyte chemokine CCL2 in dorsal root ganglion neurons causes a conditioning-like increase in neurite outgrowth and does so *via* a STAT3 dependent mechanism. *Exp. Neurol.* 275, 25–37. doi: 10.1016/j.expneurol.2015.09.018

O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufo, S., Haddad, D., McVeigh, R., et al. (2016). Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion and functional annotation. *Nucleic Acids Res.* 4, D733–D745. doi: 10.1016/j.expneurol.2015.09.018

Painter, M. W., Brosius Lutz, A., Cheng, Y. C., Latremoliere, A., Duong, K., Miller, C. M., et al. (2014). Diminished Schwann cell repair responses underlie age-associated impaired axonal regeneration. *Neuron* 83, 331–343. doi: 10.1016/j.neuron.2014.06.016

Panee, J. (2012). Monocyte chemoattractant protein 1 (MCP-1) in obesity and diabetes. *Cytokine* 60, 1–12. doi: 10.1016/j.cyto.2012.06.018

Pestronk, A., Drachman, D. B., and Griffin, J. W. (1980). Effects of aging on nerve sprouting and regeneration. *Exp. Neurol.* 70, 65–82. doi: 10.1016/0014-4886(80)90006-0

Pieraut, S., Lucas, O., Sangari, S., Sar, C., Boudes, M., Bouffi, C., et al. (2011). An autocrine neuronal interleukin-6 loop mediates chloride accumulation and NKCC1 phosphorylation in axotomized sensory neurons. *J. Neurosci.* 31, 13516–13526. doi: 10.1523/JNEUROSCI.3382-11.2011

Pimentel, H., Bray, N. L., Puente, S., Melsted, P., and Pachter, L. (2017). Differential analysis of RNA-seq incorporating quantification uncertainty. *Nat. Methods* 14, 687–690. doi: 10.1038/nmeth.4324

Plemel, J. R., Stratton, J. A., Michaels, N. J., Rawji, K. S., Zhang, E., Sinha, S., et al. (2020). Microglia response following acute demyelination is heterogeneous and limits infiltrating macrophage dispersion. *Sci. Adv.* 6:eaay6324. doi: 10.1126/sciadv.aay6324

Rubio-Ruiz, M. E., Pérez-Torres, I., Soto, M., Pastelín, G., and Guarner-Lans, V. (2014). Aging in blood vessels. Medicinal agents FOR systemic arterial hypertension in the elderly. *Ageing Res. Rev.* 18, 132–147. doi: 10.1016/j.arr.2014.10.001

Scheib, J. L., and Höke, A. (2016). An attenuated immune response by Schwann cells and macrophages inhibits nerve regeneration in aged rats. *Neurobiol. Aging* 45, 1–9. doi: 10.1016/j.neurobiolaging.2016.05.004

Stratton, J. A., Holmes, A., Rosin, N. L., Sinha, S., Vohra, M., Burma, N., et al. (2018). Macrophages regulate schwann cell maturation after nerve injury. *Cell Rep.* 24, 2561.e6–2572.e6. doi: 10.1016/j.celrep.2018.08.004

Subang, M. C., and Richardson, P. M. (2001). Influence of injury and cytokines on synthesis of monocyte chemoattractant protein-1 mRNA in peripheral nervous tissue. *Eur. J. Neurosci.* 13, 521–528. doi: 10.1046/j.1460-9568.2001.01425.x

Tanaka, K., and Webster, H. D. (1991). Myelinated fiber regeneration after crush injury is retarded in sciatic nerves of aging mice. *J. Comp. Neurol.* 308, 180–187. doi: 10.1002/cne.903080205

Taskinen, H., and Röyttä, M. (2001). Increased expression of chemokines (MCP-1, MIP-1α, RANTES) after peripheral nerve transection. *J. Peripher. Nerv. Syst.* 5, 75–81. doi: 10.1046/j.1529-8027.2000.00009.x

Tofaris, G. K., Patterson, P. H., Jessen, K. R., and Mirsky, R. (2002). Denervated schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF. *J. Neurosci.* 22, 6696–6703. doi: 10.1523/JNEUROSCI.22-15-06696.2002

Vaughan, D. W. (1992). Effects of advancing age on peripheral nerve regeneration. *J. Comp. Neurol.* 323, 219–237. doi: 10.1002/cne.903230207

Verdú, E., Butí, M., and Navarro, X. (1995). The effect of aging on efferent nerve fibers regeneration in mice. *Brain Res.* 696, 76–82. doi: 10.1016/0006-8993(95)00762-f

Verdú, E., Ceballos, D., Vilches, J. J., and Navarro, X. (2000). Influence of aging on peripheral nerve function and regeneration. *J. Peripher. Nerv. Syst.* 5, 191–208. doi: 10.1046/j.1529-8027.2000.00026.x

Wang, D., Wang, X., Geng, S., and Bi, Z. (2015). Axonal regeneration in early stages of sciatic nerve crush injury is enhanced by α7nAChR in rats. *Mol. Biol. Rep.* 42, 603–609. doi: 10.1007/s11033-014-3805-2

Ydens, E., Amann, L., Asselbergh, B., Scott, C. L., Martens, L., Sichien, D., et al. (2020). Profiling peripheral nerve macrophages reveals two macrophage subsets with distinct localization, transcriptome and response to injury. *Nat. Neurosci.* 23, 676–689. doi: 10.1038/s41593-020-0618-6

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Genistein Attenuates Acute Cerebral Ischemic Damage by Inhibiting the NLRP3 Inflammasome in Reproductively Senescent Mice

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Postmenopausal women have a higher incidence of stroke compared to the age-matched males, and the estrogen was thought to be the main cause of such difference. However, estrogen replacement therapy for the prevention of postmenopausal stroke shows controversial results and is widely disputed because of its serious side effects after chronic administration. Genistein (Gen), a natural phytestrogen with fewer side effects, has a protective effect against cerebral ischemia damage. However, whether Gen could effectively prevent postmenopausal stroke has not been elucidated. In the current study, reproductively senescent mice were treated with Gen (10 mg/kg) for 2 weeks before having transient cerebral ischemia insults. Neurological scores, infarct volumes, and cell apoptosis were evaluated 24 h after reperfusion. The levels of inflammatory factors and nod-like receptor protein 3 (NLRP3) inflammasome-related proteins were also examined. The results showed that Gen treatment reduced infarct volumes, improved neurological scores, attenuated apoptosis, and decreased inflammatory factor release. The expression of NLRP3 inflammasome-related proteins in microglia was downregulated by Gen. However, the overexpression of NLRP3 in microglia abrogated the Gen-induced inhibition of inflammatory factor release and reversed the neuroprotective effect of Gen. Taken together, the results suggest that Gen treatment could attenuate the acute injury induced by cerebral ischemia in reproductively senescent mice *via* the inhibition of the NLRP3 inflammasome in microglia, indicating that Gen could be a candidate drug for the treatment of stroke in postmenopausal women.

Keywords: genistein, cerebral ischemic injury, microglia, NLRP3, reproductively senescent mice

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INTRODUCTION

Compared with the age-matched men, the incidence of stroke in women is lower in premenopausal period but significantly increases after menopause (Haast et al., 2012). Estrogen is the main cause of the sex differences in stroke incidence. Many animal studies have confirmed that estrogen has a strong neuroprotective function. However, clinical studies have come out with controversial results. Although the hormone therapy could decrease the risk of stroke in postmenopausal women (Henderson and Lobo, 2012), and the treatment with conjugated equine estrogens for 7.2 years was not associated with risk of all-cause, cardiovascular, or cancer mortality during a

cumulative follow-up of 18 years (Manson et al., 2017), hormone therapy increases the risk of other undesired consequences, such as invasive breast cancer, intracerebral hemorrhage, and venous thromboembolism (Gartlehner et al., 2017). Fortunately, natural food-derived compounds with structures and functions similar to estrogen could be alternatives to postmenopausal hormone therapy with fewer side effects. As a well-studied plant estrogen, Genistein (Gen) is a kind of isoflavone phytestrogen and has the greatest proportion and accounts for approximately half of total isoflavones found in soy foods. Previous studies showed that Gen could reduce the damages of both focal and global cerebral ischemia (GCI) in overiectomized female mice (Schreihofer and Oppong-Gyebi, 2019). Therefore, we hypothesized that Gen supplementation could potentially be effective in protecting against stroke in the reproductively senescent animals.

Inflammation plays a crucial role in the pathophysiology of ischemic injury. Ischemia-initiated inflammatory responses have various characteristics and are gender-dependent (Dotson and Offner, 2017). This gender difference is mainly attributed to the level of sex hormones, including estrogen, which are important regulators of the inflammatory responses (Bereshchenko et al., 2018). A recently discovered inflammasome, nod-like receptor protein 3 (NLRP3), plays a key role in the mediation of inflammatory responses in ischemic stroke. The activation of the NLRP3 inflammasome aggravates the cerebral damage of stroke, whereas NLRP3 depletion protects mice from cerebral ischemia injury (Gao et al., 2017). However, whether the NLRP3 inflammasome is involved in the neuroprotective effect of Gen against reproductively senescent stroke remains to be confirmed.

In the current study, we intended to determine whether pretreatment with Gen could reduce the damage induced by ischemic stroke in reproductively senescent mice and to explore the underlying role of the NLRP3 inflammasome in the postmenopausal neuroprotection of Gen.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, 17–18 months old, weighing 28–32 g, were purchased from the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). All animals were housed in an environment with a temperature of 22 ± 2°C, a relative humidity of 50% ± 1%, and a light–dark cycle of 12/12 h. Vaginal smears were obtained from the mice to confirm the cessation of the estrous cycle for seven consecutive days. All animal studies (including the mice euthanasia procedure) were performed in compliance with the regulations and guidelines of Fourth Military Medical University institutional animal care and according to the Accreditation of Laboratory Animal Care International and the Institutional Animal Care and Use Committee guidelines.

Experimental Design

In the first part of the experiment, to examine the effect of Gen on focal cerebral ischemia in reproductively senescent mice, the animals were randomly divided into four groups: sham, control, Gen, and vehicle groups. All mice except for the sham group were subjected to unilateral middle cerebral artery occlusion (MCAO). In the Gen group, Gen was administered intraperitoneally at a dose of 10 mg/kg, once daily, for 2 weeks, before MCAO as previously described (Wang et al., 2014). Neurological scores, infarct volume, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) level were analyzed 24 h after reperfusion. Inflammatory cytokines and NLRP3 inflammasome–related proteins were also investigated after ischemia.

To confirm the involvement of microglial NLRP3 in Gen-induced neuroprotection, cultured primary microglia were randomly divided into the following groups: blank (cultured cells with no additional treatment), Gen blank (cultured cells treated with Gen for 24 h), OGD (cells subjected to oxygen glucose deprivation/reperfusion), Gen (cells treated with Gen for 24 h and then subjected to OGD/reperfusion), and vehicle (cells treated with vehicle for 24 h and then subjected to OGD/reperfusion). The expression of NLRP3, pro–caspase-1 and the release of inflammatory cytokines were analyzed.

In the third part, to elucidate the role of microglial NLRP3 in the neuroprotective effect of Gen, cocultured N9 microglia and HT22 cells were randomly assigned to the following treatments: blank (cultured cells received no treatment), OGD (cells subjected to OGD/reperfusion), Gen (N9 microglia cells treated with Gen for 24 h and then cocultured with HT22 cells and subjected to OGD/reperfusion), Gen + NLRP3 (N9 microglia overexpressing NLRP3 were treated with Gen for 24 h and then cocultured with HT22 cells and subjected to OGD/reperfusion), and Gen + control (N9 microglia infected with control virus and then cocultured with HT22 cells and subjected to OGD/reperfusion).

Drugs Dilution and Treatment

Gen purchased from Selleck Chemicals, Houston, Texas, USA was dissolved in dimethyl sulfoxide and then diluted with saline. For the *in vitro* experiments, the final concentration of Gen was 5 μg/ml. The reproductively senescent mice received intraperitoneal injection of 10 mg/kg Gen or the same volume of vehicle, once per day, for 2 weeks, prior to MCAO (Wang et al., 2014).

Focal Cerebral Ischemia and Reperfusion

Mice were allowed free access to food and tap water before surgery. Cerebral ischemia was induced by MCAO as previously described (Wang et al., 2014). Briefly, the mice were anesthetized with 1.5% isoflurane. A silicon-coated suture (RWD Life Science, Shenzhen, Guangdong, China) was then inserted into the right external carotid artery and advanced through the internal carotid artery to obstruct the middle cerebral artery. The suture remained in position for 1 h during the arterial occlusion and was then removed to allow subsequent reperfusion. The body temperature of the mice was monitored by a rectal probe and maintained at 37°C ± 0.5°C by using a heating pad. A laser Doppler sensor for blood flow monitoring was placed on the surface of the skull (2 mm caudal and 4 mm lateral to the Bregma). A procedure with 80% decrease and 70% recovery of

the regional cerebral blood flow was considered to be a successful ischemic injury. The mice in sham group received the same intervention except that no suture bolt was inserted.

Assessment of Neurological Deficit

Based on the scoring system described by Garcia et al. (1995), the neurological behavior of mice was assessed 24 h after reperfusion by an observer blinded to the animal groups. The scoring consists of six tests: spontaneous activity, symmetrical movements, symmetry of forelimbs, climbing wall of wire cage, reaction on touch on either side of trunk, and response to vibrissal touch. Each behavior was ranked based on a scale between 0 and 3 points, and a total score was the sum of all six individual tests. In the first three tests, behaviors were classified into no movement (0), slight movement (1), slow movement (2), and normal movement (3). In the last three tests, behaviors were classified into no movement or response (1), weak movement or response (2), and normal movement or response (3). The higher scores represented better neurological outcomes.

Measurement of Infarct Size

After the mice were euthanized, the brains were removed. The brains were first sectioned into 1-mm slices. Then, the sections were incubated in a 2% solution of 2,3,5-triphenyltertrazolium chloride at 37°C for 15 min and fixed in 4% formalin. The stained sections were photographed using a digital camera and measured in a blinded manner with image analysis software. In consideration of tissue edema, the ratio of infarct volume was calculated according to the following equation: infarct ratio = (contralateral hemispheres − noninfarcted areas of ipsilateral hemispheres)/contralateral hemispheres (Wang et al., 2014).

OGD/Reperfusion

Briefly, the cell culture medium was changed to Dulbecco modified eagle medium (DMEM) without glucose, glutamine, and sodium pyruvate (Gibco, Grand Island, New York, USA), and the cells were transferred to a modular incubator chamber and flushed with 3 l/min of a 95% N_2 and 5% CO_2 gas mixture for 15 min at room temperature. The chamber was then sealed and placed in a 37°C container. OGD was carried out for 2 h, and then the cells were incubated with normal growth medium for an additional 12 h of reperfusion under normal conditions (Liu et al., 2018).

Cell Viability Assay

A cell counting kit 8 (CCK-8; 7Sea Biotech, Shanghai, China) was used to assess cell survival according to the manufacturer's instructions. Briefly, 50 μl of CCK-8 solution was added into 500 μl of medium solution in each culture well of a 24-well plate and incubated for 4 h at 37°C. The absorbance at 450 nm was measured with a microplate reader (Infinite M200; TECAN, Switzerland).

Lactate Dehydrogenase Release Assay

The lactate dehydrogenase (LDH) cytotoxicity colorimetric assay kit (K313-500; Biovision, San Francisco, California, USA) was used to detect cell injury. The assessment was performed according to the manufacturer's instructions. Briefly, medium (50 μl per well) from each cell culture well of a 24-well plate was added to an optically clear 96-well plate. Then 50 μl of LDH reaction mix was added to each well, mixed, and incubated for 30 min at room temperature. The absorbance at 490 nm (in reference to 690 nm) was measured with a microplate reader (Infinite M200; TECAN, Switzerland).

TUNEL Staining

Cellular apoptosis was evaluated at 24 h after reperfusion. TUNEL staining was performed using an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Mice brains were fixed with 4% Paraformaldehyde. The tissue was then cut into 12-μm-thick coronal sections from 0.5 mm prior to Bregma. Three slices for each mice were used for TUNEL staining. Three fields from the penumbra zone for each slice were observed using a 40× objective lens. The ratio of TUNEL and NeuN double-positive cells to NeuN-positive cells was considered the apoptosis index. The ischemic penumbra area was defined as previously described (Ashwal et al., 1998). Briefly, the brain was sectioned into three slices: section 1 was 2 mm from the anterior tip of the frontal lobe, section 2 was 4 mm, and section 3 was 2 mm. Section 2 that corresponded to the ischemic core and penumbra was dissected. The midline between the two hemispheres was identified, and a longitudinal cut (from top to bottom) approximately 1 mm from the midline through infarct hemisphere was made. We then made a transverse diagonal cut at approximately the 2-o'clock position to separate the core (i.e., striatum and overlying cortex) from the penumbra (adjacent cortex).

Primary Microglia Culture and Neuron–Microglia Coculture

Primary mouse microglia cultures were harvested from 1- to 2-day-old neonatal C57BL/6J pups. Briefly, the cortical tissues were subjected to enzymatic digestion and mechanical isolation. The mixed cortical cells were then passed through a 70-μm nylon mesh cell strainer and seeded into a cell culture flask in DMEM containing 10% FBS (Gibco) and 1% penicillin/streptomycin. Seven days later, the mixed glial cultures were shaken on an orbital shaker at 200 revolutions/min (rpm) for 2 h. Then, the detached microglial cells in the supernatant were collected and reseeded into cell culture containers. The purity of the microglia in culture was more than 95% as confirmed by staining with the microglia marker Iba-1.

For the indirect neuron–microglia coculture, neurons were seeded in 24-well plates and incubated for 10 days. Primary microglia (microglia: neurons = 1:2) were added to Transwell inserts with 0.4-μm pores (Costar, Shanghai, China) for 3 days. Then, the cocultured neurons and microglia were subjected to different treatments.

N9-HT22 Coculture

For the indirect N9-HT22 coculture, HT22 cells were seeded in 24-well plates, and N9 microglia were added to Transwell inserts with 0.40 μm pores (Costar, USA). After incubation for

3 days, the cocultured N9 microglia and HT22 cells received the subsequent treatments.

Immunofluorescence Staining

Immunofluorescence staining was performed on frozen coronal sections of mouse brains or on cultured cells. The mouse brains were fixed with 4% paraformaldehyde. After fixation and concentration gradient dehydration, the brains were cut into 12-μm-thick sections. The cultured cells were fixed with 4% paraformaldehyde for 10 min. The brain sections and cell cover slips were washed three times with phosphate-buffered saline (PBS) and then incubated with primary antibodies overnight at 4°C in a humidified atmosphere. The following primary antibodies were used: mouse anti-NLRP3 (1:100; Adipogen, San Diego, California, USA), rabbit anti-NeuN (1:300; Millipore, Massachusetts, USA), goat anti–Iba-1 (1:200; Abcam, Cambridge, London, UK), and chicken anti–MAP-2 (1:200; Abcam, Cambridge, London, UK). Then, the samples were incubated with a mixture of Alexa-488–conjugated donkey anti-mouse (1:300; Abcam, Cambridge, London, UK), Alexa-594-conjugated donkey anti-rabbit (1:300; Abcam, Cambridge, London, UK), Alexa-594–conjugated donkey anti-chicken (1:300; Jackson ImmunoResearch, Pennsylvania, PA, USA), and Alexa-405–conjugated donkey (1:300; Jackson ImmunoResearch, Pennsylvania, PA, USA) anti-goat secondary antibodies for 2 h in the dark at room temperature. Finally, the sections were photographed using an Olympus BX51 (Tokyo, Japan) fluorescence microscope.

Western Blotting

Penumbra was dissected from brain ischemia and then homogenized in the RIPA lysis buffer (Beyotime, Nantong, China) containing a whole proteinase inhibitor cocktail. A BCA protein assay kit (Beyotime) was used to determine the protein concentration. The extracted proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrically transferred to polyvinylidene difluoride membranes. Then, the membranes were blocked with 5% nonfat milk for 1 h at room temperature. The following primary antibodies were used: mouse anti-NLRP3 (1:1,000; Adipogen, San Diego, California, USA), rabbit anti-pro–caspase-1 (1:1,000; Santa Cruz, California, USA), rabbit anti-cleaved–caspase-1 (1:3,000; Adipogen, San Diego, California, USA), goat anti–Iba-1 (1:2,000; Abcam, Cambridge, London, UK), and mouse anti-GAPDH (1:1,000; Cell Signaling Technology, Boston, Massachusetts, USA). The membranes were shaken at 60 rpm at 4°C overnight and incubated with a secondary anti-rabbit or mouse antibodies (1:10,000; Thermo Scientific, Massachusetts, USA) for 2 h at room temperature. The protein bands were visualized using Bio-Rad system.

Evaluation of Inflammatory Factors

The penumbra tissue was homogenized in cold normal saline after dissociated and weighted. The homogenate was centrifuged at 10,000 *g* for 15 min, and the supernatant was collected and frozen at −80°C for later detection. In order to test the release of inflammatory factors in cultured cells, the culture medium was collected and frozen at −80°C for later detection. Enzyme linked immunosorbent assay kits (Nanjing Jianchen Bioengineering Institute, Nanjing, Jiangsu, China) were used to assess the content of inflammatory factors [tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, IL-18, and cleaved caspase-1] in strict accordance with the manufacturer's protocols.

Lentivirus Transfection

The lentivirus for NLRP3overexpression and the control lentivirus was obtained commercially from Genechem Company (Shanghai, China). The component sequence was as follows: ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin. N9 microglia were seeded in a 25-cm^2 culture flask and transfected continuously with LV-NLRP3 or LV control for 12 h. Subsequently, the medium was replaced with fresh medium. The gene overexpression of NLRP3 in N9 microglia was verified using Western blotting. N9 microglia infected with lentivirus were collected and frozen after the medium was changed.

Flow Cytometric Analysis of Cell Apoptosis

The apoptotic index was detected by flow cytometry with an apoptosis detection kit (7Sea Biotech). Briefly, following the corresponding treatments, the cells were digested with 0.25% trypsin, collected from 6-well culture plates, washed twice with PBS, and centrifuged at 3,000 *g* at 4°C for 10 min. Then, the cells were incubated with 5 μl of fluorescein isothiocyanate–conjugated annexin V dye at room temperature for 15 min, which was followed by 10 μl of propidium iodide dye for 5 min in the dark. Finally, the cells were analyzed by fluorescence-activated cell sorting *via* a flow cytometric analysis (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and CytExpert 1.0 software (Beckman Coulter, Inc., Brea, CA, USA).

Statistical Analysis

Statistical analyses were performed using SPSS (version 19.0; IBM Corp, Armonk, NY, USA). All data except for neurological scores were presented as the mean with standard deviation (mean ± SD). Multiple comparisons of infarct volume were conducted with one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test. Neurological scores were presented as medians with ranges. The multiple comparisons of neurological scores and other biological tests with small samples ($n = 4$) were analyzed by the Kruskal–Wallis test followed by Dunn test. $P < 0.05$ was considered statistically different.

RESULTS

Gen Treatment Alleviated Cerebral Ischemic Injury in Reproductively Senescent Mice

The effect of Gen administration on infarct volume and neurological deficit in reproductively senescent animals 24 h after cerebral ischemia was observed. As shown in **Figure 1A**, there was no significant difference between the vehicle group [7.5 (1.5)] and control group [8.5 (2)]. Treatment with Gen induced an increase in neurological scores [11 (2; $P < 0.05$ vs. vehicle)]. As shown in **Figure 1B**, there was no significant difference of

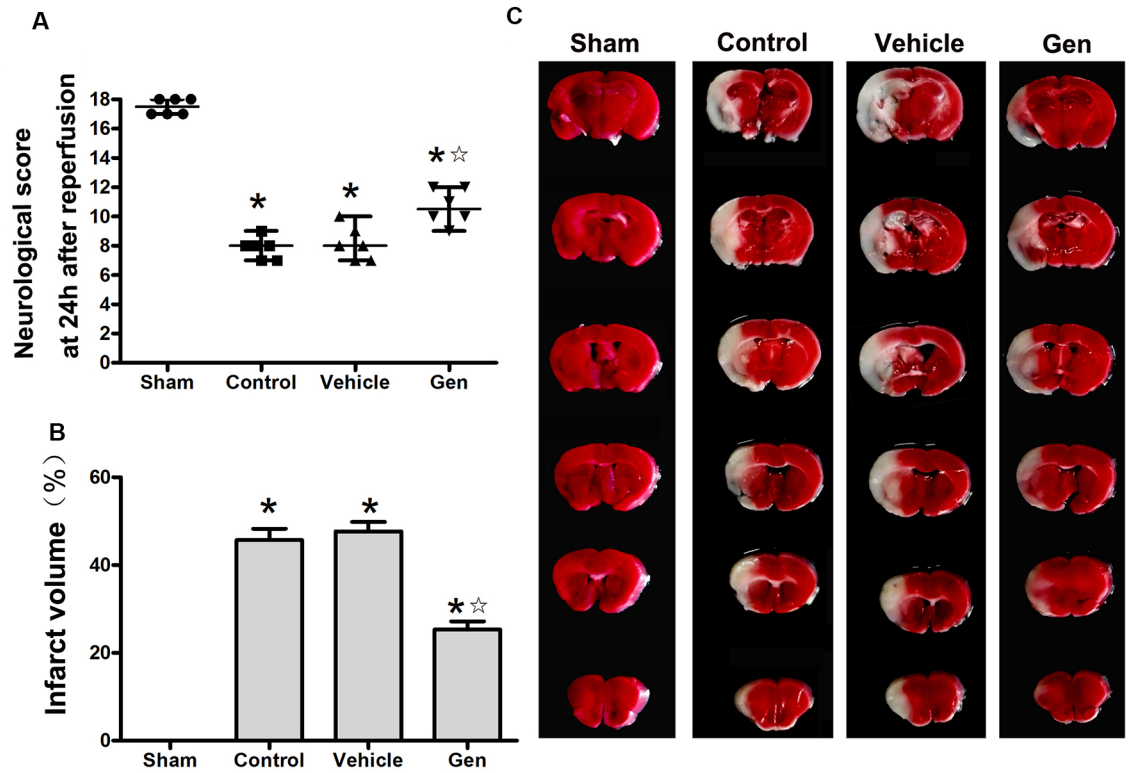

FIGURE 1 | Genistein pretreatment exerted neuroprotective effect against cerebral ischemia injury in reproductively senescent mice. **(A)** The neurological deficit scores evaluated 24 h after reperfusion in reproductively senescent mice after middle cerebral artery occlusion (MCAO). The data are presented as the median with range and analyzed by the Kruskal–Wallis test followed by Dunn test. **(B)** The infarct volumes as percentages of the contralateral hemisphere are presented as the mean ± SD and analyzed by one-way analysis of variance (ANOVA) with Tukey *post hoc* test. **(C)** Representative photographs of brain slices showing infract volume assessed 24 h after reperfusion in reproductively senescent mice. $^{*}P < 0.05$ compared to the sham group, $^{\star}P < 0.05$ compared to the vehicle group, $n = 6$ per group.

infarct volume between the vehicle group and control group (46.8% ± 5.7% vs. 45.6% ± 6.4%, $P > 0.05$).Compared with the vehicle group, Gen pretreatment decreased the infarct size (25.3% ± 4.6%, $P < 0.05$). Representative photomicrographs of cerebral infarct are shown in **Figure 1C**.

The apoptosis indicated by TUNEL was assessed 24 h after stroke. Representative photomicrographs of TUNEL staining in the ischemic penumbra area are shown in **Figure 2A**. Treatment with Gen decreased the number of TUNEL-positive neurons compared to the vehicle groups (30.5% ± 7.5% vs. 53.6% ± 9.2%, $P < 0.05$). No significant difference in the number of TUNEL-positive neurons was observed between the control (52.1% ± 9.2%) and vehicle groups ($P > 0.05$), as shown in **Figure 2B**.

Gen Pretreatment Reduced Both the Inflammatory Response and Microglial Expression of the NLRP3 Inflammasome in the Cerebral Ischemia Penumbra

To verify the anti-inflammatory function of Gen after reproductively senescent stroke, we evaluated inflammatory factors, including TNF-α, IL-1β, IL-18, and IL-6 in ischemic penumbra area 24 h after reperfusion (**Figure 3A**). Compared with the vehicle group, Gen treatment reduced all these inflammatory factors: TNF-α (190.2 ± 28.0 vs. 383.9 ± 73.5 pg/mg, $P < 0.05$), IL-1β (102.2 ± 24.1 vs. 206.2 ± 51.2 pg/mg, $P < 0.05$), IL-18 (213.48 ± 43.7 vs. 384.2 ± 58.4 pg/mg, $P < 0.05$), and IL-6 (60.0 ± 22.4 vs. 129.9 ± 35.8 pg/mg, $P < 0.05$). There was no differences between the vehicle group and control group: TNF-α (290.4 ± 38.3 vs. 383.9 ± 73.5 pg/mg, $P > 0.05$), IL-1β (203.5 ± 26.3 vs. 206.2 ± 51.2 pg/mg, $P > 0.05$), IL-18 (364.8 ± 33.6 vs. 384.2 ± 58.4 pg/mg, $P > 0.05$), and IL-6 (110.0 ± 28.6 vs. 129.9 ± 35.8 pg/mg, $P > 0.05$).

The expression and cellular localization of the NLRP3 inflammasome were detected within 24 h after reperfusion (**Figures 3B–D**). The protein expression of NLRP3 began to increase at 6 h after MCAO (1.58 ± 0.14 vs. 0.76 ± 0.22, $P < 0.05$) and was further increased at 24 h (3.06 ± 0.5 vs. 1.91 ± 0.21, 24-h group vs. 12-h group, $P < 0.05$, **Figure 3B**). Although NLRP3 gradually increased over the 24 h after MCAO, NLRP3 was continuously colocalized with Iba-1–positive microglia (>80%) during this period (**Figures 3C,D**), indicating that NLRP3 was primarily activated in the microglia after stroke.

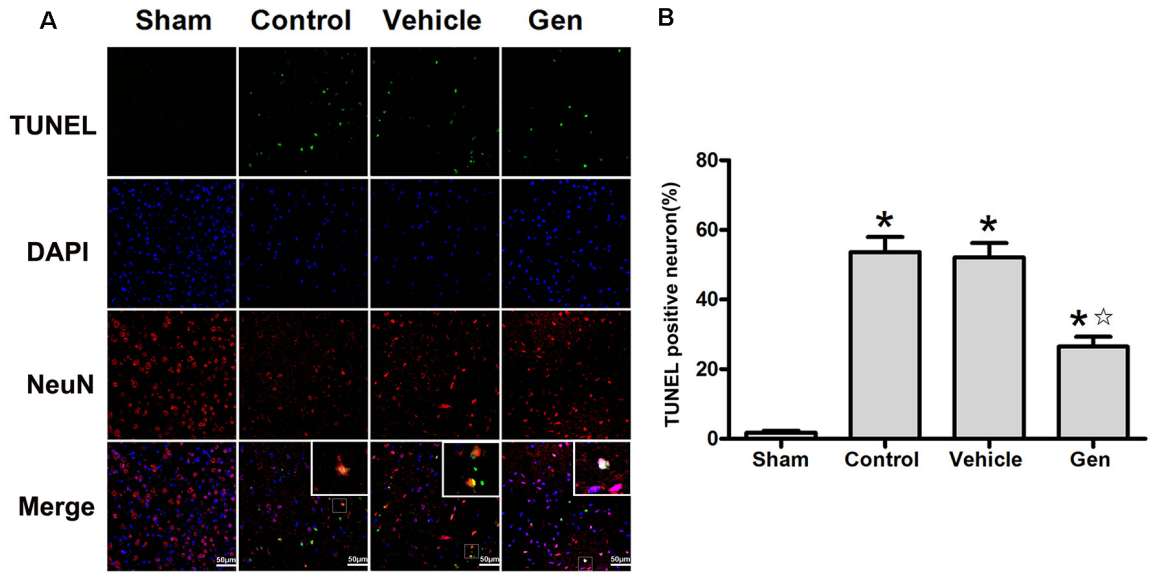

FIGURE 2 | Genistein pretreatment alleviated neuronal injury in the ischemic penumbra. **(A)** Representative photomicrographs showing TUNEL staining in the ischemic penumbra of reproductively senescent mice at 24 h after reperfusion. **(B)** The percentages of TUNEL-positive cells in the ischemic penumbra. Data are presented as the mean ± SD and analyzed by the Kruskal–Wallis test followed by Dunn test. *$P < 0.05$ compared to the sham group, ☆$P < 0.05$ compared to the vehicle group, $n = 4$.

NLRP3 expression in microglia was significantly reduced by Gen pretreatment 24 h after reperfusion (**Figure 4A**). Pro–caspase-1 and cleaved–caspase-1 expression represents the activity of NLRP3 inflammasome. As shown in **Figure 4B**, compared with the vehicle treatment, Gen treatment decreased the expression of NLRP3 (2.93 ± 0.70 vs. 4.99 ± 0.70, $P < 0.05$), pro–caspase-1 (1.88 ± 0.50 vs. 3.03 ± 0.67, $P < 0.05$), and cleave–caspase-1 (1.39 ± 0.41 vs. 3.23 ± 0.45, $P < 0.05$). The vehicle had no effect on the levels of these proteins: NLRP3 (4.33 ± 0.708 vs. 4.99 ± 0.70, $P > 0.05$), pro–caspase-1 (2.88 ± 0.53 vs. 3.03 ± 0.67, $P > 0.05$), and cleave–caspase-1 (3.41 ± 0.51 vs. 3.23 ± 0.45, $P > 0.05$). Notably, Gen did not reduce the activation of microglia, as the IBA-1 in penumbra zone in Gen group was not different from control (**Figure 4B**).

Gen Reduced the Expression and Activity of the NLRP3 Inflammasome in Microglia After OGD/Reperfusion

Because NLRP3 was mainly expressed in the microglia 24 h after MCAO *in vivo*, we further investigated the specific involvement of microglial NLRP3 in Gen-induced neuroprotection by using an *in vitro* OGD model. As shown in **Figure 5A**, the expression of NLRP3 began to increase at 6 h (1.91 ± 0.15 vs. 1.33 ± 0.10, 6 h vs. 3 h, $P < 0.05$) and reached its peak at 12 h (3.84 ± 0.32 vs. 1.91 ± 0.15, $P < 0.05$) after OGD/reperfusion. The expression of NLRP3 showed no significant change between 12 and 24 h. In cultured cells, because the expression of NLRP3 remained unchanged between 12 and 24 h after OGD/reperfusion, we chose 12 h as the observation time point for the comparison of inflammasome activity *in vitro*. The expression of NLRP3 at 12 h in microglia after OGD/reperfusion was assessed by immunofluorescence and Western blotting as shown in **Figures 5B,C**. NLRP3 (2.62 ± 0.57 vs. 4.68 ± 0.67, $P < 0.05$) and pro–caspase-1 (2.27 ± 0.74 vs. 3.85 ± 0.77, $P < 0.05$) were decreased in the Gen-treated group compared to the vehicle group; there was no difference between the control group and vehicle group: NLRP3 (4.42 ± 0.53 vs. 4.68 ± 0.67, $P < 0.05$) and pro–caspase-1 (3.27 ± 0.84 vs. 3.85 ± 0.77, $P < 0.05$). As shown in **Figure 5D**, the inflammatory factors and cleaved–caspase-1 were decreased in Gen group compared with the vehicle group: TNF-α (98.7 ± 13.7 vs. 153.3 ± 13.7, $P < 0.05$), IL-1β (157.9 ± 15.6 vs. 256.9 ± 13.4, $P < 0.05$), IL-18 (80.3 ± 11.1 vs. 123.7 ± 10.1, $P < 0.05$), IL-6 (85.7 ± 10.8 vs. 139.8 ± 9.5, $P < 0.05$), and cleaved–caspase-1 (51.7 ± 8.3 vs. 92.1 ± 12.5, $P < 0.05$); there was no difference between control group and vehicle group. We also measured the effects of Gen-pretreated microglia on neuronal damage after OGD and found that pretreating microglia with Gen could reduce the degree of neuronal damage after OGD in neuron–microglia coculture system (**Supplementary Figure S1**).

NLRP3 Overexpression Reversed the Protective Effect of Gen

To determine the role of the NLRP3 inflammasome in Gen-induced neuroprotection, we overexpressed NLRP3 with lentivirus in N9 microglia in an HT22-N9 cell coculture system. In each group, NLRP3 expression in N9 cells in the coculture system was examined after OGD

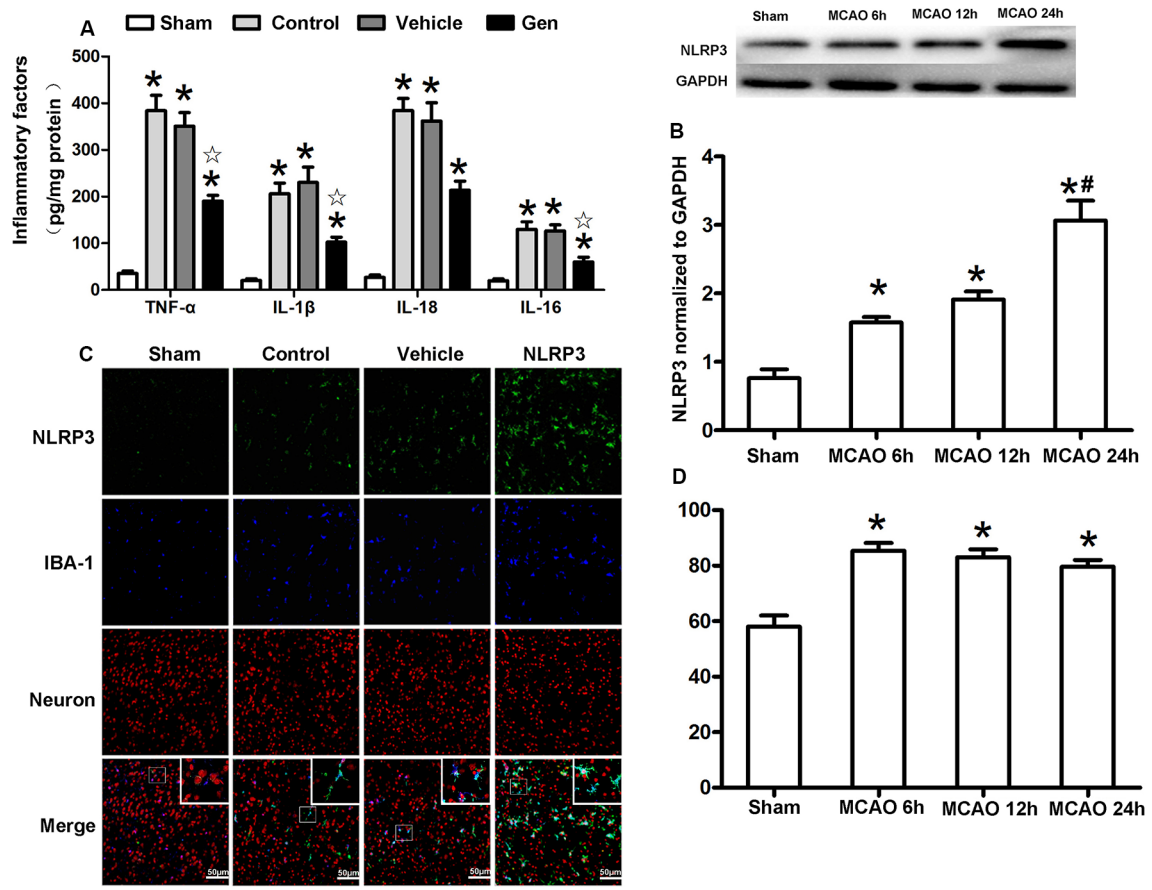

FIGURE 3 | Cellular localization and the time effect of NLRP3 after MCAO in cerebral ischemia penumbra. **(A)** The release of inflammatory factors in the ischemic penumbra of reproductively senescent mice at 24 h after reperfusion. **(B)** Western blotting analysis of the NLRP3 protein expression at different time points after cerebral ischemia. **(C)** The expression and localization of NLRP3 in microglia and neurons at 6, 12, and 24 h after reperfusion. **(D)** The percentages of NLRP3 colocalized with microglia in ischemia penumbra at different time points after cerebral ischemia. Data are presented as the mean ± SD and analyzed by the Kruskal–Wallis test followed by Dunn test. $^{*}P < 0.05$ compared to the sham group, $^{\star}P < 0.05$ compared to the vehicle group, $^{\#}P < 0.05$ compared to the group of MCAO 12 h, $n = 4$ per group.

(**Supplementary Figure S2**). The HT22 cell apoptosis, cell viability, LDH release, and inflammatory factor expression were observed after OGD/reperfusion is shown in **Figure 6**. Representative cytometry analysis of HT22 cells apoptosis after OGD/reperfusion was shown in **Figure 6A**. The quantification of apoptotic HT22 cells was illustrated in **Figure 6B**. Compared to OGD group, Gen pretreatment reduced HT22 cells apoptosis (37.43% ± 2.42%, $P < 0.05$). Although the overexpression of NLRP3 in N9 microglia abrogated the Gen-induced decrease in neuronal apoptosis (48.46% ± 5.24% $P < 0.05$), the control virus had no effects on neuronal apoptosis ($P > 0.05$). The cell viability and LDH release of HT22 cells were also measured. Compared to OGD group, Gen pretreatment increased cell viability (70.73% ± 3.57% vs. 53.70% ± 3.87%, $P < 0.05$) and decreased LDH release (1.77 ± 0.19 vs. 3.27 ± 0.37, $P < 0.05$). In contrast, the overexpression of NLRP3 in N9 microglia completely counteracted the Gen-induced increase in cell viability (48.66% ± 2.13% vs. 70.73% ±3.57%, $P < 0.05$) and reduction in LDH release (4.05 ± 0.40 vs. 1.77 ± 0.18, $P < 0.05$), whereas the control virus had no effects on cell viability (72.66% ±7.13% vs. 70.73% ± 3.57%, $P > 0.05$) or LDH release (1.95 ± 0.14 vs. 1.77 ± 0.18, $P > 0.05$).

Figure 6E presents the inflammatory factor release results; the inflammatory factors were decreased in the Gen group compared to the OGD group: TNF-α (88.80 ± 11.10 vs. 160.92 ± 9.81 pg/mg, $P < 0.05$), IL-1β (220.86 ± 30.34 vs. 331.34 ± 31.42 pg/mg, $P < 0.05$), IL-18 (82.6 ± 8.19 vs. 147.96 ± 11.04 pg/mg, $P < 0.05$), and IL-6 (80.66 ± 9.51 vs. 144.98 ± 16.83 pg/mg, $P < 0.05$). However, the overexpression of NLRP3 in N9 microglia counteracted the Gen-induced decrease in inflammatory factors: TNF-α (165.32 ± 9.19 vs. 88.80 ± 11.10 pg/mg, $P < 0.05$), IL-1β (220.86 ± 30.34 vs. 331.34 ± 31.42 pg/mg, $P < 0.05$), IL-18 (179.84 ± 43.72 vs. 82.60 ± 8.19 pg/mg, $P < 0.05$), and IL-6 (173.7 ± 24.2 vs. 80.66 ± 9.51 pg/mg, $P < 0.05$), while compared with Gen treatment alone, the control virus had no effect on these inflammatory factors.

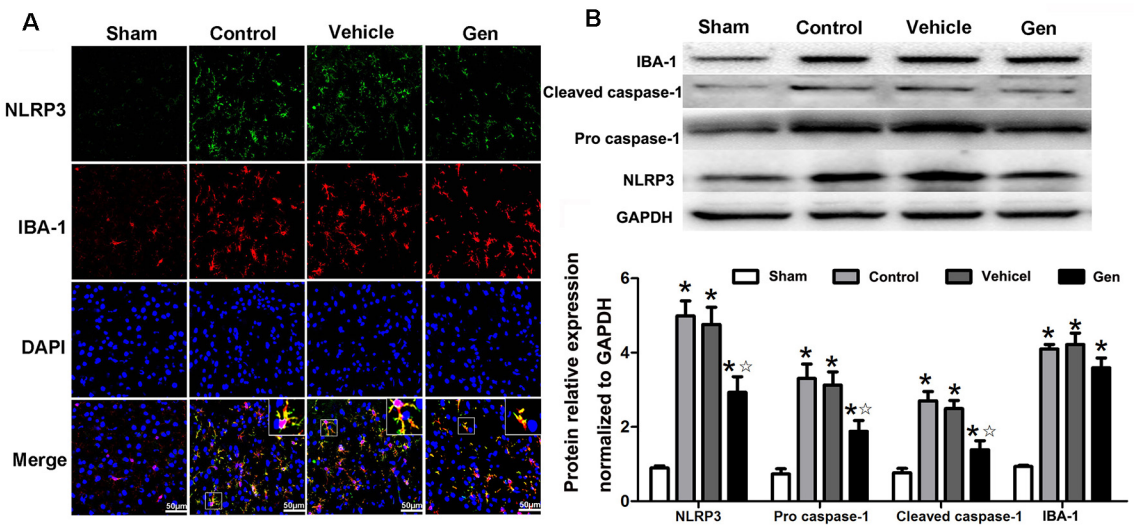

FIGURE 4 | The effect of Gen on expressions of NLRP3 inflammasome related proteins. **(A)** Representative photomicrographs of NLRP3 staining in the ischemic penumbra. **(B)** Western blotting analysis of the related proteins' expressions at 24 h after reperfusion. Data are presented as the mean ± SD and analyzed by the Kruskal–Wallis test followed by Dunn test. $^{*}P < 0.05$ compared to the sham group, $^{\star}P < 0.05$ compared to the vehicle group, $n = 4$ per group.

DISCUSSION

The current study demonstrated that the administration of Gen in the reproductively senescent mice could reduce the infarct volume, improve the neurological scores, decrease the neuronal apoptosis, and reduce the release of inflammatory factor release. In addition, Gen also inhibited the NLRP3 inflammasome, as evidenced by the decrease in protein expression of NLRP3, pro–caspase-1, and cleaved caspase-1 in microglia. By using a cell coculture system, Gen-induced neuroprotection against OGD/reperfusion injury was found to be associated with the inhibition of the microglial NLRP3 inflammasome. These results indicate that Gen administration is a preventive approach for stroke in the reproductively senescent mice, and its neuroprotection mechanism involves microglial NLRP3 inflammasome inhibition.

Estrogen decline is considered the key causation of increased risks of postmenopausal stroke. Many studies have documented the neuroprotective effects of estrogen in ischemic stroke (Shao et al., 2012). Then, estrogen replacement therapy, consisting of estrogen alone, or in combination with a progestogen could have been an ideal method for the treatment and prevention of postmenopausal stroke (Grodstein et al., 1996). However, clinical evidences come out with controversial results (Sohrabji et al., 2019). Some researchers found that estrogen replacement therapy has no effect on stroke, and some even reported the increased risk of ischemia. The variance of dosage, administration approach, timing, and patient age could be the contributors to the difference of results. The controversial results and discrepancy between animal studies and clinical data emphasize the importance of performing further investigations using appropriate animal models, modification of estrogen-based therapy, and gaining a deeper understanding of the mechanisms of estrogen-mediated neuroprotection (Manson et al., 2017). Phytestrogen may shed a light on estrogen-based method for postmenopausal stroke, as some natural compounds sharing structural and functional similarities with steroid hormones, particularly estrogens, may have fewer side effects than does estrogen in the prevention of postmenopausal stroke. Genistein is one of the most studied natural compounds. Substantial amounts of Gen are found in soybeans or soy foods such as tofu and soymilk. Interestingly, the frequent intake of foods containing high levels of Gen is associated with a reduced risk of stroke (Kokubo et al., 2007; Liang et al., 2009). As Gen could pass through the blood–brain barrier with few toxic effects (Ganai and Farooqi, 2015) and was also reported to protect rodents from both focal and GCI in male or ovariectomized female animals (Donzelli et al., 2010; Wang et al., 2013, 2014), the use of Gen for the prevention of postmenopausal stroke is promising. Fortunately, in the current study, we found that Gen pretreatment improved neurological outcome, reduced infarct volume, and decreased cellular apoptosis in naturally reproductively senescent mice, suggesting its preclinical potential in postmenopausal stroke prevention.

We found that Gen treatment could potently inhibit the release of inflammatory factors both in MCAO model and OGD model as described by the previous reports (Schreihofer and Oppong-Gyebi, 2019). As Gen is able to bind to estrogen receptors (ERs) and mimic the effect of estrogen, three ERs, ERα, ERβ (Cooke et al., 2006), and G protein–coupled ER (GPR30; Maggiolini et al., 2004), are all involved in the actions of Gen. Moreover, the activation of these receptors could inhibit inflammatory responses (Vegeto et al., 2008), which plays a pivotal role in the ischemia/reperfusion (I/R) injury (Dziedzic, 2015). In addition to its actions on ERs, Gen

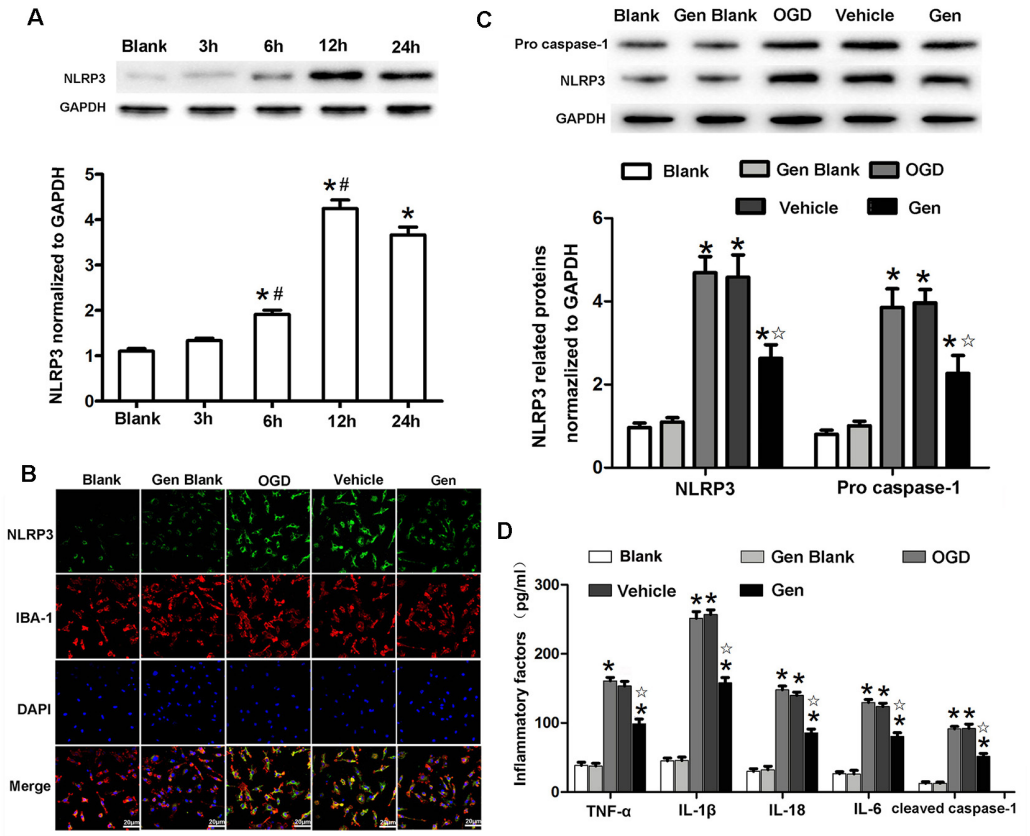

FIGURE 5 | Genistein reduced the expression of NLRP3 inflammasome related proteins in microglia after OGD/R. **(A)** Western blotting analysis of the NLRP3 protein expression at different time points after OGD/R. **(B)** Representative photomicrographs showing NLRP3 staining in microglia at 12 h after OGD/R. **(C)** Western blotting analysis of the inflammasome related proteins' expressions at 12 h after reperfusion. **(D)** The effect of Gen on the expression of inflammatory factors at 12 h after OGD/R. Data are presented as the mean ± SD and analyzed by the Kruskal–Wallis test followed by Dunn test. $^{*}P < 0.05$ compared to the sham group, $^{\star}P < 0.05$ compared to the vehicle group, $^{\#}P < 0.05$ compared to previous group, $n = 4$ per group.

also interacts with several other receptors in neuroprotection. Genistein could activate the aryl hydrocarbon receptor, which negatively regulates NLRP3 inflammasome activity by inhibiting NLRP3 transcription (Huai et al., 2014; Bialesova et al., 2015). Interleukin R4, which activates NLRP3 inflammasome, could be inhibited by Gen. The anti-inflammatory effects of Gen could also be attributed to the activation of Peroxisome proliferator–activated receptor γ (Dang et al., 2003). Given the extensive actions of Gen, although it is no easy to deduce which receptor or pathway was involved in the anti-inflammatory effect of Gen pretreatment on reproductively senescent mice, the NLRP3 inflammasome took an important part as the results demonstrated.

The NLRP3 inflammasome is the key mediator of inflammatory responses and cellular damage after I/R injury in different organs (Guo et al., 2016). Under immune challenge, the NLRP3 protein is activated and interacts with ASC and pro–caspase-1 to form the NLRP3 inflammasome. The NLRP3 inflammasome induces the transformation of the pro–caspase-1 to caspase-1, catalyzing the formation of mature IL-1β and IL-18 (Sutterwala et al., 2014). More importantly, the activation of the NLRP3 inflammasome aggravates ischemic stroke injury, whereas NLRP3 depletion reduces brain damage after cerebral ischemia (Guo et al., 2016; Alishahi et al., 2019). Therefore, the modulation of NLRP3 inflammasome activation at the molecular level may give us an insight into the development of new therapeutics for ischemic stroke. However, the cell-specific expression of NLRP3 was not well understood, as diverse reports have previously indicated different cell-specific expression of NLRP3 after experimental stroke (Fann et al., 2013; Yang et al., 2014; Gustin et al., 2015). NLRP3 was mainly reported to express in microglia after both ischemic stroke and hemorrhagic stroke (Lu et al., 2016; Ye et al., 2017; Luo et al., 2019). There were some studies indicating that NLRP3 was also increased in endothelia cells after MCAO in the acute phase (Yang et al., 2014). However, the expression of NLRP3 in neurons after cerebral ischemia is probably chronological. Some studies reported the neuronal increase of NLRP3 at the third day after stroke (Jiang et al., 2019), but some demonstrated no expression within 24 h (Zuloaga et al., 2015). Whether there is NLRP3 in astrocytes after stroke is still questionable, although very few *in vitro* studies suggested the NLRP3 increased in astrocytes,

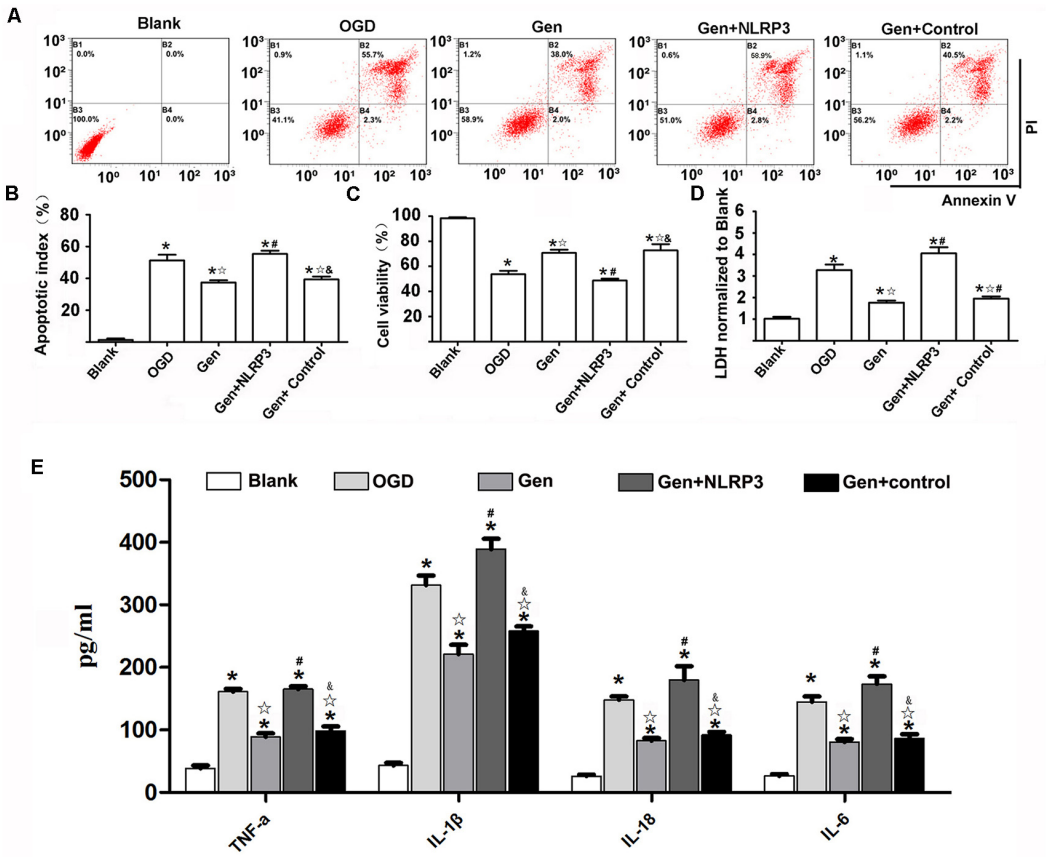

FIGURE 6 | NLRP3 overexpression in microglia partially reversed the protective effect of Gen. **(A)** The representative apoptosis cytometry analysis of HT22 in N9 microglia–HT22–coculture system after OGD/R. **(B)** The apoptosis analysis of HT22 in different groups at 12 h after OGD/R. **(C)** The cell viability of HT22 in different groups at 12 h after OGD/R. **(D)** The release of lactate dehydrogenase (LDH) in different groups at 12 h after OGD/R. **(E)** The release of inflammatory factors at 12 h after OGD/R. Data are presented as the mean ± SD and analyzed by the Kruskal–Wallis test followed by Dunn test. $^{*}P < 0.05$ compared to the sham group, $^{\star}P < 0.05$ compared to OGD group, $^{\#}P < 0.05$ compared to Gen group, $^{\&}P < 0.05$ compared to Gen + NLRP3 group, $n = 4$ per group.

under lipopolysaccharide stimulation (Alfonso-Loeches et al., 2014). In the current study, we found greater than 80% of NLRP3 was expressed in the microglia within 24 h after MCAO in the reproductively senescent mice, we subsequently chose the microglia as the target of this study. But, we cannot exclude the possible roles of NLRP3 in neurons and endothelia cells, because we observed only very few colocalization of NLRP3 with neurons (**Figure 3C**) and slight expression of NLRP3 in astrocytes (data not shown).

A recent study revealed that the activated NLRP3 inflammasome was first formed in microglia 6 h after cerebral injury and was subsequently activated in neurons and vascular endothelial cells in the ischemic core at 24 h (Gong et al., 2018). This indicates that the NLRP3 inflammasome is dynamically regulated after brain damage. Regarding the dynamic change in NLRP3 after stroke in reproductively senescent mice, we found a gradual increase in NLRP3 over 24 h in microglia after brain ischemia. This result is different from the previous ones, and this difference might be attributed to the sex, age (the most important factor) of the animals, and the sampling location. More interestingly, we found that Gen inhibited the NLRP3 inflammasome in reproductively senescent mice 24 h after reperfusion. The inhibitory effect of Gen on NLRP3 after brain ischemia injury has rarely been reported, particularly in postmenopausal stroke.

It has been reported that the suppression of NLRP3 inflammasome in microglia could protect neurons from inflammatory damage (Xu et al., 2018). To further verify the role of microglia NLRP3 in Gen-induced neuroprotection, we performed *in vitro* experiments in a neuron–microglia coculture system. In microglia, we found that the level of NLPP3 was increased at 6 h after OGD/reperfusion and reached the maximum level at 12 h after OGD/reperfusion. This finding is consistent with other reports (Qiu et al., 2016). We selected 12 h after OGD/reperfusion as the subsequent research time point for cell cultures. Similar to animal study, we found that Gen could inhibit the NLRP3 inflammasome in microglia after OGD/reperfusion. We also confirmed that Gen pretreatment exerted a neuroprotective effect on the neurons in the coculture system. The overexpression of NLRP3 in N9 microglia by lentivirus reversed the effect of Gen pretreatment on the release of inflammatory factors and on neuronal injury. This

demonstrated that NLRP3 inflammasome inhibition was necessary for Gen-induced protection. Therefore, we speculate that the NLRP3 inflammasome pathway in microglia might be the underlying mechanism of the beneficial effects of Gen.

Some limitations of this study need to be noted. Because the investigation was focused on the acute outcome of Gen in reproductively senescent mice, we only observed the first 24 h after ischemia, and no additional time points were considered. In addition, we tried to overexpress NLRP3 using a virus tool, however, neither the AAV nor the lentivirus could infect microglia *in vivo* or in primary microglia *in vitro*.

CONCLUSION

By using the reproductively senescent mice in an *in vivo* MCAO model and an *in vitro* OGD model, our study shows that Gen attenuated the inflammatory response after ischemia by inhibiting the NLRP3 inflammasome in microglia within the acute phase of postmenopausal stroke, suggesting that Gen could be a promising neuroprotective agent for postmenopausal stroke.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Ethics Review Committee at Air Force Medical University.

AUTHOR CONTRIBUTIONS

SW and QY were responsible for experimental design, experiment conduction, data collection and analysis. SW and HW wrote the article. TG and JW helped in performing the study and participated in data collection. QY supervised the experiment and improved the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/article/10.3389/fnagi.2020.00153/full#supplementary-material.

REFERENCES

Alfonso-Loeches, S., Ureña-Peralta, J. R., Morillo-Bargues, M. J., Oliver-De, L. C. J., and Guerri, C. (2014). Role of mitochondria ROS generation in ethanol-induced NLRP3 inflammasome activation and cell death in astroglial cells. *Front. Cell. Neurosci.* 8:216. doi: 10.3389/fncel.2014.00216

Alishahi, M., Farzaneh, M., Ghaedrahmati, F., Nejabatdoust, A., Sarkaki, A., and Khoshnam, S. E. (2019). $NLRP_3$ inflammasome in ischemic stroke: as possible therapeutic target. *Int. J. Stroke* 14, 574–591. doi: 10.1177/1747493019841242

Ashwal, S., Tone, B., Tian, H. R., Cole, D. J., and Pearce, W. J. (1998). Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion. *Stroke* 29, 1037–1046, 1047. doi: 10.1161/01.str.29.5.1037

Bereshchenko, O., Bruscoli, S., and Riccardi, C. (2018). Glucocorticoids, sex hormones, and immunity. *Front. Immunol.* 9:1332. doi: 10.3389/fimmu.2018.01332

Bialesova, L., Novotna, A., Macejova, D., Brtko, J., and Dvorak, Z. (2015). Agonistic effect of selected isoflavones on arylhydrocarbon receptor in a novel AZ-AhR transgenic gene reporter human cell line. *Gen. Physiol. Biophys.* 34, 331–334. doi: 10.4149/gpb_2015008

Cooke, P. S., Selvaraj, V., and Yellayi, S. (2006). Genistein, estrogen receptors, and the acquired immune response. *J. Nutr.* 136, 704–708. doi: 10.1093/jn/136.3.704

Dang, Z. C., Audinot, V., Papapoulos, S. E., Boutin, J. A., and Löwik, C. W. (2003). Peroxisome proliferator-activated receptor γ (PPARγ) as a molecular target for the soy phytoestrogen genistein. *J. Biol. Chem.* 278, 962–967. doi: 10.1074/jbc.M209483200

Donzelli, A., Braida, D., Finardi, A., Capurro, V., Valsecchi, A. E., Colleoni, M., et al. (2010). Neuroprotective effects of genistein in Mongolian gerbils: estrogen receptor-β involvement. *J. Pharmacol. Sci.* 114, 158–167. doi: 10.1254/jphs.10164fp

Dotson, A. L., and Offner, H. (2017). Sex differences in the immune response to experimental stroke: implications for translational research. *J. Neurosci. Res.* 95, 437–446. doi: 10.1002/jnr.23784

Dziedzic, T. (2015). Systemic inflammation as a therapeutic target in acute ischemic stroke. *Expert Rev. Neurother.* 15, 523–531. doi: 10.1586/14737175.2015.1035712

Fann, D. Y.-W., Lee, S. Y., Manzanero, S., Tang, S. C., Gelderblom, M., Chunduri, P., et al. (2013). Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke. *Cell Death Dis.* 4:e790. doi: 10.1038/cddis.2013.326

Ganai, A. A., and Farooqi, H. (2015). Bioactivity of genistein: a review of *in vitro* and *in vivo* studies. *Biomed. Pharmacother.* 76, 30–38. doi: 10.1016/j.biopha.2015.10.026

Gao, L., Dong, Q., Song, Z., Shen, F., Shi, J., and Li, Y. (2017). NLRP3 inflammasome: a promising target in ischemic stroke. *Inflamm. Res.* 66, 17–24. doi: 10.1007/s00011-016-0981-7

Garcia, J. H., Wagner, S., Liu, K. F., and Hu, X. J. (1995). Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke* 26, 627–634, 635. doi: 10.1161/01.str.26.4.627

Gartlehner, G., Patel, S. V., Feltner, C., Weber, R. P., Long, R., Mullican, K., et al. (2017). Hormone therapy for the primary prevention of chronic conditions in postmenopausal women: evidence report and systematic review for the US preventive services task force. *JAMA* 318, 2234–2249. doi: 10.1001/jama.2017.16952

Gong, Z., Pan, J., Shen, Q., Li, M., and Peng, Y. (2018). Mitochondrial dysfunction induces NLRP3 inflammasome activation during cerebral ischemia/reperfusion injury. *J. Neuroinflammation* 15:242. doi: 10.1186/s12974-018-1282-6

Grodstein, F., Stampfer, M. J., Manson, J. E., Colditz, G. A., Willett, W. C., Rosner, B., et al. (1996). Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. *N. Engl. J. Med.* 335, 453–461. doi: 10.1056/NEJM199608153350701

Guo, Z., Yu, S., Chen, X., Ye, R., Zhu, W., and Liu, X. (2016). NLRP3 is involved in Ischemia/Reperfusion injury. *CNS Neurol Disord. Drug Targets* 15, 699–712. doi: 10.2174/1871527315666160321111829

Gustin, A., Kirchmeyer, M., Koncina, E., Felten, P., Losciuto, S., Heurtaux, T., et al. (2015). NLRP3 inflammasome is expressed and functional in mouse brain microglia but not in astrocytes. *PLoS One* 10:e130624. doi: 10.1371/journal.pone.0130624

Haast, R. A., Gustafson, D. R., and Kiliaan, A. J. (2012). Sex differences in stroke. *J. Cereb. Blood Flow Metab.* 32, 2100–2107. doi: 10.1038/jcbfm.2012.141

Henderson, V. W., and Lobo, R. A. (2012). Hormone therapy and the risk of stroke: perspectives 10 years after the Women's Health Initiative trials. *Climacteric* 15, 229–234. doi: 10.3109/13697137.2012.656254

Huai, W., Zhao, R., Song, H., Zhao, J., Zhang, L., Zhang, L., et al. (2014). Aryl hydrocarbon receptor negatively regulates NLRP3 inflammasome activity by inhibiting NLRP3 transcription. *Nat. Commun.* 5:4738. doi: 10.1038/ncomms5738

Jiang, T., Wu, M., Zhang, Z., Yan, C., Ma, Z., He, S., et al. (2019). Electroacupuncture attenuated cerebral ischemic injury and neuroinflammation through α7nAChR-mediated inhibition of NLRP3 inflammasome in stroke rats. *Mol. Med.* 25:22. doi: 10.1186/s10020-019-0091-4

Kokubo, Y., Iso, H., Ishihara, J., Okada, K., Inoue, M., Tsugane, S., et al. (2007). Association of dietary intake of soy, beans and isoflavones with risk of cerebral and myocardial infarctions in Japanese populations: the Japan Public Health Center-based (JPHC) study cohort I. *Circulation* 116, 2553–2562. doi: 10.1161/CIRCULATIONAHA.106.683755

Liang, W., Lee, A. H., Binns, C. W., Huang, R., Hu, D., and Shao, H. (2009). Soy consumption reduces risk of ischemic stroke: a case-control study in southern china. *Neuroepidemiology* 33, 111–116. doi: 10.1159/000222093

Liu, X., Ma, Y., Wei, X., and Fan, T. (2018). Neuroprotective effect of licochalcone a against oxygen-glucose deprivation/reperfusion in rat primary cortical neurons by attenuating oxidative stress injury and inflammatory response *via* the SIRT1/Nrf2 pathway. *J. Cell. Biochem.* 119, 3210–3219. doi: 10.1002/jcb.26477

Lu, Y., Xiao, G., and Luo, W. (2016). Minocycline suppresses NLRP3 inflammasome activation in experimental ischemic stroke. *Neuroimmunomodulation* 23, 230–238. doi: 10.1159/000452172

Luo, Y., Reis, C., and Chen, S. (2019). NLRP3 inflammasome in the pathophysiology of hemorrhagic stroke: a review. *Curr. Neuropharmacol.* 17, 582–589. doi: 10.2174/1570159x17666181227170053

Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A. G., Sisci, D., Pezzi, V., et al. (2004). The G protein-coupled receptor GPR30 mediates C-Fos up-regulation by 17β-estradiol and phytoestrogens in breast cancer cells. *J. Biol. Chem.* 279, 27008–27016. doi: 10.1074/jbc.m403588200

Manson, J. E., Aragaki, A. K., Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., et al. (2017). Menopausal hormone therapy and long-term All-Cause and Cause-Specific mortality: the women's health initiative randomized trials. *JAMA* 318, 927–938. doi: 10.1001/jama.2017.11217

Qiu, J., Wang, M., Zhang, J., Cai, Q., Lu, D., Li, Y., et al. (2016). The neuroprotection of Sinomenine against ischemic stroke in mice by suppressing NLRP3 inflammasome *via* AMPK signaling. *Int. Immunopharmacol.* 40, 492–500. doi: 10.1016/j.intimp.2016.09.024

Schreihofer, D. A., and Oppong-Gyebi, A. (2019). Genistein: mechanisms of action for a pleiotropic neuroprotective agent in stroke. *Nutr. Neurosci.* 22, 375–391. doi: 10.1080/1028415x.2017.1391933

Shao, B., Cheng, Y., and Jin, K. (2012). Estrogen, neuroprotection and neurogenesis after ischemic stroke. *Curr. Drug Targets* 13, 188–198. doi: 10.2174/138945012799201702

Sohrabji, F., Okoreeh, A., and Panta, A. (2019). Sex hormones and stroke: beyond estrogens. *Horm. Behav.* 111, 87–95. doi: 10.1016/j.yhbeh.2018.10.010

Sutterwala, F. S., Haasken, S., and Cassel, S. L. (2014). Mechanism of NLRP3 inflammasome activation. *Ann. N Y Acad. Sci.* 1319, 82–95. doi: 10.1111/nyas.12458

Vegeto, E., Benedusi, V., and Maggi, A. (2008). Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. *Front. Neuroendocrinol.* 29, 507–519. doi: 10.1016/j.yfrne.2008.04.001

Wang, R., Tu, J., Zhang, Q., Zhang, X., Zhu, Y., Ma, W., et al. (2013). Genistein attenuates ischemic oxidative damage and behavioral deficits *via* eNOS/Nrf2/HO-1 signaling. *Hippocampus* 23, 634–647. doi: 10.1002/hipo.22126

Wang, S., Wei, H., Cai, M., Lu, Y., Hou, W., Yang, Q., et al. (2014). Genistein attenuates brain damage induced by transient cerebral ischemia through up-regulation of ERK activity in ovariectomized mice. *Int. J. Biol. Sci.* 10, 457–465. doi: 10.7150/ijbs.7562

Xu, X., Zhang, L., Ye, X., Hao, Q., Zhang, T., Cui, G., et al. (2018). Nrf2/ARE pathway inhibits ROS-induced NLRP3 inflammasome activation in BV2 cells after cerebral ischemia reperfusion. *Inflamm. Res.* 67, 57–65. doi: 10.1007/s00011-017-1095-6

Yang, F., Wang, Z., Wei, X., Han, H., Meng, X., Zhang, Y., et al. (2014). NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke. *J. Cereb. Blood Flow Metab.* 34, 660–667. doi: 10.1038/jcbfm.2013.242

Ye, X., Shen, T., Hu, J., Zhang, L., Zhang, Y., Bao, L., et al. (2017). Purinergic 2X7 receptor/NLRP3 pathway triggers neuronal apoptosis after ischemic stroke in the mouse. *Exp. Neurol.* 292, 46–55. doi: 10.1016/j.expneurol.2017.03.002

Zuloaga, K. L., Zhang, W., Roese, N. E., and Alkayed, N. J. (2015). Soluble epoxide hydrolase gene deletion improves blood flow and reduces infarct size after cerebral ischemia in reproductively senescent female mice. *Front. Pharmacol.* 5:290. doi: 10.3389/fphar.2014.00290

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Lactulose and Melibiose Attenuate MPTP-Induced Parkinson's Disease in Mice by Inhibition of Oxidative Stress, Reduction of Neuroinflammation and Up-Regulation of Autophagy

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Parkinson's disease (PD) is a common neurodegenerative disease characterized by the progressive loss of dopaminergic (DAergic) neurons in the ventral brain. A disaccharide trehalose has demonstrated the potential to mitigate the DAergic loss in disease models for PD. However, trehalose is rapidly hydrolyzed into glucose by trehalase in the intestine, limiting its potential for clinical practice. Here, we investigated the neuroprotective potential of two trehalase-indigestible analogs, lactulose and melibiose, in sub-chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD. Treatment with MPTP generated significant motor deficits, inhibited dopamine levels, and down-regulated dopamine transporter (DAT) in the striatum. Expression levels of genes involved in anti-oxidative stress pathways, including superoxide dismutase 2 (SOD2), nuclear factor erythroid 2-related factor 2 (NRF2), and NAD(P)H dehydrogenase (NQO1) were also down-regulated. Meanwhile, expression of the oxidative stress marker 4-hydroxynonenal (4-HNE) was up-regulated along with increased microglia and astrocyte reactivity in the ventral midbrain following MPTP treatment. MPTP also reduced the activity of autophagy, evaluated by the autophagosomal marker microtubule-associated protein 1 light chain 3 (LC3)-II. Lactulose and melibiose significantly rescued motor deficits, increased dopamine in the striatum, reduced microglia and astrocyte reactivity as well as decreased levels of 4-HNE. Furthermore, lactulose and melibiose up-regulated SOD2, NRF2, and NQO1 levels, as well as enhanced the LC3-II/LC3-I ratio in the ventral midbrain with MPTP treatment. Our findings indicate the potential of lactulose and melibiose to protect DAergic neurons in PD.

Keywords: Parkinson's disease, lactulose and melibiose, MPTP mice, oxidative stress, neuroinflammation, autophagy

INTRODUCTION

Parkinson's disease (PD), characterized by resting tremor, rigidity, bradykinesia, and postural instability, is a common neurodegenerative disease in the elderly (Jankovic, 2008). The pathological studies find a massive loss of dopaminergic (DAergic) neurons in the pars compacta of the substantia nigra (Surmeier et al., 2017). The neurodegeneration of PD could be caused by a complex interaction of genetic and environmental factors (Kalia and Lang, 2015). Genetic mutations involved in the oxidative stress pathway, such as synuclein alpha (*SNCA*), parkin RBR E3 ubiquitin-protein ligase (*PRKN*), Parkinsonism associated deglycase (*DJ1*), PTEN induced kinase 1 (*PINK1*) and leucine-rich repeat kinase 2 (*LRRK2*), are reported in patients with familial PD (Dias et al., 2013; Zuo and Motherwell, 2013). Genetic variants in glucosylceramidase β (*GBA*), proved to be the main risk for developing PD (Murphy et al., 2014), affecting autophagy activities (Aharon-Peretz et al., 2004; Gan-Or et al., 2015). A variety of environmental insults, including pesticides and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), specifically increase oxidative stress, damage DAergic neurons and produce parkinsonism similar to the main features to PD (Tuite and Krawczewski, 2007), although only prolonged chronic but not acute or sub-acute MPTP exposure in mice triggers the formation of α-synuclein inclusion pathology (reviewed in Konnova and Swanberg, 2018). Therefore, compounds that reduce oxidative stress and up-regulate autophagy may be therapeutic strategies for PD patients.

Trehalose, a disaccharide found in plants and animals, demonstrates the potential to assist protein folding during environmental stress (Elbein et al., 2003). In cell and rodent models of Alzheimer's disease (AD), trehalose protects neurons by reducing aggregation of Aβ and could be a therapeutic candidate for AD (Liu et al., 2005; Du et al., 2013). Trehalose also demonstrates neuroprotective potential in other aggregation-prone neurodegenerative diseases such as Huntington's disease (Tanaka et al., 2004), amyotrophic lateral sclerosis (Castillo et al., 2013) and spinocerebellar ataxia (SCA) type 17 (Chen et al., 2015). Neuroprotective and anti-neuroinflammatory effects of trehalose were also observed in a chronic MPTP-induced PD mouse model (Sarkar et al., 2014). Also, trehalose could accelerate the clearance of mutant huntingtin/α-synuclein (Sarkar et al., 2007), TATA-box binding protein (Lee et al., 2015) and ataxin 3 (Lin et al., 2016) by enhancement of autophagy. However, trehalose is rapidly hydrolyzed by trehalase in the intestine (Dahlqvist, 1968), limiting its application for disease treatment.

Previously two trehalase-indigestible analogs, lactulose, and melibiose were found to up-regulate autophagy in aggregation-associated SCA type 3 and 17 cell models (Lee et al., 2015; Lin et al., 2016). In the present study, we examined the neuroprotective potential of trehalose and these two disaccharides in the MPTP-induced PD mouse model. Our findings provide new drug candidates for PD *via* up-regulating anti-oxidative stress and autophagy pathways as well as reducing neuroinflammation.

MATERIALS AND METHODS

Test Disaccharides

Trehalose and melibiose were obtained from Sigma–Aldrich Company (St. Louis, MO, USA). Lactulose was purchased from ACROS Organics (Geel, Belgium).

Sub-chronic MPTP Mouse Model

The animal experiments were conducted following the guidelines and were approved by the National Taiwan Normal University (NTNU) Research Committee. Male C57BL/6 mice (8 weeks old, 18–22 g) were purchased from the National Laboratory Animal Center (Tainan City, Taiwan). The mice were kept in individually ventilated cages under controlled temperature (25 ± 2°C), relative humidity (50%), and 12 h on/off light cycle with *ad libitum* access to food and water at the Animal House Facility of NTNU.

After 2-week habituation, mice were randomly divided into five groups (n = 8). Regular drinking water or drinking water with 2% trehalose, lactulose, or melibiose was applied to the mice for 42 days. Experimental parkinsonism was established by i.p. injections of 15 total doses of MPTP (30 mg/kg in 0.9% saline; Toronto Research Chemicals, Toronto, ON, Canada) along with probenecid (250 mg/kg in 0.1 M NaOH; Sigma–Aldrich), while the control group received injections of saline. Probenecid was administered 1 h before MPTP administration as it decreases the clearance of MPTP and intensifies its neurotoxicity (Lau et al., 1990). The 15 dose regimen was administered over 3 weeks with five doses per week (once daily for five consecutive days, see flow chart in **Figure 1A**). Appropriate guidelines were abided in handling MPTP. The water was changed once a week and mouse body weight, blood glucose, and drinking amount were monitored every week for 4 weeks. There was no notable difference in terms of mouse body weight, blood glucose, and drinking amount among these five groups. Behavioral analyses were performed during the period to evaluate the treatment effect.

Behavioral Test

The pole test is a practical method to detect the degree of bradykinesia in the PD mouse model (Ogawa et al., 1985). Mice were placed head down on top of a vertical wooden pole (diameter 8 mm, height 50 cm), which was wrapped in gauze to prevent slipping (Yang et al., 2011). The time it took for the mice to climb down with all four feet on the floor was measured. Each mouse was required to perform three successive trials at 5 min interval. This test was performed at days 14, 21, 28, 35, and 42 (see flow chart). All the mice were pre-trained three times before the formal tests.

Also, stride length was measured in a gait test (Klapdor et al., 1997). To obtain footprints, the front and back paws were painted with nontoxic red and blue paints, respectively. Mice were allowed to walk along a narrow, paper-covered corridor (50 × 10 cm) toward a goal box, and stride length were measured manually as the distance between two paw prints using a digital vernier caliper. This test was performed on day 42, and the average of three strides was taken for each animal.

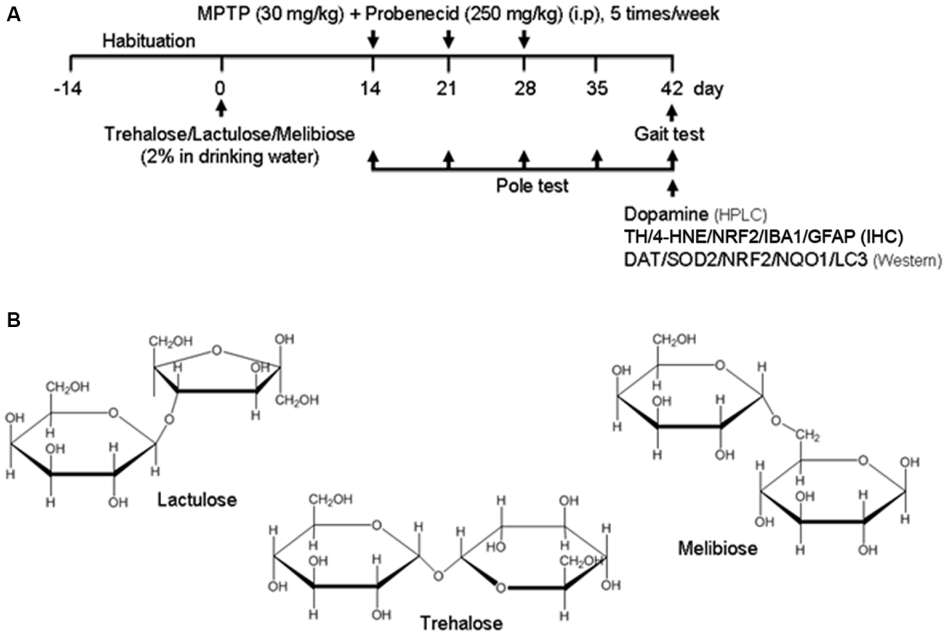

FIGURE 1 | Sub-chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model. **(A)** Experimental procotol. Parkinsonism was established by MPTP injections in C57BL/6 mice on the 14th day (15 dose regimen administered over 3 weeks) of 42-day duration of experiment. Mice received tested disaccharides from day 0 for 42 days. Saline-injected mice served as the control group. Pole test was performed on days 14, 21, 28, 35 and 42, and gait test was performed on day 42. Subsequently, mice were sacrificed for dopamine (by HPLC), tyrosine hydroxylase (TH), 4-hydroxynonenal (4-HNE), nuclear factor erythroid 2-related factor 2 (NRF2), ionized calcium-binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP; by IHC), and dopamine transporter (DAT), superoxide dismutase 2 (SOD2), NRF2, NQO1 and light chain 3 (LC3; by Western) analyses. **(B)** Structure of trehalose, lactulose and melibiose (formula $C_{12}H_{22}O_{11}$, molar mass 342.30).

HPLC Analysis of Dopamine

Levels of dopamine in the striatum were determined by high-performance liquid chromatography (HPLC) analysis. Briefly, the isolated brain striatum was homogenized in 500 μl of PRO-PREP^{TM} protein extraction solution (iNtRON Biotechnology Inc., Gyeonggi-do, Korea). The samples were centrifuged at 10,000× *g* for 30 min and then filtered through a 0.45 μm syringe membrane. Dopamine from the supernatant was analyzed by the HPLC system using a C18 column with a UV detector at 254 nm. The sample was passed through the HPLC system using a mobile phase of 87.5% 90 mM of sodium phosphate, 40 mM of citric acid, 10 mM of octane sulfonic acid, 3 mM of ethylenediaminetetraacetic acid and 12.5% acetonitrile (pH 3.0) at a flow rate of 1.0 ml/min.

Immunohistochemistry Analysis

Brains of mice were washed in PBS, fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose in PBS, and embedded in optimal cutting temperature (OCT) compound before cryosectioning. Three 20-μm thick sections of midbrain were cut, washed twice with PBS, and fixed in 4% PFA in PBS for 20 min at room temperature. After two rinses with PBS + 0.2% Triton (PBST) for 5 min each, sections were blocked in PBST with 3% normal serum followed by incubation with tyrosine hydroxylase (TH; 1:50; MyBioSource, San Diego, CA, USA), 4-hydroxynonenal (4-HNE; 1:50; Cell Biolabs, San Diego, CA, USA), nuclear factor erythroid 2-related factor 2 (NRF2; 1:50; Boster Biological Technology, Pleasanton, CA, USA), ionized calcium-binding adapter molecule 1 (IBA1; 1:1,000; Wako, Osaka, Japan) or glial fibrillary acidic protein (GFAP; 1:1,000; Invitrogen, Waltham, MA, USA) primary antibody in blocking solution overnight at 4°C. After the incubation, cells were washed three times with PBST for 20 min and then incubated for 3 h with the secondary antibody (anti-goat or anti-rabbit IgG, 1:1,000; Invitrogen) in blocking solution in the dark. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1,000; Enzo Life Sciences, Farmingdale, NY, USA) for 1 h. Quantitative analysis of TH, 4-HNE, NRF2, IBA1, or GFAP positive cells was carried out as the number of immune-positive cells with a clearly defined nucleus (identified by DAPI). MetaXpress software was applied for the determination of positive TH/4-HNE/NRF2/IBA1/GFAP cells. At least 500 cells were counted in each of the tested animals. The fluorescent intensities of IBA1 and GFAP were analyzed with ImageJ software (National Institutes of Health, ImageJ 1.40).

Western Blot Analysis

The ventral midbrain was removed immediately after the mouse was sacrificed. The tissue was homogenized by Bullet Blender (Next Advance, Averill Park, NY, USA) with zirconium oxide grinding beads (1 mm; Next Advance) for 3 min in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5% sodium

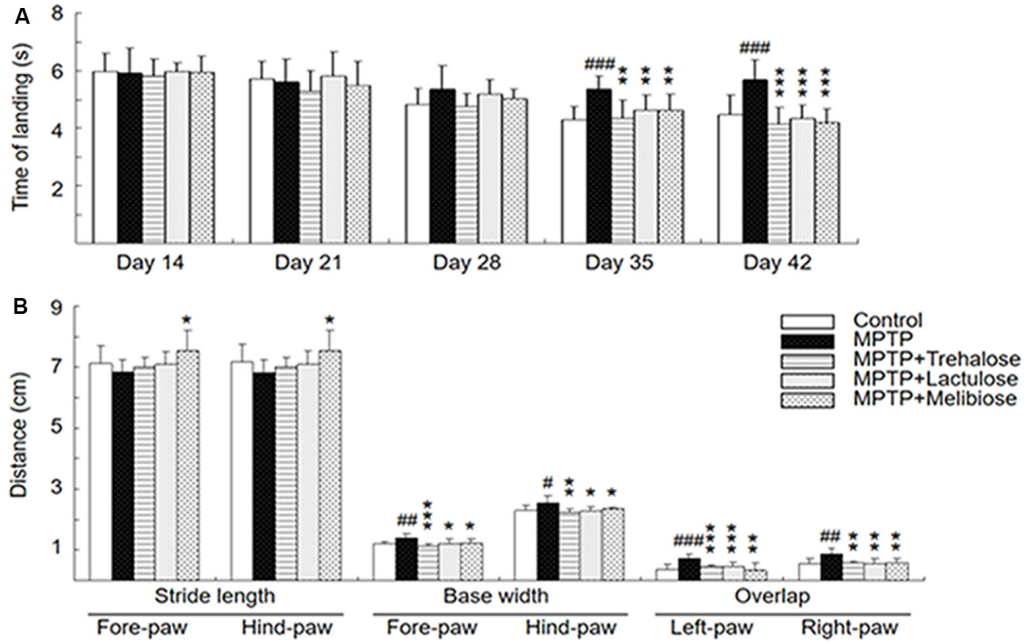

FIGURE 2 | Neuroprotective effects of trehalose and analogs in the MPTP-induced Parkinson's disease (PD) mouse model. **(A)** Pole test was conducted at days 14, 21, 28, 35 and 42. The time for mice to turn completely downward and land on the floor was recorded (n = 8). **(B)** Gait test was conducted at day 42. Stride length (fore-paw and hind-paw), base width (fore-paw and hind-paw) and overlap (left-paw and right-paw) were measured manually as the distance between two paw prints (n = 8). P-values, ANOVA with LSD *post hoc* test, MPTP vs. control ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$) and disaccharide-treated vs. untreated ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

deoxycholate, 0.1% SDS) containing protease inhibitor (Sigma–Aldrich). The samples were incubated in ice for 30 min and then centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant was collected and quantified by Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins were separated on SDS-polyacrylamide gel electrophoresis and blotted on to polyvinylidene fluoride membranes (Pall Corporation, Port Washington, NY, USA) by reverse electrophoresis. After blocking, the membrane was probed with anti-dopamine transporter (DAT; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-superoxide dismutase 2 (SOD2; 1:500; Santa Cruz Biotechnology), anti-NRF2 (1:1,000; Boster Biological Techology), anti-NQO1 (NAD(P)H dehydrogenase, quinone 1; 1:1,000; Abcam, Cambridge, UK), anti-LC3 (microtubule-associated protein 1 light chain 3; 1:2,000; MBL international corporation, Woburn, MA, USA) or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 1:5,000; MDBio, Taipei, Taiwan) at 4°C overnight. The immune complexes were subsequently detected by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000; GeneTex, Irvine, CA, USA) and chemiluminescent substrate (Millipore, Billerica, MA, USA).

Statistical Analysis

For each set of values, three independent experiments were performed and data were expressed as the means ± standard deviation (SD). Differences between groups were evaluated by student's *t*-test or ANOVA followed by an LSD *post hoc* test where appropriate. All *P*-values were two-tailed, with values of $P < 0.05$ considered significant.

RESULTS

Effects of Trehalose, Lactulose, and Melibiose on MPTP-Induced Motor Behavior in Mice

MPTP, a prodrug to the neurotoxin MPP^+ which selectively destroys DAergic neurons in the brain, was frequently applied to establish a mouse model for PD (Blandini and Armentero, 2012). MPTP treatment in mice also down-regulated autophagy and increased the level of α-synuclein, while enhancement of autophagy reduced the loss of DAergic neurons (Liu et al., 2013). Given that trehalose could up-regulate autophagy and demonstrate neuroprotective potential in MPTP-treated mice (Sarkar et al., 2007, 2014), we established a sub-chronic MPTP mouse model (**Figure 1A**) to examine the neuroprotective effects of trehalose and its analogs lactulose and melibiose (**Figure 1B**) on PD. Trehalose is formed by a 1,1-glycosidic bond between two α-glucose units. Lactulose is a synthetic disaccharide comprising fructose and galactose. It is produced by the isomerization of lactose with chemical or enzymatic methods (Aider and de Halleux, 2007). Melibiose exists in natural plants such as cacao beans and is formed by an α-1,

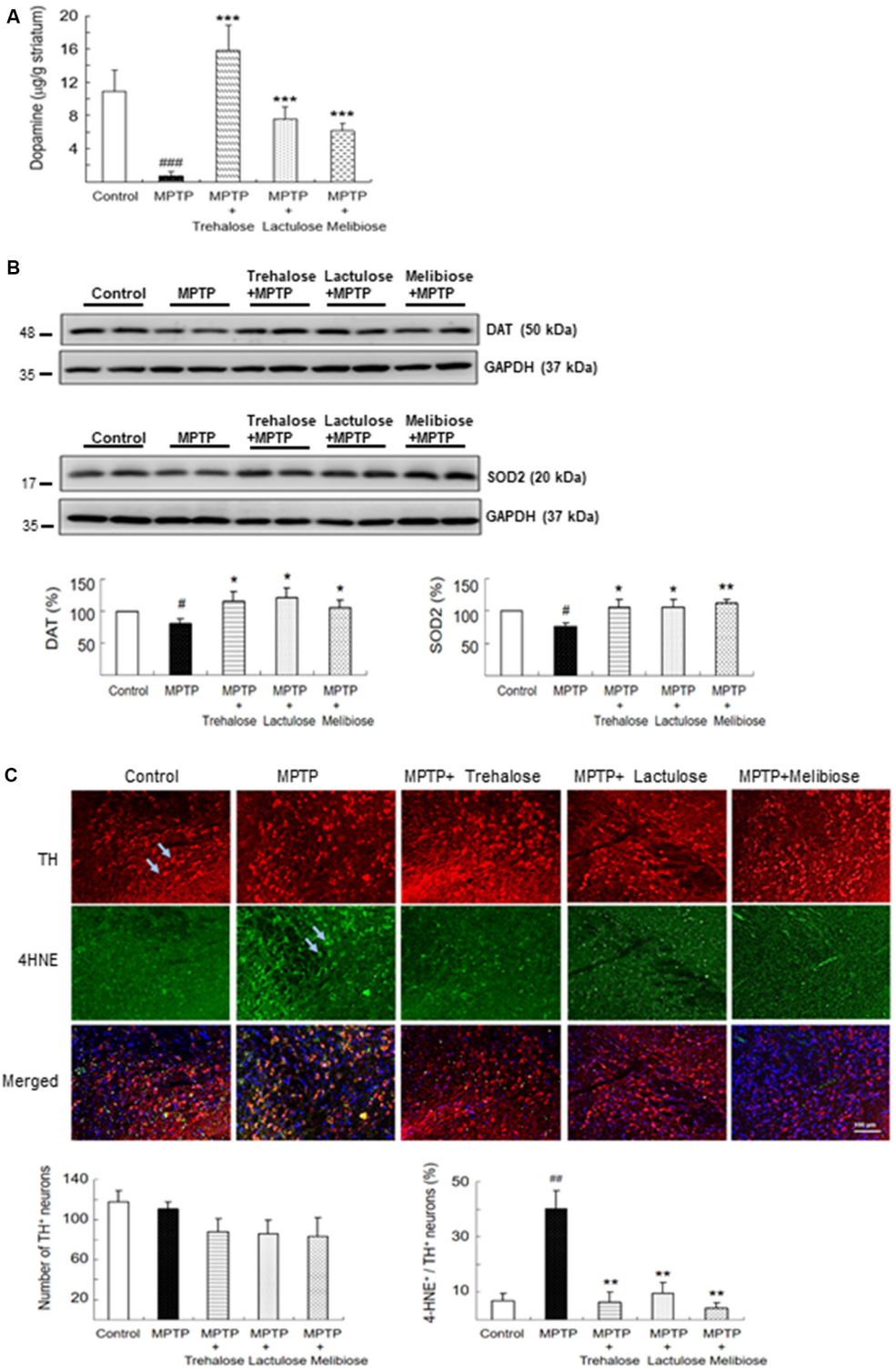

FIGURE 3 | Effects of trehalose and analogs in dopamine secretion and oxidative damage on dopaminergic neurons. **(A)** Relative levels of dopamine determined by HPLC assay in mouse striatum ($n = 8$). **(B)** Western blotting to examine altered protein levels of DAT and SOD2 in ventral midbrain ($n = 8$, divided into four batches). **(C)** Immunohistochemistry of TH (red) and 4-HNE (green) positive neurons in ventral midbrain with MPTP/trehalose/lactulose/melibiose treatment. Nuclei were counter stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Percentage of dopaminergic neurons with oxidative damages, based on TH and 4-HNE co-localization, were shown below ($n = 8$). *P*-values, ANOVA with LSD *post hoc* test, MPTP vs. control ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$) and disaccharide-treated vs. untreated ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

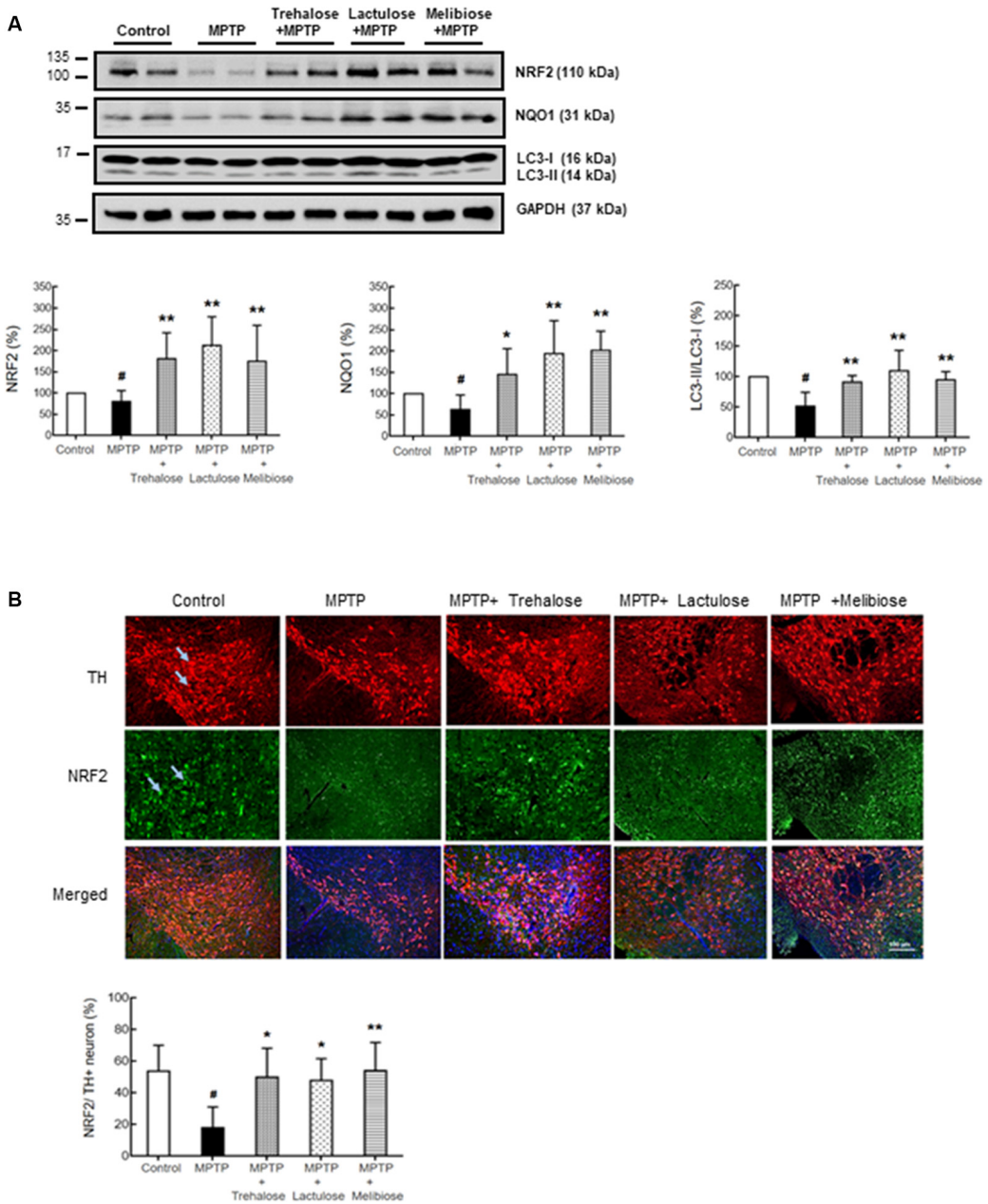

FIGURE 4 | Trehalose and both analogs enhanced autophagy and decreased oxidative stress on dopaminergic neurons. **(A)** Western blotting to examine the altered protein levels of NRF2, NQO1 and LC3-II/I in ventral midbrain (n = 8, divided into four batches). **(B)** Immunohistochemistry of TH (red) and NRF2 (green) positive neurons in ventral midbrain with MPTP/trehalose/lactulose/melibiose treatment. Nuclei were counter stained with DAPI (blue). Percentages of dopaminergic neurons with anti-oxidative damage, identified by TH and NRF2 co-localization, were shown below (n = 8). P-values, ANOVA with LSD *post hoc* test, MPTP vs. control ($^{\#}P < 0.05$) and disaccharide-treated vs. untreated ($^{*}P < 0.05$ and $^{**}P < 0.01$).

6 linkage between galactose and glucose. In the pole test, before MPTP administration (day 14), there were no differences in the time of landing between the five groups (control group: 6.0 ± 0.6 s, MPTP group: 5.9 ± 0.9 s, trehalose-treated group: 5.8 ± 0.6 s, lactulose-treated group: 6.0 ± 0.3 s, and melibiose group: 5.9 ± 0.6 s; $P > 0.05$), indicating the presence of similar baselines for all groups (**Figure 2A**). After neurotoxin injection, MPTP-treated mice showed a marked motor deficit (24–27% increase of landing time) as compared with the control group (5.4 ± 0.4 s vs. 4.3 ± 0.5 s at day 35, 5.7 ± 0.7 s vs. 4.5 ± 0.7 s at day 42; $P < 0.001$). On the other hand, mice with trehalose treatment displayed recovery (4.3 ± 0.7 s at day 35, $P < 0.01$; 4.1 ± 0.6 s at day 42, $P < 0.001$) in comparison to mice with MPTP only. Moreover, treatment of lactulose or melibiose also exhibited significant improvement on landing time (decrease of time to

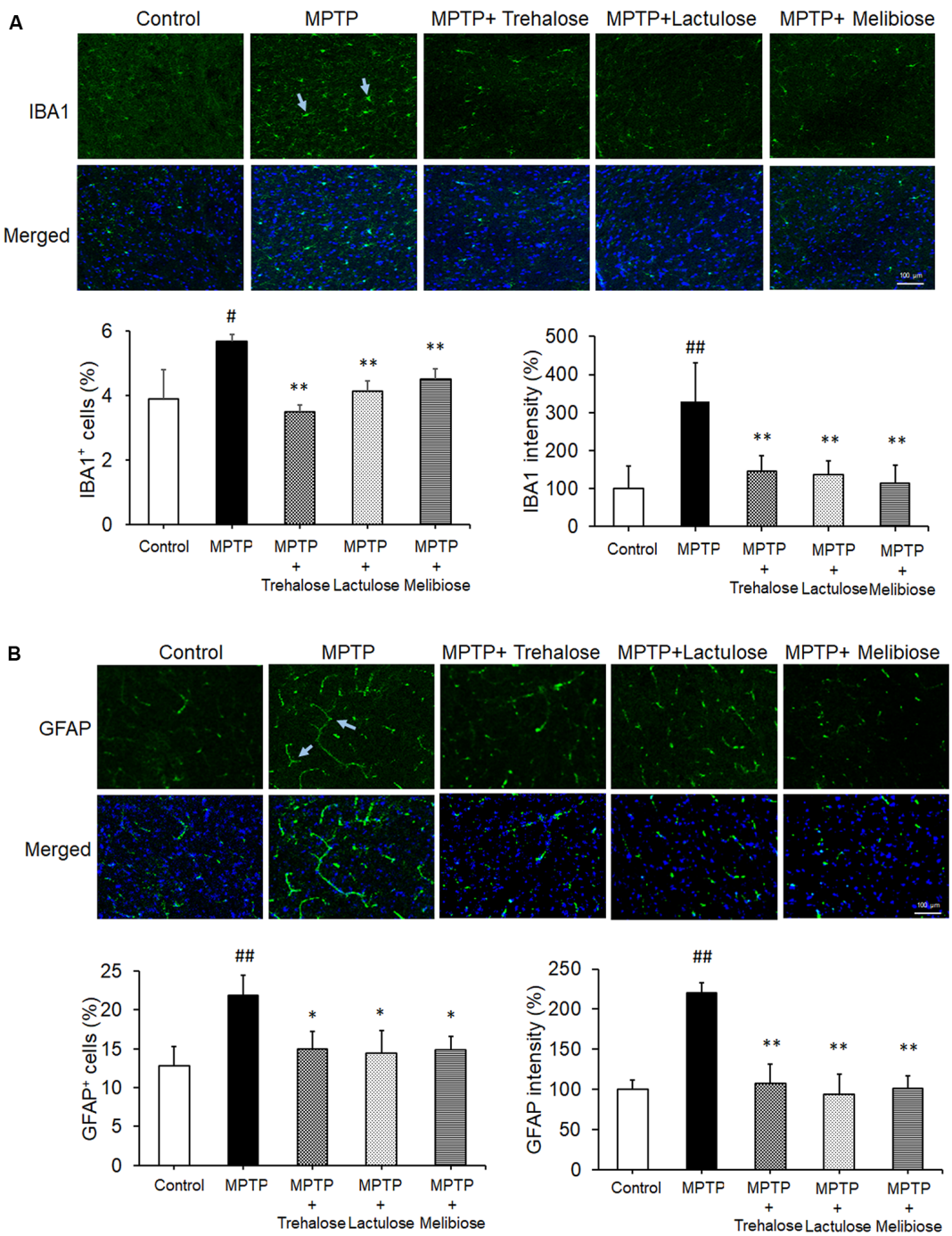

FIGURE 5 | Trehalose and both analogs reduced neuroinflammation in the ventral midbrain. **(A)** Immunohistochemistry of IBA1 positive microglia in the ventral midbrain with MPTP/trehalose/lactulose/melibiose treatment. Nuclei were counter stained with DAPI (blue). Percentages of IBA1$^+$ cells and fluorescent intensity of IBA1 were shown below (n = 8). **(B)** Immunohistochemistry of GFAP positive astrocytes in the ventral midbrain with MPTP/trehalose/lactulose/melibiose treatment. Nuclei were counter stained with DAPI (blue). Percentages of GFAP$^+$ cells and fluorescent intensity of GFAP were shown below (n = 8). P-values, ANOVA with LSD *post hoc* test, MPTP vs. control ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) and disaccharide-treated vs. untreated ($^{*}P < 0.05$ and $^{**}P < 0.01$).

reach the floor: 13% at day 35, $P < 0.01$; 24–26% at day 42, $P < 0.001$).

On the gait test, MPTP injection led to a shorter stride length at day 42 compared to the normal control (fore-paw: 6.8 ± 0.4 vs. 7.1 ± 0.6 cm; hind-paw: 6.8 ± 0.4 vs. 7.2 ± 0.6 cm; $P > 0.05$; **Figure 2B**). Although not significant, there was also a trend toward improving gait distance for both fore-paw and hind-paw in the trehalose (7.0 ± 0.3 cm) and lactulose

(7.1 ± 0.4 cm)-treated groups ($P > 0.05$) compared to the MPTP only group. However, treatment with melibiose markedly prevented the decrease of the stride length (7.5 ± 0.7 cm for both fore-paw and hind-paw, $P < 0.05$). For base width, MPTP injection led to a significant increase compared to the normal control (fore-paw: 1.4 ± 0.1 vs. 1.2 ± 0.1 cm, $P < 0.01$; hind-paw: 2.5 ± 0.2 vs. 2.3 ± 0.1 cm, $P < 0.05$; **Figure 2B**). Treatment with trehalose, lactulose and melibiose markedly decreased base width for both fore-paw (1.1 ± 0.1 cm for trehalose, $P < 0.01$; 1.2 ± 0.1 cm for lactulose, $P < 0.05$; 1.2 ± 0.1 cm for melibiose, $P < 0.05$) and hind-paw (2.2 ± 0.1 cm for trehalose, $P < 0.01$; 2.3 ± 0.2 cm for lactulose, $P < 0.05$; 2.4 ± 0.1 cm for melibiose, $P < 0.05$). Similar trends of stride overlap in left-paw and right-paw with MPTP injection (increase of stride overlap: right-paw, $P < 0.01$; left-paw, $P < 0.001$) and disaccharide treatment (decrease of stride overlap, right-paw, $P < 0.01$; left-paw, $P < 0.001$) were also observed (**Figure 2B**). Thus, through pole and gait tests, neuroprotective effects of trehalose, lactulose and melibiose were observed in sub-chronic MPTP-induced PD mouse model.

Effects of Trehalose, Lactulose and Melibiose on Dopamine, TH, DAT, SOD2 and 4-HNE Levels in MPTP-Treated Mice

In mice, MPTP treatment promotes the formation of reactive free radicals and the reduction of dopamine production (Blandini and Armentero, 2012). By examining the dopamine levels of the striatum with HPLC, we consistently found that administration of MPTP significantly reduced dopamine levels (0.66 ± 0.61 μg/g tissue, $P < 0.001$) compared with controls (10.95 ± 2.44 μg/g tissue), while treatment with trehalose (15.85 ± 2.96 μg/g tissue, $P < 0.001$), lactulose (7.61 ± 1.43 μg/g tissue, $P < 0.001$) and melibiose (6.14 ± 0.91 μg/g tissue, $P < 0.001$) successfully rescued the reduction of striatal dopamine level caused by MPTP (**Figure 3A**). Interestingly, treatment with trehalose improved striatal dopamine levels greater than that of lactulose ($P < 0.001$) and melibiose ($P < 0.001$). In addition, MPTP administration significantly reduced DAT (80%, $P < 0.05$) and SOD2 (77%, $P < 0.05$) levels, and treatment with lactulose and melibiose successfully rescued the reduction of DAT (106–121% vs. 80%, $P < 0.05$) and SOD2 (106% vs. 77%, $P < 0.05$; 112% vs. 77%, $P < 0.01$) levels in the ventral midbrain (**Figure 3B**). Although the number of TH^+ neurons was not significantly changed by MPTP and/or trehalose/lactulose/melibiose treatment, administration of MPTP significantly up-regulated the oxidative stress marker 4-HNE in TH^+ neurons in the ventral midbrain (from 7% to 40%, $P < 0.01$), while treatment with trehalose, lactulose, and melibiose successfully rescued the up-regulation of 4-HNE in TH^+ neurons (4–10% vs. 40%, $P < 0.01$; **Figure 3C**). Consistent with other studies (Fornai et al., 2005; Konnova and Swanberg, 2018), we did not find any intracellular inclusions immunoreactive for α-synuclein (data not shown). These results suggest the potential of trehalose, lactulose, and melibiose in ameliorating MPTP-induced damage on DAergic neurons in the ventral midbrain and the capacity to recover dopamine levels in the striatum.

Enhancement of Autophagy, Anti-oxidant Stress Components, and Reduction of Neuroinflammation by Trehalose, Lactulose, and Melibiose in MPTP-Treated Mice

We further investigated the potential effects of trehalose, lactulose and melibiose on anti-oxidative stress and autophagic pathways, as well as anti-neuroinflammation by examining the expression levels of NRF2 and NQO1 (anti-oxidative markers), LC3 (autophagic marker), IBA1 (microglial activation marker) and GFAP (astrocyte activation marker) in the ventral midbrain. Treatment with trehalose, lactulose and melibiose significantly rescued the down-regulation of NRF2 (trehalose: 180%, $P < 0.05$; lactulose: 212%, $P < 0.01$; melibiose: 174%, $P < 0.05$) and NQO1 (trehalose: 145%, $P < 0.05$; lactulose: 193%, melibiose: 201%, $P < 0.01$) in the ventral midbrain of mice treated with MPTP (MPTP only: NRF2: 80%, NQO1: 63%, $P < 0.05$; **Figure 4A**). The immunohistochemical study consistently showed that NRF2 in TH-positive DAergic neurons in the ventral midbrain was down-regulated by MPTP (19%, $P < 0.01$), while treatment with trehalose/lactulose/melibiose rescued this down-regulation (trehalose: 50%, lactulose: 47%, melibiose: 54%, $P < 0.01$; **Figure 4B**). In the ventral midbrain, the LC3-II/I ratio, an indicator of autophagy activity, was reduced by MPTP (52%, $P < 0.01$), while treatment with trehalose/lactulose/melibiose rescued this reduction of LC3-II/I ratio (trehalose: 91%, lactulose: 110%, melibiose: 95%, $P < 0.01$; **Figure 4A**). MPTP increased the percentage of $IBA1^+$ microglia (from 3.9% to 5.7%, $P < 0.05$), while treatment with trehalose/lactulose/melibiose reduced this microglial activation (trehalose: 3.5%, lactulose: 4.1%, melibiose: 4.5%, $P < 0.01$; **Figure 5A**). Consistently, IBA1 fluorescent intensity was up-regulated by MPTP treatment (328%, $P < 0.01$), while treatment with trehalose/lactulose/melibiose reduced IBA1 fluorescent intensity (trehalose: 145%, lactulose: 136%, melibiose: 113%, $P < 0.01$). The percentage of $GFAP^+$ astrocytes was increased by MPTP treatment (from 12.8% to 21.9%, $P < 0.01$). Treatment with trehalose/lactulose/melibiose reduced the percentage of $GFAP^+$ astrocytes (trehalose: 15.0%, lactulose: 14.5%, melibiose: 14.9%, $P < 0.05$; **Figure 5B**). GFAP fluorescent intensity was also up-regulated by MPTP treatment (221%, $P < 0.01$), while treatment with trehalose/lactulose/melibiose reduced fluorescent intensity of GFAP (trehalose: 107%, lactulose: 97%, melibiose: 101%, $P < 0.01$). Taken together, trehalose, lactulose and melibiose improved the down-regulation of anti-oxidative stress pathways and autophagy activity, as well as decreased neuroinflammation induced by MPTP.

DISCUSSION

Increased oxidative stress and decreased antioxidant capacity including reduced SOD and increased 4-HNE are among pathological findings in postmortem brains of human PD and the MPTP-induced PD mouse model (Yoritaka et al.,

1996; Castellani et al., 2002; Sofic et al., 2006; Li and Pu, 2011; Lv et al., 2012). Recently, *in vitro* studies showed that treatment with trehalose significantly reduced oxidative stress induced by chloroquine or cadmium *via* activating the NRF2 pathway, suggesting its strong anti-oxidant effect (Mizunoe et al., 2018; Wang et al., 2018). It is important to note that trehalose is readily digested by trehalase in the gut of humans (Dahlqvist, 1968), which implicates trehalase-indigestible analogs rather than trehalose as the potential treatments for aggregation-associated neurodegenerative disease. Here, we demonstrated the anti-oxidative and neuroprotective effects of two trehalase-indigestible analogs, lactulose, and melibiose, in the MPTP-induced PD mouse model. Although the elevations of striatal dopamine levels by lactulose and melibiose may be lower compared with trehalose, both of them still demonstrate improvements of motor deficits similar to trehalose. Furthermore, lactulose and melibiose increased DAT, SOD2, NRF2, and NQO1, and decreased 4-HNE, IBA1, and GFAP in the ventral midbrain of MPTP-induced PD mice. These findings suggest that lactulose and melibiose, similar to trehalose, may exert their anti-oxidative and anti-neuroinflammatory capacity to provide neuroprotection. Consistent with our findings, Sarkar et al. (2014) also demonstrate that trehalose can reduce the activation of microglia and astrocytes in the MPTP-induced PD mouse model.

Lines of evidence implicate targeting autophagy as a potential PD therapeutic strategy (Moors et al., 2017; Zhu et al., 2019). The depletion of autophagy gives rise to neurotoxicity accumulation and causes the loss of nerve cells (Hara et al., 2006; Komatsu et al., 2006). It has been proved that α-synuclein fibrils or aggregates are cleared by the autophagy-lysosomal pathways (Bae et al., 2014). Moreover, PD-associated proteins including LRRK2 (Orenstein et al., 2013; Manzoni et al., 2016), PINK1 (Lazarou et al., 2015), PRKN (Narendra et al., 2008) and ATP13A2 (ATPase cation transporting 13A2; Bento et al., 2016) are involved in autophagy-processing modulation as well. As an autophagy inducer, trehalose has the therapeutic potential on cellular and animal models of aggregation-prone neurodegenerative diseases (Sarkar et al., 2007; Rodríguez-Navarro et al., 2010; Casarejos et al., 2011; Lan et al., 2012; Schaeffer et al., 2012; Lee et al., 2015; Lin et al., 2016). In SCA17 and SCA3 cell models, we found that lactulose and melibiose demonstrate anti-aggregation and neuroprotection effects mainly through autophagy-activation (Lee et al., 2015; Lin et al., 2016). Our results showed MPTP treatment down-regulated autophagy function by reducing the conversion of LC3-II from LC3-I. Similar to trehalose, lactulose and melibiose increased the ratio of LC3-II/LC3-I in the ventral midbrain of MPTP-treated mice, suggesting their potential to up-regulate autophagy in PD.

This study demonstrates the neuroprotective potential of lactulose and melibiose in the MPTP-induced PD mouse model, by activating NRF2 and autophagy pathways. However, their neuroprotective effects may not be better than trehalose, even though they are trehalase-indigestible. Although not broken down by human enzymes, lactulose and melibiose can be metabolized in the colon by *Bifidobacterium*, *Lactobacillus* or *Saccharomyces* species (Ostergaard et al., 2000; Bouhnik et al., 2004; De Souza Oliveira et al., 2011), which may lead to less concentration of lactulose and melibiose in the brain. Further investigations to refine their metabolism by intestinal flora of microorganisms will be necessary to enhance their neuroprotective effects.

In conclusion, our results show that lactulose and melibiose reduce motor deficits, inhibit the loss of striatal dopamine, increase DAT, decrease 4-HNE level, reduce activation of microglia and astrocytes, and enhance anti-oxidative and autophagy functions in the ventral brain of MPTP-induced PD mice. Future studies in different PD models will be warranted to confirm their potentials as treatments for human PD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by National Taiwan Normal University (NTNU) Research Committee.

AUTHOR CONTRIBUTIONS

C-HL and P-CW: execution of experiments, data analysis and interpretation, and wrote the article. Y-TH, J-LanL, Y-SL, J-LiL and C-YL: execution of experiments. Y-RW: concept design and data analysis and interpretation. C-MC, K-HC, and G-JL-C: concept and design, data analysis and interpretation, obtained funding, wrote and finalized the article.

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REFERENCES

Aharon-Peretz, J., Rosenbaum, H., and Gershoni-Baruch, R. (2004). Mutations in the glucocerebrosidase gene and Parkinson's disease in Ashkenazi Jews. *N. Engl. J. Med.* 351, 1972–1977. doi: 10.1056/NEJMoa033277

Aider, M., and de Halleux, D. (2007). Isomerization of lactose and lactulose production: review. *Trends Food Sci. Technol.* 18, 356–364. doi: 10.1016/j.tifs.2007.03.005

Bae, E. J., Lee, H. J., Jang, Y. H., Michael, S., Masliah, E., Min, D. S., et al. (2014). Phospholipase D1 regulates autophagic flux and clearance of α-

synuclein aggregates. *Cell Death Differ.* 21, 1132–1141. doi: 10.1038/cdd.2014.30

Bento, C. F., Ashkenazi, A., Jimenez-Sanchez, M., and Rubinsztein, D. C. (2016). The Parkinson's disease-associated genes ATP13A2 and SYT11 regulate autophagy *via* a common pathway. *Nat. Commun.* 7:11803. doi: 10.1038/ncomms11803

Blandini, F., and Armentero, M. T. (2012). Animal models of Parkinson's disease. *FEBS J.* 279, 1156–1166. doi: 10.1111/j.1742-4658.2012.08491.x

Bouhnik, Y., Attar, A., Joly, F. A., Riottot, M., Dyard, F., and Flourié, B. (2004). Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans. *Eur. J. Clin. Nutr.* 58, 462–466. doi: 10.1038/sj.ejcn.1601829

Casarejos, M. J., Solano, R. M., Gómez, A., Perucho, J., de Yébenes, J. G., and Mena, M. A. (2011). The accumulation of neurotoxic proteins, induced by proteasome inhibition, is reverted by trehalose, an enhancer of autophagy, in human neuroblastoma cells. *Neurochem. Int.* 58, 512–520. doi: 10.1016/j.neuint.2011.01.008

Castellani, R. J., Perry, G., Siedlak, S. L., Nunomura, A., Shimohama, S., Zhang, J., et al. (2002). Hydroxynonenal adducts indicate a role for lipid peroxidation in neocortical and brainstem Lewy bodies in humans. *Neurosci. Lett.* 319, 25–28. doi: 10.1016/s0304-3940(01)02514-9

Castillo, K., Nassif, M., Valenzuela, V., Rojas, F., Matus, S., Mercado, G., et al. (2013). Trehalose delays the progression of amyotrophic lateral sclerosis by enhancing autophagy in motoneurons. *Autophagy* 9, 1308–1320. doi: 10.4161/auto.25188

Chen, Z. Z., Wang, C. M., Lee, G. C., Hsu, H. C., Wu, T. L., Lin, C. W., et al. (2015). Trehalose attenuates the gait ataxia and gliosis of spinocerebellar ataxia type 17 mice. *Neurochem. Res.* 40, 800–810. doi: 10.1007/s11064-015-1530-4

Dahlqvist, A. (1968). Assay of intestinal disaccharidases. *Anal. Biochem.* 22, 99–107. doi: 10.1016/0003-2697(68)90263-7

De Souza Oliveira, R. P., Rodrigues Florence, A. C., Perego, P., De Oliveira, M. N., and Converti, A. (2011). Use of lactulose as prebiotic and its influence on the growth, acidification profile and viable counts of different probiotics in fermented skim milk. *Int. J. Food Microbiol.* 145, 22–27. doi: 10.1016/j.ijfoodmicro.2010.11.011

Dias, V., Junn, E., and Mouradian, M. M. (2013). The role of oxidative stress in Parkinson's disease. *J. Parkinsons Dis.* 3, 461–491. doi: 10.3233/JPD-130230

Du, J., Liang, Y., Xu, F., Sun, B., and Wang, Z. (2013). Trehalose rescues Alzheimer's disease phenotypes in APP/PS1 transgenic mice. *J. Pharm. Pharmacol.* 65, 1753–1756. doi: 10.1111/jphp.12108

Elbein, A. D., Pan, Y. T., Pastuszak, I., and Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology* 13, 17R–27R. doi: 10.1093/glycob/cwg047

Fornai, F., Schlüter, O. M., Lenzi, P., Gesi, M., Ruffoli, R., Ferrucci, M., et al. (2005). Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and α-synuclein. *Proc. Natl. Acad. Sci. U S A* 102, 3413–3418. doi: 10.1073/pnas.0409713102

Gan-Or, Z., Dion, P. A., and Rouleau, G. A. (2015). Genetic perspective on the role of the autophagy-lysosome pathway in Parkinson disease. *Autophagy* 11, 1443–1457. doi: 10.1080/15548627.2015.1067364

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., et al. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885–889. doi: 10.1038/nature04724

Jankovic, J. (2008). Parkinson's disease: clinical features and diagnosis. *J. Neurol. Neurosurg. Psychiatry* 79, 368–376. doi: 10.1136/jnnp.2007.131045

Kalia, L. V., and Lang, A. E. (2015). Parkinson's disease. *Lancet* 386, 896–912. doi: 10.1016/S0140-6736(14)61393-3

Klapdor, K., Dulfer, B. G., Hammann, A., and Van der Staay, F. J. (1997). A low-cost method to analyze footprint patterns. *J. Neurosci. Methods* 75, 49–54. doi: 10.1016/s0165-0270(97)00042-3

Komatsu, M., Kominami, E., and Tanaka, K. (2006). Autophagy and neurodegeneration. *Autophagy* 2, 315–317. doi: 10.4161/auto.2974

Konnova, E. A., and Swanberg, M. (2018). "Animal models of Parkinson's disease," in *Parkinson's Disease: Pathogenesis and Clinical Aspects*, eds T. B. Stoker and J. C. Greenland (Brisbane: Codon Publications), 83–106.

Lan, D. M., Liu, F. T., Zhao, J., Chen, Y., Wu, J. J., Ding, Z. T., et al. (2012). Effect of trehalose on PC12 cells overexpressing wild-type or A53T mutant α-synuclein. *Neurochem. Res.* 37, 2025–2032. doi: 10.1007/s11064-012-0823-0

Lau, Y. S., Trobough, K. L., Crampton, J. M., and Wilson, J. A. (1990). Effects of probenecid on striatal dopamine depletion in acute and long-term 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. *Gen. Pharmacol.* 21, 181–187. doi: 10.1016/0306-3623(90)90898-v

Lazarou, M., Sliter, D. A., Kane, L. A., Sarraf, S. A., Wang, C., Burman, J. L., et al. (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 524, 309–314. doi: 10.1038/nature14893

Lee, G. C., Lin, C. H., Tao, Y. C., Yang, J. M., Hsu, K. C., Huang, Y. J., et al. (2015). The potential of lactulose and melibiose, two novel trehalose-indigestible and authophagy-inducing disaccharides, for polyQ-mediated neurodegenerative disease treatment. *Neurotoxicology* 48, 120–130. doi: 10.1016/j.neuro.2015.03.009

Li, S., and Pu, X. P. (2011). Neuroprotective effect of kaempferol against a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease. *Biol. Pharm. Bull.* 34, 1291–1296. doi: 10.1248/bpb.34.1291

Lin, C. H., Wu, Y. R., Yang, J. M., Chen, W. L., Chao, C. Y., Chen, I. C., et al. (2016). Novel lactulose and melibiose targeting autophagy to reduce polyQ aggregation in cell models of spinocerebellar ataxia 3. *CNS Neurol. Disord. Drug Targets* 15, 351–359. doi: 10.2174/1871527314666150821101522

Liu, R., Barkhordarian, H., Emadi, S., Park, C. B., and Sierks, M. R. (2005). Trehalose differentially inhibits aggregation and neurotoxicity of β-amyloid 40 and 42. *Neurobiol. Dis.* 20, 74–81. doi: 10.1016/j.nbd.2005.02.003

Liu, K., Shi, N., Sun, Y., Zhang, T., and Sun, X. (2013). Therapeutic effects of rapamycin on MPTP-induced Parkinsonism in mice. *Neurochem. Res* 38, 201–207. doi: 10.1007/s11064-012-0909-8

Lv, C., Hong, T., Yang, Z., Zhang, Y., Wang, L., Dong, M., et al. (2012). Effect of quercetin in the 1-methyl-4-phenyl-1, 2, 3,6-tetrahydropyridine-induced mouse model of Parkinson's disease. *Evid. Based Complement. Alternat. Med.* 2012:928643. doi: 10.1155/2012/928643

Manzoni, C., Mamais, A., Roosen, D. A., Dihanich, S., Soutar, M. P., Plun-Favreau, H., et al. (2016). mTOR independent regulation of macroautophagy by leucine rich repeat kinase 2 *via* Beclin-1. *Sci. Rep.* 6:35106. doi: 10.1038/srep35106

Mizunoe, Y., Kobayashi, M., Sudo, Y., Watanabe, S., Yasukawa, H., Natori, D., et al. (2018). Trehalose protects against oxidative stress by regulating the Keap1-Nrf2 and autophagy pathways. *Redox Biol.* 15, 115–124. doi: 10.1016/j.redox.2017.09.007

Moors, T. E., Hoozemans, J. J., Ingrassia, A., Beccari, T., Parnetti, L., Chartier-Harlin, M. C., et al. (2017). Therapeutic potential of autophagy-enhancing agents in Parkinson's disease. *Mol. Neurodegener.* 12:11. doi: 10.1186/s13024-017-0154-3

Murphy, K. E., Gysbers, A. M., Abbott, S. K., Tayebi, N., Kim, W. S., Sidransky, E., et al. (2014). Reduced glucocerebrosidase is associated with increased α-synuclein in sporadic Parkinson's disease. *Brain* 137, 834–848. doi: 10.1093/brain/awt367

Narendra, D., Tanaka, A., Suen, D. F., and Youle, R. J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.* 183, 795–803. doi: 10.1083/jcb.200809125

Ogawa, N., Hirose, Y., Ohara, S., Ono, T., and Watanabe, Y. (1985). A simple quantitative bradykinesia test in MPTP-treated mice. *Res. Commun. Chem. Pathol. Pharmacol.* 50, 435–441.

Orenstein, S. J., Kuo, S. H., Tasset, I., Arias, E., Koga, H., Fernandez-Carasa, I., et al. (2013). Interplay of LRRK2 with chaperonemediated autophagy. *Nat. Neurosci.* 16, 394–406. doi: 10.1038/nn.3350

Ostergaard, S., Roca, C., Rønnow, B., Nielsen, J., and Olsson, L. (2000). Physiological studies in aerobic batch cultivations of Saccharomyces cerevisiae strains harboring the MEL1 gene. *Biotechnol. Bioeng.* 68, 252–259. doi: 10.1002/(sici)1097-0290(20000505)68:3<252::aid-bit3>3.0.co;2-k

Rodríguez-Navarro, J. A., Rodríguez, L., Casarejos, M. J., Solano, R. M., Gómez, A., Perucho, J., et al. (2010). Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation. *Neurobiol. Dis.* 39, 423–438. doi: 10.1016/j.nbd.2010.05.014

Sarkar, S., Chigurupati, S., Raymick, J., Mann, D., Bowyer, J. F., Schmitt, T., et al. (2014). Neuroprotective effect of the chemical chaperone, trehalose in a chronic MPTP-induced Parkinson's disease mouse model. *Neurotoxicology* 44, 250–262. doi: 10.1016/j.neuro.2014.07.006

Sarkar, S., Davies, J. E., Huang, Z., Tunnacliffe, A., and Rubinsztein, D. C. (2007). Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and α-synuclein. *J. Biol. Chem.* 282, 5641–5652. doi: 10.1074/jbc.m609532200

Schaeffer, V., Lavenir, I., Ozcelik, S., Tolnay, M., Winkler, D. T., and Goedert, M. (2012). Stimulation of autophagy reduces neurodegeneration in a mouse model of human tauopathy. *Brain* 135, 2169–2177. doi: 10.1093/brain/aws143

Sofic, E., Sapcanin, A., Tahirovic, I., Gavrankapetanovic, I., Jellinger, K., Reynolds, G. P., et al. (2006). Antioxidant capacity in postmortem brain tissues of Parkinson's and Alzheimer's diseases. *J. Neural Transm. Suppl.* 71, 39–43. doi: 10.1007/978-3-211-33328-0_5

Surmeier, D. J., Obeso, J. A., and Halliday, G. M. (2017). Selective neuronal vulnerability in Parkinson disease. *Nat. Rev. Neurosci.* 18, 101–113. doi: 10.1038/nrn.2016.178

Tanaka, M., Machida, Y., Niu, S., Ikeda, T., Jana, N. R., Doi, H., et al. (2004). Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nat. Med.* 10, 148–154. doi: 10.1038/nm985

Tuite, P. J., and Krawczewski, K. (2007). Parkinsonism: a review-of-systems approach to diagnosis. *Semin. Neurol.* 27, 113–122. doi: 10.1055/s-2007-971174

Wang, X. Y., Wang, Z. Y., Zhu, Y. S., Zhu, S. M., Fan, R. F., and Wang, L. (2018). Alleviation of cadmium-induced oxidative stress by trehalose *via* inhibiting the Nrf2-Keap1 signaling pathway in primary rat proximal tubular cells. *J. Biochem. Mol. Toxicol.* 32:e22011. doi: 10.1002/jbt.22011

Yang, J. L., Chen, J. S., Yang, Y. F., Chen, J. C., Lin, C. H., Chang, R. S., et al. (2011). Neuroprotection effects of retained acupuncture in neurotoxin-induced Parkinson's disease mice. *Brain Behav. Immun.* 25, 1452–1459. doi: 10.1016/j.bbi.2011.05.012

Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E. R., and Mizuno, Y. (1996). Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc. Natl. Acad. Sci. U S A* 93, 2696–2701. doi: 10.1073/pnas.93.7.2696

Zhu, J., Gao, W., Shan, X., Wang, C., Wang, H., Shao, Z., et al. (2019). Apelin-36 mediates neuroprotective effects by regulating oxidative stress, autophagy and apoptosis in MPTP-induced Parkinson's disease model mice. *Brain Res.* 1726:146493. doi: 10.1016/j.brainres.2019.146493

Zuo, L., and Motherwell, M. S. (2013). The impact of reactive oxygen species and genetic mitochondrial mutations in Parkinson's disease. *Gene* 532, 18–23. doi: 10.1016/j.gene.2013.07.085

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Transcriptome Analyses in BV2 Microglial Cells Following Treatment With Amino-Terminal Fragments of Apolipoprotein E

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Despite the fact that harboring the apolipoprotein E4 (*APOE4*) allele represents the single greatest risk factor for late-onset Alzheimer's disease (AD), the exact mechanism by which ApoE4 contributes to disease progression remains unknown. Recently, we demonstrated that a 151 amino-terminal fragment of ApoE4 ($nApoE4_{1-151}$) localizes within the nucleus of microglia in the human AD brain and traffics to the nucleus causing toxicity in BV2 microglia cells. In the present study, we examined in detail what genes may be affected following treatment by $nApoE4_{1-151}$. Transcriptome analyses in BV2 microglial cells following sublethal treatment with $nApoE4_{1-151}$ revealed the upregulation of almost 4,000 genes, with 20 of these genes upregulated 182- to 715-fold compared to untreated control cells. The majority of these 20 genes play a role in the immune response and polarization toward microglial M1 activation. As a control, an identical $nApoE3_{1-151}$ fragment that differed by a single amino acid at position 112 (Cys→Arg) was tested and produced a similar albeit lower level of upregulation of an identical set of genes. In this manner, enriched pathways upregulated by $nApoE3_{1-151}$ and $nApoE4_{1-151}$ following exogenous treatment included Toll receptor signaling, chemokine/cytokine signaling and apoptosis signaling. There were unique genes differentially expressed by at least two-fold for either fragment. For $nApoE3_{1-151}$, these included 16 times as many genes, many of which are involved in physiological functions within microglia. For $nApoE4_{1-151}$, on the other hand the number genes uniquely upregulated was significantly lower, with many of the top upregulated genes having unknown functions. Taken together, our results suggest that while $nApoE3_{1-151}$ may serve a more physiological role in microglia, $nApoE4_{1-151}$ may activate genes that contribute to disease inflammation associated with AD. These data support the hypothesis that the link between harboring the *APOE4* allele and dementia risk could be enhanced inflammation through activation of microglia.

Keywords: apolipoprotein E4, microglia cells, BV2 cells, Alzheimer's disease, inflammation, toxicity, RNA-seq, M1 phenotype

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and is classified as a progressive, neurodegenerative disease whose symptoms include loss of memory and higher executive functioning (DeTure and Dickson, 2019). A prominent factor for late-onset AD is apolipoprotein E (ApoE), a lipoprotein transporter encoded by a single gene with three alleles (*APOE2*, *E3*, and *E4*). The *APOE4* allele is found in approximately 40% of AD patients and represents the greatest genetic risk factor for late-onset AD (Raber et al., 2004). In contrast, harboring the *APOE3* allele is neutral in dementia risk even though this version differs by only a single amino acid from ApoE4, having a cysteine at position 112 instead of arginine (Raber et al., 2004). A central question is how does this single amino acid difference account for dementia risk on a molecular level?

Studies have suggested that the key could be enhanced proteolysis of ApoE4 into fragments that have a toxic-gain of function (Rohn, 2013). Thus, ApoE4 is proteolyzed more readily than ApoE3, and fragments of ApoE4 are more prevalent in the brains of AD patients (Huang et al., 2001; Rohn et al., 2012; Rohn, 2013). Recently, we extended these findings by demonstrating that an amino-terminal fragment of ApoE4 ($nApoE4_{1-151}$) generated following cleavage of full-length ApoE4 by extracellular cellular proteases including MMP-9 is taken up by microglia, targets to the nucleus, and can induce cytotoxicity (Love et al., 2017). We also found the presence of this fragment in the nucleus of microglia in both *E4/E4* and *E3/E3* cases of postmortem AD brain sections (Love et al., 2017). Our hypothesis is that $nApoE4_{1-151}$ acts as a transcription factor leading to the expression of genes that promote microglia activation (Pollock et al., 2019). In the present study we examined this possibility by utilizing BV2 microglia cells and demonstrated that sublethal concentrations of $nApoE4_{1-151}$ promoted upregulation of thousands of genes, many linked to the functioning of the immune system and microglia activation. Surprisingly an identical $nApoE3_{1-151}$ fragment (differing by one amino acid at position 112, C→R) also led to an upregulation of many of the same genes as $nApoE4_{1-151}$. These results suggest a novel role for ApoE and further, potentially link harboring the *APOE4* allele to inflammation and degeneration that has long been associated with AD (Streit, 2005; Graeber and Streit, 2010; Heneka et al., 2014; Das and Chinnathambi, 2019).

MATERIALS AND METHODS

Materials

Construction and purification of the amino-terminal fragments 1–151 for ApoE4 ($nApoE4_{1-151}$) or ApoE3 ($nApoE3_{1-151}$) was contracted out to GenScript (Piscataway, NJ, United States). For both fragments, a 6X-His tag was coupled to the fragments to facilitate purification. Mouse CXCL2 or IL-12β quantikine ELISA kits were purchased from R&D Systems (Minneapolis, MN, United States).

Cell Culture of BV2 Cells

BV2, murine microglial cells, were maintained at 37°C and 6% CO_2 in a humidified incubator. Cells were maintained in RPMI 1640 Media (Hyclone) supplemented with 10% standard fetal bovine serum (Hyclone), 10% Cellgro MEM Non-essential Amino Acid (Corning) and 10% Penicillin streptomycin (Hyclone). Cells were cultured in 50 mL T25 Flasks. All supplies were purchased from ThermoFisher Scientific Inc. (Waltham, MA, United States). Treatment of BV2 cells was undertaken by incubation with $nApoE3_{1-151}$ or $nApoE4_{1-151}$ fragments diluted in conditioned media at a concentration of 25 μg/ml for 5 h to assess mRNA expression. Control cells (untreated) had an equivalent amount of conditioned media added to the wells.

Total RNA Extraction and cDNA Synthesis in BV2 Microglia Cells

Total RNA was extracted from cells with the Direct-zol RNA MicroPrep Kit (Zymo Research Corp., CA, United States) according to manufacturer's instructions. Genomic DNA was eliminated using TURBO DNAse as described by the manufacturer (Life Technologies, CA, United States). RNA quality was assessed using spectrophotometry and gel electrophoresis. Total cDNA was generated from 1 (μg of total RNA using qScript cDNA SuperMix (QuantaBio, MA, United States). Prior to use in qPCR, cDNA was diluted 1:2 with water.

CXCl2 and IL-12b Quantitative PCR

Primers were designed to specifically amplify a portion of either the IL-12β or CXCL2 genes. Serine/arginine-rich splicing factor 11 (SFRS11) and EH domain-binding protein 1 (EHBP), two ultraconserved elements that have invariant copy number in mice, were used as reference genes. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). For *Il12*β, the forward sequence was TGGTTTGCCATCGTTTTGCTG and the reverse was ACAGGTGAGGTTCACTGTTTCT. For *CXCL2*, the forward sequence was CGCTGTCAATGCCTGAAGAC and the reverse was ACACTCAAGCTCTGGATGTTCTTG. Primer efficiencies (E%) were confirmed to be between 90 and 110%. Primers were confirmed to be specific based upon melting profiles:

Gene	Forward	Reverse	E%
SFRS11	AAATACCACCCAAC AGTTT	AAGCCCTATACAGA TGGAT	101
EHBP	GAGTCTCCAATATCAT CAGTAAGC	ACACATGCCACGA TCAATG	96
CXCL2	CGCTGTCAATGCCT GAAGAC	ACACTCAAGCTCTGGAT GTTCTTG	101
IL12B	TGGTTTGCCATCGTTT TGCTG	ACAGGTGAGGTTCACTG TTTCT	99

The total volume for each reaction was 20 μl and included 10 μl Forget-Me-Not EvaGreen qPCR Master Mix (Biotium

Inc., Ca, United States), 1 μl of each appropriate primer (10 μM), 4 μl of nuclease free water, and 4 μl of template cDNA. Each PCR reaction also included a reverse transcription negative control to confirm the absence of genomic DNA in triplicate and a non-template negative control to confirm the absence of primer dimerization in triplicate. Real-time qPCR was run on a LightCycler 96 (Roche, Basel, Switzerland). The cycling conditions were 1 cycle of denaturation at 95°C for 2 min, followed by 40 cycles of amplification (95°C for 5 s, 55°C for 10 s, and 72°C for 15 s) and one cycle of product melting (95°C for 10 s, 65°C for 60 s, and 97°C for 1 s). All samples were amplified in triplicate, and the Cq value for each reaction was determined by the LightCycler 96 SW1.1. Relative differences in expression between treatments were determined by the LightCycler 96 SW1.1 and confirmed with the $\Delta\Delta$ Ct method.

RNA-Sequencing, Mapping, and Analysis

RNA-Sequencing

RNA-sequencing was performed by the Molecular Research Core Facility at Idaho State University (Pocatello, ID). All samples were sequenced using an Illumina HiSeq4000 Sequencer. Reads of 1 × 75 bp were demultiplexed and adapter sequences were removed using Trim Galore v0.5.0[1]. Trimmed reads were then assessed for quality using FASTQC v0.11.8[2]. Reads were then mapped to a mouse reference genome (version GRCm38.p6) using Hisat2 v2.1.0 (Kim et al., 2015). Gene counts were determined using HTSeq v0.11.0 (Anders et al., 2015) after which, counts were normalized using the median-of-ratios method with Deseq2 v1.22.2 (Love et al., 2014). Deseq2 was then used to calculate *p*-values using a Wald test with a Benjamini-Hochberg *post hoc* correction. Genes with an adjusted *p*-value < 0.05 and a fold-change >2 were considered to be differentially expressed. Differentially expressed genes (DEGs) were enriched for gene ontologies (GO) using PANTHER[3]. Analyses were conducted to assess biological processes, molecular functions, and cellular components.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE153454[4].

Gene Ontology Analysis

A list of every influenced gene and their respective fold changes was imputed into the PANTHER classification system (Shannon et al., 2003; Bindea et al., 2009). Statistical enrichment tests were performed against the musculus genome, and the Bonferroni correction was used. This process was performed for biological processes and pathways (Mi et al., 2019a), and the respective outputs were used. All data were statistically significant with a corrected *p*-value < 0.05. In addition, raw data was used to create a list of all genes upregulated by either $nApoE3_{1-151}$ or $nApoE4_{1-151}$ and a separate list of all genes downregulated by $nApoE_{1-151}$ fragments was imputed into the PANTHER classification system (Mi and Thomas, 2009; Mi et al., 2019b) and a functional classification analysis was performed.

Statistical Analysis

Transcriptome data was used for analysis by averaging three independent measurements. Treatment group data was then referenced as a percent fold change increase from controls. Data was segregated and organized by gene and treatment group. The organization of data was to utilize the $nApoE4_{1-151}$ (E4 group) as a standard, organizing the data by highest to lowest change for the E4 treatment group and aligning the fold change for the $nApoE3_{1-151}$ (E3 group) to those values for a matched pairs system. Data was then pulled into the statistical analysis program, R, as a.csv file and checked for normality assumptions using a Shapiro-Wilks Normality test on each treatment group. Data failed to conform to normality. A Spearman Rank Correlation was used as opposed to Pearson's Correlation due to the lack of normality in data and extreme values. The Spearman Rank Correlation was then run on the top 500 highest fold change genes for the E4 group in comparison to the matched genes in the E3 group to identify similarities in trends. The resulting rho value was then referenced alongside data to generate **Figure 6**.

Confocal Microscopy

Following treatment studies, BV2 cells were fixed by incubating cells in 4% paraformaldehyde for 23 min. For antibody labeling, cells were washed with 0.1 M Tris–buffered saline (TBS), pH 7.4, and pretreated with 3% hydrogen peroxide in 10% methanol to block endogenous peroxidase activity. Slides were subsequently washed in TBS with 0.1% Triton X-100 (TBS-A) and then blocked for 30 min in TBS-A with 3% bovine serum albumin (TBS-B). Slides were further incubated overnight at room temperature with the anti-His rabbit antibody (1:5,000). Following two washes with TBS-A and a wash in TBS-B, slides were incubated with the anti-rabbit HRP-conjugated secondary antibody. Visualization was accomplished by using a tyramide signal amplification kit (Molecular Probes, Eugene, OR, United States) consisting of Alexa Fluor 488-labeled tyramide (green, Ex/Em = 495/519). Slides were mounted using ProLong Gold Antifade Mountant with DAPI (Molecular Probes). Images were taken with a Zeiss LSM 510 Meta system combined with the Zeiss Axiovert Observer Z1 inverted microscope and ZEN 2009 imaging software (Carl Zeiss, Inc., Thornwood, NY, United States). Confocal Z-stack and single plane images were acquired with an Argon (488 nm) and a HeNe (543 nm) laser source. Z-stack images were acquired utilizing the Plan-Apochromat 63 × /NA 1.4 and alpha Plan-Fluar 100 × /NA1.45 Oil objectives and with the diode (405 nm) and Argon (488 nm) laser sources, emission band passes of 505–550 nm for the detection of the nApoE1–151 (green channel, Alexa Fluor 488). Orthogonal projection images were constructed from Z-stacks in order to demonstrate the staining is nuclear.

[1] https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

[2] www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3] pantherdb.org

[4] https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153454

RESULTS

Confirmation of Nuclear Localization of Amino-Terminal Fragments of apoE in BV2 Microglial Cells

Our previous findings have demonstrated the nuclear localization of amino-terminal fragments of apoE4 within microglia both *in vivo*, in the AD brain and *in vitro* following exogenous treatment of BV2 microglia cells (Love et al., 2017). To confirm and extend these findings, we exogenously treated BV2 microglia cells with both nApoE3$_{1-151}$ and nApoE4$_{1-151}$ fragments, which differ by a single amino acid (C→R). As shown in **Figure 1, uptake** and **trafficking** to the nucleus was apparent for both fragments, based on orthogonal projections taken from Z-stack images (**Figure 1**, crosshairs, panels B and G). These results are consistent with our previous findings (Love et al., 2017).

Transcriptome Analysis of BV2 Microglia Cells Following Treatment With ApoE4$_{1-151}$

We hypothesize the nApoE4$_{1-151}$ fragment is taken up by microglia cells, traffics to the nucleus and may act as a transcription factor leading to the change in gene expression. For example, we have recently demonstrated that treatment of BV2 cells with nApoE4$_{1-151}$ led to an increase in the expression and release of the inflammatory cytokine, TNFα, a key trigger of microglia activation (Pollock et al., 2019). In addition, we demonstrated a specific binding interaction of nApoE4$_{1-151}$

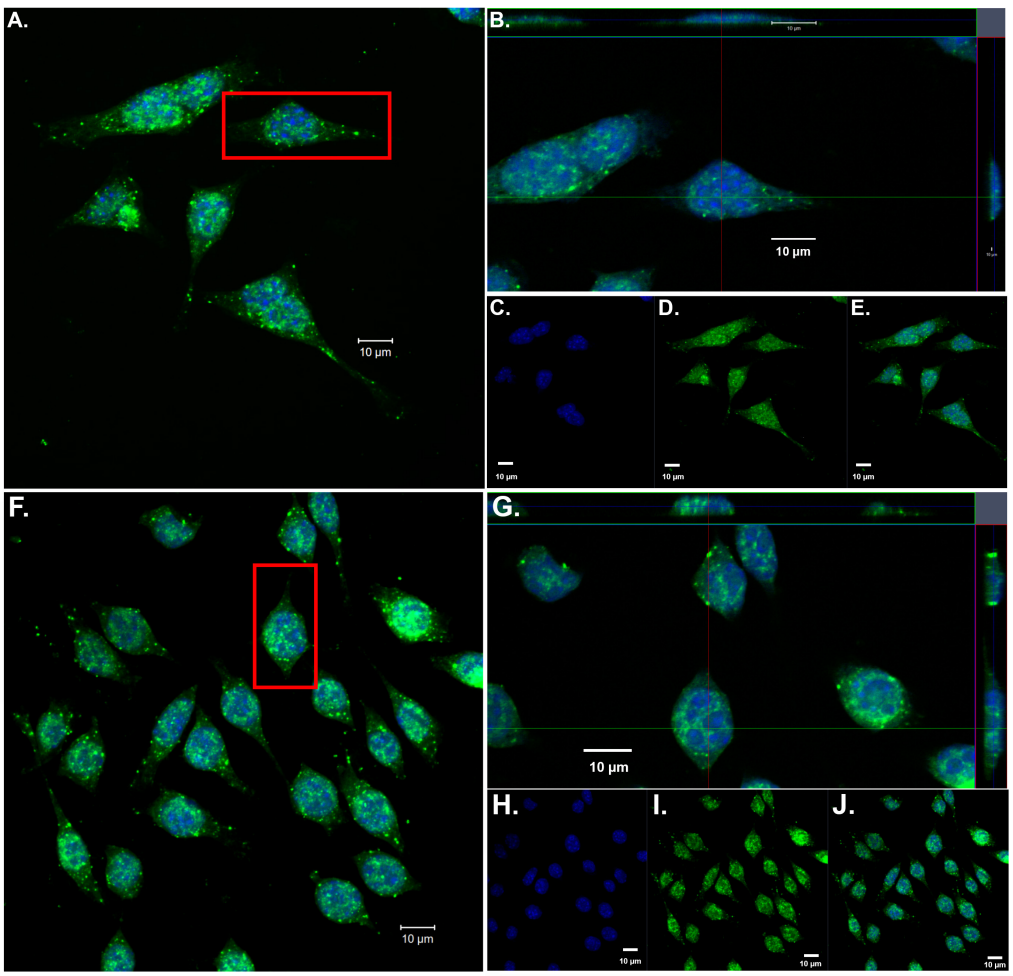

FIGURE 1 | Orthogonal projections of confocal Z-stacks show nuclear localization of amino-terminal fragments of nApoE3 and E4 following exogenous treatment in BV2 microglia cells. BV2 microglia cells were treated with either 25 µg/ml of nApoE3$_{1-151}$ **(A–E)** or with nApoE4$_{1-151}$ **(F–J)** for 24 h, fixed, and immunostained with anti-His antibody to detect the localization of the apoE fragments. Confocal images were captured and Z-stacks were constructed showing the subcellular localization of nApoE fragments. **(A)** High magnification merged image depicting nApoE3$_{1-151}$ in green and DAPI nuclear staining in blue. **(B)** Inset orthogonal projection from red rectangle in panel **A** demonstrating nuclear localization of nApoE3$_{1-151}$ in a single BV2 microglia cell (crosshair). **(C–E)** Low magnification of nApoE3$_{1-151}$ labeling with DAPI (blue, **C**), nApoE3$_{1-151}$ (green, **D**), and the overlapped image **(E)**. All scale bars represent 10 µm. **(F–J):** Identical to panels **A–E** except staining is representative of nApoE4$_{1-151}$ labeling. As with nApoE3$_{1-151}$, orthogonal images clearly demonstrate the nuclear labeling of nApoE4$_{1-151}$ (crosshair, **G**). Data are representative of five individual experiments.

with the TNFα promoter region (Pollock et al., 2019). In the current study, to test whether $nApoE4_{1-151}$ leads to the induction of a broader array of inflammatory genes or other pertinent genes, we performed a transcriptome analysis following treatment of BV2 microglia cells with a sublethal concentration of $nApoE4_{1-151}$. In this regard, a concentration of 25 μg/ml showed no toxic effects following morphological examination and LDH measurements. Cells were viable, healthy, and displayed no indication of degeneration. LDH values for controls (relative units) were 0.34 ± 0.096 SEM and 0.370 ± 104 SEM for treated cells ($N = 3$, p-value = 0.80). In comparison to untreated control BV2 cells nearly 8000 genes were determined to be differentially expressed in the presence of $nApoE4_{1-151}$. The raw data file can be accessed through **Supplementary Data File S1**. In addition, files containing the raw data are available in the GEO repository (GEO accession number, GSE153454). Although there were a similar number of up-regulated and down-regulated genes, the up-regulated genes had an average of a 6.6-fold change and the down-regulated genes had an average of a 2.2-fold change. Of the twenty most up-regulated genes, thirteen are known to be involved in the inflammatory immune response (**Table 1**). Biological processes, cellular components, molecular functions, and pathways for each gene were assigned by the PANTHER classification system (**Figure 2**). Involved cellular components included the cell, the extracellular region, the membrane etc. For each cellular component, there were many more associated genes that were upregulated than downregulated. The biological processes associated with genes differentially expressed in the presence of $nApoE4_{1-151}$ included "cellular processes," "biological regulation," "response to stimulus," and "immune system processes." Each given biological process contained more up-regulated genes than down-regulated genes, with "cellular component organization" and "reproduction" being the least up-regulated.

To verify transcriptome results, two genes were chosen and independently verified by both RT-qPCR and ELISA assays. In comparison to untreated controls, the differential expression of two genes, *CXCL2* and *IL-12B*, and their corresponding proteins were confirmed with qPCR and ELISA (**Figure 3**). In this regard following treatment of BV2 microglia cells with $nApoE4_{1-151}$, both *CXCL2* and *IL-12B* showed large increases in the fold mRNA levels (**Figures 3A,C**) as well as in the secreted protein in conditioned media (**Figures 3B,D**).

Many biological processes involved in the inflammatory immune response were enriched by the $nApoE4_{1-151}$ fragment, while many processes involved in cell division were down regulated (**Figure 4A**). The molecular functions of regulated genes included binding, catalytic activity and molecular transducer activity. Each molecular function contained more related up-regulated genes than down-regulated genes. Numerous pathways related to the inflammatory immune response were also enriched, including the apoptosis signaling pathway (**Figure 4B**). Of the 72 genes in the apoptosis signaling pathway (P00006), 66 were differentially expressed following the introduction of $nApoE4_{1-151}$.

Transcriptome Analysis of BV2 Microglia Cells Following Treatment With $ApoE3_{1-151}$

We have previously documented the nuclear presence of $nApoE_{1-151}$ fragments within microglia of the human AD brain

TABLE 1 | The twenty most up-regulated genes and their functions following treatment of BV2 microglia cells with $nApoE4_{1-151}$.

Gene symbol	Gene name	Fold change	*p*-value	Function
Il12b	Interleukin 12B	715	1.59E-89	Cytokine
Gm41236	Predicted gene, 41236	692	2.50E-14	lncRNA located near Acod1
Gbp5	Guanylate binding protein 5	672	3.79E-149	Innate immune response
Cxcl2	C-X-C Motif chemokine ligand 2	505	0	Chemokine, may suppress cell proliferation
IFI44	Interferon induced protein 44	479	1.48E-12	Immune response
Hc	Hemolytic complement	459	3.93E-31	Innate immune response
Gm41236	Predicted Gene, 41236	337	5.74E-22	lncRNA located near Il7 gene
Acod1	Aconitate decarboxylase 1	322	0	Innate immune response
Adora2a	Adenosine A2a receptor	277	3.66E-27	Adenosine signaling
1700025C18Rik	RIKEN cDNA 1700025C18 gene	268	5.57E-10	lncRNA located near Cd40 gene
Lad1	Ladinin 1	262	4.94E-10	Cell structure
Gm41647	Predicted gene, 41647	225	2.34E-09	lncRNA near socs5 gene
Serpinb2	Serpin family B member 2	219	3.12E-09	Immune response
Il1b	Interleukin 1 beta	216	0	Cytokine
Zbp1	Z-DNA Binding protein 1	213	1.46E-18	Interferon production
Gbp2	Guanylate binding protein 2	213	0	Innate immune response
LOC105245043	Uncharacterized LOC105245043	199	2.97E-09	Uncharacterized
Traf1	TNF Receptor associated Factor 1	187	0	TNF signal transduction
Il27	Interleukin 27	183	7.26E-71	Cytokine
Cd69	Cd69 Molecule	182	7.75E-50	Immune response

Of the known genes, >80% are associated with the immune system.

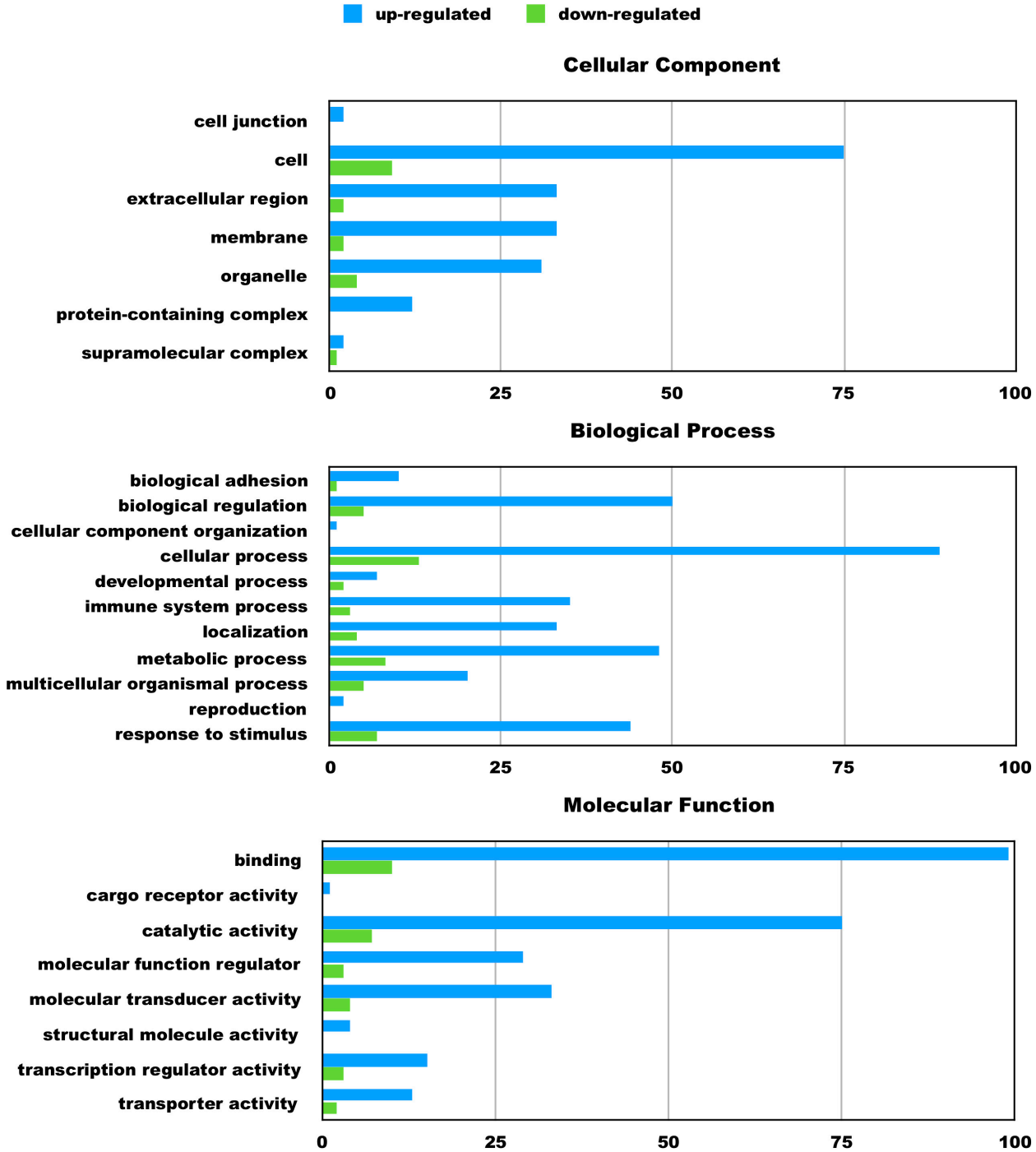

FIGURE 2 | Functional categorization of up- and downregulated genes in BV2 microglia cells treated with an amino-terminal fragment of apoE4. BV2 cells were left untreated or treated for 5 h with nApoE4$_{1-151}$ followed by total RNA purification and transcriptome analyses. The *x*-axis reflects the number of genes included for each component, process, or function. For example, there were a total of 75 upregulated genes categorized as being a part of the cellular component. Functional characterization was based on gene ontology (GO) annotations. Detailed information is shown in **Supplementary Data File S1**.

whose genotype was *E3/E3* (Love et al., 2017). This would suggest that nApoE3$_{1-151}$ may also act as a transcription factor leading to changes in gene expression. Therefore, we also performed an identical transcriptome analysis following treatment of BV2 microglia cells with nApoE3$_{1-151}$. It is important to note, that in our previous study, treatment of nApoE3$_{1-151}$ did not lead to cell toxicity (Love et al., 2017). As an initial approach, a broad overview of the similarities and differences between the two

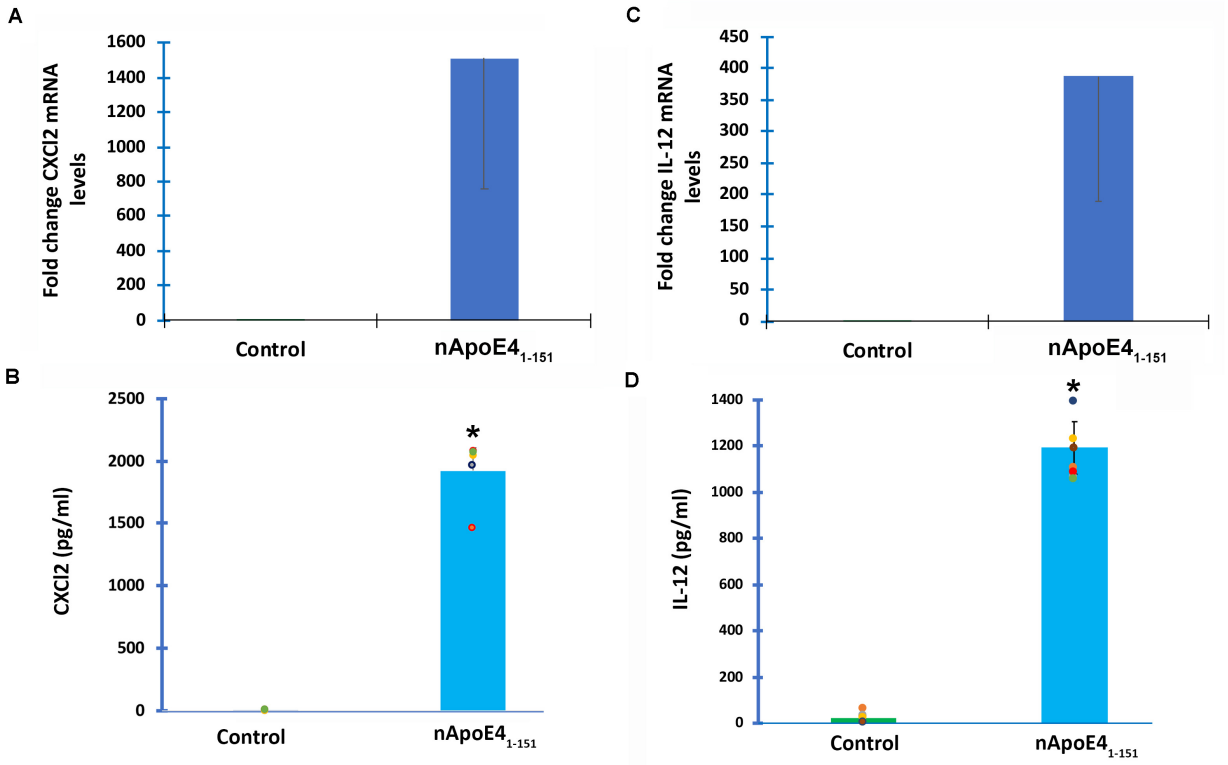

FIGURE 3 | Validation of transcriptome analysis by RT-PCR and ELISA of two identified upregulated genes. Two genes (*CXCL2* and *IL12B*) identified by transcriptome analyses to be significantly upregulated in BV2 cells following treatment with 25 μg/ml apoE4$_{1-151}$ (**Table 1**) were independently tested by RT-PCR **(A,C)** or ELISA **(B,D)** in order to validate transcriptome findings. **(A)** BV2 cells were left untreated (control, green bar) or treated for 5 h with 25 μg/ml nApoE4$_{1-151}$ (blue bar), and RNA was extracted. RT-PCR analysis indicated a ∼1,500-fold increase in the expression of the inflammatory chemokine, CXCl2. Data are representative of two independent experiments. **(B)** Secreted CXCl2 levels are significantly elevated following treatment of BV2 cells with nApoE4$_{1-151}$ (blue bar) as compared to untreated controls (green bar). Data are representative of five independent experiments ± SEM *denotes *p*-value is <0.0001 between control and nApoE4$_{1-151}$. **(C)** BV2 cells were left untreated (control, green bar) or treated for 5 h with 25 μg/ml nApoE4$_{1-151}$ (blue bar), and RNA was extracted. RT-PCR analysis indicated a ∼388-fold increase in the expression of the inflammatory cytokine, IL-12b. Data are representative of two independent experiments. **(D)** Secreted IL-12b levels are significantly elevated following treatment of BV2 cells with nApoE4$_{1-151}$ (blue bar) as compared to untreated controls (green bar). Data are representative of eight independent experiments ± SEM *denotes *p*-value is < 0.00001 between control and nApoE4$_{1-151}$.

fragments was investigated. Treatment with nApoE3$_{1-151}$ led to a differential upregulation of 2,262 genes by at least two-fold as compared to 1,617 genes for nApoE4$_{1-151}$. Examining the top 20 genes that were induced by nApoE3$_{1-151}$, the representative fold change ranged from 118- to 410-fold increased as compared to non-treated control cells (**Table 2**). This is in contrast to a representative fold change of 182- to 714-fold increase following treatment by nApoE4$_{1-151}$ (**Table 1**). Of these 20 genes, 7 were also in the top 20 for nApoE4$_{1-151}$-induced genes including *Il12B*, *GM41236*, *GBP5*, *CXCl2*, *ACOD1*, *Il1B*, and *GBP2*. Of the known top 20 genes, 70% are known to be associated with immune function (**Table 2**).

Examining the enriched upregulated pathways, many of those pathways are linked to the immune system, similar to what was found for the nApoE4$_{1-151}$ fragment (**Figure 5A**). These enriched upregulated pathways included cellular responses to interferon-beta, regulation of viral life cycle, activation of the innate immune response and defense to viruses (**Figure 5A**). Our previous findings documented the toxicity of the nApoE4$_{1-151}$ fragment, while the nApoE3$_{1-151}$ showed no toxicity under identical treatment conditions. Therefore, like for the nApoE4$_{1-151}$ (**Figure 3B**), we examined whether any genes related to apoptosis were upregulated. As shown in **Figure 5B**, there was a mix of anti-apoptotic genes induced (Bcl-2 related), Bax inhibitor-1, and cellular inhibitors of apoptosis of protein 1 and 2 as well as pro-apoptotic genes including TNF related protein, caspases, and cytochrome c (**Figure 4B**). Comparing the fold enrichment between the two fragments, there was a 3.66-fold increase in apoptosis signaling for nApoE4$_{1-151}$ fragment versus 2.68 for the nApoE3$_{1-151}$ (**Table 3**). However, these data cannot explain the difference in our previously observed toxicity between the two fragments (Love et al., 2017).

We also found similar fold increases in enrichment pathways for both fragments with regards to Toll receptor signaling and inflammation mediated by chemokine and cytokine signaling. The only difference was that for integrin signaling pathways, which was found to be specifically enriched (2.6-fold) for only the nApoE4$_{1-151}$ fragment (**Table 3**).

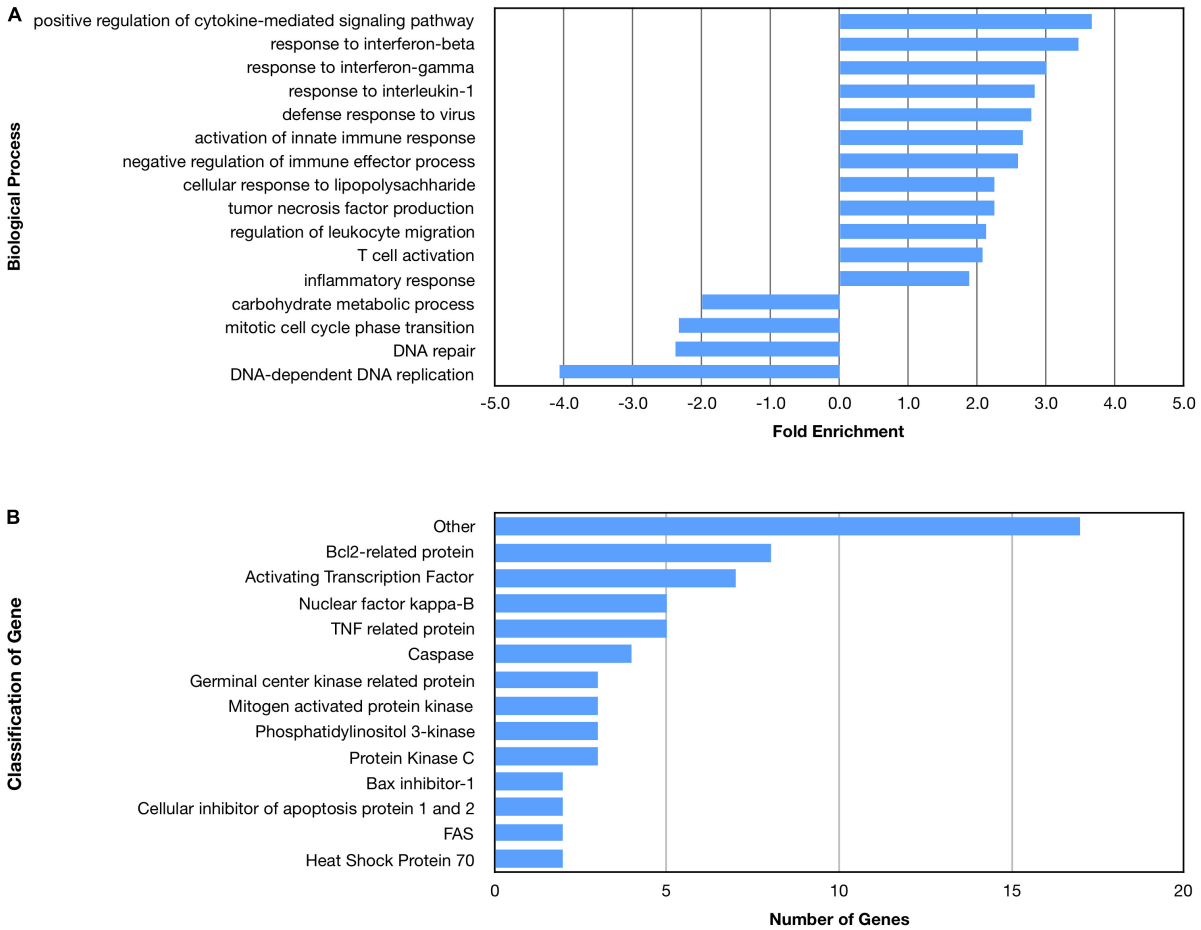

FIGURE 4 | Enriched biological processes and classification of apoptosis related genes regulated by an amino terminal fragment of nApoE4. BV2 cells were plated onto 6-well plates to confluency and treated with or without ApoE4$_{1-151}$ for 5 h. Following treatment, total RNA was extracted and transcriptome analysis was carried out as described in the Materials and Methods. **(A)** Data are expressed as fold enrichment of biological processes in BV2 microglia cells in the presence of the ApoE4$_{1-151}$. Up-regulated processes are involved in the inflammatory immune response and the activation of BV2 microglia, while down-regulated processes are involved in cell division. **(B)** Classification of genes in the apoptosis signaling pathway regulated by ApoE4$_{1-151}$. A total of 66 out of 72 genes in the apoptosis signaling pathway (P00006) were influenced by the introduction of ApoE4$_{1-151}$. Enrichment and classification analyses were conducted using the PANTHER classification system (pantherdb.org).

Looking at gene pathways downregulated, there was a significant difference found between the two fragments. The nApoE3$_{1-151}$ led to the down regulation of genes related to aerobic respiration, mitochondrial translation, and in mitochondrial respiratory chain complex 1 assembly (**Figure 5A**). In contrast, many processes involved in cell division were downregulated by the nApoE4$_{1-151}$ fragment (**Figure 4A**).

There was a significant degree of overlap in the genes differentially expressed by both nApoE fragments. Following a correlation analysis with a pool of the top 500 upregulated nApoE4$_{1-151}$ and nApoE3$_{1-151}$ genes, the trajectories of the fold changes were similar for many of the genes (S = 9651720, ρ = 0.537, p-value < 0.00). These results indicate that while the degree of change may not always align for the two treatment groups, the direction of impact follows a weak positive correlation path (**Figure 6**). In most cases, even when genes were impacted in a similar fashion, the percent fold change in reference to the control was much higher for the nApoE4$_{1-151}$ fragment than for the nApoE3$_{1-151}$ fragment (**Table 4**).

Unique Genes Differentially Regulated Following Treatment of BV2 Microglia Cells With Either nApoE3$_{1-151}$ or nApoE4$_{1-151}$

For the nApoE3$_{1-151}$ fragment, using a minimum of two-fold increase/decrease change as our criteria, a total of 1,010 genes were upregulated by this fragment alone and 644 genes downregulated (**Figure 7**). Examining the putative functions of the top 10 unique upregulated genes, there was a diverse array of genes involved in cellular processes including, mitosis, regulation of the cytoskeleton, and cell signaling. The specific proteins expressed included those for a dopamine receptor (*DRD3* gene), potassium channel (*KCNF1* gene), collagen (*COL6A1* gene),

TABLE 2 | The twenty most up-regulated genes and their functions following treatment of BV2 microglia cells with $nApoE3_{1-151}$.

Gene symbol	Gene name	Fold change	*p*-value	Function
Gm41236	Predicted gene, 41236	410	7.59E-17	lncRNA located near Acod1
Cxcl2	C-X-C Motif chemokine Ligand 2	293	0	Chemokine, may suppress cell proliferation
Nos2	Nitric oxide synthase 2	290	1.10E-180	Produces nitric oxide
Gbp5	Guanylate binding protein 5	279	2.64E-198	Innate immune response
Csf3	Colony stimulating factor 3	224	9.65E-126	Cytokine
Il12b	Interleukin 12B	221	2.56E-88	Cytokine
Gm33055	Predicted gene, 33055	201	4.22E-09	lncRNA with uncharacterized function
Acod1	Aconitate decarboxylase 1	191	0	Innate immune response
Il1b	Interleukin 1 beta	180	9.60E-159	Cytokine
Gbp6	Guanylate binding protein 6	169	4.28E-41	Innate immune response
Gbp2	Guanylate binding protein 2	164	0	Innate immune response
Il1f9	Interleukin 1F9	163	8.62E-12	Cytokine
Rsad2	Radical S-adenosyl methionine domain-containing protein 2	153	1.41E-93	Innate immune response
Ifi205	Interferon activated gene 205	149	1.13E-124	Innate immune response
Rnase10	RNase A family, 10	144	1.02E-07	Ribonuclease activity
Gm4951	Predicted gene, 4951	132	2.86E-20	Response to cytokine
Vcam1	Vascular cell adhesion molecule 1	130	6.19E-138	Immune response
Zfp811	Zinc Finger protein 811	122	1.72E-59	Nucleic acid binding
Kcnf1	Potassium voltage-gated channel modifier subfamily F member 1	118	6.50E-07	Putative voltage-gated potassium channel
Mefv	MEFV Innate immunity regulator	118	6.56E-07	Innate immune response

Of the known genes, 70% are associated with the immune system.

G-protein signaling (*HCAR1* gene), and actin stability (*NES* and *XIRP1* genes).

Using the same criteria of at least a two-fold increase/decrease change in gene expression, a total of 81 genes were upregulated by the $nApoE4_{1-151}$ fragment (**Figure 7**). For those genes downregulated following treatment with $nApoE4_{1-151}$, a total of 21 genes were identified (**Figure 7**). **Table 5** depicts the top 20 genes either upregulated or downregulated following treatment of BV2 microglia cells with the $nApoE4_{1-151}$ fragment. In both cases, these changes represent significantly fewer numbers of genes differentially expressed as compared to $nApoE3_{1-151}$. For the upregulated genes, examining the top twenty genes, only 6 (*ETNK2*, *H2-Q6*, *EVA1B*, *NAT8*, and *KCNQ2*) have known functions. The top five genes (*1700025C18Rik*, *1700003M07Rik*, *GM32846*, *GM36778*, *G930009F23RIK*) representing a fold change of 43 to 268-fold), have no known function. Other genes in this top 20 list have been associated with a number of different diseases including cancer (*SRMS*), epilepsy (*KCNQ2*), frontotemporal dementia (*TMEM252*), and amyotrophic lateral sclerosis (*TMEM252*).

DISCUSSION

ApoE is polymorphic with three major isoforms, ApoE2, ApoE3, and ApoE4, which differ by single amino acid substitutions involving cysteine-arginine replacements at positions 112 and 158 (Weisgraber et al., 1981). Harboring the *APOE3* allele is believed to neither increase nor decrease one's risk of AD, whereas inheritance of the *APOE4* allele increases disease risk upward to tenfold (Eisenstein, 2011). It is noteworthy that 65–80% of all AD patients have at least one *APOE4* allele (Saunders et al., 1993; Farrer et al., 1997). The classic function of ApoE resides in the CNS, where it is produced by a variety of cells including microglia, and transports cholesterol to neurons via apoE receptors, which are members of the low-density lipoprotein (LDL) receptor family (Pitas et al., 1987; Michikawa et al., 2000). Although ApoE3 and ApoE4 differ by a single amino acid at position 112, none of the known actions of ApoE4 sufficiently explain how harboring this allele enhances AD risk. Recent evidence from our group has suggested that this single amino acid change leads to enhanced proteolysis of the full-length protein in several fragments, including a 151 amino-terminal fragment of ApoE4 ($nApoE4_{1-151}$). We have demonstrated that this fragment is present in the human AD brain where it localizes to nuclei of microglia cells (Love et al., 2017). In addition, $nApoE4_{1-151}$ is toxic *in vitro* in BV2 microglia cells and may induce toxicity by leading to the expression of inflammatory genes including TNFα (Love et al., 2017; Pollock et al., 2019). Moreover, $nApoE4_{1-151}$ binds directly to the promoter region of TNFα and can induce expression and release of the TNFα following treatment of BV2 microglia cells (Pollock et al., 2019). The purpose of the present study was to determine in greater detail the ability of $nApoE4_{1-151}$ to induce changes in gene expression following treatment of BV2 microglia cells. As a control, we directly compared transcriptome results with an identical $nApoE3_{1-151}$ that differs by a single amino acid at position 112 (C > R). It is noteworthy that we could not test the role of an ApoE2 fragment in terms of changes in gene expression because a 1-151 amino-terminal fragment of ApoE2 would be identical to the $nApoE3_{1-151}$ fragment. Bearing this in mind, it is

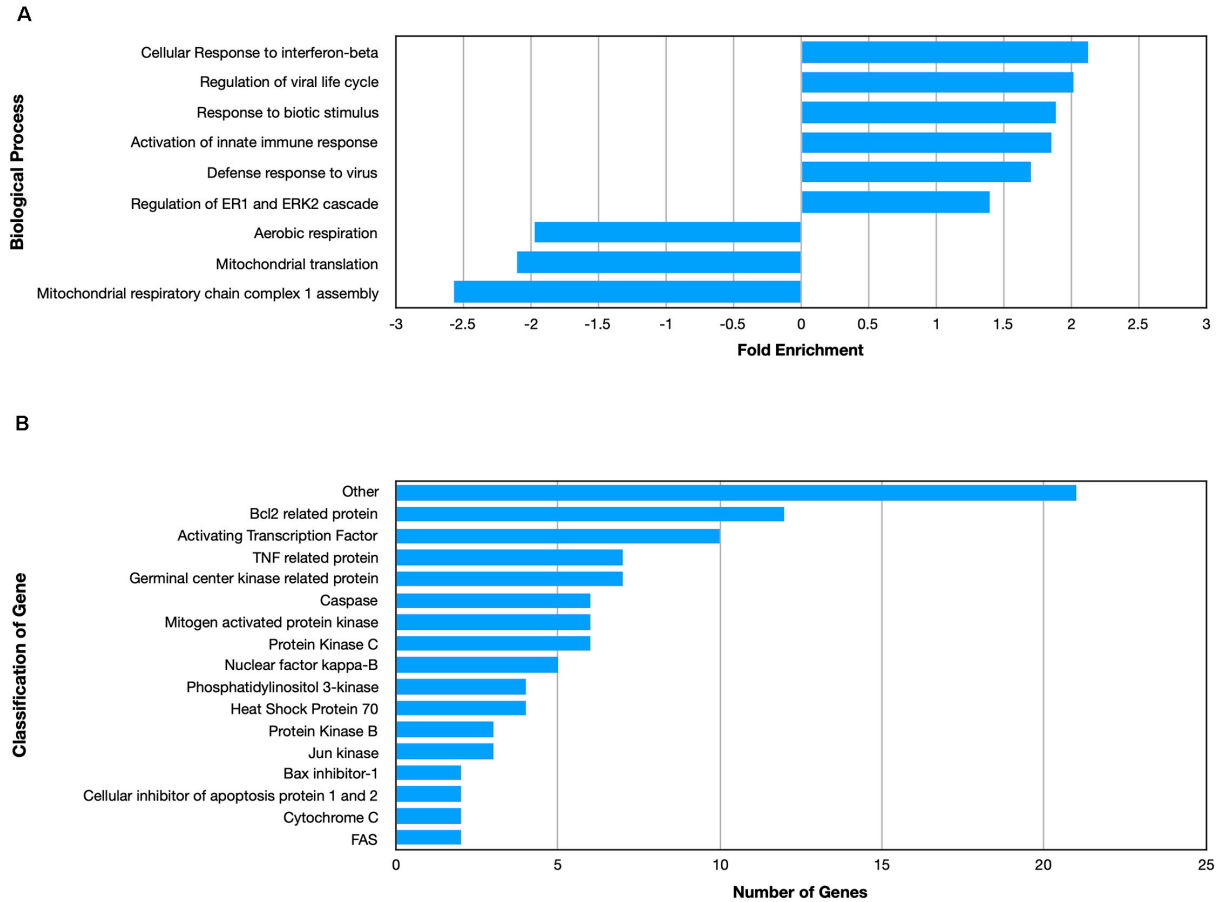

FIGURE 5 | Enriched biological processes and classification of apoptosis related genes regulated by an amino terminal fragment of nApoE3. BV2 cells were plated onto 6-well plates to confluency and treated with or without $nApoE3_{1-151}$ for 5 h. Following treatment, total RNA was extracted and transcriptome analyses was carried out as described in the Materials and Methods. **(A)** Data are expressed as fold enrichment of biological processes in BV2 microglia cells in the presence of the $nApoE3_{1-151}$. Up-regulated processes are involved in the inflammatory immune response of BV2 microglia, while down-regulated processes are involved in mitochondrial oxidative phosphorylation. **(B)** Classification of genes in the apoptosis signaling pathway regulated by $nApoE3_{1-151}$. Numerous genes involved in cell signaling pathways were upregulated following treatment with the E3 fragment. Enrichment and classification analyses were conducted using the PANTHER classification system (pantherdb.org).

TABLE 3 | Enriched pathways upregulated by $nApoE3_{1-151}$ and $nApoE4_{1-151}$.

Pathway	Enrichment by E3 fragment	Enrichment by E4 fragment
Toll receptor signaling	5.22	6.5
Chemokine/cytokine signaling	3.3	3.38
Apoptosis signaling	2.68	3.66
Integrin signaling		2.6

difficult for us to speculate on what potential structural effects of ApoE2 could potentially modulate gene expression. However, it is possible that amino-terminal ApoE2 fragments may also have an effect on gene expression, but further studies will be necessary to address this issue.

Treatment of BV2 microglia cells with $nApoE4_{1-151}$ led to the differential expression of a vast array of thousands of genes including a 715-fold increase in the expression of *IL12B* gene as well as a 504-fold increase in the *CXCL1* gene, both key mediators of inflammatory immune responses. We independently confirmed the increase in gene expression and protein secretion of IL12b, CxCl1 (**Figure 3**), and TNFα (Pollock et al., 2019) in BV2 cells by RT-PCR and ELISA assays. Overall, many biological processes involved in the inflammatory immune response were enriched by the fragment, while many processes involved in cell division were down regulated. In addition, apoptotic pathways were enriched by the $nApoE4_{1-151}$ fragment providing a possible link to the observed toxicity *in vitro* (Love et al., 2017).

Examining the top five genes induced by $nApoE4_{1-151}$ and their possible connection to AD, the top gene was *IL12B*, which increased 715-fold following treatment of BV2 microglia cells with $nApoE4_{1-151}$. This cytokine has been implicated in the AD process. Elevated levels of the p40 subunit of IL-12 have been detected in AD brains (Pitas et al., 1987), and in AD animal models, inhibition of p40 alleviates the cognitive impairments

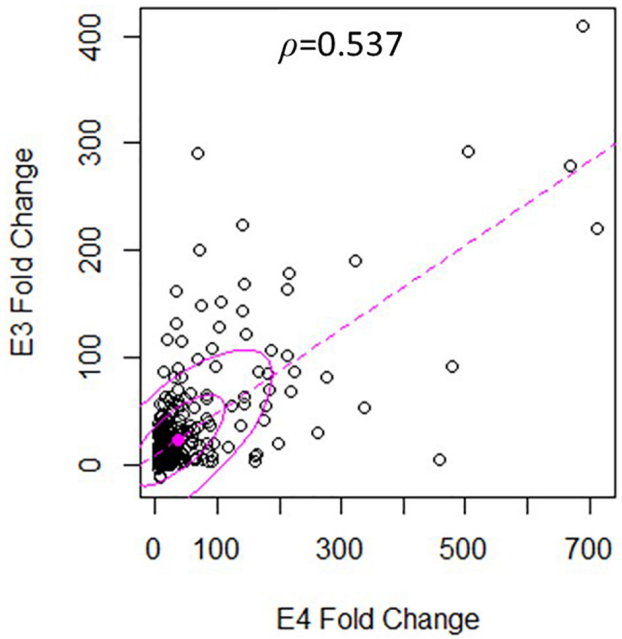

FIGURE 6 | Gene expression following treatment with fragments displays similar patterns of upregulation. The scatterplot analysis displays a moderate positive relationship between the $nApoE3_{1-151}$ and $nApoE4_{1-151}$ treatment groups (S = 9651720, ρ = 0.537, p-value < 0.00). Data is representative of three independent trials that were averaged and expressed as a percentage change in comparison to the control versus each treatment. A Spearman Rank Correlation was utilized to overcome dissonance in normality from extreme data points. Ellipses represent 0.5 and 0.95 confidence intervals and the dotted line is a linear trendline.

and AD-related pathology (Vom Berg et al., 2012; Tan et al., 2014). Rs568408 and rs3212227 SNPs, which are located in *IL-12A* and *IL-12B*, respectively, have recently been reported to influence AD risk in the Han Chinese population (Zhu et al., 2014). The *GBP5* gene increased 672-fold following treatment with $nApoE4_{1-151}$ and this gene belongs to the TRAFAC class dynamin-like GTPase superfamily. The encoded protein acts as an activator of NLRP3 inflammasome assembly and has a role in innate immunity and inflammation (Latz et al., 2013). The NLRP3 inflammasome is an important contributor to inflammatory diseases, including AD (Latz et al., 2013; Heneka, 2017; Ising et al., 2019). Finally, the *CXCl2* gene expression increased 505-fold following treatment. This gene is part of a chemokine superfamily that encodes secreted proteins involved in immunoregulatory and inflammatory processes. The CXCl2 protein and its receptor has been identified in AD patient brains and may promote Aβ-stimulated microglia activation (Boddeke et al., 1999; Van Coillie et al., 1999; El Khoury et al., 2003).

In summary, exogenous treatment of BV2 microglia cells with $nApoE4_{1-151}$ led to the upregulation of thousands of genes and of the top 20, 80% are associated with microglia activation and inflammation. These data support a possible linkage between harboring the *APOE4* allele and inflammation that has long been associated with AD (Streit, 2005; Graeber and Streit, 2010; Heneka et al., 2014; Das and Chinnathambi, 2019).

We compared the transcriptome results of $nApoE4_{1-151}$ with $nApoE3_{1-151}$ which differ by a single amino acid at position 112. It is noteworthy that in a previous study, we identified

TABLE 4 | The top 15 overlapping genes upregulated by $nApoE3_{1-151}$ and $nApoE4_{1-151}$.

Gene symbol	E3 Fold change	E4 Fold change
Il12b	221	715
Gm41236	410	691
Gbp5	279	672
CXCl2	293	505
Ifi44	92	479
Hc	5.36	459
LOC102634900	53.2	337
Acod1	191	322
Adora2a	82.7	277
Lad1	30.2	262
Gm41647	86.3	225
Serpinb2	68.1	219
Il1b	180	216
Zbp1	102	213
Gbp2	164	213

$nApoE3_{1-151}$ in postmortem human AD sections, although to a lower extent as to $nApoE4_{1-151}$ (Love et al., 2017). In BV2 cells, although $nApoE3_{1-151}$ appears to somewhat traffic to the nucleus, it does not lead to cell toxicity nor does it lead to a change of morphology indicative of activation (Love et al., 2017; Pollock et al., 2019). Therefore, we were somewhat surprised at the number of genes differentially regulated by the $nApoE3_{1-151}$ fragment. Of the top twenty genes induced,

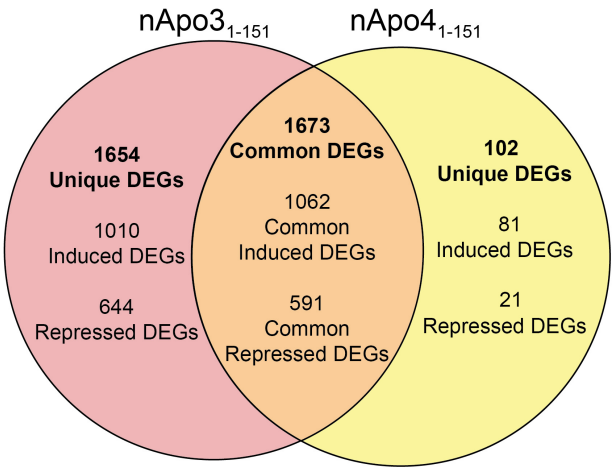

FIGURE 7 | Venn diagram demonstrating common and divergent gene regulation between nApoE3$_{1-151}$ and nApoE4$_{1-151}$ fragments.
A comparison of genes regulated more than two-fold by treatment with nApoE3$_{1-151}$ and nApoE4$_{1-151}$. nApoE3$_{1-151}$ regulated the expression of 1654 genes that were not regulated by nApoE4$_{1-151}$. Of these 1654 genes, 1010 were upregulated and 644 were downregulated. Conversely, there were 102 genes uniquely regulated by nApoE4$_{1-51}$. Of these 102 genes, 81 were upregulated, and 21 were downregulated. There were 1673 genes that were regulated by both nApoE4$_{1-151}$ and nApoE3$_{1-151}$. Also included are common differentially expressed genes (DEGs) for both fragments including 1,062 induced DEGs and 591 repressed DEGs (orange intersecting region).

TABLE 5 | Top 20 genes differentially regulated by the nApoE4$_{1-151}$ fragment.

Genes upregulated	Fold change	Genes downregulated	Fold change
1700025C18Rik	268	Adamtsl2	−38.0
1700003M07Rik	146	Treml1	−24.9
Gm32846	62.3	Zdhhc8	−20.1
Gm36778	42.7	Gm34741	−19.9
G930009F23Rik	38.9	Gm30624	−19.6
Etnk2	36.8	Gm38809	−18.4
H2-Q6	35.5	BE949265	−10.1
Eva1b	35.3	Gm26808	−9.35
Nat8	33.2	Gm34515	−7.94
Gm8075	31.5	Fam19a5	−7.61
Tmem252	31.3	Gm6416	−7.03
4930524O08Rik	29.6	Igdcc4	−6.51
LOC108168675	29.2	Pxt1	−5.61
Srms	28.0	4833415N18Rik	−5.30
Kcnq2	27.8	Pnpla5	−5.24
LOC100862202	27.6	LOC102632901	−4.63
LOC108167381	25.7	Mcidas	−3.82
Gm34542	25.6	Tpbgl	−3.21
Gm26509	25.4	Apbb1	−2.87
A530046M15Rik	25.3	Shc2	−2.72

70% are involved in immune function. In addition, six of the top twenty genes upregulated were listed in the top twenty genes induced by the nApoE4$_{1-151}$ fragment (**Tables 1**, **2**). Like nApoE4$_{1-151}$, nApoE3$_{1-151}$ led to a 2.68-fold enrichment in apoptotic pathways, compared to 3.66-fold for nApoE4$_{1-151}$ (**Table 3**). Therefore, at this time we cannot reconcile the lack of cell death as caused by treatment with nApoE3$_{1-151}$ in BV2 cells (Love et al., 2017) and the upregulation of apoptosis signaling pathways shown in the present study. It is also important to note that our read depth was sufficient so that results are not biased, meaning that the differences in the significant genes that appear in one treatment versus another are not due to a difference in coverage, but that they are actually significant.

A significant difference between the two fragments was that only nApoE4$_{1-151}$ led to enrichment in integrin signaling pathways (2.6-fold). Another interesting difference was the finding that the nApoE3$_{1-151}$ uniquely led to the downregulation of pathways involved in mitochondrial function including aerobic respiration, mitochondrial translation and mitochondrial respiratory chain complex 1 assembly (**Figure 5**). Multiple lines of evidence suggest that mitochondrial integrity and function, and innate immunity are closely interlinked processes. Mitochondria are intracellular organelles required for numerous cellular functions including energy metabolism, regulation of reactive oxygen species (ROS) signaling, Ca^{2+} homeostasis, and apoptosis (Culmsee et al., 2018). Therefore, a potential consequence of down regulating genes associated with mitochondrial function may be a dampening of the production of ROS and an overall effect of moving microglia away from activation.

We also examined those genes whose expression changed with the treatment of nApoE3$_{1-151}$ but did not change with the treatment of nApoE4$_{1-151}$ and vice versa. Using a criterion of at least a two-fold change in expression, there were ∼16 times more genes differentially upregulated by nApoE3$_{1-151}$ (1,010 genes) in comparison to nApoE4$_{1-151}$ (81 genes). Many of these nApoE3$_{1-151}$-induced genes appeared to be involved in an array of microglia processes including regulation of the cytoskeleton, cell signaling, and extracellular matrix pathways. In addition, one gene of interest was uniquely downregulated by nApoE3$_{1-151}$ by ∼20-fold. This gene, *SLC25A11* encodes for mitochondrial, 2-oxoglutarate/malate carrier (Monne et al., 2013). A recent study demonstrated that this carrier plays a key role in regulation of the NLRP3 inflammasome, a multiprotein complex, which is involved in a pro-inflammatory form of cell death (Shuvarikov et al., 2018). Thus, the downregulation of SLC25A11 protein may prevent activation of this inflammasome and thus limit this form of cell death as well as subsequent inflammation.

In contrast to those genes specifically regulated by nApoE3$_{1-151}$, many of the genes differentially expressed by nApoE4$_{1-151}$ have unknown functions. For example, of the 20 top upregulated genes, only 6 (*ETNK2*, *H2-Q6*, *EVA1B*, *NAT8*, and *KCNQ2*) have known functions. The top five genes (*1700025C18RIK*, *1700003M07Rik*, *GM32846*, *GM36778*, *G930009F23RIK*) representing a 43-268-fold change), have no known function. Although no known function has been assigned to any of these top 5 genes, one gene where there is existing evidence is *1700025C18RIK*, which increased 146-fold following nApoE4$_{1-151}$ treatment. *1700003M07Rik* encodes for AK005651 mRNA and exhibits decreased expression in thymus and spleen in non-obese diabetic mice. A type 1 diabetes susceptibility locus, *IDD11*, and recombination hotspot were

found within this gene. As a result, this gene may play a role in diabetes (Tan et al., 2010; Hamilton-Williams et al., 2013). Limited data is also available on another relatively unknown gene, *G930009F23RIK* upregulated 39-fold by the $nApoE4_{1-151}$ fragment. *G930009F23RIK* is transcribed to AK145170 lncRNA. Previous studies have shown AK145170 is upregulated 21-fold following treatment with negative factor (Nef) in mouse astrocytes (Zhou et al., 2018). Nef is a myristoylated, HIV-encoded protein released through exosomes that induces the production of various cytokines and chemokines in astrocytes to promote neuron death (van Marle et al., 2004). The effects of HIV-1 infection, especially through the neurotoxicity of Nef, may contribute to HIV-associated neurocognitive disorders (HAND) pathogenesis (Torres-Munoz et al., 2001). Thus, *G930009F23RIK* expression may play a role in the pathogenesis of HAND.

It is interesting to speculate on what role these top five genes may have and if they contribute somehow to the toxicity produced by $nApoE4_{1-151}$, which was not observed with $nApoE3_{1-151}$ (Love et al., 2017). Due to the paucity of unique genes induced by $nApoE4_{1-151}$, we were unable to analyze any enriched pathways, however, the data did reveal two genes involved in the AD-presenilin pathway and one gene involved in the AD-Aβ secretase pathway. On the other hand, enriched pathways for $nApoE3_{1-151}$, were easily calculated given the large number of genes upregulated and included collagen biosynthesis and modifying enzymes (5.17-fold enrichment), collagen formation (4.34-fold enrichment), class A/1 Rhodopsin-like receptors (2.54-fold enrichment), and GPCR ligand binding (2.23-fold enrichment). Taken together, these results can be interpreted to suggest that $nApoE3_{1-151}$ may play a more physiological role in microglia cells as compared to $nApoE4_{1-151}$, which may lead to activation, inflammation, and activation of AD pathways.

CONCLUSION

Herein, we provide transcriptome data supporting a new role for ApoE3 and E4 as regulators of gene expression. Traditionally, ApoE has an important role in regulating the metabolism of lipids by directing their transport, delivery, and distribution from one cell type to another through ApoE receptors within the CNS (Verghese et al., 2011). Our previous findings supported a role for amino-terminal fragments of ApoE4 consisting of 1–151 amino acids ($nApoE4_{1-151}$) that can traffic to the nucleus and bind to enhancer elements such as for TNFα, leading to expression, activation and cytotoxicity of BV2 microglia cells (Love et al., 2017). Full-length ApoE4 was without effect in this respect, and treatment of $nApoE3_{1-151}$ did not lead to cellular toxicity. In the current study, we now extend these findings and demonstrate $nApoE4_{1-151}$ and $nApoE3_{1-151}$ can lead to large changes in gene expression following exogenous treatment of BV2 microglial cells. Our results suggest that while $nApoE3_{1-151}$ may serve a more physiological role in this manner, $nApoE4_{1-151}$ may activate genes with a more pathological purpose. These data support the hypothesis that the link between harboring the *APOE4* allele and dementia risk could be enhanced inflammation through activation of microglia to the inflammatory M1 phenotype (see below). Future studies are warranted to understand the set of genes uniquely upregulated by the $nApoE_{1-151}$ fragment, many of which have an unknown function at this time. BV2 cells are a well-characterized, extensively employed model system for microglia. Studies have demonstrated that BV2 cells are a valid substitute for primary microglia in many experimental settings, including complex cell-cell interaction studies (Henn et al., 2009). However, there are clear limitations using these cells including that they are murine in origin and represent transformed cells, which could change their phenotype (Timmerman et al., 2018). Therefore, further studies will be needed in human iPSC-induced microglia cells or other primary cultures of microglia to confirm that similar sets of genes are expressed in human microglia cells following treatment with E3 or E4 fragments.

RNA transcriptome analyses in BV2 microglia cells following sublethal treatment with $nApoE4_{1-151}$ indicated an upregulation of almost 4,000 genes with 20 of these genes upregulated 182-715-fold as compared to untreated control cells. The majority of these 20 genes play a role in the immune response and polarization toward the microglial M1 activation phenotype. For example several studies have supported that a M1 phenotype is characterized by the upregulation of proinflammatory pathways including TNFα, IL-12, IL-1B, CCl2, whereas a shift to the M2 antinflammatory phenotype leads to the downregulation of genes such as CD86 and CD163 (Shin et al., 2014; Gensel and Zhang, 2015; Yang et al., 2016; Li et al., 2019). Treatment of BV2 cells with $nApoE4_{1-151}$ led to a similar pattern of gene regulation supporting the conclusion of a conversion to a M1 phenotype. Recently, a strong case has been made for ApoE as a pivotal regulator for microglia phenotypes (Krasemann et al., 2017). In this regard, the authors identify a subset termed microglia neurodegenerative phenotype (MGnD) that is characterized by suppressed microglial homeostatic genes such as *P2ry12, Tmem119, Olfml3, Csf1r, Rhob, Cx3cr1, Tgfb1, Mef2a, Mafb, Sall1* and upregulated inflammatory molecules included *Spp1, Itgax, Axl, Lilrb4, Clec7a, Csf1*, and *Apoe*, The authors concluded that as disease progresses, the gene expression profile of microglia switches to MGnD type. We examined the differential gene expression following treatment of BV2 cells with the $nApoE4_{1-151}$ fragment in the context of this study and found a similar increase in *Axl* (eight-fold), *CCl2* (5.6-fold), *Lilrb4* (4.43-fold), and *Spp1* (two-fold). On the other hand, we found a similar decrease in *Tmem119* (−2-fold), *CSF1r* (−1.11-fold), *Rhob* (−1.8-fold), and *Mafb* (−8.22-fold). Therefore, we believe our data support a similar phenotypic change to MGnD in our model system suggesting that $nApoE4_{1-151}$ may lead to a similar conversion of microglia in the AD brain. In a separate study, a novel type of disease-associated microglia (DAM) was characterized and was associated with beta-amyloid plaques in AD, and is also present in ALS (Keren-Shaul et al., 2017). Their analysis indicated that all of the microglia with a DAM signature expressed CD11c and showed an upregulation of other genes including *CD9*, *Clec7a*, and *CD63* (Keren-Shaul et al., 2017). In

the present study, we did not find a similar gene expression profile or other DAM-type changes in BV2 cells treated with nApoE fragments, and therefore, we do not believe $nApoE4_{1-151}$ switches microglia to this phenotype in the AD brain.

In conclusion, the $nApoE4_{1-151}$ fragment is a powerful regulator of gene expression, leading to the upregulation of numerous genes linked to inflammation associated with AD. Therefore, preventing the nuclear localization of $nApoE4_{1-151}$, perhaps through the use of structure-corrector molecules (Chen et al., 2012; Mahley and Huang, 2012) may prevent nuclear localization, blocking transcriptional effects and thus, preventing the subsequent inflammation accorded by microglia in AD (Webers et al., 2020).

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE153454: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153454.

AUTHOR CONTRIBUTIONS

TR and TP designed experiments, analyzed the data, helped construct tables and figures, and contributed to writing of the manuscript. MM analyzed the data, constructed figures, and contributed to writing of the manuscript. NI, RD, and ES helped carry out experiments. TS and GC helped analyze the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq–a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., et al. (2009). ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 25, 1091–1093. doi: 10.1093/bioinformatics/btp101

Boddeke, E. W., Meigel, I., Frentzel, S., Gourmala, N. G., Harrison, J. K., Buttini, M., et al. (1999). Cultured rat microglia express functional beta-chemokine receptors. *J. Neuroimmunol.* 98, 176–184. doi: 10.1016/s0165-5728(99)00096-x

Chen, H. K., Liu, Z., Meyer-Franke, A., Brodbeck, J., Miranda, R. D., McGuire, J. G., et al. (2012). Small molecule structure correctors abolish detrimental effects of apolipoprotein E4 in cultured neurons. *J. Biol. Chem.* 287, 5253–5266. doi: 10.1074/jbc.m111.276162

Culmsee, C., Michels, S., Scheu, S., Arolt, V., Dannlowski, U., and Alferink, J. (2018). Mitochondria, microglia, and the immune system-how are they linked in affective disorders? *Front. Psychiatry* 9:739. doi: 10.3389/fpsyt.2018.00739

Das, R., and Chinnathambi, S. (2019). Microglial priming of antigen presentation and adaptive stimulation in Alzheimer's disease. *Cell Mol. Life. Sci.* 76, 3681–3694. doi: 10.1007/s00018-019-03132-2

DeTure, M. A., and Dickson, D. W. (2019). The neuropathological diagnosis of Alzheimer's disease. *Mol. Neurodegener.* 14:32.

Eisenstein, M. (2011). Genetics: finding risk factors. *Nature* 475, S20–S22.

El Khoury, J. B., Moore, K. J., Means, T. K., Leung, J., Terada, K., Toft, M., et al. (2003). CD36 mediates the innate host response to beta-amyloid. *J. Exp. Med.* 197, 1657–1666. doi: 10.1084/jem.20021546

Farrer, L. A., Cupples, L. A., Haines, J. L., Hyman, B., Kukull, W. A., Mayeux, R., et al. (1997). Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. a meta-analysis. APOE and Alzheimer disease meta analysis consortium. *JAMA* 278, 1349–1356. doi: 10.1001/jama.278.16.1349

Gensel, J. C., and Zhang, B. (2015). Macrophage activation and its role in repair and pathology after spinal cord injury. *Brain Res.* 1619, 1–11. doi: 10.1016/j.brainres.2014.12.045

Graeber, M. B., and Streit, W. J. (2010). Microglia: biology and pathology. *Acta Neuropathol.* 119, 89–105. doi: 10.1007/s00401-009-0622-0

Hamilton-Williams, E. E., Rainbow, D. B., Cheung, J., Christensen, M., Lyons, P. A., Peterson, L. B., et al. (2013). Fine mapping of type 1 diabetes regions Idd9.1 and Idd9.2 reveals genetic complexity. *Mamm. Genome* 24, 358–375. doi: 10.1007/s00335-013-9466-y

Heneka, M. T. (2017). Inflammasome activation and innate immunity in Alzheimer's disease. *Brain Pathol.* 27, 220–222. doi: 10.1111/bpa.12483

Heneka, M. T., Kummer, M. P., and Latz, E. (2014). Innate immune activation in neurodegenerative disease. *Nat. Rev. Immunol.* 14, 463–477. doi: 10.1038/nri3705

Henn, A., Lund, S., Hedtjarn, M., Schrattenholz, A., Porzgen, P., and Leist, M. (2009). The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *ALTEX* 26, 83–94. doi: 10.14573/altex.2009.2.83

Huang, Y., Liu, X. Q., Wyss-Coray, T., Brecht, W. J., Sanan, D. A., and Mahley, R. W. (2001). Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. *Proc. Natl. Acad. Sci. U S A.* 98, 8838–8843. doi: 10.1073/pnas.151254698

Ising, C., Venegas, C., Zhang, S., Scheiblich, H., Schmidt, S. V., Vieira-Saecker, A., et al. (2019). NLRP3 inflammasome activation drives tau pathology. *Nature* 575, 669–673.

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., et al. (2017). The TREM2-APOE pathway drives the transcriptional phenotype

of dysfunctional microglia in neurodegenerative diseases. *Immunity* 47, 566–581.e9. doi: 10.1016/j.immuni.2017.08.008

Latz, E., Xiao, T. S., and Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nat. Rev. Immunol.* 13, 397–411. doi: 10.1038/nri3452

Li, Z., Liu, F., He, X., Yang, X., Shan, F., and Feng, J. (2019). Exosomes derived from mesenchymal stem cells attenuate inflammation and demyelination of the central nervous system in EAE rats by regulating the polarization of microglia. *Int. Immunopharmacol.* 67, 268–280. doi: 10.1016/j.intimp.2018.12.001

Love, J. E., Day, R. J., Gause, J. W., Brown, R. J., Pu, X., Theis, D. I, et al. (2017). Nuclear uptake of an amino-terminal fragment of apolipoprotein E4 promotes cell death and localizes within microglia of the Alzheimer's disease brain. *Int. J. Physiol. Pathophysiol. Pharmacol.* 9, 40–57.

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550.

Mahley, R. W., and Huang, Y. (2012). Small-molecule structure correctors target abnormal protein structure and function: structure corrector rescue of apolipoprotein E4-associated neuropathology. *J. Med. Chem.* 55, 8997–9008. doi: 10.1021/jm3008618

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., et al. (2017). A unique microglia type associated with restricting development of Alzheimer's disease. *Cell* 169, 1276–1290.e17. doi: 10.1016/j.cell.2017.05.018

Mi, H., Muruganujan, A., Ebert, D., Huang, X., and Thomas, P. D. (2019a). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res.* 47, D419–D426.

Mi, H., Muruganujan, A., Huang, X., Ebert, D., Mills, C., Guo, X., et al. (2019b). Protocol Update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). *Nat. Protoc.* 14, 703–721. doi: 10.1038/s41596-019-0128-8

Mi, H., and Thomas, P. (2009). PANTHER pathway: an ontology-based pathway database coupled with data analysis tools. *Methods Mol. Biol.* 563, 123–140. doi: 10.1007/978-1-60761-175-2_7

Michikawa, M., Fan, Q. W., Isobe, I., and Yanagisawa, K. (2000). Apolipoprotein E exhibits isoform-specific promotion of lipid efflux from astrocytes and neurons in culture. *J. Neurochem.* 74, 1008–1016. doi: 10.1046/j.1471-4159.2000.0741008.x

Monne, M., Miniero, D. V., Iacobazzi, V., Bisaccia, F., and Fiermonte, G. (2013). The mitochondrial oxoglutarate carrier: from identification to mechanism. *J. Bioenerg. Biomembr.* 45, 1–13. doi: 10.1007/s10863-012-9475-7

Pitas, R. E., Boyles, J. K., Lee, S. H., Hui, D., and Weisgraber, K. H. (1987). Lipoproteins and their receptors in the central nervous system. characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. *J. Biol. Chem.* 262, 14352–14360.

Pollock, T. B., Mack, J. M., Day, R. J., Isho, N. F., Brown, R. J., Oxford, A. E., et al. (2019). A fragment of apolipoprotein E4 leads to the downregulation of a CXorf56 homologue, a novel ER-associated protein, and activation of BV2 microglial cells. *Oxid. Med. Cell Longev.* 2019:5123565.

Raber, J., Huang, Y., and Ashford, J. W. (2004). ApoE genotype accounts for the vast majority of AD risk and AD pathology. *Neurobiol. Aging* 25, 641–650. doi: 10.1016/j.neurobiolaging.2003.12.023

Rohn, T. T. (2013). Proteolytic cleavage of apolipoprotein e4 as the keystone for the heightened risk associated with Alzheimer's disease. *Int. J. Mol. Sci.* 14, 14908–14922. doi: 10.3390/ijms140714908

Rohn, T. T., Catlin, L. W., Coonse, K. G., and Habig, J. W. (2012). Identification of an amino-terminal fragment of apolipoprotein E4 that localizes to neurofibrillary tangles of the Alzheimer's disease brain. *Brain Res.* 1475, 106–115. doi: 10.1016/j.brainres.2012.08.003

Saunders, A. M., Strittmatter, W. J., Schmechel, D., George-Hyslop, P. H., Pericak-Vance, M. A., Joo, S. H., et al. (1993). Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 43, 1467–1472.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shin, J. A., Lim, S. M., Jeong, S. I., Kang, J. L., and Park, E. M. (2014). Noggin improves ischemic brain tissue repair and promotes alternative activation of microglia in mice. *Brain Behav. Immun.* 40, 143–154. doi: 10.1016/j.bbi.2014.03.013

Shuvarikov, A. A., Davis, M. A., Esser-Nobis, K., and Gate, M. J. (2018). Mitochondrial SLC25 proteins interact with NLRP3 to regulate inflammasome function. *J. Immunol.* 200(1 Suppl.). 115.8.

Streit, W. J. (2005). Microglia and neuroprotection: implications for Alzheimer's disease. *Brain Res. Brain Res. Rev.* 48, 234–239. doi: 10.1016/j.brainresrev.2004.12.013

Tan, I. K., Mackin, L., Wang, N., Papenfuss, A. T., Elso, C. M., Ashton, M. P., et al. (2010). A recombination hotspot leads to sequence variability within a novel gene (AK005651) and contributes to type 1 diabetes susceptibility. *Genome Res.* 20, 1629–1638. doi: 10.1101/gr.101881.109

Tan, M. S., Yu, J. T., Jiang, T., Zhu, X. C., Guan, H. S., and Tan, L. (2014). IL12/23 p40 inhibition ameliorates Alzheimer's disease-associated neuropathology and spatial memory in SAMP8 mice. *J. Alzheimers. Dis.* 38, 633–646. doi: 10.3233/jad-131148

Timmerman, R., Burm, S. M., and Bajramovic, J. J. (2018). An overview of in vitro methods to study microglia. *Front. Cell. Neurosci.* 12:242. doi: 10.3389/fncel.2018.00242

Torres-Munoz, J., Stockton, P., Tacoronte, N., Roberts, B., Maronpot, R. R., and Petito, C. K. (2001). Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection. *J. Neuropathol. Exp. Neurol.* 60, 885–892. doi: 10.1093/jnen/60.9.885

Van Coillie, E., Van Damme, J., and Opdenakker, G. (1999). The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth. Factor. Rev.* 10, 61–86. doi: 10.1016/s1359-6101(99)00005-2

van Marle, G., Henry, S., Todoruk, T., Sullivan, A., Silva, C., Rourke, S. B., et al. (2004). Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10. *Virology* 329, 302–318. doi: 10.1016/j.virol.2004.08.024

Verghese, P. B., Castellano, J. M., and Holtzman, D. M. (2011). Apolipoprotein E in Alzheimer's disease and other neurological disorders. *Lancet Neurol.* 10, 241–252.

Vom Berg, J., Prokop, S., Miller, K. R., Obst, J., Kalin, R. E., Lopategui-Cabezas, A. I, et al. (2012). Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. *Nat. Med.* 18, 1812–1819. doi: 10.1038/nm.2965

Webers, A., Heneka, M. T., and Gleeson, P. A. (2020). The role of innate immune responses and neuroinflammation in amyloid accumulation and progression of Alzheimer's disease. *Immunol. Cell Biol.* 98, 28–41. doi: 10.1111/imcb.12301

Weisgraber, K. H., Rall, S. C. Jr., and Mahley, R. W. (1981). Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. *J. Biol. Chem.* 256, 9077–9083.

Yang, X. W., Li, Y. H., Zhang, H., Zhao, Y. F., Ding, Z. B., Yu, J. Z., et al. (2016). Safflower Yellow regulates microglial polarization and inhibits inflammatory response in LPS-stimulated Bv2 cells. *Int. J. Immunopathol. Pharmacol.* 29, 54–64. doi: 10.1177/0394632015617065

Zhou, F., Liu, X., Zuo, D., Xue, M., Gao, L., Yang, Y., et al. (2018). HIV-1 Nef-induced lncRNA AK006025 regulates CXCL9/10/11 cluster gene expression in astrocytes through interaction with CBP/P300. *J. Neuroinflamm.* 15:303.

Zhu, X. C., Tan, L., Jiang, T., Tan, M. S., Zhang, W., and Yu, J. T. (2014). Association of IL-12A and IL-12B polymorphisms with Alzheimer's disease susceptibility in a Han Chinese population. *J. Neuroimmunol.* 274, 180–184.

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REVIEW
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Aging and Neurodegenerative Disease: Is the Adaptive Immune System a Friend or Foe?

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Neurodegenerative diseases of the central nervous system (CNS) are characterized by progressive neuronal death and neurological dysfunction, leading to increased disability and a loss of cognitive or motor functions. Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis have neurodegeneration as a primary feature. However, in other CNS diseases such as multiple sclerosis, stroke, traumatic brain injury, and spinal cord injury, neurodegeneration follows another insult, such as demyelination or ischaemia. Although there are different primary causes to these diseases, they all share a hallmark of neuroinflammation. Neuroinflammation can occur through the activation of resident immune cells such as microglia, cells of the innate and adaptive peripheral immune system, meningeal inflammation and autoantibodies directed toward components of the CNS. Despite chronic inflammation being pathogenic in these diseases, local inflammation after insult can also promote endogenous regenerative processes in the CNS, which are key to slowing disease progression. The normal aging process in the healthy brain is associated with a decline in physiological function, a steady increase in levels of neuroinflammation, brain shrinkage, and memory deficits. Likewise, aging is also a key contributor to the progression and exacerbation of neurodegenerative diseases. As there are associated co-morbidities within an aging population, pinpointing the precise relationship between aging and neurodegenerative disease progression can be a challenge. The CNS has historically been considered an isolated, "immune privileged" site, however, there is mounting evidence that adaptive immune cells are present in the CNS of both healthy individuals and diseased patients. Adaptive immune cells have also been implicated in both the degeneration and regeneration of the CNS. In this review, we will discuss the key role of the adaptive immune system in CNS degeneration and regeneration, with a focus on how aging influences this crosstalk.

Keywords: aging, adaptive immune system, neurodegenerative diseases, degeneration, regeneration

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INTRODUCTION

Neurodegenerative disease defines conditions in which there is progressive neuronal loss in the central nervous system (CNS), leading to either physical disability, cognitive deficits or both. Classical neurodegenerative diseases in which neurodegeneration is the key hallmark includes Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Dugger and Dickson, 2017). However, other diseases can be defined as neurodegenerative when a primary insult such as demyelination, ischaemia or trauma leads to neuronal loss. Multiple sclerosis (MS), stroke and traumatic injury to the CNS are all examples of secondary neurodegenerative diseases (Amor et al., 2010). Aging is a major risk factor for neurodegenerative disease, and with a growing elderly population, its prevalence is continuously increasing (Wyss-Coray, 2016). Beyond being a risk factor, aging also increases the severity of disease and results in an impaired recovery following insult. Although these diseases have different pathogenetic mechanisms such as protein aggregation, demyelination, ischaemia, or direct trauma, they all share a hallmark of neuroinflammation (Stephenson et al., 2018).

The immune system plays a key role in CNS homeostasis and disease. The innate immune system is the first line of defense against pathogens (Chaplin, 2010) and CNS-resident macrophages, microglia, are of vital importance as early respondents to CNS alterations such as damage or infection but also in development and homeostasis (Bachiller et al., 2018). Microglia activation is also an important component of neuroinflammation, aging, and different neurodegenerative diseases either directly via phagocytosis and cytokine production, as shown by the identification of disease-specific microglia, or indirectly in response to cues from the adaptive immune system (Keren-Shaul et al., 2017; Deczkowska et al., 2018). The adaptive immune system is an important component of the host defense against pathogens, through the recognition of non-self antigens (Chaplin, 2010). This defensive mechanism is mediated by B and T lymphocytes which display a diverse range of specific antigen receptors during humoral and cellular-mediated immunity (Chaplin, 2010). Although the CNS was once considered an 'immune-privileged' site, recent studies have indicated the presence and importance of the adaptive immune system in the CNS for immune-surveillance and defense against neurotropic viruses (Ellwardt et al., 2016). Studies have also highlighted the role of adaptive immunity in maintaining CNS homeostasis and integrity, promoting neurogenesis and improving cognitive function (Ziv et al., 2006; Brynskikh et al., 2008; Radjavi et al., 2014). In healthy individuals, this immune-CNS interaction is highly regulated to maintain the beneficial relationship. However, during both aging and neurodegenerative disease, the blood-brain barrier (BBB) is disrupted, leading to an increased infiltration of peripheral immune cells into the CNS, where they can potentiate further neurodegeneration or facilitate tissue regeneration. In both neurodegenerative disease and the normal aging process, there is a common theme of immune dysregulation and abnormal immune responses. This review will discuss the involvement of the adaptive immune system in neurodegenerative disease, highlighting its role in degeneration and regeneration, and the impact of aging in disease pathogenesis.

HEALTHY CNS

In addition to the key role of the adaptive immune system in CNS homeostasis and immunosurveillance, it also influences brain development and behavior, particularly affecting hippocampal neurogenesis. Severe combined immunodeficient (SCID) mice, lacking mature lymphocytes, showed impaired neurogenesis compared to wild-type (WT) mice, in which T cells infiltrate the CNS (Ziv et al., 2006). SCID mice also show a reduced learning capacity and impaired memory (Brynskikh et al., 2008; Luo et al., 2019), possibly associated with a higher expression of neurotransmission-related genes, indicating dysfunctional synaptic connectivity or an imbalance of neurotransmitters as a result of lymphocyte deficiency (Luo et al., 2019). Adaptive immune cells in particular are important for normal learning and memory function, as $Rag2^{-/-}$ mice, which lack mature lymphocytes, show impaired cognitive function (Radjavi et al., 2014). This effect is T cell-mediated as function was restored upon adoptive transfer of splenocytes but not T cell-depleted splenocytes (Kipnis et al., 2004; Ziv et al., 2006; Brynskikh et al., 2008). Similarly, nude mice lacking mature lymphocytes show diminished neurogenesis and impaired cognitive function (Kipnis et al., 2004; Ziv et al., 2006). T cell activation and a simultaneous increase in corticosterone levels also leads to a transient increase in proliferation of hippocampal progenitor cells and neurogenesis (Wolf et al., 2009b).

Adoptive transfer of $CD3^+$, $CD4^+$, and $CD8^+$ T cells derived from human umbilical cord blood mononuclear cells into rats was shown to enhance the proliferation and survival of neural stem cells (Shahaduzzaman et al., 2013). However, other studies suggest that $CD4^+$ T cells, but not $CD8^+$ T cells or B cells, are involved in promoting hippocampal neurogenesis and maintaining cognitive function, as only $CD4^+$ T cell depletion impaired these processes (Wolf et al., 2009a). Even if the role for $CD4^+$ T cells in neurogenesis is widely accepted, the involvement of antigen-specificity has been debated. Wolf et al. suggest a systemic mechanism of $CD4^+$ T cell-mediated neurogenesis, showing that repopulation of $Rag2^{-/-}$ mice with non-specific $CD4^+$ T cells increases neural stem cell proliferation (Wolf et al., 2009a). Others, however, have shown the requirement for CNS antigen specificity, as increased neurogenesis and improved cognitive function was only observed with the adoptive transfer of MBP-specific T cells and not ovalbumin (OVA)-specific T cells, reactive toward a foreign protein (Ziv et al., 2006). Mice which only have OVA-specific T cells present learning and memory deficits, which were restored upon transfer of MOG-specific $CD4^+$ T cells (Radjavi et al., 2014). Nonetheless, both studies agreed on the $CD4^+$ T cell-mediated promotion of brain-derived neurotrophic factor (BDNF), which is required for neuronal survival and differentiation (Ziv et al., 2006; Wolf et al., 2009a).

In contrast, neurogenesis is impacted by aging, where neurogenic niches become dysfunctional, in part due to an increased infiltration of $CD8^+$ T cells (Dulken et al., 2019).

Gene expression changes in $CD8^+$ T cells and neuronal stem cells in aged neurogenic niches show increased genes related to the IFN-γ pathway and IFN-γ signaling, respectively (Dulken et al., 2019). This highlights the dynamic balance between the beneficial and detrimental impacts of the adaptive immune system in the CNS, depending on the environment (summarized in **Figure 1**). The interaction between the CNS, adaptive immune system and aging is further modified by disease states. We next address these relationships in the context of several common neurodegenerative diseases (summarized in **Tables 1**, **2**).

ALZHEIMER'S DISEASE

Alzheimer's disease is a progressive neurodegenerative disease with key hallmarks of cognitive dysfunction, memory loss and behavioral disturbances in the elderly population (Chen and Mobley, 2019). AD is primarily characterized by the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT) of hyperphosphorylated tau in the brain (Chen and Mobley, 2019), leading to synaptic loss, reduced dendritic spines and neuronal death (Paulson et al., 2008). These protein aggregates not only cause neurodegeneration but also lead to the dysfunction of other glial cells such as oligodendrocytes, astrocytes, and microglia (Jantaratnotai et al., 2003; Desai et al., 2011). Accumulation is also associated with microglial and astrocyte activation, which induces inflammation and oxidation, promoting further neuronal dysfunction and apoptosis (Hardy and Allsop, 1991; Kametani and Hasegawa, 2018). Age is the most important risk factor for AD, with ∼90% of cases being late-onset and ∼10% early-onset (Tanzi, 2012; Bature et al., 2017). However, genetics is also a risk factor, particularly in early-onset patients who are more likely to have familial AD with mutations in the Aβ precursor protein (APP) and the presenilin genes (PSEN1 and PSEN2) (Bature et al., 2017; Chen and Mobley, 2019).

Neuroinflammation has been implicated in the pathogenesis of AD, with the innate immune system thought to play a dominating role in the recruitment of microglia to the site of damage (Nordengen et al., 2019). However, the role of the adaptive immune system in AD remains poorly understood. Evidence has shown increased numbers of T lymphocytes in the post-mortem brain tissue of AD patients compared to healthy controls (Rogers et al., 1988; Togo et al., 2002). The increased number of $CD3^+$ T cells in AD patients brains were mostly $CD8^+$ T cells, which significantly correlated with tau but not Aβ burden, suggesting a role for T cells in NFT development (Merlini et al., 2018). In an animal model of AD, in which mutations in APP and PS1 cause elevated levels of Aβ, there was similarly an increase of T cells in the brain parenchyma (Browne et al., 2013). Evaluation of T cell subsets in the peripheral blood of AD patients showed decreased regulatory T cells (Tregs) (Ciccocioppo et al., 2019), increased Th17 cells (Oberstein et al., 2018) and increased $CD8^+$ T cells (Gate et al., 2020). Another study showed similar proportions of Tregs in the peripheral blood of patients and controls, and established a correlation between circulating Tregs and tau in the CSF as an indication of less severe disease (Oberstein et al., 2018).

Aβ-specific Th1 cells adoptively transferred into APP/PS1 mice leads to microglial activation, production of pro-inflammatory cytokines, Aβ deposition and impaired cognitive function (Browne et al., 2013). This pathogenic effect is thought to be mediated by IFN-γ, with IFN-γ neutralization attenuating the Th1-associated detrimental effect (Browne et al., 2013). However, a contradictory study shows an IFN-γ-producing $CD4^+$ T cell-dependent mechanism of macrophage recruitment and Aβ clearance, leading to improved cognitive function in an AD mouse model (5xFAD) (Baruch et al., 2016). These contradictions may be due to the different methods used, with one transferring Aβ-specific Th1 cells and the other inducing a natural increase in Th1 cells due to treatment with a PD-1 immune checkpoint inhibitor. Induction of AD in rats through the injection of Aβ into the hippocampus also causes Th17 cell infiltration and the upregulation of IL-17 and IL-22 in the hippocampus, blood and CSF (Zhang J. et al., 2013). Th17 cells are similarly thought to promote neurodegeneration by acting directly on neurons via the Fas/FasL apoptotic pathway (Zhang J. et al., 2013) and are also implicated in the dysfunctional neurogenesis seen in AD; which can be rescued by the genetic deletion of IL-17 (Liu et al., 2014).

There is further evidence of $CD8^+$ T cell trafficking in AD, with clonally expanded antigen-specific $CD8^+$ T cells in the CSF of AD patients (Gate et al., 2020). In THY-Tau22 mice, which develop tau pathology and cognitive dysfunction, activation of microglia and astrocytes was accompanied by an infiltration of $CD8^+$ T cells linked to an early CCL3 chemokine response (Laurent et al., 2017). Similarly, $CD8^+$ T cell infiltration was also observed in AD patients with a P301L tau mutation (Laurent et al., 2017). T cell depletion reversed cognitive deficits without affecting tau burden, indicating a detrimental T cell response to tau burden (Laurent et al., 2017). These studies therefore support a role of $CD8^+$ T cells in neuroinflammation and neurodegeneration in a model of tauopathy.

Although the adaptive immune system, specifically T lymphocytes, have been implicated in AD pathogenesis, the presence of these cells can also be beneficial. Adaptive immune cells were genetically deleted in the 5xFAD animal model, enhancing AD pathology (Rag-5xFAD) (Marsh et al., 2016). Rag-5xFAD mice showed increased Aβ pathology and neuroinflammation, following the upregulation of pro-inflammatory cytokines implicated in neurodegeneration, plaque formation and cognitive impairments (IL-1β, IL-6, and TNFα) (Cacabelos et al., 1994; Huell et al., 1995; Tweedie et al., 2012; Marsh et al., 2016). Bone marrow transplantation to replace the adaptive immune cells negated the AD pathology, mainly through microglia modulation (Marsh et al., 2016).

Research into T cell subsets has highlighted a role for Th2 cells and Tregs in the reduction of AD pathology and regeneration. Aβ-specific polarized Th2 cells adoptively transferred into APP/PS1 mice showed reduced plaque-associated microglia, reduced Aβ deposits, and a reversal of cognitive dysfunction, with a memory and identification ability recovery similar to WT animals (Cao et al., 2009). Th2-mediated reversal of AD pathology may be IL-4-dependent, as $IL\text{-}4^{-/-}$ mice show cognitive impairments which are reversed upon the

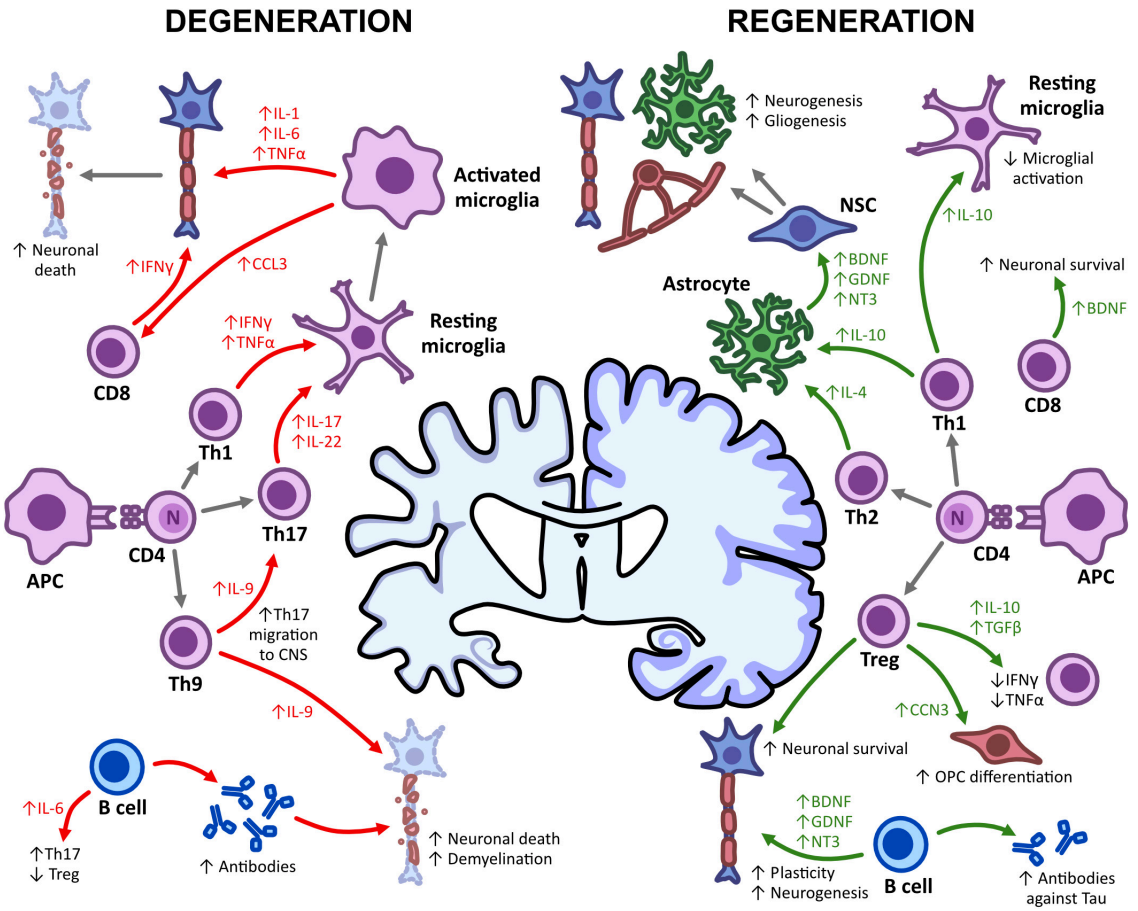

FIGURE 1 | Adaptive immunity in CNS degeneration and regeneration. The damage and repair of the CNS is mediated by immune mechanisms, with both T and B cells having detrimental and regenerative effects. Upon APC-T lymphocyte interactions, T effector cells such as Th1, Th17, and Th9 promote microglial activation through the expression of pro-inflammatory cytokines (IFN-γ, TNFα, IL-9, and IL-17). This subsequently enhances neuronal death and demyelination and leads to a decline in cognitive functioning. Th1, Th2, and Treg cells, however, can also promote regeneration, enhancing neurogenesis, gliogenesis and remyelination upon the secretion of IL-4, IL-10, and TGFβ. Somewhat similar to Th1, $CD8^+$ T cells, and B cells also increase neuronal death but can promote survival through neurotrophic production (BDNF, GDNF, and NT3) and an increased antibody repertoire. This shows the impact of immune-mediated mechanisms on degeneration/regeneration and some possible targets for immunotherapies.

adoptive transfer of WT T cells (Derecki et al., 2010). This is further supported by studies showing that T cell-derived IL-4 can modulate meningeal myeloid cells, increasing astrocyte expression of BDNF, associated with reduced cognitive deficits and neurodegeneration in a mouse model of tauopathy (Derecki et al., 2010; Jiao et al., 2016).

Tregs delay the progression of AD pathology, with Treg depletion accelerating cognitive decline (Baek et al., 2016; Dansokho et al., 2016). Increasing Treg numbers reversed the cognitive deficits observed in APP/PS1 mice and increased microglial numbers in plaques (Dansokho et al., 2016). Adoptive transfer of Tregs into 3xTg-AD mice similarly improved cognitive function and reduced Aβ deposition and plaque formation (Baek et al., 2016). Although total depletion is detrimental, the transient genetic ablation of Tregs resulted in improved cognitive functioning and a reduction in Aβ plaque area, through an increased infiltration of macrophages and $CD4^+$ T cells (Baruch et al., 2015). This suggests that controlled Treg depletion may be neuroprotective and instead contribute to AD mitigation by minimizing the suppression of other T cell subsets.

PARKINSON'S DISEASE

Parkinson's disease is another example of a classical neurodegenerative disease of the CNS, characterized by motor dysfunction and neuropsychiatric symptoms. The hallmarks of PD pathogenesis are Lewy body/neurite formation and death of dopamine-secreting neurons in the substantia nigra, a region which modulates motor movement (Eriksen et al., 2005). Lewy bodies and neurites are formed by the aggregation of α-synuclein within neuronal cell bodies and neuronal processes, respectively (Eriksen et al., 2005). Although the cause of PD is unknown, there are hypotheses of disease pathogenesis including neuroinflammation, mitochondrial defects and dysfunctional protein clearance (DeMaagd and Philip, 2015).

Neuroinflammation in PD is a result of activated microglia and monocytes in response to misfolded protein α-synuclein (Mosley et al., 2012). These innate immune cells secrete pro-inflammatory and neurotoxic cytokines and chemokines, leading to BBB disruption and the infiltration of lymphocytes to the site of damage (Mosley et al., 2012). Although evidence for the role of the immune system can be distinguished through blood and post-mortem brain samples, and through the efficacy of treatment trials, much of the research refers to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) PD mouse model. This model employs a toxin-induced death of dopaminergic neurons to mimic the damage seen in PD patient brains (Meredith and Rademacher, 2011).

Systemic inflammation increases the risk of PD (Chen et al., 2003), however, the numbers of lymphocytes within the periphery and the CNS remains controversial. Studies have shown a decrease in T and B lymphocytes in the peripheral blood of PD patients (Bas et al., 2001; Baba et al., 2005; Stevens et al., 2012). While there are fewer B lymphocytes, the levels of α-synuclein-specific autoantibodies are increased in the blood and CSF of PD patients (Horvath et al., 2017; Shalash et al., 2017; Akhtar et al., 2018). The decreased number of B lymphocytes is also associated with alterations in the expression of B cell-related genes in peripheral blood leukocytes in PD patients (Kobo et al., 2016). However, it is unclear whether the change in number or functionality of B lymphocytes are causal or secondary to CNS injury in PD (Sabatino et al., 2019). Evaluation of T lymphocyte subsets showed a decrease in $CD4^+$ T cells with an increase in $CD8^+$ T cells (Baba et al., 2005; Saunders et al., 2012; Stevens et al., 2012). Despite this decrease in $CD4^+$ T cells, there is an evident shift in cell phenotype proportions, with decreased naïve and increased effector and memory cells (Fiszer et al., 1994; Bas et al., 2001; Saunders et al., 2012). There is further controversy on the proportions of $CD4^+$ T cell subsets in the circulation of PD patients, as one study highlights an increase in Tregs (Bas et al., 2001) and others show a decrease and loss in functionality (Baba et al., 2005; Saunders et al., 2012; Chen et al., 2015; Sommer et al., 2019). Levels of Th1 cells are also debated, some studies show a shift toward a Th1 immune response with IFN-γ production, correlating with disease rating scores (Baba et al., 2005; Chen et al., 2015). Others, however, show decreased Th1 cells and no evidence of subset dominance in PD patients (Niwa et al., 2012; Sommer et al., 2019). An increase in Th17 cells has also been shown in the peripheral blood of PD patients (Chen et al., 2015; Sommer et al., 2019). While these studies do not always agree on lymphocyte levels, there is consistent evidence for immune cell dysregulation in the circulation of PD patients. $CD3^+$ T cells infiltrate the brains of PD patients, with post-mortem brain tissue showing cells localized around damaged neurons (Sommer et al., 2019). The immune infiltrate consists of both $CD4^+$ and $CD8^+$ T cells, but not B cells, suggesting a role for T lymphocytes in the pathogenesis or repair of PD (Brochard et al., 2009). Although B cells were not identified in PD post-mortem tissue, another study showed IgG deposits on Lewy bodies and dopaminergic neurons, highlighting a role for B cell antibody production in PD (Orr et al., 2005).

Neuroinflammation has proven to be pathogenic in PD through inhibition of the JAK/STAT pathway, which is critical in the modulation of the immune system (Qin et al., 2016). Inhibiting the JAK/STAT pathway in rats overexpressing α-synuclein prevented the loss of dopaminergic neurons and neuroinflammation, following the suppression of both microglial activation and $CD4^+$ T cell infiltration (Qin et al., 2016). Lymphocytes are also implicated in the loss of dopaminergic neurons, with recent investigations suggesting a potential autoimmune role for T lymphocytes in PD, as patient-derived T cells recognize α-synuclein in pre-clinical and early PD cases (Sulzer et al., 2017; Lindestam Arlehamn et al., 2020).

The role of the adaptive immune system in the pathogenesis of PD was shown using different MPTP models. In an MPTP monkey model of PD, treatment with the antiviral oral drug Maraviroc led to a reduced infiltration of T lymphocytes into the CNS, protecting from nigrostriatum neuronal cell death and improving locomotor activities (Mondal et al., 2019). Similarly, SCID, $Rag1^{-/-}$ and $Tcrb^{-/-}$ mice, all lacking mature lymphocytes, show attenuated dopaminergic cell death following MPTP treatment, an effect abolished upon reconstitution with WT splenocytes (Benner et al., 2008; Brochard et al., 2009). This detrimental effect is mediated by $CD4^+$ T cells, as $CD4^{-/-}$ mice show attenuated neuronal cell death after MPTP induction, but $CD8^{-/-}$ mice do not (Brochard et al., 2009). Reconstitution of SCID mice with WT $CD4^+$ T cells abolishes this attenuated neuronal death (Gonzalez et al., 2013). $CD4^+$ T cell-mediated neurodegeneration requires the expression of FasL but not IFN-γ, suggesting cytotoxic mechanisms of cell death (Brochard et al., 2009).

Besides Th1 cells, Th17 cells and the cytokine IL-17 have also been associated with PD. Immunization of animals with nitrated α-synuclein induces an adaptive immune response in the MPTP model. The adoptive transfer of immune cells from immunized mice into recipients prior to MPTP exacerbated neuroinflammation and neurodegeneration, a mainly Th17-mediated effect (Reynolds et al., 2010). $CD4^+$ T cells isolated from immunized mice displayed a shift toward a Th17/Th1 phenotype, through the production of pro-inflammatory and neurotoxic cytokines such as IL-17, TNF-α, and IFN-γ (Reynolds et al., 2010). Adoptive transfer of *ex vivo* polarized T cells from immunized mice similarly showed that Th17 cells exacerbate neuronal loss, while Th1 cells show only a slight increase in neurodegeneration (Reynolds et al., 2010). Further supporting the role of IL-17 in PD pathogenesis, autologous co-cultures of iPSC-derived midbrain neurons and activated T cells from PD patients showed increased neuronal cell death, associated with an increased production of T cell-derived IL-17 and upregulated IL-17 receptor on neurons (Sommer et al., 2019). This degenerative effect was abolished following the pre-treatment of neurons with IL-17 or IL-17 receptor neutralizing antibodies, suggesting a role for Th17 cells in PD neuronal death (Sommer et al., 2019). These studies highlight a disrupted balance of $CD4^+$ T cell subsets, with a shift toward pro-inflammatory Th17/Th1 cells, causing the dopaminergic neurodegeneration seen in PD.

On the other hand, the regenerative capacity of the adaptive immune system in PD is predominantly mediated by Tregs.

Mice immunized with copolymer 1 (Cop-1) to generate T cells which are non-encephalitic and MBP-specific are protected from neurodegeneration in response to neurotoxicity (Schori et al., 2001). Moreover, adoptive transfer of splenocytes from mice immunized with Cop-1 to MPTP recipient mice led to protection against dopaminergic neuronal cell death (Benner et al., 2004). Dopaminergic protection is mediated by the infiltration of donor T lymphocytes to the area of damage as the adoptive transfer of T cell-depleted splenocytes shows no protective effect (Benner et al., 2004). T lymphocyte secretion of IL-4 and IL-10 suppresses microglial activation and induces astrocytic production of glial cell-derived neurotrophic factor (GDNF), a factor contributing to neuroprotection (Benner et al., 2004). Adoptive transfer of activated Tregs following MPTP induction resulted in over 90% survival of dopaminergic neurons, whilst the adoptive transfer of effector T cells showed no effect (Reynolds et al., 2007). Treg-mediated neuroprotection was conferred by modulation of neuroinflammation, increased neurotrophic production and suppressed microglial responses to stimuli, including aggregated α-synuclein (Reynolds et al., 2007). This was further validated *in vitro*, where co-cultures of microglia activated by α-synuclein and T cell subsets reveal that Tregs modulate microglial production of reactive oxygen species and the activation of nuclear factor kappa B (NFKB) (Reynolds et al., 2009). In contrast, effector T cells aggravate microglial inflammation and neurotoxicity (Reynolds et al., 2009).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is also considered neuroprotective in both animal models of PD and in PD patients, with neuroprotection primarily mediated through elevated Treg levels (Kosloski et al., 2013; Gendelman et al., 2017; Schutt et al., 2018). Treatment of mice with GM-CSF prior to MPTP intoxication protected against dopaminergic neuronal loss, with increased proportions of Tregs and reduced microgliosis (Kosloski et al., 2013). Adoptive transfer of $CD4^+$ T cells or Tregs from GM-CSF treated mice following MPTP intoxication protected dopaminergic neurons and attenuated microglial activation (Kosloski et al., 2013). Clinical trials using recombinant GM-CSF (sagramostim) have also shown increased levels of Tregs and improved motor function in PD patients (Gendelman et al., 2017). Although these studies highlight the protective role of Tregs in preventing dopaminergic neuronal damage, they do not assess the role of T cells in PD-related regeneration.

Similarly to AD, age is a major risk factor for PD, with older onset being associated with a more severe disease phenotype and disability status (Pringsheim et al., 2014). Aging results in greater motor dysfunction, dopaminergic disturbances and a reduction of α-synuclein and total tau in the CSF (Mehanna et al., 2014; Szewczyk-Krolikowski et al., 2014; Pagano et al., 2016). Although age does not influence the common pathological final stages of PD, it does impact disability milestones over the early and progressive phases (Kempster et al., 2010). The mechanistic impact on disease progression and severity remains to be elucidated, with speculation primarily given to how aging impacts cell numbers and signaling in patients. The number of Tregs increase with age, however, no difference was observed in total Treg numbers or functionality in healthy elderly controls and elderly PD patients (Rosenkranz et al., 2007). Neurotoxin exposure occurring in PD exacerbates the neuroinflammation and oxidative stress seen during normal aging, leading to downregulation of the Wnt/β-catenin signaling pathway, which is essential for dopaminergic neurogenesis (Marchetti et al., 2020). This suggests that immune alterations in aging patients could potentially contribute to age-associated PD pathology.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis is a classical neurodegenerative disease of the CNS, mainly characterized by motor dysfunction including spasticity, muscle weakness and dysphagia (Hardiman et al., 2017). ALS causes degeneration of motor neurons, resulting in progressive muscle weakness and subsequent paralysis, ultimately ending in respiratory failure and death (Hardiman et al., 2017). Affecting both upper and lower motor neurons, ALS mainly leads to motor symptoms. However, a growing body of literature has recently shown that non-motor symptoms such as cognitive and behavioral deficits, similar to those observed in frontotemporal dementia (FTD), can be seen in ALS patients (Ferrari et al., 2011; Phukan et al., 2012; Hardiman et al., 2017). The exact pathophysiological mechanism of ALS is unknown, however, protein aggregation in motor neurons and surrounding oligodendrocytes represents a hallmark of the disease. TAR DNA-binding protein 43 (TDP-43) is the main component of these aggregates and is detected in most ALS patients (Neumann et al., 2006). The accumulation of other misfolded proteins has also been found in specific ALS subtypes, such as misfolded superoxide dismutase 1 (SOD1) and fused in sarcoma (FUS) proteins (Morgan and Orrell, 2016). Similar to other neurodegenerative diseases, aging is a major risk factor for ALS development, with peak incidence between 75 and 79 years of age (Alonso et al., 2009).

Although adaptive immunity is not the central pathogenic mechanism of ALS, T lymphocytes displaying pro-inflammatory features contribute to ALS progression and severity. Infiltrated T lymphocytes were found in the post-mortem spinal cord of ALS patients (Engelhardt et al., 1993). Studies evaluating immune cell profiles in the peripheral blood of healthy controls and ALS patients showed no differences in levels of total lymphocytes or $CD3^+$ T lymphocytes, however, highlighted differences within specific T cell subsets (Saresella et al., 2013; Shin et al., 2013; Chen et al., 2014; Jin et al., 2020). ALS patients have significantly lower proportions of $CD4^+$ T cells with unchanged or elevated $CD8^+$ T cell proportions (Chen et al., 2014; Jin et al., 2020). Despite this decreased CD4:CD8 T cell ratio, only a higher $CD4^+$ T cell percentage in ALS patients correlates with disease severity and progression (Shi et al., 2007; Chen et al., 2014). This correlation was associated with a shift toward a Th1/Th17 cell-mediated pro-inflammatory immune response (Saresella et al., 2013; Jin et al., 2020). Even though the percentages of anti-inflammatory Th2 cells and Tregs were decreased (Saresella et al., 2013; Jin et al., 2020), higher percentages of IL-13-producing $CD4^+$ T cells, a Th2-related cytokine, were detected in ALS patients, correlating with disease severity (Shi et al., 2007). Adding to

TABLE 1 | Contribution of the adaptive immune system in primary neurodegenerative diseases.

Primary neurodegenerative diseases	Neurodegeneration	Regeneration
Alzheimer's disease	Rag-5xfAD mice show enhanced Aβ pathology and neuroinflammation (Marsh et al., 2016).	AB-specific Th2 cells promote neurological recovery (Cao et al., 2009).
	IFN-y producing Th1 cells enhance microglial activation and Aβ deposition (McQuillan et al., 2010; Browne et al., 2013).	IL-17 depletion enhances neural precursor cell expression and synaptic transmission (Liu et al., 2014).
	Depletion of Tregs in mice accelerates AD-related cognitive dysfunction (Baek et al., 2016; Dansokho et al., 2016).	Adoptive transfer of Tregs reduces Aβ deposition and reverses cognitive deficits (Baek et al., 2016).
Parkinson's disease	SCID, Rag1 KO, TCR B KO, CD4 KO but not CD8 KO mice show attenuated dopaminergic cell death (Benner et al., 2008; Brochard et al., 2009; Gonzalez et al., 2013).	Copolymer 1 immunized T cells administered to MPTP mice limited neuronal loss (Schori et al., 2001; Benner et al., 2004).
	Adoptive transfer of T cells from mice immunized with α-synuclein exacerbate MTPT (Reynolds et al., 2010).	Adoptive transfer of activated Tregs to MPTP provides 90% neuronal protection (Reynolds et al., 2007).
	IL-17 increases cell death in iPSC-derived neurons from PD patients (Sommer et al., 2019).	GM-CSF administration increases Tregs, limits inflammation and increases neuroprotection (Kosloski et al., 2013; Gendelman et al., 2017; Schutt et al., 2018).
	Suppression of CD4 T cell infiltration ameliorates PD symptoms (Qin et al., 2016).	
Amyotrophic lateral sclerosis	A Th1/Th17 immune response correlates with disease progression and severity (Saresella et al., 2013; Jin et al., 2020).	Reconstitution with $CD4^+$ T cells in SOD1G93A mice increases neuroprotection (Beers et al., 2008).
	In the $SOD1^{G93A}$ model, $CD8^+$ T cell ablation leads to a reduction in motor neuron loss (Coque et al., 2019).	Disease progression reduces Tregs (Beers et al., 2011).
	A2BG2 glycan is increased in IgG antibodies for SOD1G93A mice, increasing neuronal cytotoxicity and death (Edri-Brami et al., 2015).	Adoptive transfer of activated Tregs to SOD1G93A mice delays motor function loss and enhances survival (Banerjee et al., 2008).

the pro-inflammatory profile of ALS patients, there were also decreased proportions of BDNF-producing $CD8^+$ T cells, which promote neuronal survival (Saresella et al., 2013). Furthermore, a study evaluating peripheral blood mononuclear cells (PBMCs) of monozygotic twins, one which had ALS and the other healthy, showed that the ALS twin had a more pro-inflammatory profile than her sister, with only her PBMC supernatant proving toxic to rat cortical neurons (Lam et al., 2016). Interestingly, although the findings show the presence of memory T cells in both twins, effector T cells were only found in the ALS twin, suggesting a role for effector T cells in this pro-inflammatory neurotoxic response (Lam et al., 2016).

The $SOD1^{G93A}$ mouse model of ALS is an important tool for elucidating the role of the adaptive immune system in ALS pathology. This transgenic mouse model expresses a human SOD1 transgene with a G93A mutation, resulting in an excess of SOD1 protein causing degeneration of motor neurons and paralysis (Acevedo-Arozena et al., 2011). This model has shown that $CD8^+$ T cells are involved in motor neuron loss following protein aggregation (Nardo et al., 2018; Coque et al., 2019). During the symptomatic stage of ALS pathology, $CD8^+$ T cells infiltrate the CNS and contribute to neurodegeneration, as ablation of $CD8^+$ T cells reduces motor neuron loss (Coque et al., 2019). SOD1 mutant $CD8^+$ T cells secrete IFN-γ, inducing MHC-I expression and provoking neurodegeneration through Fas and granzyme cytotoxic pathways (Coque et al., 2019). Infiltrating $CD8^+$ T cells in the spinal cord of ALS mice display a restricted T cell receptor repertoire, suggesting a self-directed immune response contributing to the selective ablation of motor neurons during ALS (Coque et al., 2019). A reduction of MHC-I expression and a lack of mature $CD8^+$ T cells in the $SOD1^{G93A}$ mice protects cervical motor neurons (Nardo et al., 2018).

The number of B lymphocytes is not altered in ALS patients and post-mortem spinal cord tissue showed a greater infiltration of T lymphocytes but not B lymphocytes (Engelhardt et al., 1993). B lymphocytes do not significantly impact ALS pathogenesis, since B cell-deficient $SOD1^{G93A}$ mice show similar motor dysfunctions and survival rates to controls, and $SOD1^{G93A}$-derived B cells show a similar phenotype to WT-derived B cells (Naor et al., 2009). In line with these findings, there are no differences in the levels of immunoglobulins in the peripheral blood of ALS patients compared to healthy controls (Chen et al., 2014). However, a unique glycan (A2BG2) which was discovered on the Fc domain of IgG antibodies in ALS patients has been implicated in ALS pathology and progression (Edri-Brami et al., 2012, 2015). These antibodies identify antigens located at the surface of motor neurons at the end stage of the disease, progressing cytotoxic neurodegeneration (Edri-Brami et al., 2015). Although there was no difference in B lymphocyte levels in ALS patients, B cells appear to have a role in neuronal cytotoxicity and death, through the production of specific IgG antibodies.

Whilst studies have shown a more pro-inflammatory immune profile in ALS patients, with correlations to disease severity, $CD4^+$ T lymphocytes have also proven to be neuroprotective and regenerative in ALS. ALS mice deficient in functional T cells have shown an accelerated disease progression, with reduced microglia reactivity and decreased levels of the neuroregenerative insulin-like growth factor (IGF-1) (Beers et al., 2008; Chiu et al., 2008). This neuroprotective role was shown to be specifically $CD4^+$ T cell-mediated, as $SOD1^{G93A}$ mice lacking functional $CD4^+$ T cells show accelerated motor neuron deficiency, reduced gliosis, increased pro-inflammatory markers and a reduction of trophic factors and glial glutamate transporters (Beers et al., 2008). Reconstitution with $CD4^+$ T cells prolonged survival, with the mediation of microglial neuroprotection leading to ALS attenuation (Beers et al., 2008). $SOD1^{G93A}$ mice also show an altered immune profile, similar to ALS patients, with decreased lymphoid numbers and T cell dysfunction (Banerjee et al., 2008). In particular, they have reduced Tregs as the disease progresses (Beers et al., 2011). Adoptive transfer of activated Tregs or activated effector T cells to $SOD1^{G93A}$ mice resulted in the delayed loss of motor function and enhanced survival, results not seen with the adoptive transfer of naïve T cells (Banerjee et al., 2008). Interestingly, only Tregs delayed the onset of neurological symptoms and only effector T cells delayed progression in the late phase of the disease (Banerjee et al., 2008); suggesting differential roles for T lymphocytes depending on disease stage. Passive transfer of Tregs from $SOD1^{G93A}$ mice at an early disease stage to $SOD1^{G93A}$ mice lacking functional lymphocytes resulted in prolonged survival, with increased IL-4 levels and microglia modulation (Beers et al., 2011). Moreover, inducing Treg expansion in the $SOD1^{G93A}$ mouse model of ALS resulted in increased neuroprotection, repression of astrocytic and microglial reactivity and increased neurotrophic factors, slowing disease progression and prolonging survival (Sheean et al., 2018).

As mentioned previously, ALS patients have a pro-inflammatory profile with decreased Tregs (Saresella et al., 2013; Jin et al., 2020). Multiple studies have shown that Tregs decrease during disease progression, with lower numbers predicting a more progressive disease course and diminished survival (Beers et al., 2011; Henkel et al., 2013; Rashid Chehreh Bargh et al., 2018; Sheean et al., 2018). These findings suggest that Tregs provide protection or regenerative properties during early stages of disease but ultimately decrease in number or functionality, allowing for disease progression. Tregs isolated from ALS patients were dysfunctional, with less effective suppression of T cell proliferation compared to healthy controls and a greater Treg dysfunction in rapidly progressing ALS patients (Beers et al., 2017). *In vitro* expansion of isolated ALS Tregs recovered suppressive capabilities, indicating a potential therapeutic target for the autologous transplant of expanded Tregs in slowing ALS progression through neuroprotection or neuroregeneration (Beers et al., 2017).

MULTIPLE SCLEROSIS

Multiple sclerosis is an immune-mediated disease of the CNS, the hallmark of which is demyelination followed by neurodegeneration. Although not a classical neurodegenerative disease, MS is characterized by neuroinflammation, demyelination, apoptosis of oligodendrocytes, astrogliosis

and finally, axonal loss (Lassmann, 2018). The immune system is intrinsically linked to the risk and development of MS, with both genetic and environmental risk factors related to immunity. Multipoint linkage screens of families with MS show significant linkage within the MHC region, highlighting the importance of antigen presentation and the adaptive immune system in MS genetic risk (Sawcer et al., 2005). This was further supported by the discovery of MS risk alleles within the MHC region relating to T cell differentiation in genome-wide association studies (Sawcer et al., 2011; Alcina et al., 2012). Although genetic risk involves adaptive immunity, familial studies highlight a modest role for genetics in MS risk, with concordance rates for monozygotic twins around 15–25% (Ramagopalan et al., 2008; Westerlind et al., 2014). Disease-modifying therapies (DMT) prevent disease progression through different mechanisms of immunomodulation. As most DMTs either deplete T and B lymphocytes and monocytes (Coles et al., 2008; Giovannoni et al., 2010; Hauser et al., 2017) or prevent lymphocyte entrance into the CNS (Polman et al., 2006; Kappos et al., 2010), this identifies a key role for the adaptive immune system in the pathogenesis and progression of the disease, at least in the early stages.

Histological examination of brain biopsies from MS patients has indicated a role for the adaptive immune system, particularly T lymphocytes, in demyelination and neurodegeneration. Demyelinating lesions show an infiltration of peripheral T lymphocytes, with both $CD4^+$ T cells and $CD8^+$ T cells found at the edge of demyelinating lesions and less immune infiltration in chronic inactive lesions (Traugott et al., 1983). The T cell-mediated pathogenic mechanism has been thought to be a Th1 response, due to the expression of IFN-γ within lesions and the association of macrophages as the effector cells of Th1 immunity and demyelination (Traugott and Lebon, 1988). Although studies indicate that immunopathology in MS is T cell-driven, it is also hypothesized that immune infiltration is secondary to CNS insult. Histopathological analysis of newly forming lesions from MS patients describes minimal myelin loss, dysfunction and apoptosis of oligodendrocytes, reactive astrocytes and activated microglia as early features of lesion formation (Rodriguez et al., 1993; Barnett and Prineas, 2004). Infiltrating immune cells were a feature of lesion progression and were found in close proximity to deteriorating myelin sheaths (Rodriguez et al., 1993). Classification of active demyelinating lesions showed the heterogeneity and complexity of the pathogenesis of MS. Although lesions showed common pathology, different demyelinating mechanisms were observed between patients (Lucchinetti et al., 2000). These findings suggest that both hypotheses for the pathogenesis of MS may be correct depending on the patient in question.

The pathogenic immune-mediated mechanisms of demyelination and neurodegeneration have largely been hypothesized using the animal model, experimental autoimmune encephalomyelitis (EAE). EAE is induced through immunization with myelin peptides, CNS tissues, or through the adoptive transfer of myelin-specific T cells, showing the key features of MS pathology. Although this model is not an exact representation of MS, it has allowed for the study and validation of DMT's (Constantinescu et al., 2011). The integral role of T lymphocytes in the induction of EAE has been highlighted, as mice depleted of T cells do not develop EAE or produce MBP-specific autoantibodies, whereas mice depleted of B cells develop normal EAE in the absence of MBP-specific autoantibodies (Ortiz-ortiz and Weigle, 1976; Hjelmström et al., 1998). Further supporting this finding, the adoptive transfer of MBP-specific clonal T cells from rats immunized with MBP induced paralysis, meningeal inflammation and demyelinated lesions in recipients (Zamvil et al., 1985). This effect is lost when cells were first depleted of $CD4^+$ T cells, and remained when depleted of $CD8^+$ T cells (Pettinelli and McFarlin, 1981). During active demyelination and recovery in EAE, inflammatory aggregates within the perivascular space predominantly contain $CD4^+$ T cells but also contain $CD8^+$ T cells and B cells (Sriram et al., 1982). Although EAE is induced by $CD4^+$ T cells, B lymphocytes are also implicated in demyelination and neurodegeneration, as shown by IgG antibody deposits present on degenerating myelin sheaths in both MS patient lesions and a primate model of EAE (Raine et al., 1999).

Experimental autoimmune encephalomyelitis induction has been further narrowed down to $CD4^+$ T cell subsets. MOG-specific T cells polarized to Th1, Th17, and Th9 all induced EAE after adoptive transfer to recipients but Th2 polarized cells did not (Jager et al., 2009). Th1 and Th17 cells showed classical inflammatory infiltration and demyelinating lesions, however, when Th17 cells were cultured with IL-23, lymphoid follicle-like structures were observed (Jager et al., 2009). Th9 cells similarly induced extensive demyelination within both the CNS and PNS (Jager et al., 2009). Adoptive transfer of Th1 cells induces 'classical' EAE with paralysis developing from tail to head while the adoptive transfer of Th17 cells induces 'atypical' EAE with ataxia, unbalanced gait and rotary defects progressing to paralysis (Domingues et al., 2010). Adoptive transfer of both, however, induces a more severe disease (Domingues et al., 2010). The differences seen in EAE may represent the heterogeneity seen in MS patients and may be indicative of different disease mechanisms in patients.

The role of the adaptive immune system in the pathogenesis of MS is well established, however, the role of the adaptive immune system in regeneration is more novel. Regeneration in MS is centered on the differentiation of oligodendrocyte progenitor cells (OPC) into oligodendrocytes, which replace myelin sheaths to prevent axon degeneration in a process known as remyelination (Smith et al., 1979; Franklin and Ffrench-Constant, 2017). T lymphocytes have been shown to have regenerative effects in the CNS, with $Rag1^{-/-}$ mice, lacking mature lymphocytes, displaying reduced remyelination of lysolecithin-induced demyelinating lesions compared to WT controls (Bieber et al., 2003). This impaired remyelination was also observed in mice deficient or depleted of either $CD4^+$ or $CD8^+$ T cells, highlighting the important role for T cells in myelin regeneration (Bieber et al., 2003). Tregs have been shown to be key in this process, promoting OPC differentiation and efficient remyelination (Dombrowski et al., 2017). Conversely, not all $CD4^+$ T cell subsets are beneficial, as Th17 cells proved detrimental for remyelination in a model of cuprizone-mediated demyelination (Baxi et al., 2015).

Similar to other neurodegenerative diseases, aging is an important risk factor for MS and is implicated in disease onset, progression and evolution. However, the role of aging in disease susceptibility and severity is controversial in EAE models. Some studies suggest that aging decreases disease susceptibility (Djikić et al., 2015), whilst others report an increased susceptibility or severity with aging (Matejuk et al., 2005; Seo et al., 2015; Stojić-Vukanić et al., 2015). Further studies have also indicated that with aging, mice develop chronic EAE that is more similar to the symptoms observed in progressive MS (Ludowyk et al., 1993; Peferoen et al., 2016). Research performed with MS patients indicated that disability milestones are mainly determined by patient age and age at disease onset, indicating age-related mechanisms of disease development (Confavreux and Vukusic, 2006). The probability of incomplete recovery and relapse severity also increases with age, leading to the accumulation of disability (Cossburn et al., 2012; Kalincik et al., 2014). This is mainly mediated by age-associated impaired remyelination, as shown in lysolecithin-induced demyelinating lesions in juvenile, young and old rats. Old rats (>12 months) showed decreased remyelination of axons and thinner myelin sheaths for those that did remyelinate, compared to their young and juvenile counterparts (Gilson and Blakemore, 1993). The lesions of older animals also contained more myelin debris, indicating that remyelination failure may be due to a delayed damage response from astrocytes and macrophages, with deficient myelin debris clearance (Kotter et al., 2006; Linehan et al., 2014; Natrajan et al., 2015; Cantuti-Castelvetri et al., 2018). This aging-related failure may also be mediated by the reduced or slower recruitment of OPCs (Gilson and Blakemore, 1993), and the delayed differentiation of OPCs into oligodendrocytes (Sim et al., 2002). Recently, it has also been shown that aging impairs the response of OPCs to pro-differentiation cues such as the thyroid hormone (Neumann et al., 2019), suggesting that enhancement of OPC differentiation by the adaptive immune system may be altered with aging.

STROKE

Globally, stroke represents the second highest cause of death and commonly results in disability (Campbell et al., 2019). There are two classifications of stroke: ischaemic and haemorrhagic stroke. The majority of cases are ischaemic, which are caused by arterial occlusion, often by emboli originating in the heart or large arteries, but also by local thrombosis, vasculitis or arterial dissection (Campbell et al., 2019). Although stroke is not a classical neurodegenerative disease, tissue ischaemia causes neuronal death and is associated with a rapid local innate immune response, upregulation of pro-inflammatory cytokines, BBB breakdown and infiltration of peripheral immune cells (Kamel and Iadecola, 2012).

Tissue ischaemia promotes activation of adaptive immune networks with increased infiltration of antigen-presenting cells in the CNS (Felger et al., 2010), expansion of CNS antigen-specific T cells (Jin et al., 2018) and increased immunoglobulin synthesis in the CSF (Pruss et al., 2012). There is mounting evidence that the adaptive immune system contributes to the pathogenesis and evolution of acute ischaemic stroke. Patients that die following a stroke (<24 h) show an increased infiltration of neutrophils, B lymphocytes, $CD3^+$ T cells and $CD4^+$ T cells in the infarcted area (Gelderblom et al., 2012; Clarkson et al., 2014; Doyle et al., 2015). This is supported in experimental stroke animal models, with higher T lymphocyte numbers at the edge of the infarcted tissue early after induction (Schroeter et al., 1994). Moreover, IL-$17A^+$ T lymphocytes were detected in the post-mortem tissue of patients that died shortly after their stroke (Gelderblom et al., 2012) and IL-21-producing $CD4^+$ T cells, potentially Th17, Tfh, or Th9 cells, were found surrounding the infarcted tissue area in post-mortem tissue of patients with acute stroke (Clarkson et al., 2014).

Much of the evidence linking the adaptive immune system to stroke is from the common animal model used to study its pathogenesis: the middle cerebral artery occlusion model (MCAO). In MCAO, a surgical filament is inserted into the external carotid artery to occlude the origin of the middle cerebral artery, resulting in blood flow cessation and brain infarction in the striatum (Chiang et al., 2011). T cell involvement in the progression of neurodegeneration following experimental stroke induction has been studied by the depletion of adaptive immune cell subsets. SCID mice, deficient in mature lymphocytes, showed a reduction in total infarct size after MCAO with little effect on the infarct core, suggesting that lymphocytes promote the progression of ischaemic neurodegeneration following initial vascular insult (Hurn et al., 2007). Similar results were also observed in lymphocyte-deficient $Rag1^{-/-}$ mice (Kleinschnitz et al., 2010). The susceptibility of these mice to MCAO was restored with the adoptive transfer of $CD3^+$ T cells but not with the transfer of B cells, highlighting the role of T lymphocytes in progressive ischaemic neurodegeneration (Kleinschnitz et al., 2010). Despite not having a direct role in stroke pathogenesis, B lymphocyte responses have been shown to cause a delayed cognitive impairment in stroke mouse models, without impacting infarct size (Doyle et al., 2015).

Depletion of $CD8^+$ T cells has been shown to reduce infarct size and behavioral deficits following MCAO, whilst the adoptive transfer of $CD8^+$ T cells in $Rag1^{-/-}$ mice increased infarct size (Mracsko et al., 2014). $CD4^+$ T cells also contribute to neurodegeneration following stroke, as antibody-mediated depletion of either $CD4^+$ or $CD8^+$ T lymphocytes decreased infarct size, outlining a role for both subsets (Liesz et al., 2011). This study demonstrated the role of both humoral and cytotoxic T cell responses in promoting ischaemic infarction, whilst the inhibition of lymphocyte trafficking to the CNS resulted in a protective effect (Shichita et al., 2009; Liesz et al., 2011). The use of different knockout transgenic mouse models, has shown that this early pathogenic role of T lymphocytes can occur independently of classical adaptive immune mechanisms, such as antigen-recognition or TCR co-stimulation (Kleinschnitz et al., 2010). Although these mechanisms do not seem to be required for the initial stages of T cell-mediated ischaemic brain injury, the

role of T lymphocytes in the progression of neurodegeneration does appear to be antigen-dependent (Kleinschnitz et al., 2010; Mracsko et al., 2014).

The study of T cell-derived cytokines provides an insight into the degenerative effects of the adaptive immune system following a stroke. Depletion of $CD4^+$ T cells 3 days post-stroke induction improved behavioral outcomes without affecting infarct size, through the reduction of inflammatory cytokines such as IFN-γ and IFN-γ-inducible protein (IP-10) (Harris et al., 2020). In addition, IL-17-producing γδ T cells, have been found at the infarct edge and have been implicated in the delayed phase of infarction, with IL-17-deficient mice showing reduced neuronal death and improved neurological outcome (Shichita et al., 2009). Furthermore, neutralization of IL-17A following MCAO improved neurological outcome and reduced infarct size (Gelderblom et al., 2012). The blockade of IL-21, produced by infiltrating $CD4^+$ T cells, either before or after MCAO induction showed reduced infarct size and increased locomotor function (Clarkson et al., 2014). Despite the knowledge gained from the use of experimental stroke in rodents, there has so far been little success in translating immunosuppressive therapies into the clinic, with natalizumab, a drug preventing lymphocyte infiltration into the CNS, having no effect in stroke patients during phase II clinical trials (Elkins et al., 2017).

There is also evidence that systemic inflammation during stroke can lead to long-term autoimmunity with further neurodegeneration. BBB disruption during ischaemic tissue injury promotes secretion of pro-inflammatory cytokines and sensitisation to CNS antigens, associated with poorer neurological outcomes (Javidi and Magnus, 2019). The long-term autoimmune response is exacerbated in the context of systemic inflammation, as mice treated with lipopolysaccharides (LPS) are more likely to be sensitized to MBP after MCAO and show greater neurological deficits when compared to non-LPS treated mice (Becker et al., 2005). In addition, T cells isolated from patients following acute ischaemic stroke were more likely to react to CNS myelin antigens than those of control patients with other neurological diseases (Wang et al., 1992). Following stroke there is an increase in antibody titres recognizing CNS self-antigens, indicating a B cell response following ischaemic brain injury (Dambinova et al., 2003). The relationship between poorer neurological outcomes and systemic inflammation during stroke has also been highlighted, as patients who developed an infection following their stroke were more likely to have MBP and GFAP-specific T cells 3 months post-stroke; associated to a Th1 response (Becker et al., 2011). Establishing immune-tolerance can mitigate some of the adverse effects of the adaptive immune response, with the generation of oral tolerance to MBP in rats reducing infarct size following subsequent MCAO (Becker et al., 1997). In line with this finding, it has also been shown that IL-10-producing $CD4^+$ T cells are important for mediating mucosal tolerance to CNS antigens and can limit damage after an ischaemic stroke (Frenkel et al., 2005).

The adaptive immune system is, however, also partly involved in neuroprotection and neuroregeneration following ischaemic tissue injury, due to the infiltration of Tregs; though there is debate about the timing of infiltration and the nature of these Tregs in ischaemic stroke (Ito et al., 2019; Javidi and Magnus, 2019). Tregs that accumulate post-damage are molecularly distinct from those in other tissues and regulate neurotoxic astrogliosis, promoting neurological recovery during the chronic stroke phase (Ito et al., 2019). The beneficial role of T lymphocytes has also been demonstrated by treatment with fingolimod to sequester T cells in lymph nodes 6–13 days following stroke, which delayed neurological recovery (Ito et al., 2019). Depletion of Tregs a week following stroke similarly showed a poorer neurological outcome without affecting infarct size (Ito et al., 2019), whereas depletion of Tregs 48 h before MCAO resulted in the increased activation of microglia and T cells, sources of the pro-inflammatory cytokines TNF-α and IFN-γ (Liesz et al., 2009). Treg depletion led to increased chronic ischaemic brain damage and a worsened neurological outcome with increased infarct size after 7 days (Liesz et al., 2009). Treatment with IL-10, on the other hand, reduced TNF-α and IFN-γ cytokine overexpression and prevented secondary infarct progression (Liesz et al., 2009). Additionally, administration of $CD34^+$ immune progenitor cells, which can differentiate into both innate and adaptive immune cells, promoted revascularisation and enhanced neurogenesis after MCAO induction (Taguchi et al., 2004), being now further evaluated in clinical trials (Sargento-Freitas et al., 2018). Although these studies highlight the ability of adaptive immune cells to reduce neurodegeneration, it can be difficult to establish whether these effects are through neuroprotection or by promoting neuroregeneration and plasticity.

Aging is also an important risk factor for stroke, with the vast majority of cases occurring in people over the age of 75 (Engstad et al., 2012). Elderly patients have a greater risk of fatality, are more likely to require longer hospitalization and are less likely to return home following stroke (Saposnik et al., 2008). Although it has been shown that older stroke patients make a less complete neurological recovery than their younger counterparts, this data is complicated due to other cofounding morbidities in elderly patients. Behavioral recovery has been shown to be delayed in older mice, despite infarct size being lower than younger controls; potentially caused by an enhanced innate immune response and increased reactive gliosis (Manwani et al., 2011). The immunological response to stroke is also exacerbated in older animals (Manwani et al., 2013), with bone marrow transplants from young mice improving stroke outcomes (Ritzel et al., 2018). The numbers of $CD8^+$ T cells in the CNS are increased in aged animals, regardless of tissue injury (Ritzel et al., 2016). Following the induction of experimental stroke, these resident $CD8^+$ T cells exacerbate ischaemic brain injury by potentiating further leukocyte recruitment from the periphery and amplifying pro-inflammatory cytokines in aged animals (Ritzel et al., 2016). Older animals with chronic systemic infection similarly show increased immune cell recruitment, upregulation of pro-inflammatory cytokines and increased infarct size compared to young controls with systemic infection (Dhungana et al., 2013). This suggests that the inflammatory response of elderly patients, which tends to be dysregulated, is an important parameter in the severity of ischaemic tissue injury.

TRAUMATIC CNS INJURY

Traumatic CNS injury is a broad term encompassing damage to the brain (TBI) or spinal cord (SCI) commonly caused by a sudden, external impact. The primary insult is a determinant of the extent of neurodegeneration and outcome for patients, however, neuronal loss is often the result of secondary neurodegeneration. In SCI, damage is often caused by displaced surrounding structures causing bruising or tearing of the spinal cord, leading to either complete or incomplete classifications of SCI (Alizadeh et al., 2019). Secondary neurodegeneration often occurs as a result of the altered tissue environment following damage, with ischaemia, oxidative stress, glial activation, matrix remodeling and neuroinflammation all contributing to progressive damage (Alizadeh et al., 2019). TBI, on the other hand, comprises a diverse range of pathologies, including focal damage due to bruising, laceration or traumatic hemorrhage, as well as more diffuse effects resulting from acceleration/deceleration injuries (Werner and Engelhard, 2007). Secondary neurodegeneration is a common feature, with ischaemia, oedema formation, intracranial hypertension and neuroinflammation causing neuronal death (Werner and Engelhard, 2007). TBI is also a risk factor for chronic neurodegeneration, particularly chronic traumatic encephalopathy (McKee et al., 2009). Although it is necessary to evaluate both the brain and spinal cord in the inflammatory response to traumatic injury, many researchers focus on SCI due to the greater inflammatory response seen in SCI animal models (Schnell et al., 1999).

There is little evidence that the adaptive immune system exacerbates pathology acutely following TBI, however, there is evidence of it contributing to TBI progression. TBI patients show progressive degeneration of the white matter and persistent inflammation many years after the initial injury (Johnson et al., 2013). Both $CD4^+$ and $CD8^+$ T cells infiltrate the CNS following contusion TBI (Holmin et al., 1995). Inflammation persists several months after focal TBI, with upregulation of MHC-II, phagocytes and pro-inflammatory cytokines IL-1β and TNF-α (Holmin and Mathiesen, 1999). Brain biopsies from patients undertaking surgeries for brain contusions showed a limited inflammatory response <24 h post-injury, however, by 3–5 days there was a substantial immune infiltrate of reactive microglia, macrophages, polymorphonuclear cells, and both $CD4^+$ and $CD8^+$ T lymphocytes (Holmin et al., 1998). Neuroinflammation is known to be involved in progressive brain damage with pre-clinical data for several immunosuppressive therapies showing promising results. However, this has not translated to give clear benefits in clinical trials, with some drugs worsening neurological outcome (Bergold, 2016; Russo and McGavern, 2016).

Progressive neurodegeneration following TBI is mediated by adaptive immunity. Inhibition of antigen processing and presentation impairs immune cell infiltration to the CNS, resulting in less neurodegeneration and a smaller lesion size following fluid percussion TBI (Tobin et al., 2014). Other models argue against a role for the adaptive immune system in neurodegeneration after TBI (Weckbach et al., 2012; Mencl et al., 2014). $Rag1^{-/-}$ mice, lacking functional lymphocytes, show a similar extent of neurological injury compared to controls following closed head injury (Weckbach et al., 2012). Additionally, fingolimod treatment to sequester T cells and prevent CNS infiltration had no impact of lesion size or functional outcome following focal cortical cryo-lesion TBI (Mencl et al., 2014). This study also observed a reduced number of neutrophils and activated microglia/macrophages in lesions, highlighting an adaptive immune role in sustaining neuroinflammation (Mencl et al., 2014). These divergent results may be due to the pathologically diverse lesions induced through a wide range of animal models, representing the spectrum of TBI. Autoimmunity is also a feature of adaptive immunity following TBI, with myelin-reactive circulating T cells in patients and CNS autoantibodies developing upon contusion injury in rats (Cox et al., 2006; Rudehill et al., 2006).

Similarly, SCI patients show an altered immune profile with suppressed and defective immune responses, known as CNS injury-induced immunodepression (CIDS), a phenomenon which also occurs in TBI patients (Meisel et al., 2005; Zhang Y. et al., 2013; Gucluler et al., 2017). SCI patients have a higher susceptibility to infections due to a decline in both innate and adaptive immune responses (Gucluler et al., 2017). SCI insult induces a potent inflammatory response with subsequent anti-inflammatory mechanisms systemically and within the damaged area, leading to suppression of immunity. Immune cells' functionality continues to evolve as the injury progresses from primary insult to secondary neurodegeneration (Schwab et al., 2014). For example, in a transection mouse model of SCI, neutrophils and T lymphocytes persist at the acute phase of injury, with a 50% reduction after 1–3 days, whilst microglia and macrophages persist into the chronic phase, falling by 50% only after 55 days (Pruss et al., 2011). Despite this early reduction, 10% of T lymphocytes persisted after 4 weeks, adding to the sustained neuroinflammation seen following acute SCI (Pruss et al., 2011). Other studies have highlighted different dynamics of lymphocyte infiltration after injury, although studies agree on the persistence of T lymphocytes several weeks after injury (Popovich et al., 1997; Schnell et al., 1997; Sroga et al., 2003). A similar response was seen in post-mortem spinal cord tissue from SCI patients with different survival times of up to 1 year post-trauma (Fleming et al., 2006). The innate immune response occurred quickly following SCI and T lymphocytes increased in number at 1–6 months post-injury, predominantly $CD8^+$ T cells (Fleming et al., 2006).

Similar to other CNS neurodegenerative diseases, BBB disruption following injury can lead to autoimmunity. This effect is seen in patients and mouse models of SCI, with detection of MBP-specific T cells and the activation of MBP-reactive T lymphocytes from SCI donors promoting neuroinflammation and inducing transient paralysis in donor mice (Popovich et al., 1996). In patients with SCI, the frequency of MBP-reactive T cells reach levels similar to that seen in MS patients (Kil et al., 1999). These MBP-reactive T lymphocytes have been suggested to contribute to neurodegeneration and impede recovery following SCI, with SCI induced in 2D2 mice, with >95% of all $CD4^+$ T cells reactive to MBP, showing exacerbated neuropathology (Jones et al., 2002). Likewise, induction of contusion SCI in

TABLE 2 | Contribution of the adaptive immune system in neurodegeneration and regeneration secondary to other pathology.

Secondary neurodegenerative diseases	Neurodegeneration	Regeneration
Multiple sclerosis	T cell depleted mice do not develop EAE (Ortiz-ortiz and Weigle, 1976). Adoptive transfer of MBP-specific $CD4^+$T cells induce EAE (Pettinelli and McFarlin, 1981; Zamvil et al., 1985). Adoptive transfer of Th1 and Th17 cells induce classical and atypical EAE (Jager et al., 2009; Domingues et al., 2010).	Mice deficient in $CD4^+$ or $CD8^+$ T cells show impaired remyelination following lysolecithin-induced demyelination (Bieber et al., 2003). Tregs promote OPC differentiation and efficient remyelination (Dombrowski et al., 2017).
Stroke	Rag1 KO and SCID mice show a reduced infarct size after MCAO (Hurn et al., 2007; Kleinschnitz et al., 2010). Adoptive transfer of CD8 T cells into Rag1 KO mice increases infarct size (Mracsko et al., 2014). Specific antibody-mediated depletion of either $CD4^+$ or $CD8^+$ T cells decreased infarct size (Liesz et al., 2011). B lymphocytes mediate a delayed cognitive impairment following stroke in mice (Doyle et al., 2015).	Tregs accumulate following ischaemia and have a role in suppressing astrogliosis and promoting neurological recovery (Ito et al., 2019). Administration of $CD34^+$ immune progenitor cells promoted revascularisation and neurogenesis (Taguchi et al., 2004).
Traumatic CNS injury	**In SCI:** SCID mice show better functional recovery after SCI (Luo et al., 2019). Rag2 KO mice, BCKO mice, Athymic nude rats and B cell depletion show less degeneration and improved recover (Potas et al., 2006; Ankeny et al., 2009; Wu et al., 2012; Casili et al., 2016). MBP-reactive lymphocytes contribute to SCI neurodegeneration (Jones et al., 2002). Antibodies against CXCL10 ameliorate SCI (Gonzalez et al., 2003). Rag1 KO mice administered with IgM antibodies show exacerbated pathology (Narang et al., 2017). $CD8^+$ T cells inhibit neurite outgrowth *in vitro* (Pool et al., 2012). **In TBI:** $Rag1^{-/-}$ mice have a similar injury extent to controls in the closed head injury model (Weckbach et al., 2012). Inhibition of antigen processing/presentation reduces lesion size in a fluid percussion trauma model (Tobin et al., 2014).	**In SCI:** CD4 T cells promote neurite outgrowth *in vitro* (Pool et al., 2012). Adoptive transfer of Th1 cells promotes locomotor recovery in SCI (Ishii et al., 2012). Adoptive transfer of $CD4^+$ T cells into IL-4 deficient mice promotes neuronal survival and regeneration (Walsh et al., 2015). Active immunization with MBP or transfer of MBP-T cells enhance SCI locomotor recovery and regeneration (Hauben et al., 2000). Myelin and spinal cord homogenate immunization improves regeneration and recovery (Huang et al., 1999; Sicotte et al., 2003). **In TBI:** Vaccination with Cop-1 (a synthetic mimic of MBP epitopes) reduced neuronal loss and promoted recovery (Kipnis et al., 2003).

mice leads to a dysregulation of B lymphocytes, with increased numbers and production of neurotoxic CNS autoantibodies (Ankeny et al., 2006).

To address the functional role of T lymphocyte infiltrates in SCI, mice lacking components of the adaptive immune system were utilized. Researchers showed that lymphocytes contribute to neurodegeneration and worsened functional recovery following crush SCI in SCID mice (Luo et al., 2019). Post-damage, animals showed a reduced inflammatory response, reduced immune function-related gene expression, increased neural transmission-related gene expression, smaller lesion size and an improved recovery of motor function after injury (Luo et al., 2019). Similar results were obtained in Rag2$^{-/-}$ mice and athymic nude rats, which lack mature lymphocytes or T cells, respectively, with decreased secondary neurodegeneration, increased regeneration and improved functional recovery (Potas et al., 2006; Wu et al., 2012). Dorsal hemisection SCI in mice leads to an elevated expression of a T cell chemoattractant, CXCL10 (Gonzalez et al., 2003). Its neutralization 1 day post-injury led to reduced T cell accumulation in the CNS, with decreased lesion size and reduced behavioral deficits, supporting the detrimental role of T cells in SCI (Gonzalez et al., 2003).

B lymphocytes and immunoglobulins have also been associated with progressive neuronal degeneration following traumatic CNS injury, with large antibody deposits detected at sites of axon degeneration and demyelination following contusion SCI (Ankeny et al., 2009). Depletion of B cells after compression SCI caused immunomodulatory effects with decreased neuronal death and delayed motor dysfunction (Casili et al., 2016). BCKO mice, lacking B lymphocytes, also showed reduced lesion pathology and improved locomotor recovery associated with lack of immunoglobulins in the CSF of injured mice (Ankeny et al., 2009). Previously mentioned expression of new epitopes expressed following SCI are detected by clonally specific IgM antibodies, resulting in complement activation and worse pathology (Narang et al., 2017). Rag1$^{-/-}$ mice, which are less susceptible to neurodegeneration following injury, administered with specific IgM antibodies, showed exacerbated pathology and a worsened functional outcome (Narang et al., 2017). These findings together support a role for both, B and T lymphocytes in the development and progression of traumatic CNS injury.

Despite the role in progressing secondary neurodegeneration and neuroinflammation following traumatic injury to the CNS, adaptive immunity and specifically autoimmunity have also shown neuroprotective and regenerative properties. Co-culture of neurons and immune cell subtypes, highlight the regenerative capacity of CD4$^+$ T cells which augment neurite outgrowth (Pool et al., 2012). CD8$^+$ T cells inhibited this process, showing the heterogeneity of lymphocytes in CNS repair (Pool et al., 2012). Adoptive transfer of *ex vivo* polarized Th1 cells following SCI in WT mice enhanced recovery of locomotor function, promoting regeneration of the corticospinal tract, serotonergic fibers and myelin (Ishii et al., 2012). This regenerative effect may have been mediated by the Th1 production of NT3 or IL-10, as IL-10 neutralization attenuated the positive effects while adoptive transfer of either Th2 or Th17 cells did not show the same effects (Ishii et al., 2012). Another study has shown that adoptive transfer of CD4$^+$ T cells into IL-4-deficient mice after CNS injury promotes neuronal survival and regeneration of injured neurons, an effect not seen with the adoptive transfer of IL-4-deficient CD4$^+$ T cells (Walsh et al., 2015). The IL-4-producing T cell-mediated effect, indicative of Th2 cells, was shown to be MHC-II-independent and MyD88-dependent (Walsh et al., 2015). In line with these studies, vaccination with Cop-1 following closed head TBI caused reduced neuronal loss and promoted recovery, thought to be mediated by increased immune repair mechanisms by Cop-1 treatment (Kipnis et al., 2003).

Autoimmunity induced following traumatic CNS injury is observed in both the brain and spinal cord, however, there is debate on whether this is detrimental or beneficial in CNS repair. Active immunization with MBP or transfer of MBP-specific T lymphocytes following contusion SCI promoted neuronal regeneration and improved motor function (Hauben et al., 2000). Similar results were seen in dorsal hemisection injury, with myelin or spinal cord homogenate immunization promoting long-distance regeneration of axons, corticospinal tract sprouting and improved locomotor activity (Huang et al., 1999; Sicotte et al., 2003). Immunized mice can develop autoantibodies against endogenous inhibitors of neurite growth found in the adult CNS, which can in turn promote neurite growth *in vitro* (Huang et al., 1999). These studies highlight the neuroprotective and neuroregenerative roles of T cell-mediated immune activity following CNS injury.

As traumatic CNS injury is dependent on the external trauma, it is the only neurodegenerative disease discussed here for which aging is not a risk factor. However, older patients with TBI show a less complete recovery at 1 year post-injury, with more severe consequences following trauma (Rothweiler et al., 1998); suggesting that age-associated regeneration failure will also influence recovery in traumatic CNS injury.

AGING – A KEY FACTOR MODULATING CNS AND IMMUNE SYSTEM INTERACTIONS

As discussed above, aging is a major risk factor in neurodegenerative diseases, with aged individuals often presenting with more severe forms of disease or incomplete recovery after damage. Studies have discussed the impact or mechanisms of aging-related pathogenesis in specific neurodegenerative diseases, however, research into the normal physiological changes that occur during aging can facilitate our understanding of disease development or progression with age. Like all biological entities, the CNS and the adaptive immune system functionally decline with healthy aging, with brain atrophy and increased levels of neuroinflammation. Although the CNS was previously considered an 'immune-privileged site,' with immune infiltration only occurring after BBB disruption caused by damage or aging, studies have shown that the adaptive immune system is present and beneficial in the healthy CNS (Ellwardt et al., 2016). CD4$^+$ and CD8$^+$ T lymphocytes are present in the healthy CNS and are required

for immune-surveillance and protection against neurotropic viruses (Kivisäkk et al., 2003; Smolders et al., 2013, 2018). Infiltrating T lymphocytes regulate the integrity and homeostasis of the CNS, inducing hippocampal neurogenesis and improving cognitive function in healthy rats (Ziv et al., 2006). As cognitive function declines during aging, this may suggest a dysfunctional CNS-resident T cell population.

Functional decline of the aging adaptive immune system is termed immunosenescence and is associated with greater susceptibility to infections. Although cellular senescence occurs, aging also induces dysfunctional systemic inflammation (inflammaging) through immune cell alterations. This dysfunction may be due to heterogeneous epigenetic modifications identified in PBMC's of older individuals, showing a clear aging signature (Ucar et al., 2017; Cheung et al., 2018). During aging, there is an increased number of inflammatory cells in the CNS, contributing to higher levels of neuroinflammation (Stichel and Luebbert, 2007; Dulken et al., 2019). Increased infiltration may result from aging-related alterations of the BBB and its transport mechanisms, leading to increased BBB permeability (Shah and Mooradian, 1997; Bake et al., 2009; Blau et al., 2012; Lee et al., 2012). Alongside increased permeability, the aged rat brain also showed decreased perfusion, promoting microglial activation and infiltration of macrophages which express lymphocyte and monocyte chemo-attractants, IP-10 and MCP-1 (Blau et al., 2012). Compounding this reduction in vascular perfusion, the aging brain is also less efficiently drained by meningeal lymphatic vessels, associated with impaired clearance of macromolecules and dysregulated inflammation-associated gene expression in lymphatic endothelial cells (Da Mesquita et al., 2018). Together, these mechanisms could contribute to the greater infiltration of lymphocytes into the aging brain. Greater infiltration was confirmed by single-cell analysis of young and old mice showing an increased T cell population, predominantly $CD8^+$ T cells, within neurogenic niches in aged animals (Dulken et al., 2019). This age-associated increase in $CD8^+$ T cells leads to increased IFN-γ signaling and a subsequent decrease in neurogenesis (Dulken et al., 2019). A similar study showed an aging-related increase in number and accumulation of $CD3^+$ T cells and dendritic cells in the brain parenchyma from 12 months onward, whilst B lymphocytes were not detected at any age (Stichel and Luebbert, 2007). Aged mice display an increase in numbers of naïve and memory $CD4^+$ T cells which recognize foreign pMHC-II, however, decreased numbers were detected in the brain and draining lymph nodes of aged mice following neurotropic virus infection (Deshpande et al., 2018). This highlights the dysfunctional immune response in aged animals, with increased responding cell numbers not translating into a greater clearance of infection (Deshpande et al., 2018).

The aging-related deterioration of T cell homeostasis has been linked to the age-dependent involution of the thymus (Linton and Dorshkind, 2004). This chronic inflammation leads to an increased rate of brain CNS aging which in turn initiates systemic aging, highlighting the co-dependency of the two systems (Coder et al., 2017). This co-dependency is supported by the greater response of microglia and astrocytes in the aged brain to pro-inflammatory cytokines such as IFN-γ and TNF-α (Xu et al., 2010), which also shows an age-related increase in intercellular adhesion molecule (ICAM)-1 expression on astrocytes and microglia, promoting further T cell recruitment to the CNS (Xu et al., 2010). As a result, increased $CD4^+$ and $CD8^+$ T cells are observed in the brain parenchyma and choroid plexus during aging (Xu et al., 2010). Thymus involution and aging results in a decline of naïve T cells and peripheral B cells with an increase in the number of terminally differentiated T cells (Pulko et al., 2016; Cao and Zheng, 2018). Thymic involution also causes autoreactive T cell clones to be released into the periphery, as they are no longer depleted by the thymus, increasing susceptibility to autoimmunity and contributing to chronic inflammation (Coder et al., 2015). Aged naïve $CD8^+$ T cells show defective expansion and differentiation following bacterial infection, with increased apoptosis of effector cells (Smithey et al., 2011). A large proportion of memory $CD8^+$ T cells in aged mice develop without antigen stimulation and are the result of a switch from naïve T cells to a memory cell phenotype (Chiu et al., 2013; Moskowitz et al., 2017). These high-avidity memory-like virtual T cells are important compensatory cells during aging as aged naïve T cells have a less diverse repertoire of T cell receptors (Rudd et al., 2011).

Diminished numbers of circulating B lymphocytes during aging are associated with an altered and less diverse antibody repertoire (Gibson et al., 2009; Tabibian-Keissar et al., 2016; Riley et al., 2017; Nikolich-Zugich, 2018). Although numbers are decreased, the proportion of age-associated B cells (ABCs) increases (Hao et al., 2011; Riley et al., 2017). ABCs are pro-inflammatory cells which inhibit normal B cell differentiation and have a distinct antibody repertoire that is more reactive to self-antigens (Ratliff et al., 2013; Riley et al., 2017). There are alterations of B cell subsets in the peripheral blood of aged individuals, with increased proportions of late/exhausted B cells, similar to ABCs, expressing senescence-associated secretory phenotype (SASP) markers and activating NF-κB (Frasca et al., 2017; Frasca, 2018). Aging-associated disruption of B cell subsets results in a diminished response to exogenous antigens and vaccines with a greater susceptibility to infection (Pinti et al., 2016; Frasca et al., 2017). This diminished response may be mediated by a higher expression of TNF-α by ABCs, which in turn negatively affects other B cell subsets (Frasca et al., 2014). Aging-related B lymphocyte dysregulation is thought to occur over the age of 60, with genome-wide expression profiles of B cells from young and aged donors up to 60 years of age showing no differences in gene expression (Knight et al., 2016).

Analysis of aged individual's blood showed increased pro-aging and immune factors, such as CCL11 and B2M, resulting in the development of an aging phenotype negatively affecting memory function and neurogenesis (Villeda et al., 2011; Smith et al., 2015). The main barrier between the circulation and the CNS, the choroid plexus, is home to CNS-specific $CD4^+$ T cells. During aging and immunosenescence, these cells are shifted to a Th2 response, resulting in elevated levels of IL-4 and CCL11 which are detrimental for cognitive function (Baruch et al., 2013). Restoration of the cytokine balance between IL-4 and IFN-γ,

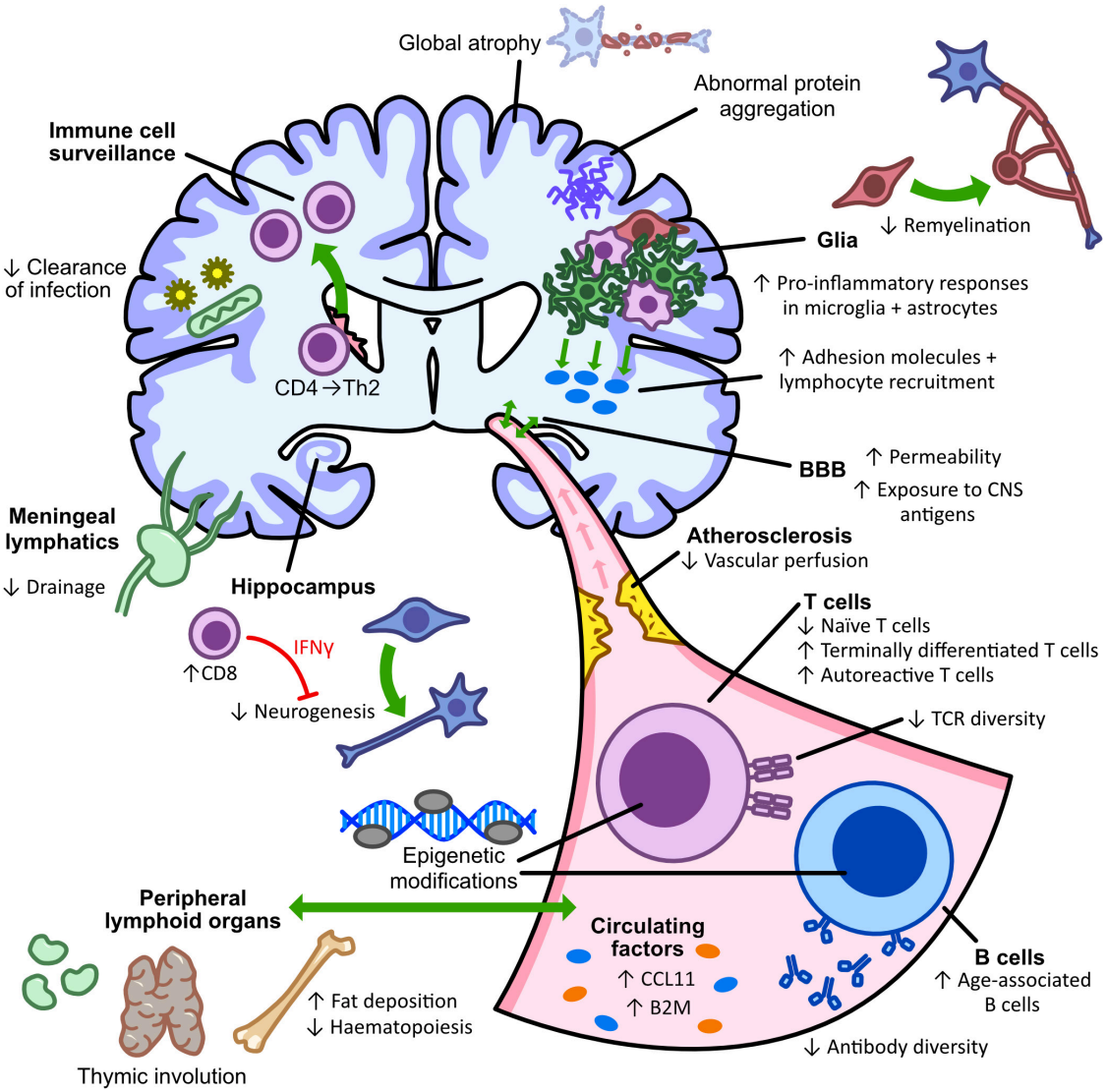

FIGURE 2 | Aging alters CNS-immune interactions in health and disease. Aging is a major risk factor for neurodegeneration that is accompanied by progressive immunosenescence, inflammaging, atrophy, and neuroinflammation. Microglia and astrocytes are thought to respond more to pro-inflammatory cytokines, such as IFNγ and TNFα, and become prone to abnormal inflammatory activation; leading to reduced remyelination and enhanced lymphocytic recruitment. Subsequent aging-related changes in BBB permeability and lymphatic drainage also increase the infiltration of cytotoxic $CD8^+$ T cells to the brain, which can inhibit neurogenesis through IFN-γ signaling. In the periphery, processes such as thymic involution and epigenetic modifications similarly alter the number of naïve $CD4^+$ T cells and can diminish the antibody repertoire following an increase in age-associated pro-inflammatory B cells. By contributing to poor infection clearance, protein aggregation and altered immune cell surveillance, aging therefore has a detrimental impact on both the immune system and cognitive functioning.

with blockage of type I interferon signaling, can restore cognitive function, highlighting the disruption the aged immune system has on the CNS (Baruch et al., 2013, 2014).

These studies stress the detrimental relationship of the CNS and the adaptive immune system during aging, which modulates the functional connection between the two (summarized in **Figure 2**). Aging leads to an increase in pro-inflammatory cytokines and a decrease in T cell repertoire and pro-regenerative functions. Therefore, aging tilts the balance in favor of the degenerative role of the adaptive immune system in the CNS at the expense of its regenerative functions.

CONCLUDING REMARKS

This review has detailed the role of the adaptive immune system in both degeneration and regeneration in neurodegenerative diseases. In these diseases, the adaptive immune system is often dysfunctional, with altered levels of immune cells and shifts to a more pro-inflammatory immune profile, both of which are associated with neurodegeneration. Other studies have also shown a neuroprotective and regenerative role for the adaptive immune system in animal models of disease. However, despite research suggesting a greater role for adaptive immunity in

progressing neuronal loss, it is debatable whether this is truly due to a greater detrimental role of the adaptive immune system or rather due to the less abundant research in neuroregeneration; a newer and currently growing field. There is also difficulty in establishing a definitive regenerative effect for the adaptive immune system, with many studies failing to distinguish between neuroprotection and neuroregeneration. This may be due to the variety of animal models used to study these diseases, and their limitations in evaluating regeneration.

Interestingly, the normal biological process of aging causes similar dysregulation of the adaptive immune system. As aging is a major risk factor of neurodegenerative diseases, often influencing progression and evolution of disease severity, immunosenescence and the declining function of the CNS may influence neurodegeneration. This is of particular importance when evaluating the dual role of the adaptive immune system in the CNS. The switch of the adaptive immune system between degenerative and regenerative effects may have an environmental or age-associated trigger. This reveals a need to expand research on neurodegenerative diseases into aging animal models, furtherly delving into the opposing sides of adaptive immunity in the CNS. Future research into the mechanisms of the adaptive immune system in neuroregeneration is also required, and whether this regenerative role is lost during disease progression and aging before establishing therapeutics. To date, immunotherapies for neurodegenerative diseases have focused on targeting protein aggregation, however, there may be scope for immunotherapies which regulate the pro-inflammatory profiles seen in disease and aging, or immunotherapies focused on boosting the neuroprotective and neuroregenerative function of the adaptive immune system.

AUTHOR CONTRIBUTIONS

KM wrote the manuscript. CM designed and created the figures. KM, JAW, CM, FJR, and AdlF contributed to literature research and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Acevedo-Arozena, A., Kalmar, B., Essa, S., Ricketts, T., Joyce, P., Kent, R., et al. (2011). A comprehensive assessment of the SOD1G93A low-copy transgenic mouse, which models human amyotrophic lateral sclerosis. *Dis. Model. Mech.* 4, 686–700. doi: 10.1242/dmm.007237

Akhtar, R. S., Licata, J. P., Luk, K. C., Shaw, L. M., Trojanowski, J. Q., and Lee, V. M.-Y. (2018). Measurements of auto-antibodies to alpha-synuclein in the serum and cerebral spinal fluids of patients with Parkinson's disease. *J. Neurochem.* 145, 489–503. doi: 10.1111/jnc.14330

Alcina, A., Abad-Grau, M. D. M., Fedetz, M., Izquierdo, G., Lucas, M., Fernández, O., et al. (2012). Multiple sclerosis risk variant HLA-DRB1*1501 associates with high expression of DRB1 gene in different human populations. *PLoS One* 7:e29819. doi: 10.1371/journal.pone.0029819

Alizadeh, A., Dyck, S. M., and Karimi-Abdolrezaee, S. (2019). Traumatic spinal cord injury: an overview of pathophysiology, models and acute injury mechanisms. *Front. Neurol.* 10:282. doi: 10.3389/fneur.2019.00282

Alonso, A., Logroscino, G., Jick, S. S., and Hernán, M. A. (2009). Incidence and lifetime risk of motor neuron disease in the United Kingdom: a population-based study. *Eur. J. Neurol.* 16, 745–751. doi: 10.1111/j.1468-1331.2009.02586.x

Amor, S., Puentes, F., Baker, D., and van der Valk, P. (2010). Inflammation in neurodegenerative diseases. *Immunology* 129, 154–169. doi: 10.1111/j.1365-2567.2009.03225.x

Ankeny, D. P., Guan, Z., and Popovich, P. G. (2009). B cells produce pathogenic antibodies and impair recovery after spinal cord injury in mice. *J. Clin. Invest.* 119, 2990–2999. doi: 10.1172/JCI39780

Ankeny, D. P., Lucin, K. M., Sanders, V. M., McGaughy, V. M., and Popovich, P. G. (2006). Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis. *J. Neurochem.* 99, 1073–1087. doi: 10.1111/j.1471-4159.2006.04147.x

Baba, Y., Kuroiwa, A., Uitti, R. J., Wszolek, Z. K., and Yamada, T. (2005). Alterations of T-lymphocyte populations in Parkinson disease. *Parkinsonism Relat. Disord.* 11, 493–498. doi: 10.1016/j.parkreldis.2005.07.005

Bachiller, S., Jiménez-Ferrer, I., Paulus, A., Yang, Y., Swanberg, M., Deierborg, T., et al. (2018). Microglia in neurological diseases: a road map to brain-disease dependent-inflammatory response. *Front. Cell. Neurosci.* 12:488. doi: 10.3389/fncel.2018.00488

Baek, H., Ye, M., Kang, G.-H., Lee, C., Lee, G., Choi, D., et al. (2016). Neuroprotective effects of CD4+CD25+Foxp3+ regulatory T cells in a 3xTg-AD Alzheimer's disease model. *Oncotarget* 7, 69347–69357. doi: 10.18632/oncotarget.12469

Bake, S., Friedman, J. A., and Sohrabji, F. (2009). Reproductive age-related changes in the blood brain barrier: expression of IgG and tight junction proteins. *Microvasc. Res.* 78, 413–424. doi: 10.1016/j.mvr.2009.06.009

Banerjee, R., Mosley, R. L., Reynolds, A. D., Dhar, A., Jackson-Lewis, V., Gordon, P. H., et al. (2008). Adaptive immune neuroprotection in G93A-SOD1 amyotrophic lateral sclerosis mice. *PLoS One* 3:e2740. doi: 10.1371/journal.pone.0002740

Barnett, M. H., and Prineas, J. W. (2004). Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann. Neurol.* 55, 458–468. doi: 10.1002/ana.20016

Baruch, K., Deczkowska, A., David, E., Castellano, J. M., Miller, O., Kertser, A., et al. (2014). Aging. Aging-induced type I interferon response at the choroid plexus negatively affects brain function. *Science* 346, 89–93. doi: 10.1126/science.1252945

Baruch, K., Deczkowska, A., Rosenzweig, N., Tsitsou-Kampeli, A., Sharif, A. M., Matcovitch-Natan, O., et al. (2016). PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. *Nat. Med.* 22, 135–137. doi: 10.1038/nm.4022

Baruch, K., Ron-Harel, N., Gal, H., Deczkowska, A., Shifrut, E., Ndifon, W., et al. (2013). CNS-specific immunity at the choroid plexus shifts toward destructive Th2 inflammation in brain aging. *Proc. Natl. Acad. Sci. U.S.A.* 110, 2264–2269. doi: 10.1073/pnas.1211270110

Baruch, K., Rosenzweig, N., Kertser, A., Deczkowska, A., Sharif, A. M., Spinrad, A., et al. (2015). Breaking immune tolerance by targeting Foxp3(+) regulatory T cells mitigates Alzheimer's disease pathology. *Nat. Commun.* 6:7967. doi: 10.1038/ncomms8967

Bas, J., Calopa, M., Mestre, M., Mollevi, D. G., Cutillas, B., Ambrosio, S., et al. (2001). Lymphocyte populations in Parkinson's disease and in rat models of parkinsonism. *J. Neuroimmunol.* 113, 146–152. doi: 10.1016/s0165-5728(00)00422-7

Bature, F., Guinn, B.-A., Pang, D., and Pappas, Y. (2017). Signs and symptoms preceding the diagnosis of Alzheimer's disease: a systematic scoping review of literature from 1937 to 2016. *BMJ Open* 7:e015746. doi: 10.1136/bmjopen-2016-015746

Baxi, E. G., DeBruin, J., Tosi, D. M., Grishkan, I. V., Smith, M. D., Kirby, L. A., et al. (2015). Transfer of myelin-reactive th17 cells impairs endogenous remyelination in the central nervous system of cuprizone-fed mice. *J. Neurosci.* 35, 8626–8639. doi: 10.1523/JNEUROSCI.3817-14.2015

Becker, K. J., Kalil, A. J., Tanzi, P., Zierath, D. K., Savos, A. V., Gee, J. M., et al. (2011). Autoimmune responses to the brain after stroke are associated with worse outcome. *Stroke* 42, 2763–2769. doi: 10.1161/STROKEAHA.111.619593

Becker, K. J., Kindrick, D. L., Lester, M. P., Shea, C., and Ye, Z.-C. (2005). Sensitization to brain antigens after stroke is augmented by lipopolysaccharide. *J. Cereb. Blood Flow Metab.* 25, 1634–1644. doi: 10.1038/sj.jcbfm.9600160

Becker, K. J., McCarron, R. M., Ruetzler, C., Laban, O., Sternberg, E., Flanders, K. C., et al. (1997). Immunologic tolerance to myelin basic protein decreases stroke size after transient focal cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.* 94, 10873–10878. doi: 10.1073/pnas.94.20.10873

Beers, D. R., Henkel, J. S., Zhao, W., Wang, J., and Appel, S. H. (2008). CD4+ T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15558–15563. doi: 10.1073/pnas.0807419105

Beers, D. R., Henkel, J. S., Zhao, W., Wang, J., Huang, A., Wen, S., et al. (2011). Endogenous regulatory T lymphocytes ameliorate amyotrophic lateral sclerosis in mice and correlate with disease progression in patients with amyotrophic lateral sclerosis. *Brain* 134, 1293–1314. doi: 10.1093/brain/awr074

Beers, D. R., Zhao, W., Wang, J., Zhang, X., Wen, S., Neal, D., et al. (2017). ALS patients' regulatory T lymphocytes are dysfunctional, and correlate with disease progression rate and severity. *JCI Insight* 2:e89530. doi: 10.1172/jci.insight.89530

Benner, E. J., Banerjee, R., Reynolds, A. D., Sherman, S., Pisarev, V. M., Tsiperson, V., et al. (2008). Nitrated alpha-synuclein immunity accelerates degeneration of nigral dopaminergic neurons. *PLoS One* 3:e1376. doi: 10.1371/journal.pone.0001376

Benner, E. J., Mosley, R. L., Destache, C. J., Lewis, T. B., Jackson-Lewis, V., Gorantla, S., et al. (2004). Therapeutic immunization protects dopaminergic neurons in a mouse model of Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9435–9440. doi: 10.1073/pnas.0400569101

Bergold, P. J. (2016). Treatment of traumatic brain injury with anti-inflammatory drugs. *Exp. Neurol.* 275, 367–380. doi: 10.1016/j.expneurol.2015.05.024

Bieber, A. J., Kerr, S., and Rodriguez, M. (2003). Efficient central nervous system remyelination requires T cells. *Ann. Neurol.* 53, 680–684. doi: 10.1002/ana.10578

Blau, C. W., Cowley, T. R., O'Sullivan, J., Grehan, B., Browne, T. C., Kelly, L., et al. (2012). The age-related deficit in LTP is associated with changes in perfusion and blood-brain barrier permeability. *Neurobiol. Aging* 33, 1005.e23–1005.e35. doi: 10.1016/j.neurobiolaging.2011.09.035

Brochard, V., Combadiere, B., Prigent, A., Laouar, Y., Perrin, A., Beray-Berthat, V., et al. (2009). Infiltration of CD4+ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease. *J. Clin. Invest.* 119, 182–192. doi: 10.1172/JCI36470

Browne, T. C., McQuillan, K., McManus, R. M., O'Reilly, J.-A., Mills, K. H. G., and Lynch, M. A. (2013). IFN-gamma Production by amyloid beta-specific Th1 cells promotes microglial activation and increases plaque burden in a mouse model of Alzheimer's disease. *J. Immunol.* 190, 2241–2251. doi: 10.4049/jimmunol.1200947

Brynskikh, A., Warren, T., Zhu, J., and Kipnis, J. (2008). Adaptive immunity affects learning behavior in mice. *Brain Behav. Immun.* 22, 861–869. doi: 10.1016/j.bbi.2007.12.008

Cacabelos, R., Alvarez, X. A., Fernandez-Novoa, L., Franco, A., Mangues, R., Pellicer, A., et al. (1994). Brain interleukin-1 beta in Alzheimer's disease and vascular dementia. *Methods Find Exp. Clin. Pharmacol.* 16, 141–151.

Campbell, B. C. V., De Silva, D. A., Macleod, M. R., Coutts, S. B., Schwamm, L. H., Davis, S. M., et al. (2019). Ischaemic stroke. *Nat. Rev. Dis. Prim.* 5:70. doi: 10.1038/s41572-019-0118-8

Cantuti-Castelvetri, L., Fitzner, D., Bosch-Queralt, M., Weil, M.-T., Su, M., Sen, P., et al. (2018). Defective cholesterol clearance limits remyelination in the aged central nervous system. *Science* 359, 684–688. doi: 10.1126/science.aan4183

Cao, C., Arendash, G. W., Dickson, A., Mamcarz, M. B., Lin, X., and Ethell, D. W. (2009). Abeta-specific Th2 cells provide cognitive and pathological benefits to Alzheimer's mice without infiltrating the CNS. *Neurobiol. Dis.* 34, 63–70. doi: 10.1016/j.nbd.2008.12.015

Cao, W., and Zheng, H. (2018). Peripheral immune system in aging and Alzheimer's disease. *Mol. Neurodegener.* 13:51. doi: 10.1186/s13024-018-0284-2

Casili, G., Impellizzeri, D., Cordaro, M., Esposito, E., and Cuzzocrea, S. (2016). B-cell depletion with CD20 antibodies as new approach in the treatment of inflammatory and immunological events associated with spinal cord injury. *Neurotherapeutics* 13, 880–894. doi: 10.1007/s13311-016-0446-2

Chaplin, D. D. (2010). Overview of the immune response. *J. Allergy Clin. Immunol.* 125, S3–S23. doi: 10.1016/j.jaci.2009.12.980

Chen, H., Zhang, S. M., Hernan, M. A., Schwarzschild, M. A., Willett, W. C., Colditz, G. A., et al. (2003). Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. *Arch. Neurol.* 60, 1059–1064. doi: 10.1001/archneur.60.8.1059

Chen, X., Feng, W., Huang, R., Guo, X., Chen, Y., Zheng, Z., et al. (2014). Evidence for peripheral immune activation in amyotrophic lateral sclerosis. *J. Neurol. Sci.* 347, 90–95. doi: 10.1016/j.jns.2014.09.025

Chen, X.-Q., and Mobley, W. C. (2019). Alzheimer disease pathogenesis: insights from molecular and cellular biology studies of oligomeric abeta and tau species. *Front. Neurosci.* 13:659. doi: 10.3389/fnins.2019.00659

Chen, Y., Qi, B., Xu, W., Ma, B., Li, L., Chen, Q., et al. (2015). Clinical correlation of peripheral CD4+cell subsets, their imbalance and Parkinson's disease. *Mol. Med. Rep.* 12, 6105–6111. doi: 10.3892/mmr.2015.4136

Cheung, P., Vallania, F., Warsinske, H. C., Donato, M., Schaffert, S., Chang, S. E., et al. (2018). Single-cell chromatin modification profiling reveals increased epigenetic variations with aging. *Cell* 173, 1385.e14–1397.e14. doi: 10.1016/j.cell.2018.03.079

Chiang, T., Messing, R. O., and Chou, W.-H. (2011). Mouse model of middle cerebral artery occlusion. *J. Vis. Exp.* 48:2761. doi: 10.3791/2761

Chiu, B.-C., Martin, B. E., Stolberg, V. R., and Chensue, S. W. (2013). Cutting edge: central memory CD8 T cells in aged mice are virtual memory cells. *J. Immunol.* 191, 5793–5796. doi: 10.4049/jimmunol.1302509

Chiu, I. M., Chen, A., Zheng, Y., Kosaras, B., Tsiftsoglou, S. A., Vartanian, T. K., et al. (2008). T lymphocytes potentiate endogenous neuroprotective inflammation in a mouse model of ALS. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17913–17918. doi: 10.1073/pnas.0804610105

Ciccocioppo, F., Lanuti, P., Pierdomenico, L., Simeone, P., Bologna, G., Ercolino, E., et al. (2019). The characterization of regulatory T-cell profiles in alzheimer's disease and multiple sclerosis. *Sci. Rep.* 9:8788. doi: 10.1038/s41598-019-45433-3

Clarkson, B. D. S., Ling, C., Shi, Y., Harris, M. G., Rayasam, A., Sun, D., et al. (2014). T cell-derived interleukin (IL)-21 promotes brain injury following stroke in mice. *J. Exp. Med.* 211, 595–604. doi: 10.1084/jem.20131377

Coder, B., Wang, W., Wang, L., Wu, Z., Zhuge, Q., and Su, D.-M. (2017). Friend or foe: the dichotomous impact of T cells on neuro-de/re-generation during aging. *Oncotarget* 8, 7116–7137. doi: 10.18632/oncotarget.12572

Coder, B. D., Wang, H., Ruan, L., and Su, D.-M. (2015). Thymic involution perturbs negative selection leading to autoreactive T cells that induce chronic inflammation. *J. Immunol.* 194, 5825–5837. doi: 10.4049/jimmunol.1500082

Coles, A. J., Compston, A. S., Selmaj, K. W., Lake, S. L., Moran, S., Margolin, D. H., et al. (2008). Alemtuzumab vs. interferon Beta-1a in early multiple sclerosis. *N. Engl. J. Med.* 359, 1786–1801. doi: 10.1056/NEJMoa0802670

Confavreux, C., and Vukusic, S. (2006). Age at disability milestones in multiple sclerosis. *Brain* 129, 595–605. doi: 10.1093/brain/awh714

Constantinescu, C. S., Farooqi, N., O'Brien, K., and Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br. J. Pharmacol.* 164, 1079–1106. doi: 10.1111/j.1476-5381.2011.01302.x

Coque, E., Salsac, C., Espinosa-Carrasco, G., Varga, B., Degauque, N., Cadoux, M., et al. (2019). Cytotoxic CD8(+) T lymphocytes expressing ALS-causing SOD1 mutant selectively trigger death of spinal motoneurons. *Proc. Natl. Acad. Sci. U.S.A.* 116, 2312–2317. doi: 10.1073/pnas.1815961116

Cossburn, M., Ingram, G., Hirst, C., Ben-Shlomo, Y., Pickersgill, T. P., and Robertson, N. P. (2012). Age at onset as a determinant of presenting phenotype and initial relapse recovery in multiple sclerosis. *Mult. Scler.* 18, 45–54. doi: 10.1177/1352458511417479

Cox, A. L., Coles, A. J., Nortje, J., Bradley, P. G., Chatfield, D. A., Thompson, S. J., et al. (2006). An investigation of auto-reactivity after head injury. *J. Neuroimmunol.* 174, 180–186. doi: 10.1016/j.jneuroim.2006.01.007

Da Mesquita, S., Louveau, A., Vaccari, A., Smirnov, I., Cornelison, R. C., Kingsmore, K. M., et al. (2018). Functional aspects of meningeal lymphatics in ageing and Alzheimer's disease. *Nature* 560, 185–191. doi: 10.1038/s41586-018-0368-8

Dambinova, S. A., Khounteev, G. A., Izykenova, G. A., Zavolokov, I. G., Ilyukhina, A. Y., and Skoromets, A. A. (2003). Blood test detecting autoantibodies to N-methyl-D-aspartate neuroreceptors for evaluation of patients with transient ischemic attack and stroke. *Clin. Chem.* 49, 1752–1762. doi: 10.1373/49.10.1752

Dansokho, C., Ait Ahmed, D., Aid, S., Toly-Ndour, C., Chaigneau, T., Calle, V., et al. (2016). Regulatory T cells delay disease progression in Alzheimer-like pathology. *Brain* 139, 1237–1251. doi: 10.1093/brain/awv408

Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., and Amit, I. (2018). Disease-associated microglia: a Universal immune sensor of neurodegeneration. *Cell* 173, 1073–1081. doi: 10.1016/j.cell.2018.05.003

DeMaagd, G., and Philip, A. (2015). Parkinson's disease and its management: part 1: disease entity, risk factors, pathophysiology, clinical presentation, and diagnosis. *P T* 40, 504–532.

Derecki, N. C., Cardani, A. N., Yang, C. H., Quinnies, K. M., Crihfield, A., Lynch, K. R., et al. (2010). Regulation of learning and memory by meningeal immunity: a key role for IL-4. *J. Exp. Med.* 207, 1067–1080. doi: 10.1084/jem.20091419

Desai, M. K., Guercio, B. J., Narrow, W. C., and Bowers, W. J. (2011). An Alzheimer's disease-relevant presenilin-1 mutation augments amyloid-beta-induced oligodendrocyte dysfunction. *Glia* 59, 627–640. doi: 10.1002/glia.21131

Deshpande, N. R., Uhrlaub, J. L., Way, S. S., Nikolich-Zugich, J., and Kuhns, M. S. (2018). A disconnect between precursor frequency, expansion potential, and site-specific CD4+ T cell responses in aged mice. *PLoS One* 13:e0198354. doi: 10.1371/journal.pone.0198354

Dhungana, H., Malm, T., Denes, A., Valonen, P., Wojciechowski, S., Magga, J., et al. (2013). Aging aggravates ischemic stroke-induced brain damage in mice with chronic peripheral infection. *Aging Cell* 12, 842–850. doi: 10.1111/acel.12106

Djikić, J., Nacka-Aleksić, M., Pilipović, I., Kosec, D., Arsenović-Ranin, N., Stojić-Vukanić, Z., et al. (2015). Age-related changes in spleen of Dark Agouti rats immunized for experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 278, 123–135. doi: 10.1016/j.jneuroim.2014.12.014

Dombrowski, Y., O'Hagan, T., DIttmer, M., Penalva, R., Mayoral, S. R., Bankhead, P., et al. (2017). Regulatory T cells promote myelin regeneration in the central nervous system. *Nat. Neurosci.* 20, 674–680. doi: 10.1038/nn.4528

Domingues, H. S., Mues, M., Lassmann, H., Wekerle, H., and Krishnamoorthy, G. (2010). Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *PLoS One* 5:e15531. doi: 10.1371/journal.pone.0015531

Doyle, K. P., Quach, L. N., Sole, M., Axtell, R. C., Nguyen, T.-V. V., Soler-Llavina, G. J., et al. (2015). B-lymphocyte-mediated delayed cognitive impairment following stroke. *J. Neurosci.* 35, 2133–2145. doi: 10.1523/JNEUROSCI.4098-14.2015

Dugger, B. N., and Dickson, D. W. (2017). Pathology of Neurodegenerative Diseases. *Cold Spring Harb. Perspect. Biol.* 9:a028035. doi: 10.1101/cshperspect.a028035

Dulken, B. W., Buckley, M. T., Navarro Negredo, P., Saligrama, N., Cayrol, R., Leeman, D. S., et al. (2019). Single-cell analysis reveals T cell infiltration in old neurogenic niches. *Nature* 571, 205–210. doi: 10.1038/s41586-019-1362-5

Edri-Brami, M., Rosental, B., Hayoun, D., Welt, M., Rosen, H., Wirguin, I., et al. (2012). Glycans in sera of amyotrophic lateral sclerosis patients and their role in killing neuronal cells. *PLoS One* 7:e35772. doi: 10.1371/journal.pone.0035772

Edri-Brami, M., Sharoni, H., Hayoun, D., Skutelsky, L., Nemirovsky, A., Porgador, A., et al. (2015). Development of stage-dependent glycans on the Fc domains of IgG antibodies of ALS animals. *Exp. Neurol.* 267, 95–106. doi: 10.1016/j.expneurol.2015.02.023

Elkins, J., Veltkamp, R., Montaner, J., Johnston, S. C., Singhal, A. B., Becker, K., et al. (2017). Safety and efficacy of natalizumab in patients with acute ischaemic stroke (ACTION): a randomised, placebo-controlled, double-blind phase 2 trial. *Lancet Neurol.* 16, 217–226. doi: 10.1016/S1474-4422(16)30357-X

Ellwardt, E., Walsh, J. T., Kipnis, J., and Zipp, F. (2016). Understanding the Role of T Cells in CNS Homeostasis. *Trends Immunol.* 37, 154–165. doi: 10.1016/j.it.2015.12.008

Engelhardt, J. I., Tajti, J., and Appel, S. H. (1993). Lymphocytic infiltrates in the spinal cord in amyotrophic lateral sclerosis. *Arch. Neurol.* 50, 30–36. doi: 10.1001/archneur.1993.00540010026013

Engstad, T., Engstad, T. T., Viitanen, M., and Ellekjćr, H. (2012). Epidemiology of stroke in the elderly in the Nordic countries. Incidence, survival, prevalence and risk factors. *Nor. Epidemiol.* 22, 121–126. doi: 10.5324/nje.v22i2.1557

Eriksen, J. L., Wszolek, Z., and Petrucelli, L. (2005). Molecular pathogenesis of parkinson disease. *Arch. Neurol.* 62, 353–357. doi: 10.1001/archneur.62.3.353

Felger, J. C., Abe, T., Kaunzner, U. W., Gottfried-Blackmore, A., Gal-Toth, J., McEwen, B. S., et al. (2010). Brain dendritic cells in ischemic stroke: time course, activation state, and origin. *Brain Behav. Immun.* 24, 724–737. doi: 10.1016/j.bbi.2009.11.002

Ferrari, R., Kapogiannis, D., Huey, E. D., and Momeni, P. (2011). FTD and ALS: a tale of two diseases. *Curr. Alzheimer Res.* 8, 273–294. doi: 10.2174/156720511795563700

Fiszer, U., Mix, E., Fredrikson, S., Kostulas, V., and Link, H. (1994). Parkinson's disease and immunological abnormalities: increase of HLA-DR expression on monocytes in cerebrospinal fluid and of CD45RO+ T cells in peripheral blood. *Acta Neurol. Scand.* 90, 160–166. doi: 10.1111/j.1600-0404.1994.tb02699.x

Fleming, J. C., Norenberg, M. D., Ramsay, D. A., Dekaban, G. A., Marcillo, A. E., Saenz, A. D., et al. (2006). The cellular inflammatory response in human spinal cords after injury. *Brain* 129, 3249–3269. doi: 10.1093/brain/awl296

Franklin, R. J. M., and Ffrench-Constant, C. (2017). Regenerating CNS myelin - from mechanisms to experimental medicines. *Nat. Rev. Neurosci.* 18, 753–769. doi: 10.1038/nrn.2017.136

Frasca, D. (2018). Senescent B cells in aging and age-related diseases: their role in the regulation of antibody responses. *Exp. Gerontol.* 107, 55–58. doi: 10.1016/j.exger.2017.07.002

Frasca, D., Diaz, A., Romero, M., and Blomberg, B. B. (2017). Human peripheral late/exhausted memory B cells express a senescent-associated secretory phenotype and preferentially utilize metabolic signaling pathways. *Exp. Gerontol.* 87, 113–120. doi: 10.1016/j.exger.2016.12.001

Frasca, D., Diaz, A., Romero, M., Landin, A. M., and Blomberg, B. B. (2014). High TNF-alpha levels in resting B cells negatively correlate with their response. *Exp. Gerontol.* 54, 116–122. doi: 10.1016/j.exger.2014.01.004

Frenkel, D., Huang, Z., Maron, R., Koldzic, D. N., Moskowitz, M. A., and Weiner, H. L. (2005). Neuroprotection by IL-10-producing MOG CD4+ T cells following ischemic stroke. *J. Neurol. Sci.* 233, 125–132. doi: 10.1016/j.jns.2005.03.022

Gate, D., Saligrama, N., Leventhal, O., Yang, A. C., Unger, M. S., Middeldorp, J., et al. (2020). Clonally expanded CD8 T cells patrol the cerebrospinal fluid in Alzheimer's disease. *Nature* 577, 399–404. doi: 10.1038/s41586-019-1895-7

Gelderblom, M., Weymar, A., Bernreuther, C., Velden, J., Arunachalam, P., Steinbach, K., et al. (2012). Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* 120, 3793–3802. doi: 10.1182/blood-2012-02-412726

Gendelman, H. E., Zhang, Y., Santamaria, P., Olson, K. E., Schutt, C. R., Bhatti, D., et al. (2017). Evaluation of the safety and immunomodulatory effects of sargramostim in a randomized, double-blind phase 1 clinical Parkinson's disease trial. *NPJ Park. Dis.* 3:10. doi: 10.1038/s41531-017-0013-5

Gibson, K. L., Wu, Y.-C., Barnett, Y., Duggan, O., Vaughan, R., Kondeatis, E., et al. (2009). B-cell diversity decreases in old age and is correlated with poor health status. *Aging Cell* 8, 18–25. doi: 10.1111/j.1474-9726.2008.00443.x

Gilson, J., and Blakemore, W. F. (1993). Failure of remyelination in areas of demyelination produced in the spinal cord of old rats. *Neuropathol. Appl. Neurobiol.* 19, 173–181. doi: 10.1111/j.1365-2990.1993.tb00424.x

Giovannoni, G., Comi, G., Cook, S., Rammohan, K., Rieckmann, P., Soelberg Sørensen, P., et al. (2010). A placebo-controlled trial of oral cladribine for relapsing multiple sclerosis. *N. Engl. J. Med.* 362, 416–426. doi: 10.1056/NEJMoa0902533

Gonzalez, H., Contreras, F., Prado, C., Elgueta, D., Franz, D., Bernales, S., et al. (2013). Dopamine receptor D3 expressed on CD4+ T cells favors neurodegeneration of dopaminergic neurons during Parkinson's disease. *J. Immunol.* 190, 5048–5056. doi: 10.4049/jimmunol.1203121

Gonzalez, R., Glaser, J., Liu, M. T., Lane, T. E., and Keirstead, H. S. (2003). Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury. *Exp. Neurol.* 184, 456–463. doi: 10.1016/s0014-4886(03)00257-7

Gucluler, G., Adiguzel, E., Gungor, B., Kahraman, T., Gursel, M., Yilmaz, B., et al. (2017). Impaired toll like receptor-7 and 9 induced immune activation in chronic spinal cord injured patients contributes to immune dysfunction. *PLoS One* 12:e0171003. doi: 10.1371/journal.pone.0171003

Hao, Y., O'Neill, P., Naradikian, M. S., Scholz, J. L., and Cancro, M. P. (2011). A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. *Blood* 118, 1294–1304. doi: 10.1182/blood-2011-01-330530

Hardiman, O., Al-Chalabi, A., Chio, A., Corr, E. M., Logroscino, G., Robberecht, W., et al. (2017). Amyotrophic lateral sclerosis. *Nat. Rev. Dis. Prim.* 3:17071. doi: 10.1038/nrdp.2017.71

Hardy, J., and Allsop, D. (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol. Sci.* 12, 383–388. doi: 10.1016/0165-6147(91)90609-v

Harris, N. M., Roy-O'Reilly, M., Ritzel, R. M., Holmes, A., Sansing, L. H., O'Keefe, L. M., et al. (2020). Depletion of CD4 T cells provides therapeutic benefits in aged mice after ischemic stroke. *Exp. Neurol.* 326:113202. doi: 10.1016/j.expneurol.2020.113202

Hauben, E., Butovsky, O., Nevo, U., Yoles, E., Moalem, G., Agranov, E., et al. (2000). Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J. Neurosci.* 20, 6421–6430.

Hauser, S. L., Bar-Or, A., Comi, G., Giovannoni, G., Hartung, H. P., Hemmer, B., et al. (2017). Ocrelizumab versus interferon Beta-1a in relapsing multiple sclerosis. *N. Engl. J. Med.* 376, 221–234. doi: 10.1056/NEJMoa1601277

Henkel, J. S., Beers, D. R., Wen, S., Rivera, A. L., Toennis, K. M., Appel, J. E., et al. (2013). Regulatory T-lymphocytes mediate amyotrophic lateral sclerosis progression and survival. *EMBO Mol. Med.* 5, 64–79. doi: 10.1002/emmm.201201544

Hjelmström, P., Juedes, A. E., Fjell, J., and Ruddle, N. H. (1998). Cutting edge: B cell-deficient mice develop experimental allergic encephalomyelitis with demyelination after myelin oligodendrocyte glycoprotein sensitization. *J. Immunol.* 161, 4480–4483.

Holmin, S., and Mathiesen, T. (1999). Long-term intracerebral inflammatory response after experimental focal brain injury in rat. *Neuroreport* 10, 1889–1891. doi: 10.1097/00001756-199906230-00017

Holmin, S., Mathiesen, T., Shetye, J., and Biberfeld, P. (1995). Intracerebral inflammatory response to experimental brain contusion. *Acta Neurochir.* 132, 110–119. doi: 10.1007/BF01404857

Holmin, S., Soderlund, J., Biberfeld, P., and Mathiesen, T. (1998). Intracerebral inflammation after human brain contusion. *Neurosurgery* 42, 291–299. doi: 10.1097/00006123-199802000-00047

Horvath, I., Iashchishyn, I. A., Forsgren, L., and Morozova-Roche, L. A. (2017). Immunochemical detection of alpha-synuclein autoantibodies in Parkinson's disease: correlation between plasma and cerebrospinal fluid levels. *ACS Chem. Neurosci.* 8, 1170–1176. doi: 10.1021/acschemneuro.7b00063

Huang, D. W., McKerracher, L., Braun, P. E., and David, S. (1999). A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. *Neuron* 24, 639–647. doi: 10.1016/s0896-6273(00)81118-6

Huell, M., Strauss, S., Volk, B., Berger, M., and Bauer, J. (1995). Interleukin-6 is present in early stages of plaque formation and is restricted to the brains of Alzheimer's disease patients. *Acta Neuropathol.* 89, 544–551. doi: 10.1007/bf00571510

Hurn, P. D., Subramanian, S., Parker, S. M., Afentoulis, M. E., Kaler, L. J., Vandenbark, A. A., et al. (2007). T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J. Cereb. Blood Flow Metab.* 27, 1798–1805. doi: 10.1038/sj.jcbfm.9600482

Ishii, H., Jin, X., Ueno, M., Tanabe, S., Kubo, T., Serada, S., et al. (2012). Adoptive transfer of Th1-conditioned lymphocytes promotes axonal remodeling and functional recovery after spinal cord injury. *Cell Death Dis.* 3:e363. doi: 10.1038/cddis.2012.106

Ito, M., Komai, K., Mise-Omata, S., Iizuka-Koga, M., Noguchi, Y., Kondo, T., et al. (2019). Brain regulatory T cells suppress astrogliosis and potentiate neurological recovery. *Nature* 565, 246–250. doi: 10.1038/s41586-018-0824-5

Jager, A., Dardalhon, V., Sobel, R. A., Bettelli, E., and Kuchroo, V. K. (2009). Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J. Immunol.* 183, 7169–7177. doi: 10.4049/jimmunol.0901906

Jantaratnotai, N., Ryu, J. K., Kim, S. U., and McLarnon, J. G. (2003). Amyloid beta peptide-induced corpus callosum damage and glial activation in vivo. *Neuroreport* 14, 1429–1433. doi: 10.1097/00001756-200308060-00005

Javidi, E., and Magnus, T. (2019). Autoimmunity after ischemic stroke and brain injury. *Front. Immunol.* 10:686. doi: 10.3389/fimmu.2019.00686

Jiao, S.-S., Shen, L.-L., Zhu, C., Bu, X.-L., Liu, Y.-H., Liu, C.-H., et al. (2016). Brain-derived neurotrophic factor protects against tau-related neurodegeneration of Alzheimer's disease. *Transl. Psychiatry* 6:e907. doi: 10.1038/tp.2016.186

Jin, M., Gunther, R., Akgun, K., Hermann, A., and Ziemssen, T. (2020). Peripheral proinflammatory Th1/Th17 immune cell shift is linked to disease severity in amyotrophic lateral sclerosis. *Sci. Rep.* 10:5941. doi: 10.1038/s41598-020-62756-8

Jin, W.-N., Gonzales, R., Feng, Y., Wood, K., Chai, Z., Dong, J.-F., et al. (2018). Brain ischemia induces diversified neuroantigen-specific t-cell responses that exacerbate brain injury. *Stroke* 49, 1471–1478. doi: 10.1161/STROKEAHA.118.020203

Johnson, V. E., Stewart, J. E., Begbie, F. D., Trojanowski, J. Q., Smith, D. H., and Stewart, W. (2013). Inflammation and white matter degeneration persist for years after a single traumatic brain injury. *Brain* 136, 28–42. doi: 10.1093/brain/aws322

Jones, T. B., Basso, D. M., Sodhi, A., Pan, J. Z., Hart, R. P., MacCallum, R. C., et al. (2002). Pathological CNS autoimmune disease triggered by traumatic spinal cord injury: implications for autoimmune vaccine therapy. *J. Neurosci.* 22, 2690–2700.

Kalincik, T., Buzzard, K., Jokubaitis, V., Trojano, M., Duquette, P., Izquierdo, G., et al. (2014). Risk of relapse phenotype recurrence in multiple sclerosis. *Mult. Scler.* 20, 1511–1522. doi: 10.1177/1352458514528762

Kamel, H., and Iadecola, C. (2012). Brain-immune interactions and ischemic stroke: clinical implications. *Arch. Neurol.* 69, 576–581. doi: 10.1001/archneurol.2011.3590

Kametani, F., and Hasegawa, M. (2018). Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. *Front. Neurosci.* 12:25. doi: 10.3389/fnins.2018.00025

Kappos, L., Radue, E. W., O'Connor, P., Polman, C., Hohlfeld, R., Calabresi, P., et al. (2010). A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N. Engl. J. Med.* 362, 387–401. doi: 10.1056/NEJMoa0909494

Kempster, P. A., O'Sullivan, S. S., Holton, J. L., Revesz, T., and Lees, A. J. (2010). Relationships between age and late progression of Parkinson's disease: a clinico-pathological study. *Brain* 133, 1755–1762. doi: 10.1093/brain/awq059

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., et al. (2017). A unique microglia type associated with restricting development of Alzheimer's disease. *Cell* 169, 1276.e17–1290.e17. doi: 10.1016/j.cell.2017.05.018

Kil, K., Zang, Y. C., Yang, D., Markowski, J., Fuoco, G. S., Vendetti, G. C., et al. (1999). T cell responses to myelin basic protein in patients with spinal cord injury and multiple sclerosis. *J. Neuroimmunol.* 98, 201–207. doi: 10.1016/s0165-5728(99)00057-0

Kipnis, J., Cohen, H., Cardon, M., Ziv, Y., and Schwartz, M. (2004). T cell deficiency leads to cognitive dysfunction: implications for therapeutic vaccination for schizophrenia and other psychiatric conditions. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8180–8185. doi: 10.1073/pnas.0402268101

Kipnis, J., Nevo, U., Panikashvili, D., Alexandrovich, A., Yoles, E., Akselrod, S., et al. (2003). Therapeutic vaccination for closed head injury. *J. Neurotrauma* 20, 559–569. doi: 10.1089/089771503767168483

Kivisäkk, P., Mahad, D. J., Callahan, M. K., Trebst, C., Tucky, B., Wei, T., et al. (2003). Human cerebrospinal fluid central memory CD4⁢sup>+⁢/sup> T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8389–8394. doi: 10.1073/pnas.1433000100

Kleinschnitz, C., Schwab, N., Kraft, P., Hagedorn, I., Dreykluft, A., Schwarz, T., et al. (2010). Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* 115, 3835–3842. doi: 10.1182/blood-2009-10-249078

Knight, A., Nemec, P., Bretzova, S., Valkova, L., Kolmanova, M., Vytopilova, R., et al. (2016). Do human B-lymphocytes avoid aging until 60 years? *Oncotarget* 7, 42873–42880. doi: 10.18632/oncotarget.10146

Kobo, H., Bar-Shira, A., Dahary, D., Gan-Or, Z., Mirelman, A., Goldstein, O., et al. (2016). Down-regulation of B cell-related genes in peripheral blood leukocytes of Parkinson's disease patients with and without GBA mutations. *Mol. Genet. Metab.* 117, 179–185. doi: 10.1016/j.ymgme.2015.09.005

Kosloski, L. M., Kosmacek, E. A., Olson, K. E., Mosley, R. L., and Gendelman, H. E. (2013). GM-CSF induces neuroprotective and anti-inflammatory responses in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine intoxicated mice. *J. Neuroimmunol.* 265, 1–10. doi: 10.1016/j.jneuroim.2013.10.009

Kotter, M. R., Li, W.-W., Zhao, C., and Franklin, R. J. M. (2006). Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J. Neurosci.* 26, 328–332. doi: 10.1523/JNEUROSCI.2615-05.2006

Lam, L., Chin, L., Halder, R. C., Sagong, B., Famenini, S., Sayre, J., et al. (2016). Epigenetic changes in T-cell and monocyte signatures and production of neurotoxic cytokines in ALS patients. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 30, 3461–3473. doi: 10.1096/fj.201600259RR

Lassmann, H. (2018). Multiple sclerosis pathology. *Cold Spring Harb. Perspect. Med.* 8, 1–15. doi: 10.1101/cshperspect.a028936

Laurent, C., Dorothee, G., Hunot, S., Martin, E., Monnet, Y., Duchamp, M., et al. (2017). Hippocampal T cell infiltration promotes neuroinflammation and cognitive decline in a mouse model of tauopathy. *Brain* 140, 184–200. doi: 10.1093/brain/aww270

Lee, P., Kim, J., Williams, R., Sandhir, R., Gregory, E., Brooks, W. M., et al. (2012). Effects of aging on blood brain barrier and matrix metalloproteases following controlled cortical impact in mice. *Exp. Neurol.* 234, 50–61. doi: 10.1016/j.expneurol.2011.12.016

Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., et al. (2009). Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat. Med.* 15, 192–199. doi: 10.1038/nm.1927

Liesz, A., Zhou, W., Mracskó, E., Karcher, S., Bauer, H., Schwarting, S., et al. (2011). Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain* 134, 704–720. doi: 10.1093/brain/awr008

Lindestam Arlehamn, C. S., Dhanwani, R., Pham, J., Kuan, R., Frazier, A., Rezende Dutra, J., et al. (2020). α-Synuclein-specific T cell reactivity is associated with preclinical and early Parkinson's disease. *Nat. Commun.* 11:1875. doi: 10.1038/s41467-020-15626-w

Linehan, E., Dombrowski, Y., Snoddy, R., Fallon, P. G., Kissenpfennig, A., and Fitzgerald, D. C. (2014). Aging impairs peritoneal but not bone marrow-derived macrophage phagocytosis. *Aging Cell* 13, 699–708. doi: 10.1111/acel.12223

Linton, P. J., and Dorshkind, K. (2004). Age-related changes in lymphocyte development and function. *Nat. Immunol.* 5, 133–139. doi: 10.1038/ni1033

Liu, Q., Xin, W., He, P., Turner, D., Yin, J., Gan, Y., et al. (2014). Interleukin-17 inhibits adult hippocampal neurogenesis. *Sci. Rep.* 4:7554. doi: 10.1038/srep07554

Lucchinetti, C., Bru, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* 47, 707–717.

Ludowyk, P. A., Hughes, W., Hugh, A., Willenborg, D. O., Rockett, K. A., and Parish, C. R. (1993). Astrocytic hypertrophy: an important pathological feature of chronic experimental autoimmune encephalitis in aged rats. *J. Neuroimmunol.* 48, 121–134. doi: 10.1016/0165-5728(93)90184-z

Luo, D., Ge, W., Hu, X., Li, C., Lee, C.-M., Zhou, L., et al. (2019). Unbiased transcriptomic analyses reveal distinct effects of immune deficiency in CNS function with and without injury. *Protein Cell* 10, 566–582. doi: 10.1007/s13238-018-0559-y

Manwani, B., Liu, F., Scranton, V., Hammond, M. D., Sansing, L. H., and McCullough, L. D. (2013). Differential effects of aging and sex on stroke induced inflammation across the lifespan. *Exp. Neurol.* 249, 120–131. doi: 10.1016/j.expneurol.2013.08.011

Manwani, B., Liu, F., Xu, Y., Persky, R., Li, J., and McCullough, L. D. (2011). Functional recovery in aging mice after experimental stroke. *Brain Behav. Immun.* 25, 1689–1700. doi: 10.1016/j.bbi.2011.06.015

Marchetti, B., Tirolo, C., L'Episcopo, F., Caniglia, S., Testa, N., Smith, J. A., et al. (2020). Parkinson's disease, aging and adult neurogenesis: Wnt/beta-catenin signalling as the key to unlock the mystery of endogenous brain repair. *Aging Cell* 19:e13101. doi: 10.1111/acel.13101

Marsh, S. E., Abud, E. M., Lakatos, A., Karimzadeh, A., Yeung, S. T., Davtyan, H., et al. (2016). The adaptive immune system restrains Alzheimer's disease pathogenesis by modulating microglial function. *Proc. Natl. Acad. Sci. U.S.A.* 113, E1316–E1325. doi: 10.1073/pnas.1525466113

Matejuk, A., Hopke, C., Vandenbark, A. A., Hurn, P. D., and Offner, H. (2005). Middle-age male mice have increased severity of experimental autoimmune encephalomyelitis and are unresponsive to testosterone therapy. *J. Immunol.* 174, 2387–2395. doi: 10.4049/jimmunol.174.4.2387

McKee, A. C., Cantu, R. C., Nowinski, C. J., Hedley-Whyte, E. T., Gavett, B. E., Budson, A. E., et al. (2009). Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. *J. Neuropathol. Exp. Neurol.* 68, 709–735. doi: 10.1097/NEN.0b013e3181a9d503

McQuillan, K., Lynch, M. A., and Mills, K. H. (2010). Activation of mixed glial by Aβ-specific Th1 and Th17 cells and its regulation by Th2 cells. *Brain Behav. Immun.* 24, 598–607. doi: 10.1016/j.bbi.2010.01.003

Mehanna, R., Moore, S., Hou, J. G., Sarwar, A. I., and Lai, E. C. (2014). Comparing clinical features of young onset, middle onset and late onset Parkinson's disease. *Parkinsonism Relat. Disord.* 20, 530–534. doi: 10.1016/j.parkreldis.2014.02.013

Meisel, C., Schwab, J. M., Prass, K., Meisel, A., and Dirnagl, U. (2005). Central nervous system injury-induced immune deficiency syndrome. *Nat. Rev. Neurosci.* 6, 775–786. doi: 10.1038/nrn1765

Mencl, S., Hennig, N., Hopp, S., Schuhmann, M. K., Albert-Weissenberger, C., Siren, A.-L., et al. (2014). FTY720 does not protect from traumatic brain injury in mice despite reducing posttraumatic inflammation. *J. Neuroimmunol.* 274, 125–131. doi: 10.1016/j.jneuroim.2014.07.010

Meredith, G. E., and Rademacher, D. J. (2011). MPTP mouse models of Parkinson's disease: an update. *J. Parkinsons. Dis.* 1, 19–33. doi: 10.3233/JPD-2011-11023

Merlini, M., Kirabali, T., Kulic, L., Nitsch, R. M., and Ferretti, M. T. (2018). Extravascular CD3+ T cells in brains of alzheimer disease patients correlate with tau but not with amyloid pathology: an immunohistochemical study. *Neurodegener. Dis.* 18, 49–56. doi: 10.1159/000486200

Mondal, S., Rangasamy, S. B., Roy, A., Dasarathy, S., Kordower, J. H., and Pahan, K. (2019). Low-dose maraviroc, an antiretroviral drug, attenuates the infiltration of t cells into the central nervous system and protects the nigrostriatum in hemiparkinsonian monkeys. *J. Immunol.* doi: 10.4049/jimmunol.1800587 [Epub ahead of print].

Morgan, S., and Orrell, R. W. (2016). Pathogenesis of amyotrophic lateral sclerosis. *Br. Med. Bull.* 119, 87–98. doi: 10.1093/bmb/ldw026

Moskowitz, D. M., Zhang, D. W., Hu, B., Le Saux, S., Yanes, R. E., Ye, Z., et al. (2017). Epigenomics of human CD8 T cell differentiation and aging. *Sci. Immunol.* 2:eaag0192. doi: 10.1126/sciimmunol.aag0192

Mosley, R. L., Hutter-Saunders, J. A., Stone, D. K., and Gendelman, H. E. (2012). Inflammation and adaptive immunity in Parkinson's disease. *Cold Spring Harb. Perspect. Med.* 2:a009381. doi: 10.1101/cshperspect.a009381

Mracsko, E., Liesz, A., Stojanovic, A., Lou, W. P.-K., Osswald, M., Zhou, W., et al. (2014). Antigen dependently activated cluster of differentiation 8-positive T cells cause perforin-mediated neurotoxicity in experimental stroke. *J. Neurosci.* 34, 16784–16795. doi: 10.1523/JNEUROSCI.1867-14.2014

Naor, S., Keren, Z., Bronshtein, T., Goren, E., Machluf, M., and Melamed, D. (2009). Development of ALS-like disease in SOD-1 mice deficient of B lymphocytes. *J. Neurol.* 256, 1228–1235. doi: 10.1007/s00415-009-5097-3

Narang, A., Qiao, F., Atkinson, C., Zhu, H., Yang, X., Kulik, L., et al. (2017). Natural IgM antibodies that bind neoepitopes exposed as a result of spinal cord injury, drive secondary injury by activating complement. *J. Neuroinflammation* 14, 120. doi: 10.1186/s12974-017-0894-6

Nardo, G., Trolese, M. C., Verderio, M., Mariani, A., de Paola, M., Riva, N., et al. (2018). Counteracting roles of MHCI and CD8(+) T cells in the peripheral and central nervous system of ALS SOD1(G93A) mice. *Mol. Neurodegener.* 13:42. doi: 10.1186/s13024-018-0271-7

Natrajan, M. S., Komori, M., Kosa, P., Johnson, K. R., Wu, T., Franklin, R. J. M., et al. (2015). Pioglitazone regulates myelin phagocytosis and multiple sclerosis monocytes. *Ann. Clin. Transl. Neurol.* 2, 1071–1084. doi: 10.1002/acn3.260

Neumann, B., Baror, R., Zhao, C., Segel, M., Dietmann, S., Rawji, K. S., et al. (2019). Metformin restores CNS remyelination capacity by rejuvenating aged stem cells. *Cell Stem Cell* 25, 473.e8–485.e8. doi: 10.1016/j.stem.2019.08.015

Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., et al. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 314, 130–133. doi: 10.1126/science.1134108

Nikolich-Zugich, J. (2018). The twilight of immunity: emerging concepts in aging of the immune system. *Nat. Immunol.* 19, 10–19. doi: 10.1038/s41590-017-0006-x

Niwa, F., Kuriyama, N., Nakagawa, M., and Imanishi, J. (2012). Effects of peripheral lymphocyte subpopulations and the clinical correlation with Parkinson's disease. *Geriatr. Gerontol. Int.* 12, 102–107. doi: 10.1111/j.1447-0594.2011.00740.x

Nordengen, K., Kirsebom, B.-E., Henjum, K., Selnes, P., Gisladottir, B., Wettergreen, M., et al. (2019). Glial activation and inflammation along the Alzheimer's disease continuum. *J. Neuroinflammation* 16:46. doi: 10.1186/s12974-019-1399-2

Oberstein, T. J., Taha, L., Spitzer, P., Hellstern, J., Herrmann, M., Kornhuber, J., et al. (2018). Imbalance of circulating Th17 and regulatory T cells in Alzheimer's disease: a case control study. *Front. Immunol.* 9:1213. doi: 10.3389/fimmu.2018.01213

Orr, C. F., Rowe, D. B., Mizuno, Y., Mori, H., and Halliday, G. M. (2005). A possible role for humoral immunity in the pathogenesis of Parkinson's disease. *Brain* 128, 2665–2674. doi: 10.1093/brain/awh625

Ortiz-ortiz, L., and Weigle, W. O. (1976). Cellular events in the induction of experimental allergic encephalomyelitis in rats. *J. Exp. Med.* 144, 604–616. doi: 10.1084/jem.144.3.604

Pagano, G., Ferrara, N., Brooks, D. J., and Pavese, N. (2016). Age at onset and Parkinson disease phenotype. *Neurology* 86, 1400–1407. doi: 10.1212/WNL.0000000000002461

Paulson, J. B., Ramsden, M., Forster, C., Sherman, M. A., McGowan, E., and Ashe, K. H. (2008). Amyloid plaque and neurofibrillary tangle pathology in a regulatable mouse model of Alzheimer's disease. *Am. J. Pathol.* 173, 762–772. doi: 10.2353/ajpath.2008.080175

Peferoen, L. A. N., Breur, M., van de Berg, S., Peferoen-Baert, R., Boddeke, E. H. W. G. M., van der Valk, P., et al. (2016). Ageing and recurrent episodes of neuroinflammation promote progressive experimental autoimmune encephalomyelitis in Biozzi ABH mice. *Immunology* 149, 146–156. doi: 10.1111/imm.12644

Pettinelli, C., and McFarlin, D. (1981). Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J. Immun.* 127, 1420–1423.

Phukan, J., Elamin, M., Bede, P., Jordan, N., Gallagher, L., Byrne, S., et al. (2012). The syndrome of cognitive impairment in amyotrophic lateral sclerosis: a population-based study. *J. Neurol. Neurosurg. Psychiatry* 83, 102–108. doi: 10.1136/jnnp-2011-300188

Pinti, M., Appay, V., Campisi, J., Frasca, D., Fulop, T., Sauce, D., et al. (2016). Aging of the immune system: focus on inflammation and vaccination. *Eur. J. Immunol.* 46, 2286–2301. doi: 10.1002/eji.201546178

Polman, C. H., O'Connor, P. W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D. H., et al. (2006). A randomised, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl J. Med.* 354, 899–910. doi: 10.1056/NEJMoa044397

Pool, M., Rambaldi, I., Darlington, P. J., Wright, M. C., Fournier, A. E., and Bar-Or, A. (2012). Neurite outgrowth is differentially impacted by distinct immune cell subsets. *Mol. Cell. Neurosci.* 49, 68–76. doi: 10.1016/j.mcn.2011.09.004

Popovich, P. G., Stokes, B. T., and Whitacre, C. C. (1996). Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system. *J. Neurosci. Res.* 45, 349–363.

Popovich, P. G., Wei, P., and Stokes, B. T. (1997). Cellular inflammatory response after spinal cord injury in sprague-dawley and lewis rats. *J. Comp. Neurol.* 377, 443–464.

Potas, J. R., Zheng, Y., Moussa, C., Venn, M., Gorrie, C. A., Deng, C., et al. (2006). Augmented locomotor recovery after spinal cord injury in the athymic nude rat. *J. Neurotrauma* 23, 660–673. doi: 10.1089/neu.2006.23.660

Pringsheim, T., Jette, N., Frolkis, A., and Steeves, T. D. L. (2014). The prevalence of Parkinson's disease: a systematic review and meta-analysis. *Mov. Disord.* 29, 1583–1590. doi: 10.1002/mds.25945

Pruss, H., Iggena, D., Baldinger, T., Prinz, V., Meisel, A., Endres, M., et al. (2012). Evidence of intrathecal immunoglobulin synthesis in stroke: a cohort study. *Arch. Neurol.* 69, 714–717. doi: 10.1001/archneurol.2011.3252

Pruss, H., Kopp, M. A., Brommer, B., Gatzemeier, N., Laginha, I., Dirnagl, U., et al. (2011). Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau. *Brain Pathol.* 21, 652–660. doi: 10.1111/j.1750-3639.2011.00488.x

Pulko, V., Davies, J. S., Martinez, C., Lanteri, M. C., Busch, M. P., Diamond, M. S., et al. (2016). Human memory T cells with a naive phenotype accumulate with aging and respond to persistent viruses. *Nat. Immunol.* 17, 966–975. doi: 10.1038/ni.3483

Qin, H., Buckley, J. A., Li, X., Liu, Y., Fox, T. H. III, Meares, G. P., et al. (2016). Inhibition of the JAK/STAT pathway protects against alpha-synuclein-induced neuroinflammation and dopaminergic neurodegeneration. *J. Neurosci.* 36, 5144–5159. doi: 10.1523/JNEUROSCI.4658-15.2016

Radjavi, A., Smirnov, I., and Kipnis, J. (2014). Brain antigen-reactive CD4+ T cells are sufficient to support learning behavior in mice with limited T cell repertoire. *Brain Behav. Immun.* 35, 58–63. doi: 10.1016/j.bbi.2013.08.013

Raine, C. S., Cannella, B., Hauser, S. L., and Genain, C. P. (1999). Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann. Neurol.* 46, 144–160.

Ramagopalan, S. V., Dyment, D. A., and Ebers, G. C. (2008). Genetic epidemiology: the use of old and new tools for multiple sclerosis. *Trends Neurosci.* 31, 645–652. doi: 10.1016/j.tins.2008.09.001

Rashid Chehreh Bargh, S., Tafakhori, A., Masoumi, F., Rahmani, F., Ahmadi, M., Namdar, A., et al. (2018). Evaluation of regulatory T lymphocytes and IL2Ra and FOXP3 gene expression in peripheral mononuclear cells from patients with amyotrophic lateral sclerosis. *Ir. J. Med. Sci.* 187, 1065–1071. doi: 10.1007/s11845-018-1793-2

Ratliff, M., Alter, S., Frasca, D., Blomberg, B. B., and Riley, R. L. (2013). In senescence, age-associated B cells secrete TNFalpha and inhibit survival of B-cell precursors. *Aging Cell* 12, 303–311. doi: 10.1111/acel.12055

Reynolds, A. D., Banerjee, R., Liu, J., Gendelman, H. E., and Mosley, R. L. (2007). Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease. *J. Leukoc. Biol.* 82, 1083–1094. doi: 10.1189/jlb.0507296

Reynolds, A. D., Stone, D. K., Hutter, J. A. L., Benner, E. J., Mosley, R. L., and Gendelman, H. E. (2010). Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease. *J. Immunol.* 184, 2261–2271. doi: 10.4049/jimmunol.0901852

Reynolds, A. D., Stone, D. K., Mosley, R. L., and Gendelman, H. E. (2009). Nitrated {alpha}-synuclein-induced alterations in microglial immunity are regulated by CD4+ T cell subsets. *J. Immunol.* 182, 4137–4149. doi: 10.4049/jimmunol.0803982

Riley, R. L., Khomtchouk, K., and Blomberg, B. B. (2017). Age-associated B cells (ABC) inhibit B lymphopoiesis and alter antibody repertoires in old age. *Cell Immunol.* 321, 61–67. doi: 10.1016/j.cellimm.2017.04.008

Ritzel, R. M., Crapser, J., Patel, A. R., Verma, R., Grenier, J. M., Chauhan, A., et al. (2016). Age-associated resident memory CD8 T cells in the central nervous system are primed to potentiate inflammation after ischemic brain injury. *J. Immunol.* 196, 3318–3330. doi: 10.4049/jimmunol.1502021

Ritzel, R. M., Lai, Y.-J., Crapser, J. D., Patel, A. R., Schrecengost, A., Grenier, J. M., et al. (2018). Aging alters the immunological response to ischemic stroke. *Acta Neuropathol.* 136, 89–110. doi: 10.1007/s00401-018-1859-2

Rodriguez, M., Scheithauer, B. W., Forbes, G., and Kelly, P. J. (1993). Oligodendrocyte injury is an early event in lesions of multiple sclerosis. *Mayo Clin. Proc.* 68, 627–636. doi: 10.1016/s0025-6196(12)60597-7

Rogers, J., Luber-Narod, J., Styren, S. D., and Civin, W. H. (1988). Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol. Aging* 9, 339–349. doi: 10.1016/s0197-4580(88)80079-4

Rosenkranz, D., Weyer, S., Tolosa, E., Gaenslen, A., Berg, D., Leyhe, T., et al. (2007). Higher frequency of regulatory T cells in the elderly and increased suppressive activity in neurodegeneration. *J. Neuroimmunol.* 188, 117–127. doi: 10.1016/j.jneuroim.2007.05.011

Rothweiler, B., Temkin, N. R., and Dikmen, S. S. (1998). Aging effect on psychosocial outcome in traumatic brain injury. *Arch. Phys. Med. Rehabil.* 79, 881–887. doi: 10.1016/s0003-9993(98)90082-x

Rudd, B. D., Venturi, V., Li, G., Samadder, P., Ertelt, J. M., Way, S. S., et al. (2011). Nonrandom attrition of the naive CD8+ T-cell pool with aging governed by T-cell receptor:pMHC interactions. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13694–13699. doi: 10.1073/pnas.1107594108

Rudehill, S., Muhallab, S., Wennersten, A., von Gertten, C., Al Nimer, F., Sandberg-Nordqvist, A. C., et al. (2006). Autoreactive antibodies against neurons and basal lamina found in serum following experimental brain contusion in rats. *Acta Neurochir.* 148, 199–205. doi: 10.1007/s00701-005-0673-5

Russo, M. V., and McGavern, D. B. (2016). Inflammatory neuroprotection following traumatic brain injury. *Science* 353, 783–785. doi: 10.1126/science.aaf6260

Sabatino, J. J., Pröbstel, A.-K., and Zamvil, S. S. (2019). B cells in autoimmune and neurodegenerative central nervous system diseases. *Nat. Rev. Neurosci.* 20, 728–745. doi: 10.1038/s41583-019-0233-2

Saposnik, G., Cote, R., Phillips, S., Gubitz, G., Bayer, N., Minuk, J., et al. (2008). Stroke outcome in those over 80: a multicenter cohort study across Canada. *Stroke* 39, 2310–2317. doi: 10.1161/STROKEAHA.107.511402

Saresella, M., Piancone, F., Tortorella, P., Marventano, I., Gatti, A., Caputo, D., et al. (2013). T helper-17 activation dominates the immunologic milieu of both amyotrophic lateral sclerosis and progressive multiple sclerosis. *Clin. Immunol.* 148, 79–88. doi: 10.1016/j.clim.2013.04.010

Sargento-Freitas, J., Pereira, A., Gomes, A., Amorim, P., Matos, T., Cardoso, C. M. P., et al. (2018). STROKE34 study protocol: a randomized controlled phase iia trial of intra-arterial CD34+ cells in acute ischemic stroke. *Front. Neurol.* 9:302. doi: 10.3389/fneur.2018.00302

Saunders, J. A. H., Estes, K. A., Kosloski, L. M., Allen, H. E., Dempsey, K. M., Torres-Russotto, D. R., et al. (2012). CD4+ regulatory and effector/memory T cell subsets profile motor dysfunction in Parkinson's disease. *J. Neuroimmune Pharmacol.* 7, 927–938. doi: 10.1007/s11481-012-9402-z

Sawcer, S., Ban, M., Maranian, M., Yeo, T. W., Compston, A., Kirby, A., et al. (2005). A high-density screen for linkage in multiple sclerosis. *Am. J. Hum. Genet.* 77, 454–467. doi: 10.1086/444547

Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C. A., Patsopoulos, N. A., Moutsianas, L., et al. (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 476, 214–219. doi: 10.1038/nature10251

Schnell, L., Fearn, S., Klassen, H., Schwab, M. E., and Perry, V. H. (1999). Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. *Eur. J. Neurosci.* 11, 3648–3658. doi: 10.1046/j.1460-9568.1999.00792.x

Schnell, L., Schneider, R., Berman, M. A., Perry, V. H., and Schwab, M. E. (1997). Lymphocyte recruitment following spinal cord injury in mice is altered by prior viral exposure. *Eur. J. Neurosci.* 9, 1000–1007. doi: 10.1111/j.1460-9568.1997.tb01450.x

Schori, H., Kipnis, J., Yoles, E., WoldeMussie, E., Ruiz, G., Wheeler, L. A., et al. (2001). Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3398–3403. doi: 10.1073/pnas.041609498

Schroeter, M., Jander, S., Witte, O. W., and Stoll, G. (1994). Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. *J. Neuroimmunol.* 55, 195–203. doi: 10.1016/0165-5728(94)90010-8

Schutt, C. R., Gendelman, H. E., and Mosley, R. L. (2018). Tolerogenic bone marrow-derived dendritic cells induce neuroprotective regulatory T cells in a model of Parkinson's disease. *Mol. Neurodegener.* 13:26. doi: 10.1186/s13024-018-0255-7

Schwab, J. M., Zhang, Y., Kopp, M. A., Brommer, B., and Popovich, P. G. (2014). The paradox of chronic neuroinflammation, systemic immune suppression, autoimmunity after traumatic chronic spinal cord injury. *Exp. Neurol.* 258, 121–129. doi: 10.1016/j.expneurol.2014.04.023

Seo, J.-E., Hasan, M., Han, J.-S., Kang, M.-J., Jung, B.-H., Kwok, S.-K., et al. (2015). Experimental autoimmune encephalomyelitis and age-related correlations of NADPH oxidase, MMP-9, and cell adhesion molecules: the increased disease severity and blood-brain barrier permeability in middle-aged mice. *J. Neuroimmunol.* 287, 43–53. doi: 10.1016/j.jneuroim.2015.08.005

Shah, G. N., and Mooradian, A. D. (1997). Age-related changes in the blood-brain barrier. *Exp. Gerontol.* 32, 501–519. doi: 10.1016/s0531-5565(96)00158-1

Shahaduzzaman, M., Golden, J. E., Green, S., Gronda, A. E., Adrien, E., Ahmed, A., et al. (2013). A single administration of human umbilical cord blood T cells produces long-lasting effects in the aging hippocampus. *Age* 35, 2071–2087. doi: 10.1007/s11357-012-9496-5

Shalash, A., Salama, M., Makar, M., Roushdy, T., Elrassas, H. H., Mohamed, W., et al. (2017). Elevated serum alpha-synuclein autoantibodies in patients with Parkinson's disease relative to alzheimer's disease and controls. *Front. Neurol.* 8:720. doi: 10.3389/fneur.2017.00720

Sheean, R. K., McKay, F. C., Cretney, E., Bye, C. R., Perera, N. D., Tomas, D., et al. (2018). Association of regulatory T-cell expansion with progression of amyotrophic lateral sclerosis: a study of humans and a transgenic mouse model. *JAMA Neurol.* 75, 681–689. doi: 10.1001/jamaneurol.2018.0035

Shi, N., Kawano, Y., Tateishi, T., Kikuchi, H., Osoegawa, M., Ohyagi, Y., et al. (2007). Increased IL-13-producing T cells in ALS: positive correlations with disease severity and progression rate. *J. Neuroimmunol.* 182, 232–235. doi: 10.1016/j.jneuroim.2006.10.001

Shichita, T., Sugiyama, Y., Ooboshi, H., Sugimori, H., Nakagawa, R., Takada, I., et al. (2009). Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nat. Med.* 15, 946–950. doi: 10.1038/nm.1999

Shin, B. S., Furuhashi, T., Nakamura, M., Torii, K., and Morita, A. (2013). Impaired inhibitory function of circulating CD4+CD25+ regulatory T cells in alopecia areata. *J. Dermatol. Sci.* 70, 141–143. doi: 10.1016/j.jdermsci.2013.01.006

Sicotte, M., Tsatas, O., Jeong, S. Y., Cai, C.-Q., He, Z., and David, S. (2003). Immunization with myelin or recombinant Nogo-66/MAG in alum promotes axon regeneration and sprouting after corticospinal tract lesions in the spinal cord. *Mol. Cell. Neurosci.* 23, 251–263. doi: 10.1016/s1044-7431(03)00053-8

Sim, F. J., Zhao, C., Penderis, J., and Franklin, R. J. M. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *J. Neurosci.* 22, 2451–2459.

Smith, K. J., Blakemore, W. F., and McDonald, W. I. (1979). Central remyelination restores secure conduction. *Nature* 280, 395–396. doi: 10.1038/280395a0

Smith, L. K., He, Y., Park, J.-S., Bieri, G., Snethlage, C. E., Lin, K., et al. (2015). beta2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nat. Med.* 21, 932–937. doi: 10.1038/nm.3898

Smithey, M. J., Renkema, K. R., Rudd, B. D., and Nikolich-Zugich, J. (2011). Increased apoptosis, curtailed expansion and incomplete differentiation of CD8+ T cells combine to decrease clearance of *L. monocytogenes* in old mice. *Eur. J. Immunol.* 41, 1352–1364. doi: 10.1002/eji.201041141

Smolders, J., Heutinck, K. M., Fransen, N. L., Remmerswaal, E. B. M., Hombrink, P., Ten Berge, I. J. M., et al. (2018). Tissue-resident memory T cells populate the human brain. *Nat. Commun.* 9:4593. doi: 10.1038/s41467-018-07053-9

Smolders, J., Remmerswaal, E. B. M., Schuurman, K. G., Melief, J., van Eden, C. G., van Lier, R. A. W., et al. (2013). Characteristics of differentiated CD8+ and CD4+ T cells present in the human brain. *Acta Neuropathol.* 126, 525–535. doi: 10.1007/s00401-013-1155-0

Sommer, A., Marxreiter, F., Krach, F., Fadler, T., Grosch, J., Maroni, M., et al. (2019). Th17 lymphocytes induce neuronal cell death in a human iPSC-based model of Parkinson's disease. *Cell Stem Cell* 24, 1006. doi: 10.1016/j.stem.2019.04.019

Sriram, S., Solomon, D., Rouse, R. V., and Steinman, L. (1982). Identification of T cell subsets and B lymphocytes in mouse brain experimental allergic encephalitis lesions. *J. Immunol.* 129, 1649–1651.

Sroga, J. M., Jones, T. B., Kigerl, K. A., McGaughy, V. M., and Popovich, P. G. (2003). Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. *J. Comp. Neurol.* 462, 223–240. doi: 10.1002/cne.10736

Stephenson, J., Nutma, E., van der Valk, P., and Amor, S. (2018). Inflammation in CNS neurodegenerative diseases. *Immunology* 154, 204–219. doi: 10.1111/imm.12922

Stevens, C. H., Rowe, D., Morel-Kopp, M.-C., Orr, C., Russell, T., Ranola, M., et al. (2012). Reduced T helper and B lymphocytes in Parkinson's disease. *J. Neuroimmunol.* 252, 95–99. doi: 10.1016/j.jneuroim.2012.07.015

Stichel, C. C., and Luebbert, H. (2007). Inflammatory processes in the aging mouse brain: participation of dendritic cells and T-cells. *Neurobiol. Aging* 28, 1507–1521. doi: 10.1016/j.neurobiolaging.2006.07.022

Stojić-Vukanić, Z., Nacka-Aleksić, M., Pilipović, I., Vujnović, I., Blagojević, V., Kosec, D., et al. (2015). Aging diminishes the resistance of AO rats to EAE: putative role of enhanced generation of GM-CSF Expressing CD4+ T cells in aged rats. *Immun. Ageing* 12:16. doi: 10.1186/s12979-015-0044-x

Sulzer, D., Alcalay, R. N., Garretti, F., Cote, L., Kanter, E., Agin-Liebes, J., et al. (2017). T cells from patients with Parkinson's disease recognize alpha-synuclein peptides. *Nature* 546, 656–661. doi: 10.1038/nature22815

Szewczyk-Krolikowski, K., Tomlinson, P., Nithi, K., Wade-Martins, R., Talbot, K., Ben-Shlomo, Y., et al. (2014). The influence of age and gender on motor and non-motor features of early Parkinson's disease: initial findings from the Oxford Parkinson Disease Center (OPDC) discovery cohort. *Parkinsonism Relat. Disord.* 20, 99–105. doi: 10.1016/j.parkreldis.2013.09.025

Tabibian-Keissar, H., Hazanov, L., Schiby, G., Rosenthal, N., Rakovsky, A., Michaeli, M., et al. (2016). Aging affects B-cell antigen receptor repertoire diversity in primary and secondary lymphoid tissues. *Eur. J. Immunol.* 46, 480–492. doi: 10.1002/eji.201545586

Taguchi, A., Soma, T., Tanaka, H., Kanda, T., Nishimura, H., Yoshikawa, H., et al. (2004). Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J. Clin. Invest.* 114, 330–338. doi: 10.1172/JCI20622

Tanzi, R. E. (2012). The genetics of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2:a006296. doi: 10.1101/cshperspect.a006296

Tobin, R. P., Mukherjee, S., Kain, J. M., Rogers, S. K., Henderson, S. K., Motal, H. L., et al. (2014). Traumatic brain injury causes selective, CD74-dependent peripheral lymphocyte activation that exacerbates neurodegeneration. *Acta Neuropathol. Commun.* 2:143. doi: 10.1186/s40478-014-0143-5

Togo, T., Akiyama, H., Iseki, E., Kondo, H., Ikeda, K., Kato, M., et al. (2002). Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. *J. Neuroimmunol.* 124, 83–92. doi: 10.1016/s0165-5728(01)00496-9

Traugott, U., and Lebon, P. (1988). Multiple sclerosis: involvement of interferons in lesion pathogenesis. *Ann. Neurol.* 24, 243–251. doi: 10.1002/ana.410240211

Traugott, U., Reinherz, E., and Raine, C. (1983). Multiple sclerosis: distribution of T cell subsets within active chronic lesions. *Science* 219, 308–310. doi: 10.1126/science.6217550

Tweedie, D., Ferguson, R. A., Fishman, K., Frankola, K. A., Van Praag, H., Holloway, H. W., et al. (2012). Tumor necrosis factor-alpha synthesis inhibitor 3,6'-dithiothalidomide attenuates markers of inflammation, Alzheimer pathology and behavioral deficits in animal models of neuroinflammation and Alzheimer's disease. *J. Neuroinflammation* 9:106. doi: 10.1186/1742-2094-9-106

Ucar, D., Marquez, E. J., Chung, C.-H., Marches, R., Rossi, R. J., Uyar, A., et al. (2017). The chromatin accessibility signature of human immune aging stems from CD8(+) T cells. *J. Exp. Med.* 214, 3123–3144. doi: 10.1084/jem.20170416

Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., et al. (2011). The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* 477, 90–94. doi: 10.1038/nature10357

Walsh, J. T., Hendrix, S., Boato, F., Smirnov, I., Zheng, J., Lukens, J. R., et al. (2015). MHCII-independent CD4+ T cells protect injured CNS neurons via IL-4. *J. Clin. Invest.* 125:2547. doi: 10.1172/JCI82458

Wang, W. Z., Olsson, T., Kostulas, V., Hojeberg, B., Ekre, H. P., and Link, H. (1992). Myelin antigen reactive T cells in cerebrovascular diseases. *Clin. Exp. Immunol.* 88, 157–162. doi: 10.1111/j.1365-2249.1992.tb03056.x

Weckbach, S., Neher, M., Losacco, J. T., Bolden, A. L., Kulik, L., Flierl, M. A., et al. (2012). Challenging the role of adaptive immunity in neurotrauma: Rag1(−/−) mice lacking mature B and T cells do not show neuroprotection after closed head injury. *J. Neurotrauma* 29, 1233–1242. doi: 10.1089/neu.2011.2169

Werner, C., and Engelhard, K. (2007). Pathophysiology of traumatic brain injury. *BJA Br. J. Anaesth.* 99, 4–9. doi: 10.1093/bja/aem131

Westerlind, H., Ramanujam, R., Uvehag, D., Kuja-Halkola, R., Boman, M., Bottai, M., et al. (2014). Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden. *Brain* 137, 770–778. doi: 10.1093/brain/awt356

Wolf, S. A., Steiner, B., Akpinarli, A., Kammertoens, T., Nassenstein, C., Braun, A., et al. (2009a). CD4-positive T lymphocytes provide a neuroimmunological link in the control of adult hippocampal neurogenesis. *J. Immunol.* 182, 3979–3984. doi: 10.4049/jimmunol.0801218

Wolf, S. A., Steiner, B., Wengner, A., Lipp, M., Kammertoens, T., and Kempermann, G. (2009b). Adaptive peripheral immune response increases proliferation of neural precursor cells in the adult hippocampus. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 23, 3121–3128. doi: 10.1096/fj.08-113944

Wu, B., Matic, D., Djogo, N., Szpotowicz, E., Schachner, M., and Jakovcevski, I. (2012). Improved regeneration after spinal cord injury in mice lacking functional T- and B-lymphocytes. *Exp. Neurol.* 237, 274–285. doi: 10.1016/j.expneurol.2012.07.016

Wyss-Coray, T. (2016). Ageing, neurodegeneration and brain rejuvenation. *Nature* 539, 180–186. doi: 10.1038/nature20411

Xu, Y.-Z., Nygard, M., Kristensson, K., and Bentivoglio, M. (2010). Regulation of cytokine signaling and T-cell recruitment in the aging mouse brain in response to central inflammatory challenge. *Brain. Behav. Immun.* 24, 138–152. doi: 10.1016/j.bbi.2009.09.006

Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R., et al. (1985). T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature* 317, 355–358. doi: 10.1038/317355a0

Zhang, J., Ke, K.-F., Liu, Z., Qiu, Y.-H., and Peng, Y.-P. (2013). Th17 cell-mediated neuroinflammation is involved in neurodegeneration of abeta1-42-induced Alzheimer's disease model rats. *PLoS One* 8:e75786. doi: 10.1371/journal.pone.0075786

Zhang, Y., Guan, Z., Reader, B., Shawler, T., Mandrekar-Colucci, S., Huang, K., et al. (2013). Autonomic dysreflexia causes chronic immune suppression after spinal cord injury. *J. Neurosci.* 33, 12970–12981. doi: 10.1523/JNEUROSCI.1974-13.2013

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., et al. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat. Neurosci.* 9, 268–275. doi: 10.1038/nn1629

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frontiers in Aging Neuroscience

REVIEW
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Target Dysbiosis of Gut Microbes as a Future Therapeutic Manipulation in Alzheimer's Disease

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Alzheimer's disease (AD) is commonly an age-associated dementia with neurodegeneration. The pathogenesis of AD is complex and still remains unclear. The inflammation, amyloid β (Aβ), and neurofibrillary tangles as well misfolded tau protein in the brain may contribute to the occurrence and development of AD. Compared with tau protein, Aβ is less toxic. So far, all efforts made in the treatments of AD with targeting these pathogenic factors were unsuccessful over the past decades. Recently, many studies demonstrated that changes of the intestinal environment and gut microbiota *via* gut–brain axis pathway can cause neurological disorders, such as AD, which may be involved in the pathogenesis of AD. Thus, remodeling the gut microbiota by various ways to maintain their balance might be a novel therapeutic strategy for AD. In the review article, we analyzed the characteristics of gut microbiota and its dysbiosis in AD and its animal models and investigated the possibility of targeting the gut microbiota in the treatment of the patients with AD in the future.

Keywords: Alzheimer's disease, gut microbiota, dysbiosis, microbiota-gut-brain axis, treatment

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HIGHLIGHTS

- The gut microbes communicate with the brain by several regulating pathways *via* the gut–brain axis involved in the physiological activities to maintain homeostasis of the human body. The imbalance of gut microbiota is associated with AD.
- The gut dysbiosis caused by several factors may aggravate neuroinflammation and other pathologies promoting the development and progression of AD.
- Targeting the gut dysbiosis or remodeling the gut microbiota might be a novel strategy for AD therapy.

INTRODUCTION

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disorder and the most common form of age-associated dementia. In year 2017, it has been reported that about 40 million people suffered from AD in the world (Alzheimer's Association, 2017; Esquerda-Canals et al., 2017). Despite a lot of previous intensive studies, the pathogenesis of AD

remains insufficiently understood. Pathologic changes in the brain of AD include amyloid β (Aβ) plaque deposits and neurofibrillary tangles formed by intracellular accumulation of hyperphosphorylated tau protein and neuroinflammation. The pathological characteristics are current major theory of the pathogenesis of AD (Angelucci et al., 2019). However, the great efforts in therapeutic AD basis on the pathogenesis of AD with pathogenic Aβ or tau over the past decades have witnessed continuous failure, indicating that the pathogenesis of AD should be multifactorial and is more complex than a simple pathogenic Aβ or tau would suggest. With aging of human beings, the incidence of AD is rising continuously in the world, which has become a major public health problem (Angelucci et al., 2019). In order to develop the effective treatment, we need further a better understanding of the pathogenesis of AD.

Over the past 10 years, the researchers have been very concerned and interested in the role of the gut microbiome in modulating brain function, although the results were obtained mainly from animal models (Long-Smith et al., 2020). Microbiota may be a crucial predisposing factor for AD and other neurological disorders, which has been proven by a growing number of studies (Zhuang et al., 2018; Sochocka et al., 2019; Cryan et al., 2020; Long-Smith et al., 2020). AD has been considered as a systemic disease related to inflammation, and the inflammatory–infectious hypothesis of its pathogenesis becomes more significant (Bronzuoli et al., 2016). It has been evidenced that microbes and their products from the periphery infiltrating into the brain causing chronic inflammation are an important predisposing factor of neuroinflammation and neurodegenerative changes observed in AD (Cattaneo et al., 2017; Ashraf et al., 2019). AD and cognitive decline, as well as other neurodegenerative diseases, are often associated with gastrointestinal (GI) dysfunction (Zhuang et al., 2018; Sochocka et al., 2019; Ticinesi et al., 2019). It is postulated that AD may begin in the gut and is related to the imbalance of gut microbiota, while the intestinal inflammation and infections caused by various pathogens may control the changes of the gut microbiota first, and then other factors, as described below, are also involved in controlling these changes.

Throughout the course of these diseases, the GI disturbances may occur in the different stage of the diseases as a clinical manifestation. The alteration of enteric neuroimmune system (ENIS) and dysbiosis of the gut microbiota may lead to the occurrence of GI dysfunction and neurologic disorders (Pellegrini et al., 2018; Sochocka et al., 2019). Therefore, it has been hypothesized that AD is closely related to gut microbial alteration, and it is consistent with the pivotal role of inflammation in the pathogenesis of AD (Calsolaro and Edison, 2016; Haran et al., 2019). In the review article, we clarify the characteristics of the gut microbiota, analyze the role of dysbiosis of the gut microbes in the pathogenesis of AD, and discuss the possibility whether targeting the dysbiosis of gut microbes can be as a future therapeutic manipulation in AD.

THE GUT MICROBIOME AND THEIR ROLE

Features of the Gut Microbiota

Normal microorganisms in human being consist of bacteria, fungi, viruses, etc. and 95% of them are located in the large intestine (Swidsinski et al., 2005; Galland, 2014). The microbiota refers to bacteria, fungi, viruses, etc., existing in an ecosystem/habitat, and the intestinal microbial community is named gut microbiota (Shahi et al., 2017). The amount of microorganisms is 10^{14} with a total weight of approximately 2 kg (Picca et al., 2018). In human, distribution and species of the bacteria residing in the intestinal tract are always changing and uncertain, which depend on the physiological condition of the GI tract. So far, the exact species of microbe populations are unclear.

However, the comprehensive view of human-connected microbes has been offered by the Metagenomics of the Human Intestinal Tract and the Human Microbiome Project. There are a total of 2,172 species of microbes classified into 12 phyla in human, and *Proteobacteria, Firmicutes, Actinobacteria*, and *Bacteroidetes* phyla possess 93.5% of total microbes (Li et al., 2014; Hugon et al., 2015; Bilen et al., 2018). The phyla *Firmicutes* and *Bacteroidetes* are the majority, containing the genera *Prevotella*, *Bacteroides*, and *Ruminococcus*, as well as *Verrucomicrobia* and *Actinobacteria*, but *Proteobacteria* phyla members have a small quantity (Mowry and Glenn, 2018). Because many factors impact on gut microbiota, for example, genetic factors, sex, diet, and others, such as place of residence, smoking, etc., therefore, different ethnicities have different gut microbiomes (Blum, 2017).

Role of the Gut Microbiota

The microbiota is involved in important homeostatic processes and essential for the homeostasis of intestinal intraepithelial lymphocytes (Liu L. et al., 2019). Besides the role of microbiota associated with GI function, the microbiota also contributes to inflammation and immune response, central and peripheral (enteric) neurotransmission, glucose metabolism, etc. (Liu et al., 2020). Therefore, the gut microbes play a beneficial role in maintaining homeostasis of immune systems of the host. The necessary vitamins and other substances involved in the development of the central nervous system (CNS) and immune regulation are produced by gut microbiota (Blum, 2017; Picca et al., 2018).

In a healthy organism, microbiota can also create a protective barrier against the infectious agents in the gut (Angelucci et al., 2019; Liu L. et al., 2019). Furthermore, a dynamic network is formed *via* the interaction among intestinal epithelial barrier, intestinal immune system, gut microbiota, and enteric nervous system (ENS) to coordinate the GI physiology and maintain homeostasis of gut (Pellegrini et al., 2018). The association of gut microbiota and its interaction with intestinal mucosal barrier and immune system in maintaining brain homeostasis have been demonstrated by more and more evidence (Foster et al., 2017; Fung et al., 2017).

Impact of Dysbiosis of Gut Microbes on Body

In addition to destabilizing the intestinal environment, the dysbiosis of gut microbes can affect behavior, learning, and memory, as well as neurogenesis, etc. (Fang et al., 2016; Luczynski et al., 2016; Minato et al., 2017; Tremlett et al., 2017). Therefore, the gut microbiome plays a key role in maintaining the body healthy. So far, several review articles summarized well the role of gut microbiota in the maintenance of brain homeostasis (Fung et al., 2017; Tognini, 2017; Tremlett et al., 2017; Askarova et al., 2020). Here, we describe concisely the most important results about the role of gut microbiota in the regulation of brain physiological processes.

Microbiota and Senescence

The relationship between the gut microbiota and the senescent brain is unclear, and until now, it is still an unanswerable question. As several neurodegenerative diseases occur in the elderly, it has attracted attention to the relationship between the gut microbiota and aging. However, at present, not many clinical and experimental studies evaluated this in the field. Claesson et al. (2012) studied the composition of the gut microbiota from 16 older than 65 years in the Ireland and showed more diverse gut microbiota with better health outcomes, indicating that the composition of the gut microbiota is closely related to health condition and immune function, and a diet rich in fruits and vegetables has a greater diversity of gut microbiota. Thus, one of the features of healthy aging may be the diversity of the gut microbiota (Claesson et al., 2012). Unfortunately, the study did not provide the information regarding the relationship between the gut microbiota and the senescence. The field is nascent, and so far, not many studies have been published.

The evidence concerning the relationship between gut microbiota and aging in mice showed that age-associated behavioral impairments were consistent with alterations of the microbiota (Scott et al., 2017), which is a direct evidence to confirm the close correlation between microbiota and aging. In the process of aging, the gut microbiota's composition is altered, accompanied by increasing proteobacteria and decreasing probiotics, such as bifidobacteria, and neuroprotective molecules (Lambert et al., 2009; Caracciolo et al., 2014). The probiotic bacteria called "good" microbes play an advantageous role in human health and produce the essential substances to inhibit inflammation (Mukherjee et al., 2018).

The age-associated neuroinflammation, a crucial pathogenic factor in the development of AD and the cause or consequence of most neurodegenerative diseases, was ameliorated by administration with prebiotic inulin that targets the microbiota (Games et al., 1995). Microglial activation as an inflammatory hallmark in the pathology of AD is regulated by the microbiota, which plays a key role in aging and neurodegeneration (Abbas et al., 2002; Lambert et al., 2009). Moreover, high levels of proinflammatory cytokines in healthy elderly subjects were related to the disorder of microbiome function, particularly the genes encoding short-chain fatty acids (SCFAs; Claesson et al., 2012), which is a basic characteristic for the extensive age-related pathologies, such as age-related dysbiosis of gut microbes and neurological decline (Franceschi et al., 2000). Although lots of studies demonstrated this correlation, unfortunately a direct cause effect has not yet been established (Sun et al., 2019b; Kim et al., 2020). Thus, more studies are needed to show evidence of the relationship.

LINKS BETWEEN GUT AND BRAIN *VIA* MICROBIOME–GUT–BRAIN AXIS

The gut microbiome is involved in bidirectional communication between the gut and brain, which is a significant scientific discovery recently (Erny et al., 2017; Fung, 2020). It has been suggested that human gut microbiome may be considered as the "second brain" and contributes to AD and other neurodegenerative disorders (Gershon, 1999; Schneider et al., 2019; Sochocka et al., 2019).

Communication Between Brain and Gut

The gut microbiota can modulate brain signals and activity *via* the microbiome–gut–brain axis through the nervous, endocrine, and immune systems proven by many animal and preclinical experiments. Also the chemical substances produced by themselves (monoamines and amino acids) can cross the blood–brain barrier (BBB) reaching the CNS (Collins et al., 2012; Crane et al., 2015; Yano et al., 2015) and influence brain activity with possible repercussions on behavior (Wekerle, 2016; Kowalski and Mulak, 2019). The gut microbiota can also receive signals from the brain in the form of neurotransmitters, including acetylcholine, the modified amino acids glutamate and γ-aminobutyric acid (GABA), and the biogenic amines dopamine (DA), serotonin (5-HT), and histamine, interacting with the brain (Briguglio et al., 2018). Furthermore, the concept of the microbiome–gut–brain axis has been supported by the current research data; thus, the gut microbiome can communicate with the brain and is responsible for some neurodegenerative disorders (Haran et al., 2019). The new perspective makes us realize that the gut microbiota may play an important role in this mutual relationship between brain and gut communication, as well as physiological regulation. **Figure 1** presents the microbiome–gut–brain axis containing several molecular pathways and their interactions. However, the microbiome–gut–brain axis is a complex multidirectional system between the gut microbiota, ENS, and the brain, which is still poorly understood.

Communication Through Neural Regulation Pathway

The pathways of communication between the gut and brain have been reported (Dinan and Cryan, 2017). The first pathway is neural regulation pathway, in which the vagus nerve links between the gut and the spinal cord (autonomic nervous system; Bonaz et al., 2018). The ended vagus nerve of brain stem nuclei receiving and giving afferent and efferent fibers may regulate the gut functions and send messages to other regions of CNS (Bonaz et al., 2018). The catecholamines or acetylcholine secreted from the brain affecting ENS circuits can modulate the gut functions

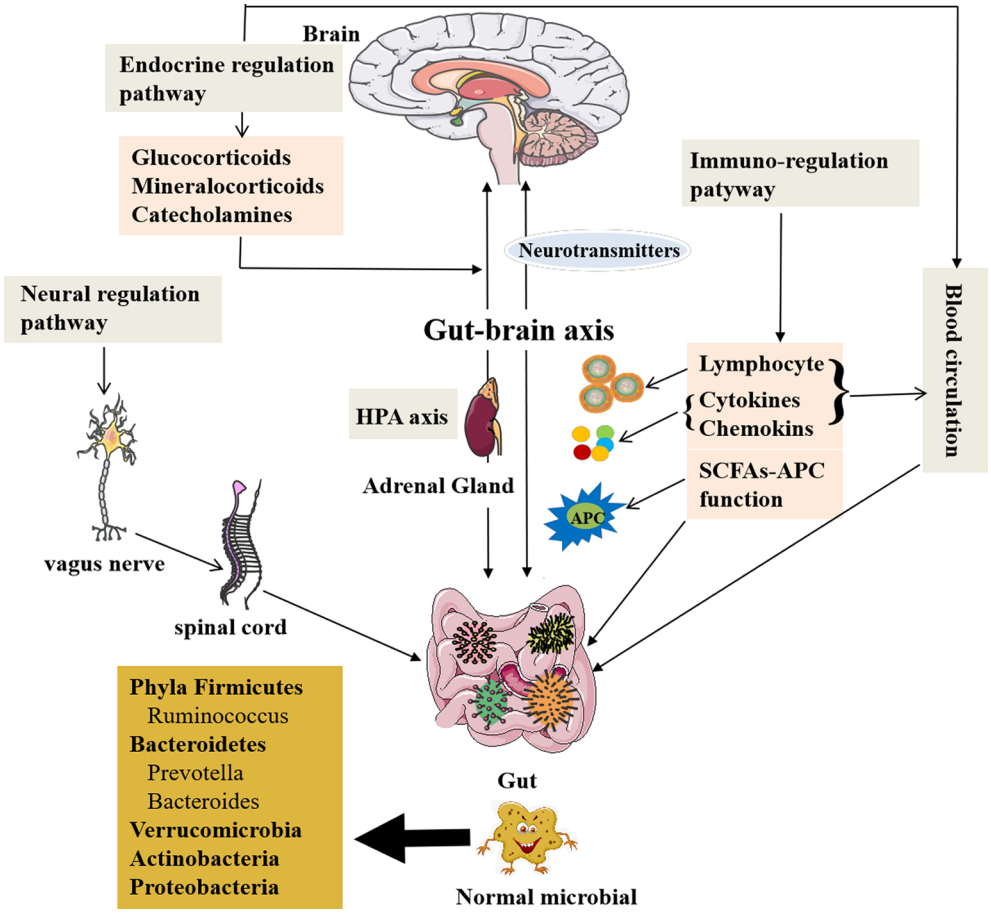

FIGURE 1 | The role of the gut microbiota and communication with the brain. A healthy gut contains large fractions of the phyla Firmicutes and Bacteroidetes, including the genera *Prevotella*, *Bacteroides*, and *Ruminococcus* followed by *Verrucomicrobia* and *Actinobacteria*, but contains a low number of Proteobacteria phyla members (Mowry and Glenn, 2018). The gut microbiota is related to GI function, but also involved in several complex modulatory processes, inflammation and immune response, and peripheral (enteric) and central neurotransmission, as well as synthetize and secrete essential substances. The gut microbiota contributes to important homeostatic processes and is essential for the homeostasis of intestinal intraepithelial lymphocytes (Liu L. et al., 2019). The gut microbiota can affect brain function and bioactivity through gut–brain axis *via* several pathways: (1) the neural regulating pathway, in which vagus nerve links between the gut and the spinal cord (autonomic nervous system; Bonaz et al., 2018). The gut microbiota can secrete and regulate neurotransmitters of the CNS. (2) The endocrine pathway. The HPA axis can release glucocorticoids, etc., after stimulations by stress or other factors, which can alter gut microbiota composition and increase gut epithelium permeability and immune responses in gut (Ait-Belgnaoui et al., 2012; Park et al., 2013; Bellavance and Rivest, 2014). (3) The immune-regulating pathway *via* lymphocyte, cytokines, chemokines, and antigen-presenting effect of SCFAs communicating with the brain. (4) The blood circulation pathway (Logsdon et al., 2018). The immune and endocrine molecules, such as cytokines and hormones, can pass BBB and intestinal mucosa to influence both gut and brain functions (Zac-Varghese et al., 2010). BBB, blood–brain barrier; CNS, central nervous system; HPA axis, hypothalamic–pituitary–adrenal axis; SCFA, short-chain fatty acids; APC, antigen-presenting cells.

(Mayer et al., 2015; Weinstein et al., 2015). It is also through the gut bacteria to exchange signals between ENS and CNS (Carabotti et al., 2015).

On the other hand, the gut microbiota is able to produce and modulate neurotransmitters in both CNS and peripheral nervous system, and intestinal environmental changes can affect lymphocytes of the gut to produce more cytokines and chemokines, such as interleukin 1 (IL-1), IL-6, IL-17, IL-22, tumor necrosis factor-α (TNF-α; Thaiss et al., 2016; Sochocka et al., 2019), and transforming growth factor β (Ma et al., 2017), as well as chemokine, fractalkine, and its receptor (CX3CR1; Merino et al., 2011), affecting the CNS through activating the endocrine or paracrine systems. The proinflammatory cytokines, IL-1, IL-6, IL-17, and TNF-α, are potentially harmful to the brain (Angelucci et al., 2019). The gut microbes secrete several important substances such as GABA, histamine, 5-HT, and DA, which contribute to neuroactive and immune regulation (Barrett et al., 2012; Lyte, 2013), and also produce toxic substances to the brain, such as ammonia and others (Galland, 2014). In addition, the microbiome–gut–brain axis can be affected by microbiota *via* immunological, neuroendocrine, and direct neural mechanisms (Logsdon et al., 2018), which insulted the brain to cause memory impairment, anxiety, and other cognitive dysfunctions (Gareau et al., 2011; Galland, 2014;

Johnson and Foster, 2018) and resulted in several diseases, such as anxiety and depression (Lach et al., 2018; Capuco et al., 2020), neurodegenerative diseases, and drug-resistant epilepsy (Braakman and van Ingen, 2018).

Communication Through Endocrine Regulation Pathway

Endocrine regulating pathway is the second pathway of communication between the gut and brain. The hypothalamic–pituitary–adrenal (HPA) axis can release glucocorticoids, mineralocorticoids, or catecholamines after stimulations by stress or other factors, which result in the changes of intestinal microbiota components and the intestinal epithelium permeability, as well as immune responses in the gut (Ait-Belgnaoui et al., 2012; Park et al., 2013; Bellavance and Rivest, 2014). Enhanced genus *Clostridium* and declined *Bacteroides* as the feature of the gut dysbiosis were caused by high corticosterone levels in the stressed mice (Bailey et al., 2011). The glucocorticoids have both proinflammatory and anti-inflammatory roles; however, inflammations are related to damaged HPA axis functionality in AD and other neurodegenerative disorders (Silverman and Sternberg, 2012; Bellavance and Rivest, 2014; Hueston and Deak, 2014).

Communication Through Immune-Regulation Pathway

Immunoregulating pathway as the third pathway also participates in this communication between the gut and brain *via* gut microbes, which could affect antigen presentations and regulate cytokines production and lymphocyte function, as well as the development of two types of immune system through the gut–brain axis (Olszak et al., 2012; Fung et al., 2017). The gut microbiota also impacts on productions of SCFAs that can activate immune response and trigger inflammation in the brain, resulting in a series of neurological symptoms. Additionally, SCFAs are related to G-protein-coupled receptor 43 (GPR43) to lead a strong anti-inflammatory reaction (Maslowski et al., 2009). The gut microbes are necessary for host immunity generation in the GI tract. The data obtained from germ-free (GF) mice have shown that the maturations of the immune, endocrine, and nervous systems are affected by gut bacteria, which is a strong evidence for the gut microbiota linking with the brain *via* microbiome–gut–brain axis (Wang and Wang, 2016; Kowalski and Mulak, 2019).

Communication Through Blood Circulation

Communication between the gut and brain is also through the blood circulation (Logsdon et al., 2018). The cytokines and hormones as well as some molecules can pass BBB and intestinal mucosa to influence both gut and brain functions (Zac-Varghese et al., 2010). Furthermore, the central, peripheral, immune, and endocrine systems are involved in the communication between gut and brain in a multifunctional network formed by the microbiome–gut–brain axis (Borre et al., 2014).

However, the mechanisms that mediate gut–brain communication remain in its infancy. There are still many questions to explore, such as the molecular and cellular mechanisms underlying the microbiome–gut–brain axis in health and under pathological conditions, etc.

DYSBIOSIS OF GUT MICROBES IN AD PATIENTS AND ITS ANIMAL MODELS

Generally, the gut microbial communities in human are stable; however, they can be altered in the different conditions by the effects of various factors through their direct action (microbial infection) or indirect actions (antimicrobial protection hypothesis, hygiene hypothesis; DiSabato et al., 2016; Ashraf et al., 2019; Kong et al., 2020). Recently, the studies of several groups have been demonstrated that various diseases, including intestinal diseases and more systemic diseases such as diabetes, metabolic syndrome, and neurodegenerative disorders, including AD and others, are related to the imbalance of gut microbiota called "dysbiosis" (Del Tredici et al., 2002; Murono et al., 2015; Hu et al., 2016; Jiang et al., 2017). Occurrence and development of AD and other neurodegenerative disorders may be accompanied by the gut microbiome dysbiosis, inflammation, and dysfunction of the gut–brain axis. It has been speculated that AD may appear during the aging of immune system based on the theory of age-related dysbiosis derived from the association between gut microbiota and AD, which has been evidenced by clinical and experimental studies (Cattaneo et al., 2017; Pellegrini et al., 2018).

Generally, the traditional ecological measures are used to characterize the composition of the gut microbiome, including richness [the number of unique operational taxonomic units (OTUs) present in a participant], alpha diversity (the richness and abundance of OTUs within each participant), and beta diversity (the similarity or difference in composition between participants). Declined microbial richness and diversity as well as a distinct composition of the gut microbiome were found in AD patients. The levels of differentially abundant genera were correlated with cerebrospinal fluid (CSF) biomarkers of AD pathology (Vogt et al., 2017). In short, definite genera as more abundant in AD were related to greater AD pathology, whereas genera as less abundant in AD were associated with less AD pathology (Vogt et al., 2017).

As mentioned previously, immune response system participates in this communication between the gut microbes and brain. There is also a close interaction between gut microbes and the local as well as systemic immune system. In general, the gut dysbiosis could lead to dysfunctions of both innate and adoptive immune through several ways, such as changing antigen presentations, cytokines production, and lymphocyte functions, as well as increasing inflammation, etc., also can cause the gut–brain axis malfunction (Levy et al., 2017). In AD patients, the molecular and cellular alterations involving immune cells, such as T cells, B cells, microglia, etc., as well as immune mediators, occur not only in the peripheral blood, but also in the brain and the CSF, which may be associated with triggering immune response by the gut dysbiosis. The gut dysbiosis impacts on innate and adoptive immune response in AD patients obviously *via* activating immune/inflammatory cells, shifting them into inflammatory type to enhance immune mediated

inflammatory response, and promoting neurodegeneration in the brain. The gut dysbiosis in AD was obviously correlated with more T helper 1 (T_H1) cell infiltration into the brain (Togo et al., 2002; Monsonego et al., 2003), and increased T-cell infiltration in the brain parenchyma and peripheral T-cell responses to Aβ have been found in AD patients (Rogers et al., 1988; Monsonego et al., 2003).

Pathologically, deposition of Aβ plaques in the brain is a major character, and it has been considered as one of the important pathogenic factors in AD (Salter and Stevens, 2017; Angelucci et al., 2019). Reactive gliosis and neuroinflammation are the histological hallmarks and key factors in the pathogenesis of AD (Salter and Stevens, 2017; Yeh et al., 2017; Zhang et al., 2020). Microglial activation in the CNS is heterogeneous and categorized into two types: proinflammatory and anti-inflammatory microglia (Tang and Le, 2016; Yu et al., 2019). Microglia plays either a cytotoxic or neuroprotective role, depending on the types activated, which can be changed in the different stages of AD. The anti-inflammatory microglia phagocytizes Aβ plaques by the Fc receptors and promotes the cleaning and degradation of Aβ by possibly increased phagocytic and lysosomal activity, as well as restriction of the inflammatory response (Kamphuis et al., 2016; Dubbelaar et al., 2018). Oppositely, proinflammatory microglia leads to Aβ accumulation, inducing cell death and worsening disease (Fakhoury, 2018). We speculated that microglial malfunction may be the basis of AD pathogenesis and precede and accelerate the onset of AD.

Table 1 compares the gut microbiota between healthy subjects and AD patients, in which alterations of the gut microbiota can be seen in the AD patients; thus, dysbiosis of gut microbes may be involved in AD-related impairments.

TABLE 1 | The comparisons of the gut microbiota between healthy subjects and Alzheimer's disease (AD) patients.

	Healthy subjects	AD patients	References
The gut microbiota	Eubacterium rectale	↑	Cattaneo et al. (2017)
	Escherichia/Shigella	↓	
	Bacteroides	↓	Zhuang et al. (2018)
	Actinobacteria	↑	
	Bacilli	↑	
	Negativicutes	↓	
	Ruminococcaceae	↑	
	Enterococcaceae	↑	
	Lactobacillaceae	↑	
	Lanchnospiraceae	↓	
	Veillonellaceae	↓	
	Firmicutes	↓	Vogt et al. (2017)
	Bifidobacterium	↓	
	Dialister	↓	
	Bacteroidetes	↑	
	Blautia	↑	
	Phylum Firmicutes	↓	Liu P. et al. (2019)
	Proteobacteria	↑	
	Gammaproteobacteria	↑	
	Enterobacteriales	↑	
	Enterobacteriaceae	↑	

↑:*increase;* ↓:*decrease.*

Dysbiosis of Gut Microbes Involved in the Pathogenesis of AD Models

So far, the evidence obtained about the role of dysbiosis of gut microbes in AD pathophysiology is mainly from its animal models. A significant decrease in the Aβ pathology was observed in GF mice, and after the control mice were exposed to the gut microbiota, the Aβ pathology occurred again (Harach et al., 2017). In addition, an obvious absence of amyloid plaque deposit and neuroinflammation were seen in GF mice when microbes were not present (Harach et al., 2017). Of course, the pathological manifestations in GF mice may not be completely attributed to gut dysbiosis, because GF mice also exhibited defects in the immune system and difficulties with energy acquisition, etc., which also impact on the pathological changes in GF mice. The changes of gut microbiota promoted Aβ protein accumulations in the gut. Evidently, a thoroughly changed gut microbiome was found in APP transgenic (Tg) mice (AβAPP) [a genetic model of AD; the mice overexpress mutated forms of human amyloid precursor protein (APP) linked to familial AD] when compared to wild-type mice (WT; Wang X.-L. et al., 2015). The removal of gut microbiome was related to central Aβ levels in AD mice; however, increased amyloid accumulation was found in the brain after transplantation by microbiota from AD mice (Harach et al., 2017). Similarly, high levels of Aβ protein of brain and related behavioral alterations were associated with the gut dysbiosis in APP/PS1 mice [a genetic model of AD; APP/PS1 mice are double Tg mice expressing a chimeric mouse/human APP (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9); Shen et al., 2017]. Moreover, both enhanced Aβ protein precursor (AβPP) accumulation in the gut and Firmicutes/Bacteroidetes ratio were found in 5xFAD mice (a transgenetic model of AD; 5xFAD mice express human APP and PSEN1 transgenes with a total of five AD-linked mutations: APP KM670/671NL, APP I716V, APP V7171, and PSEN1M146L and PSEN1L286V) following the change of the gut microbiota composition in these mice since the earliest phase of the diseases (Brandscheid et al., 2017). These data suggest that changing of gut microbiome in the animal models of AD promotes deposit of Aβ protein in the brain.

A significant study indicated that calorie restriction decreased Aβ deposition in the brain of AD mouse model. During aging process, calorie restriction could change the gut microbiome, including an increase in *Bacteroides*, which was found obviously in female Tg2576 mouse model when compared to WT mice. It has been demonstrated that the specific gut microbiota change was related to Aβ levels, and the change had a greater impact on females than males. Furthermore, long-term calorie restriction can change the gut environment and prevent the expansion of microbes that promotes age-related cognitive decline (Cox et al., 2019).

Interestingly, there was activation of immune/inflammatory cells and high expressions of Aβ and phosphorylated tau (p-tau) protein, as well as neuronal coding rearrangements in the gut of APP/PS1 mice, which feature is accompanied by lower levels of neuronal nitric oxide synthase and choline acetyltransferase,

suggesting that Aβ and p-tau protein deposits in the gut can influence local and peripheral neurogenic/inflammatory responses and promote inflammation and neurodegeneration in the brain of AD models (Haghikia et al., 2016; Feng et al., 2018). It has been shown that enhanced Aβ protein expression in the gut precedes inflammation in the brain of TgCRND8 mice (a genetic model of AD; TgCRND8 mice overexpress mutant human APP KM670/671NL and APP V717F; Semar et al., 2013). Aβ protein can also be transmitted to the CNS through myenteric neurons and nerve gut–brain axis involved in the pathogenesis of AD directly (Zhao and Lukiw, 2015; Pistollato et al., 2016). However, the causal relationship between these possible pathogenic factors is unclear; therefore, further studies are needed to investigate.

Dysbiosis of Gut Microbes Associated With Inflammation in AD Models

It is beyond doubt and has been evidenced that inflammation is a crucial factor in the pathogenesis of AD. Recent studies have shown that a strong correlation between NLRP3 inflammasomes, one of the multiprotein complexes, and initiation of inflammation and neurological diseases, is identified (Pellegrini et al., 2019). NLRP3 inflammasomes are key molecules in neuroinflammation and Aβ caused AD pathology in AD models (Heneka et al., 2013; Ising et al., 2019). Conversely, impaired NLRP3 inflammasome function lowered tau hyperphosphorylation by regulating tau kinases and phosphatases (Ising et al., 2019; Tejera et al., 2019). NLRP3 knockout (KO) mice exhibited significant difference of the composition of gut microbiota and behaviors compared with WT mice, suggesting that NLRP3 inflammasome deficiency affected the gut microbiota composition (Zhang et al., 2019). Transplantation of the gut microbiota of NLRP3 KO mice or using NLRP3's inhibitor ameliorated depressive-like behaviors *via* remodeling gut microbiota (Zhang et al., 2019). The cognitive function of AD mice was repaired by using NLRP3 inhibitor, which may be associated with altering gut microbiota (Daniels et al., 2016; Ising et al., 2019; Tejera et al., 2019). Inflammation playing a central role in AD is linked to the closed relationship between gut microbiota and AD (Calsolaro and Edison, 2016).

Dysbiosis of Gut Microbes and Enteric Inflammation or Infections in AD Patients

In AD patients, the proportion and prevalence of bacteria synthesizing butyrate were low, and the abundances of taxa were high that lead to inflammation compared to healthy or other dementia types, which evidenced that the nexus between the gut microbiome and an altered epithelial homeostasis could have an effect on AD (Daniels et al., 2016) by increases in inflammatory and decreases in anti-inflammatory microbial metabolism (Haran et al., 2019).

Several studies on dysbiosis of gut microbes in AD patients have exhibited that AD's main pathological features in the brain, such as amyloidosis and inflammation, are linked to inflammatory bacteria and their neurotoxic products, like lipopolysaccharides (Bester et al., 2015; Cattaneo et al., 2017). In AD patients, increased *Bacteroides* and *Blautia* and decreased relative abundance of the genera *SMB53* and *Dialister* were a feature of the changes of gut microbiota, which was associated with high levels of chitinase-3-like protein 1 and p-tau, accompanied by a low Aβ42/Aβ40 ratio in CSF (Vogt et al., 2017). Intestinal inflammation in AD patients was positively correlated with a high level of fecal calprotectin (Leblhuber et al., 2015). However, the clinical evidence on accumulations of Aβ protein, AβPP, and p-tau in the gut of AD patients is rare, and the data obtained are contradictory (Joachim et al., 1989; Puig et al., 2015). Meanwhile, studies with no matched healthy controls are also unable to make such conclusion; i.e., there is a causal relationship between intestinal Aβ and p-tau deposition, inflammation and gut dysbiosis.

The chronic *Helicobacter pylori* infection can trigger the release of inflammatory mediators and is associated with low Mini-Mental State Examination score in AD patients when compared with patients without infections (Roubaud-Baudron et al., 2012).

Infections by *H. pylori*, *Borrelia burgdorferi*, and *Chlamydia pneumoniae*, and so on, increased levels of Aβ40 and Aβ42 in serum of AD patients (Bu et al., 2015). *In vitro*, the neuroblastoma cell cultures treated by *H. pylori* filtrate induced tau hyperphosphorylation, which was similar to AD tau pathological changes (Wang X.-L. et al., 2015). Furthermore, the inflammatory disorders are also linked to gut dysbiosis caused by viruses, such as herpes simplex virus type 1, which can be one of the crucial risk factors for AD. Maintaining the homeostasis of intestinal intraepithelial lymphocytes required these commensal viruses; however, the sustaining intestinal homeostasis can be also destroyed by infections with bacteria and virus (Harris and Harris, 2015). Collectively, the gut dysbiosis could be a risk factor for AD due to lacking or reducing immune defenses in the seniors (Angelucci et al., 2019).

Although gut dysbiosis contributes to the pathogenesis of many neurological and neurodegenerative diseases generally, the types of microbiota changes in the gut of AD are different from those of other neurodegenerative diseases, when compared with multiple sclerosis (MS), Parkinson disease (PD), and amyotrophic lateral sclerosis as shown in **Table 2**. We speculated that some microbial changes are relatively specific to AD due to causing AD pathology different from other diseases. Increased *Bacteroides* was found in the AD patients, and *Bacteroides* colonization aggravated Aβ deposition, which is speculated to be a mechanism whereby the gut impacts AD pathogenesis (Cox et al., 2019). However, there are too many factors affecting the gut microbiota; it is difficult to determine a causal relationship and needs to be further explored.

Up to now, no study confirmed clearly that Aβ deposition or tau accumulation is related to alter a particular microbe in the gut of AD patients. However, the specific microbes resulting in inflammation may promote Aβ and p-tau protein deposits in the gut indirectly, and the two promote each other. We considered that the infections by *H. pylori* and *B. burgdorferi* and enhanced *Bacteroides* may promote Aβ deposition or tau accumulation in the gut of AD patients, which needed to be evidenced in the future studies.

TABLE 2 | The gut microbes in central nervous system (CNS) disorders and treatments by microbes or microbial products.

CNS disorders	Pathologies	Gut microbes ↑	Gut microbes ↓	Treatments	Other results
AD	Glial cell activation and inflammatory molecules production. Aβ plaques containing extracellularly deposited Aβ. Intracellular neurofibrillary tangles formed with hyperphosphorylated and misfolded tau protein (Angelucci et al., 2019).	*CMV, HSV-1, B. burgdorferi, C. pneumoniae and H. pylori* (Kountouras et al., 2009; Bu et al., 2015) *Bacteroides*, *Gemellaceae, genera Blautia, Phascolarctobacterium, and Gemella* (Vogt et al., 2017).	*Firmicutes, Actinobacteria, genera* SMB53 (family *Clostridiaceae*), and *Dialister, Clostridium, Turicibacter*, and cc115 (family *Erysipelotrichaceae*; Vogt et al., 2017).	Increased MMSE after treatments by probiotic (*Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, and Lactobacillus fermentum*, Akbari et al., 2016), or by selenium plus probiotic (Tamtaji et al., 2019a). GV-971 had cognition improvement by targeting gut dysbiosis (Wang et al., 2019). Increased MMSE after triple eradication regimen (omeprazole, clarithromycin and amoxicillin) treatment for HP+ patients (Kountouras et al., 2009).	
ALS	Tetanus and botulinum toxins, "leaky gut"; higher richness of OTUs and reduction of butyrate (Zhu et al., 2020).	*Dorea* (Fang et al., 2016). *E. coli and enterobacteria* (Mazzini et al., 2018).	*Oscillibacter, Anaerostipes, Lachnospiraceae* (Fang et al., 2016). Total yeast (Mazzini et al., 2018).		
MS	BBB integrity disruption and astrocyte pathogenicity, primary demyelination, axonal loss, and reactive gliosis in the CNS (Duffy et al., 2014; Chu et al., 2018).	*Methanobrevibacter* and *Akkermansia* (Jangi et al., 2016). *Actinobacteria,Bifidobacterium* and *Streptococcus* (Miyake et al., 2015) *Firmicutes, Archaea Euryarchaeota* (Tremlett et al., 2016) *Ruminococcus* (Cantarel et al., 2015)	*Butyricimonas* (Jangi et al., 2016) *Bacteroides, Faecalibacterium, Prevotella, Anaerostipes Clostridia* XIVa and IV *Clusters* (Miyake et al., 2015) *Fusobacteria* (Tremlett et al., 2016) *Faecalibacterium, Bacteroidaceae* (Cantarel et al., 2015)	*Prevotella, Sutterella*↑ and *Sarcina*↓ after IFN-ßor GA treatmet (Jangi et al., 2016). *Faecalibacterium*↑ after GA treatment (Cantarel et al., 2015). *C. perfringens* ↓ after Fingolimod, DMF or Teriflunomide treatment (Rumah et al., 2017).	Absence (vs. presence) of *Fusobacteria* associated with relapse risk (Tremlett et al., 2016). Inhibiting the growth of *C. perfringens* enhancing the efficacy of MS drugs (Rumah et al., 2017).
PD	Higher frequency of α-synuclein, dopaminergic neuronal loss (Zhu et al., 2020).	*Prevotellaceae* (Scheperjans et al., 2015). *Blautia, Coprococcus, and Roseburia* (Zhu et al., 2020).	*H. pylori* (Fasano et al., 2015). *E. coli, Ralstonia, Oscillospira and Bacteroides* (Zhu et al., 2020).	Decreased MDS-UPDRS after probiotic (*Lactobacillus acidophilus, Bifidobacterium bifidum, Lactobacillus reuteri, and Lactobacillus fermentum*) treatment for 12 weeks (Tamtaji et al., 2019b).	Reduced *Prevotellaceae* significantly in PD as a sensitive biomarker for PD (Zhu et al., 2020).

Abbreviations: Aβ, Amyloid β; AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; BBB, Blood-brain barrier; CNS, Central nervous system; DMF, Dimethyl fumarate; GA, Glatiramer acetate; GV-971, Sodium oligomannate; HP+, Helicobacter pylori positive; MDS-UPDRS, Movement disorder society unified Parkinson's disease rating scale; MS, Multiple sclerosis; MMSE, Mini-mental state examination; OTUs, Operational taxonomic units; PD, Parkinson's disease. ↑:increase; ↓:decrease.

Factors Caused Dysbiosis of Gut Microbes Can Target AD

The factors that disturbed the gut microbiota to lead gut dysbiosis could target AD (Stenman et al., 2013; Vogt et al., 2018; MahmoudianDehkordi et al., 2019). There was an interaction between gut microbiome and bile acid (BA) levels. The bacteria containing the abundant bile salt hydrolase can easily change BA pattern to modulate the commensal bacteria and protect the integrity of the intestinal barrier (Stenman et al., 2013; Shapiro et al., 2014; MahmoudianDehkordi et al., 2019). In the CSF of mild cognitive impairment (MCI) and AD patients, the high level of trimethylamine *n*-oxide (TMAO), a metabolite derived from gut microbiota, was related to the biomarkers of AD in CSF (Vogt et al., 2018).

A pivotal pathogenic factor, oxidative stress (OS) has been shown to contribute to the development of AD. In the CNS, the reactive oxygen species (ROS) levels and inflammation can be enhanced by a microbiota type to favors abnormal aggregation of Aβ, which speculated that high levels of CNS OS may be due to gut dysbiosis or its consequence (Jones et al., 2012; Dumitrescu et al., 2018). In short, it can be seen from the above results and summarized as follows: (1) chronic bacterial infections as a possible etiology linking AD pathogenesis; (2) obvious alteration in the compositions of gut microbiome in AD; and (3) rising proinflammatory and lowering anti-inflammatory bacteria in the gut related to systemic inflammation in the patients suffering from the brain amyloidosis and cognitive impairment, which changes might impact brain functions. Thus, microbial dysbiosis or imbalance may potentially contribute to the pathogenesis of AD.

IS TARGETING DYSBIOSIS OF GUT MICROBES AS A THERAPEUTIC MANIPULATION IN AD?

The gut microbiota may impact on AD development and progress as descripted above. Dysbiosis of gut microbes supposes to be involved in the pathogenesis of AD. Despite much disappointment in anti-AD drug discovery previously, it is still promising and possible to find new treatments basis on gut microbe impacting on AD. Modifying the microbiota composition or remodeling gut microbes using the substances or manipulations that are able to change their composition or balance gut microbes, such as antibiotics and others, may affect or provide therapy for AD and other neurological diseases (Chu et al., 2018).

Targeting Dysbiosis of Gut Microbes by Antibiotics in AD Patients and Its Models

Usually, eliminating and avoiding bacterial colonization are the main effects of antibiotics on human, rather than targeting the specific types of bacteria (Angelucci et al., 2019). After treatments by the broad-spectrum antibiotics, the composition of the gut microbiota was markedly altered, and its biodiversity was declined, as well as the colonization was temporized (Angelucci et al., 2019). Therapies with antibiotic could change the gut microbiota during the different length of time (Ianiro et al., 2016) and alter behavior we know well as brain chemistry in both humans and animals (Jernberg et al., 2007; Fröhlich et al., 2016). However, the evidences from two studies displayed that the antibiotic treatment caused also neuropsychiatric symptoms such as anxiety, psychosis, and delirium in AD patients who received antibiotic as a cocktail therapy (Loeb et al., 2004; Molloy et al., 2013), which is associated with antibiotic treatment of *H. pylori* infections, but these neuropsychiatric symptoms as side effects were not found in the general population (Neufeld et al., 2017). The effects of antibiotics on AD may be extensive or even opposite, depending on the antibiotics applied and on the role of targeted gut microbiome in the pathogenesis of AD. The antibiotic therapy was effective in the animal models of AD, but it has not yet been widely investigated in AD patients, because it is not clear which microbiomes dominate in the gut of AD patients and whether there are safe antibiotics available (Panza et al., 2019). Besides, there is a lack of such study on the effect of different antibiotics on AD pathology; further study is needed.

Obviously, lower amounts of microglia and astrocyte accumulation around amyloid plaques in the hippocampus and reduced insoluble Aβ plaques in aged APPSWE/PS1DeltaE9 Tg mice of AD model (which overexpress the Swedish mutation of APP KM670/671NL together with PS1 deleted in exon 9) after treatment with an antibiotic cocktail (Minter et al., 2017) were found, which is only circumstantial evidence that antibiotic interfered with microglial activation through reducing the amounts of microglia. However, in APP/PS1 Tg mice, the treatments with cocktail of antibiotics resulted in enhanced neuroinflammation and proinflammatory cytokine levels, and the disease itself was deteriorated (Minter et al., 2016). The harmful effects of antibiotics may break down the balance of gut bacteria, as streptozotocin and ampicillin did, which favors AD or worsens its course (Zarrinpar et al., 2018). Ampicillin increased rat serum corticosterone related to memory dysfunctions and decreased brain-derived neurotrophic factor in hippocampus, the features of AD pathology. Also, ampicillin deteriorated the anxiety-like behavior and impaired spatial memory in rats (Fröhlich et al., 2016). Surprisingly, the disorder of physiological and psychological function caused by ampicillin in rats was turned down by administration with probiotics (Wang T. et al., 2015). The clinical and experimental studies highlight that the results using antibiotics targeting and remodeling gut microbes in AD patients are controversial. Also there were some adverse consequences after antibiotic treatment, such as gut microbes coming back with their same features. According to the results of the current studies, it might be difficult to determine the effect of antibiotics in the treatment of AD. Attention should be paid to protection of the new beneficial and specific microbes and to the focus of future therapeutic trials by antibiotics in AD.

Remodeling Gut Microbes by Fecal Microbiota Transplantation

A new therapeutic method with fecal microbiota transplantation (FMT) has been applied in the neurodegenerative disorders and their animal models, as well as other diseases recently (Allegretti

et al., 2018). FMT consists of obtaining a fecal specimen from a healthy donor and administering a sample through either the mouth or the rectum of the ill person. The obtained results from FMT were encouraging and remarkably good in patients with recurrent *Clostridium difficile* infection, and FMT has become an important care option. FMT improved clinical symptoms obviously and fecal microbiome in the dog model with inflammatory bowel disease (Niina et al., 2019). In this respect, most clinical and experimental studies have been done in the patients with PD and its animal model. The exciting results with slowing down clinical progress in PD patients were obtained after reconstruction of the gut microbiome by FMT (Dutta et al., 2019). A study reported that constipation in a PD patient was clearly relieved after FMT through reconstruction of gut microbiota (Huang et al., 2019). The mechanisms behind the therapeutic effects of FMT are related to significant reduction of gut microbial dysbiosis and fecal SCFAs, as well as increment of levels of striatal DA and 5-HT, which has been evidenced in PD mice model. Furthermore, the activation of glial cells in the substantia nigra and TLR4/TNF-α signaling pathway molecules was inhibited by FMT in both gut and brain, which further evidences that gut microbial dysbiosis contributes to PD development, and FMT is beneficial to PD models (Sun et al., 2018). Therefore, the gut microbiota reconstruction may have therapeutic effects on PD patients and is a new therapeutic option (Fang, 2019). However, the study on treatment with FMT in AD and its animal models is scarce.

Recently, DeFilipp et al. (2019) reported a patient death treated with FMT due to extended-spectrum beta-lactamase-producing *Escherichia coli* bacteremia. To avoid similar accidents, it is necessary to enhance donor screening in order to reduce the transmission of microorganisms when treating patients with FMT and to properly evaluate the benefits and risks of FMT in different patient populations (DeFilipp et al., 2019), which should improve the new approaches for treatments in AD patients in the future (Blaser, 2019).

Remodeling Gut Microbes With Substances/Compounds in AD Patients and Its Models

Moreover, prebiotic fructooligosaccharides (FOSs) as dietary supplements ameliorated cognitive deficits and pathological changes in the APPSWE/PS1DeltaE9 Tg mice and increased the levels of synapsin I and synaptic plasticity markers, postsynaptic density protein 95, and decreased the phosphorylated level of c-Jun N-terminal kinase, indicating that FOS can modulate the gut microbiota-glucagon-like peptide-1 (GLP-1)/GLP-1 receptor (GLP-1R) pathway to play a beneficial role in AD (Sun et al., 2019a).

However, the results obtained from a double blind clinical trial, which was carried out in AD patients treated by probiotic supplementation (PS) and placebo, respectively, for 12 weeks, were negative, indicating that treatment with PS was ineffective in the severe AD patients, and the curative effect with PS was related to severity of AD at least (Agahi et al., 2018). In another clinical trial, treatment with multispecies probiotic for 4 weeks changed gut microbiota composition and tryptophan metabolism in serum of AD patients. Furthermore, a correlation between kynurenine/tryptophan and neopterin levels was observed indicating activation of macrophages and/or dendritic cells in AD patients (Leblhuber et al., 2018).

An exciting new drug for treatment of AD named GV-971 (sodium oligomannate) is discovered more recently (Wang et al., 2019). Polysaccharides or oligosaccharides are able to regulate gut microbiota (Thomson et al., 2018). Main therapeutic effects of GV-971 on AD are: (1) restoring the balance of gut microbiota *via* targeting and remodeling gut microbiota; and (2) inhibiting the neuroinflammation caused by gut bacterial amino acids to slow down AD progression (Wang et al., 2019). The enhanced levels of phenylalanine and isoleucine in periphery caused by dysbiosis of the gut microbiota can induce activation of proinflammatory microglia and proliferation of infiltrated inflammatory T_H1 cells from blood into the brain of AD mice deteriorating inflammation. Meanwhile, there were high levels of phenylalanine, isoleucine, and T_H1 cell in blood of MCI patients. The oligomannate sodium GV-971 is a carbohydrate-based anti-AD drug that markedly improved cognition in Chinese patients by targeting gut dysbiosis, dropping phenylalanine/isoleucine in the feces and blood, and inhibiting T_H1-associated neuroinflammation in the brain to reverse the cognition impairment (Wang et al., 2019).

In the study, the authors focused on the gut microbiota associated with neuroinflammation in AD patients and animal models through observing T_H1 and proinflammatory microglia activities. They showed that alterations of gut microbiota composition in AD were obviously correlated with more T_H1 cell infiltration into the brain. Also removing the gut microbiota by administrating antibiotic in AD mice can block T_H1 cell infiltration and proinflammatory microglia activation. Additionally, strengthened T_H1 cell infiltration and proinflammatory microglia activation in WT mice can be caused by FMT from AD mice and prolonged contact with fecal bacteria (Wang et al., 2019). By contrary, less T_H1 cell infiltration can be seen in Tg mice receiving FMT from WT mice (Wang et al., 2019). The new discoveries emphasize the abnormal phenylalanine and isoleucine induced by gut microbiota worsening T_H1 cell–mediated inflammation in AD and its models and effectively remodeling the gut microbiota is a novel strategy for AD therapy (**Figure 2**). The therapeutic strategies targeting the gut microbiota in AD patients and animal models are summarized in **Table 3**. Unfortunately, it is still difficult to determine which microbes are special targets for AD therapy currently, because many factors, including diet, place of residence, smoking, ethnicity, etc., can also influence the changes of the gut microbiota in AD.

Targeting dysbiosis of gut microbes as a future therapeutic manipulation in many diseases including AD is a promising therapeutic strategy. The advantages of this manipulation are effective to treat or alleviate diseases. It prevented the recurrence of MS by adding microbes to the daily diet for long-term use (Tremlett et al., 2016) and slowing down the clinical progress in PD patients (Dutta et al., 2019), as well as treated refractory constipation in a PD patient (Huang et al., 2019), etc., in which

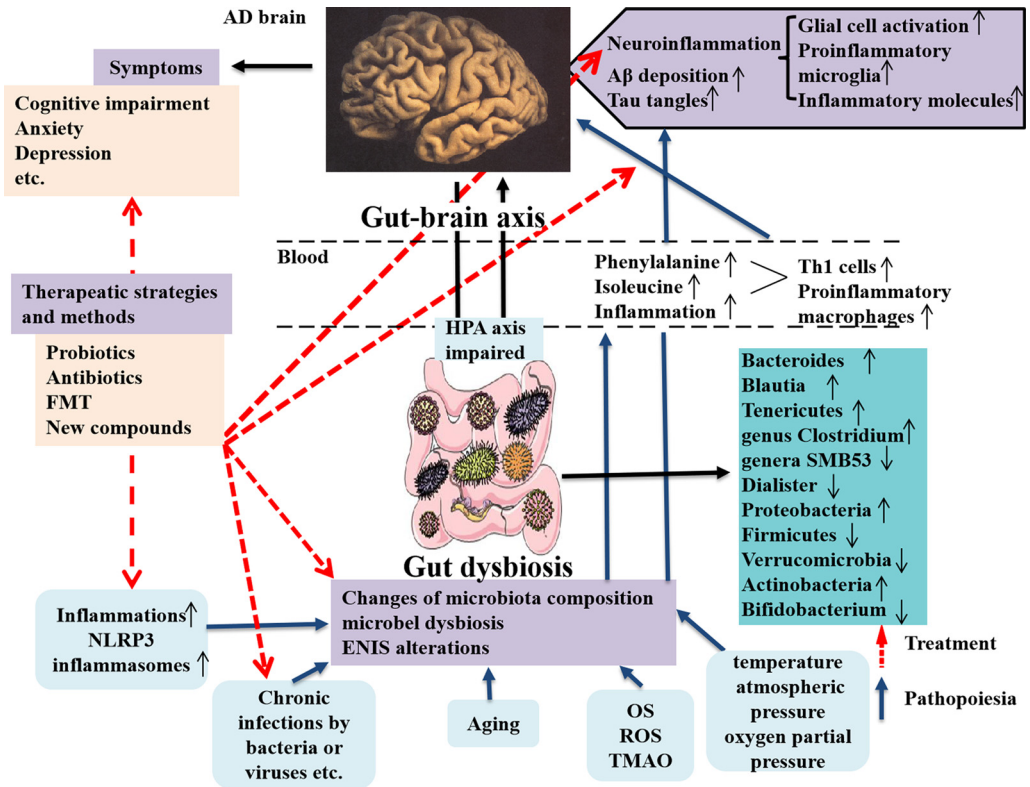

FIGURE 2 | Dysbiosis of gut microbes in AD and the intervention strategy. The communications between the gut microbes and brain is through gut–brain axis to involve in the modulatory processes, inflammation, and immune response to maintain homeostasis of body. The gut microbiota can affect brain function and bioactivity (Liu L. et al., 2019). Several factors including inflammation (such as NLPR3 inflammasomes), chronic infections by bacteria or viruses, aging, and increased inflammatory molecules (OS, ROS, and TMAO) production, etc., can cause the changes of microbiota composition, microbial dysbiosis, and ENIS alterations, which contribute to AD pathology in the brain, including neuroinflammation due to glial cell and M1 microglia activations and increased inflammatory molecules production and enhanced Aβ deposition and tau tangles in the brain, these being the classical pathological features in AD (Angelucci et al., 2019). The microbial dysbiosis also leads to the peripheral accumulation of phenylalanine and isoleucine, which promotes the differentiation and proliferation of proinflammatory T helper 1 cells that infiltrated into the brain of AD mice *via* blood circulation, associated with the M1 microglia activation, contributing to neuroinflammation in AD (Liu P. et al., 2019). AD pathological changes result in a series of clinical symptoms, as cognitive impairment, anxiety, depression, and others. The novel intervention strategies contain applying probiotics, antibiotics, and FMT, as well as using a compound, such as GV-971 and others, which are targeting and remodeling gut microbiota and suppressing gut bacterial amino acids-shaping neuroinflammation to inhibit AD progression. AD, Alzheimer disease; OS, oxidative stress; ROS, reactive oxygen species; TMAO, trimethylamine *n*-oxide; ENIS, enteric neuroimmune system; FMT, fecal microbiota transplantation.

the conventional treatments were helpless. This therapeutic manipulation overcomes the disadvantages of conventional treatments that either overlook the microbes in the mechanism of action or remove vast populations of microbes *via* antibiotics. It aims at rebalancing the gut microbiota balance, preventing dysbiosis, and keeping the internal environment stable. Thus, the therapeutic manipulation is exciting and challenging. However, there are still many problems and disadvantages in microbial treatment. First, the obtained results treated by microbes or microbial products in human disorders are contradictory. Also the sample size treated by microbes is too small, which causes doubt to the effectiveness of the treatments. Second, it is difficult to select the correct therapeutic scheme. Third, although the side effects of the microbial treatment were reported sporadically, the exact side effects were not found because of the small sample size. It is unclear whether microbial therapy can cause other diseases.

There are many ways to balance the microbiota in the gut, such as reducing or inhibiting infections and inflammation of the system and gut, eating a healthy diet, quitting smoking, increasing immunity, and exercise, etc.; thus, balancing the microbiota can be maintained for a long time.

CONCLUSIONS

The gut microbiome may contribute to the pathogenesis of AD and neurodegenerative disorders through the microbiota–gut–brain axis pathway. The dysbiosis of the gut microbiome and resulting inflammation may be important pathogenic factors in development and progression of AD. Targeting and remodeling the gut microbiome open a potential new door to an effective therapeutic strategy in AD patients. However, the field is nascent, and the data obtained are controversial, as well as many factors influence the gut microbiome. It is still difficult to establish the relationship between the gut microbiome and brain bioactivity in any specific disorder of humans *via* the microbiota–gut–brain axis pathway.

TABLE 3 | Therapeutic strategies targeting the gut microbiota in AD animal models and patients.

Pre-clinical and clinical studies	Therapeutic manipulations	Gut microbiota changes after treatments	Results	References
$APP_{SWE}/PS1_{deltaE9}$	ABX	*Lachnospiraceae and S24-7*↑	Brain Aβ deposition↓ Microglia and astrocyte accumulation around amyloid plaques↓ Blood: Foxp3+ T-regulatory cells ↑	Minter et al. (2017)
$APP_{SWE}/PS1_{deltaE9}$	ABX	*Akkermansia* ↑ *Lachnospiraceae*↑	Brain Aβ deposition↓ Brain soluble Aβ↑ Blood: CCL11, CXCL16, LIX, TIMP-1 and NFαR1↑	Minter et al. (2016)
APP/PS1 mice	probiotic	*Eubacteria, Roseburia*↑ *Clostridium*↓	Spatial memory↑ Hippocampus Aβ plaques↓ The numbers of microglia in hippocampus ↓ OOG1 in the hippocampus↓	Abraham et al. (2019)
APP/PS1 mice	OMO	*Lactobacillus, Akkermansia, Bacteroides, Adlercreutzia*, and *Desulfovibrio*↑ *Ruminococcus, Bifidobacterium, Blautia, Oscillospira, Coprococcus, Sutterella*, and *Clostridium*↓	Learning and memory impairments↓ $A\beta_{1-42}$ positive cells in brain↓	Xin et al. (2018)
APP/PS1 mice	FOS	*Epsionproteobacteria, Proteobacteria, Helicobacteraceae, Deferribacteraceae, Helicobacter*↓ *Actinobacteria, Lactobacillus*↑	Cognitive deficits↓ Aβ deposition in the brain↓ synapsin I and PSD-95 in the brain ↑ phosphorylated level of JNK in the brain↓ GLP-1, GLP-1R in the gut↑	Sun et al. (2019a)
5XFAD mice	GV-971	*remodeling the gut microbiota*	Cognitive impairment↓ Aβ and tau phosphorylation in the hippocampus ↓ Brain Th1 cells↓ IBA1 in hippocampal↓ Inhibiting neuroinflammation by harnessing amino acid metabolism	Wang et al. (2019)
D-galactose and $A\beta_{1-42}$-induced Alzheimer's rats	OMO	*Firmicutes, Bacteroidetes, Bacteroidia, Bacilli, Lactobacillales, Bacteroidales, Lactobacillaceaes*↑ *Clostridia, Clostridiales*↓	Learning and memory abilities↑ Swelling of brain tissues and neuronal apoptosis↓ Tau and $A\beta_{1-42}$ expression in brain↓ Tissue damages and inflammation induced by TNBS ↓	Chen et al. (2017)

(Continued)

TABLE 3 | Continued

Pre-clinical and clinical studies	Therapeutic manipulations	Gut microbiota changes after treatments	Results	References
ddY mice (the strain is outbred and has been maintained as a closed colony and a model of postprandial hypertriglyceridemia in response to dietary fat)	B. breve A1	*Phylum Actinobacteria and Bifidobacteriaceae*↑ *Odoribacteraceae and Lachnospiraceae*↓	Prevents Aβ-induced cognitive dysfunction Aβ-induced gene expression changes in the hippocampus↓ Plasma acetate↑	Kobayashi et al. (2017)
D-Galactose-induced Alzheimer's rats.	*Lactobacillus plantarum* MTCC1325		Cognition deficits↓ Aβ in brain↓ Ach and AChE in hippocampus and cerebral cortex↑	Nimgampalle and Kuna (2017)
Wistar rats intrahippocampal injection of $A\beta_{1-42}$	*Lactobacillus acidophilus*, *L. fermentum*, *Bifidobacterium lactis*, and *B. longum*	*Coliform*↓ *Bifidobacterial* and *lactobacilli*↑	Learning and memory abilities↑ MDA in the hippocampus ↓ SOD in the hippocampus ↓	Athari Nik Azm et al. (2018)
AD patients	Probiotic (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum)*		MMSE score↑ hs-CRP, HOMA-IR, HOMA-B and MDA in blood↓ QUICKI↑	Akbari et al. (2016)
AD patients	selenium plus probiotic *(Lactobacillus acidophilus, Bifidobacterium bifidum*, and *Bifidobacterium longum)*		MMSE score↑ hs-CRP, HOMA-IR, LDL-cholesterol, total-/HDL-cholesterol ratio, and QUICKI in blood↓	Tamtaji et al. (2019a)

Abbreviations: ABX, broad-spectrum antibiotics, including gentamicin, vancomycin, metronidazole, neomycin, ampicillin, kanamycin, colistinand and cefaperazone; Ache, Acetylcholinesterase; B. breve A1, Bifidobacterium breve strain A1; FOS, Prebiotic fructooligosaccharides; GLP-1, Glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GV-971, Sodium oligomannate; HOMA-B, Homeostatic model assessment for B-cell function; HOMA-IR, Homeostatic model of assessment for insulin resistance; hs-CRP, High sensitivity C-reactive protein; JNK, C-Jun N-terminal kinase; MDA, Malondialdehyde; OMO, Oligosaccharides from Morinda officinalis; OOG1, 8-oxoguanine DNA glycosylase-1; PSD-95, Postsynaptic density protein 95; QUICKI, Quantitative insulin sensitivity check index; SOD, Superoxide dismutase. ↑:increase; ↓:decrease.

Therefore, the longitudinal study and randomized controlled trials in humans are essential to determine the role of the gut microbiota in AD and other neurological diseases. Finding potent drugs targeting the microbiome may be more promising for future clinical therapeutic strategies.

AUTHOR CONTRIBUTIONS

FZ prepared the manuscript. CL provided views and revised the manuscript. FC and XT helped to correct the manuscript and prepared figures. JZ designed the framework of manuscript, prepared and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Alzheimer's Association. (2017). 2017 Alzheimer's disease facts and figures. *Alzheimers Dement.* 13, 325–373. doi: 10.1016/j.jalz.2017.02.001

Abbas, N., Bednar, I., Mix, E., Marie, S., Paterson, D., Ljungberg, A., et al. (2002). Up-regulation of the inflammatory cytokines IFN-γ and IL-12 and down-regulation of IL-4 in cerebral cortex regions of APP(SWE) transgenic mice. *J. Neuroimmunol.* 126, 50–57. doi: 10.1016/s0165-5728(02)00050-4

Abraham, D., Feher, J., Scuderi, G. L., Szabo, D., Dobolyi, A., Cservenak, M., et al. (2019). Exercise and probiotics attenuate the development of Alzheimer's disease in transgenic mice: role of microbiome. *Exp. Gerontol.* 115, 122–131. doi: 10.1016/j.exger.2018.12.005

Agahi, A., Hamidi, G. A., Daneshvar, R., Hamdieh, M., Soheili, M., Alinaghipour, A., et al. (2018). Does severity of Alzheimer's disease contribute to its responsiveness to modifying gut microbiota? A double blind clinical trial. *Front. Neurol.* 9:662. doi: 10.3389/fneur.2019.00667

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., et al. (2012). Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* 37, 1885–1895. doi: 10.1016/j.psyneuen.2012.03.024

Akbari, E., Asemi, Z., Daneshvar Kakhaki, R., Bahmani, F., Kouchaki, E., Tamtaji, O. R., et al. (2016). Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: a randomized, double-blind and controlled trial. *Front. Aging Neurosci.* 8:256. doi: 10.3389/fnagi.2018.00054

Allegretti, J. R., Kassam, Z., Osman, M., Budree, S., Fischer, M., and Kelly, C. R. (2018). The 5D framework: a clinical primer for fecal microbiota transplantation to treat *Clostridium difficile* infection. *Gastrointest. Endosc.* 87, 18–29. doi: 10.1016/j.gie.2017.05.036

Angelucci, F., Cechova, K., Amlerova, J., and Hort, J. (2019). Antibiotics, gut microbiota, and Alzheimer's disease. *J. Neuroinflammation* 16:108. doi: 10.1186/s12974-019-1494-4

Ashraf, G. M., Tarasov, V. V., Makhmutova, A., Chubarev, V. N., Avila-Rodriguez, M., Bachurin, S. O., et al. (2019). The possibility of an infectious etiology of Alzheimer disease. *Mol. Neurobiol.* 56, 4479–4491. doi: 10.1007/s12035-018-1388-y

Askarova, S., Umbayev, B., Masoud, A. R., Kaiyrlykyzy, A., Safarova, Y., Tsoy, A., et al. (2020). The links between the gut microbiome, aging, modern lifestyle and Alzheimer's disease. *Front. Cell. Infect. Microbiol.* 10:104. doi: 10.3389/fcimb.2020.00104

Athari Nik Azm, S., Djazayeri, A., Safa, M., Azami, K., Ahmadvand, B., Sabbaghziarani, F., et al. (2018). Lactobacilli and bifidobacteria ameliorate memory and learning deficits and oxidative stress in β-amyloid (1–42) injected rats. *Appl. Physiol. Nutr. Metab.* 43, 718–726. doi: 10.1139/apnm-2017-0648

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G., and Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav. Immun.* 25, 397–407. doi: 10.1016/j.bbi.2010.10.023

Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F., and Stanton, C. (2012). γ-aminobutyric acid production by culturable bacteria from the human intestine. *J. Appl. Microbiol.* 113, 411–417. doi: 10.1111/j.1365-2672.2012.05344.x

Bellavance, M.-A., and Rivest, S. (2014). The HPA—immune axis and the immunomodulatory actions of glucocorticoids in the brain. *Front. Immunol.* 5:136. doi: 10.3389/fimmu.2014.00136

Bester, J., Soma, P., Kell, D. B., and Pretorius, E. (2015). Viscoelastic and ultrastructural characteristics of whole blood and plasma in Alzheimer-type dementia and the possible role of bacterial lipopolysaccharides (LPS). *Oncotarget* 6, 35284–35303. doi: 10.18632/oncotarget.6074

Bilen, M., Dufour, J.-C., Lagier, J.-C., Cadoret, F., Daoud, Z., Dubourg, G., et al. (2018). The contribution of culturomics to the repertoire of isolated human bacterial and archaeal species. *Microbiome* 6:94. doi: 10.1186/s40168-018-0485-5

Blaser, M. J. (2019). Fecal microbiota transplantation for dysbiosis—predictable risks. *N. Engl. J. Med.* 381, 2064–2066. doi: 10.1056/nejme1913807

Blum, H. E. (2017). The human microbiome. *Adv. Med. Sci.* 62, 414–420. doi: 10.1016/j.advms.2017.04.005

Bonaz, B., Bazin, T., and Pellissier, S. (2018). The vagus nerve at the interface of the microbiota-gut-brain axis. *Front. Neurosci.* 12:49. doi: 10.3389/fnins.2018.00049

Borre, Y. E., Moloney, R. D., Clarke, G., Dinan, T. G., and Cryan, J. F. (2014). The impact of microbiota on brain and behavior: mechanisms and therapeutic potential. *Adv. Exp. Med. Biol.* 817, 373–403. doi: 10.1007/978-1-4939-0897-4_17

Braakman, H. M. H., and van Ingen, J. (2018). Can epilepsy be treated by antibiotics? *J. Neurol.* 265, 1934–1936. doi: 10.1007/s00415-018-8943-3

Brandscheid, C., Schuck, F., Reinhardt, S., Schäfer, K.-H., Pietrzik, C. U., Grimm, M., et al. (2017). Altered gut microbiome composition and tryptic activity of the 5xFAD Alzheimer's mouse model. *J. Alzheimers Dis.* 56, 775–788. doi: 10.3233/jad-160926

Briguglio, M., Dell'Osso, B., Panzica, G., Malgaroli, A., Banfi, G., Zanaboni Dina, C., et al. (2018). Dietary neurotransmitters: a narrative review on current knowledge. *Nutrients* 10:591. doi: 10.3390/nu10050591

Bronzuoli, M. R., Iacomino, A., Steardo, L., and Scuderi, C. (2016). Targeting neuroinflammation in Alzheimer's disease. *J. Inflamm. Res.* 9, 199–208. doi: 10.2147/jir.s86958

Bu, X.-L., Yao, X.-Q., Jiao, S.-S., Zeng, F., Liu, Y.-H., Xiang, Y., et al. (2015). A study on the association between infectious burden and Alzheimer's disease. *Eur. J. Neurol.* 22, 1519–1525. doi: 10.1111/ene.12477

Calsolaro, V., and Edison, P. (2016). Neuroinflammation in Alzheimer's disease: current evidence and future directions. *Alzheimers Dement.* 12, 719–732. doi: 10.1016/j.jalz.2016.02.010

Cantarel, B. L., Waubant, E., Chehoud, C., Kuczynski, J., DeSantis, T. Z., Warrington, J., et al. (2015). Gut microbiota in multiple sclerosis. *J. Investig. Med.* 63, 729–734. doi: 10.1097/JIM.0000000000000192

Capuco, A., Urits, I., Hasoon, J., Chun, R., Gerald, B., Wang, J. K., et al. (2020). Current perspectives on gut microbiome dysbiosis and depression. *Adv. Ther.* 37, 1328–1346. doi: 10.1007/s12325-020-01272-7

Carabotti, M., Scirocco, A., Maselli, M. A., and Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann. Gastroenterol.* 28, 203–209.

Caracciolo, B., Xu, W., Collins, S., and Fratiglioni, L. (2014). Cognitive decline, dietary factors and gut-brain interactions. *Mech. Ageing Dev.* 136–137, 59–69. doi: 10.1016/j.mad.2013.11.011

Cattaneo, A., Cattane, N., Galluzzi, S., Provasi, S., Lopizzo, N., Festari, C., et al. (2017). Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiol. Aging* 49, 60–68. doi: 10.1016/j.neurobiolaging.2016.08.019

Chen, D., Yang, X., Yang, J., Lai, G., Yong, T., Tang, X., et al. (2017). Prebiotic effect of fructooligosaccharides from *Morinda officinalis* on Alzheimer's disease in rodent models by targeting the microbiota-gut-brain axis. *Front. Aging Neurosci.* 9:403. doi: 10.3389/fnagi.2017.00403

Chu, F., Shi, M., Lang, Y., Shen, D., Jin, T., Zhu, J., et al. (2018). Gut microbiota in multiple sclerosis and experimental autoimmune encephalomyelitis: current applications and future perspectives. *Mediators Inflamm.* 2018:8168717. doi: 10.3389/fmicb.2019.00740

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., et al. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488, 178–184. doi: 10.1038/nature11319

Collins, S. M., Surette, M., and Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nat. Rev. Microbiol.* 10, 735–742. doi: 10.1038/nrmicro2876

Cox, L. M., Schafer, M. J., Sohn, J., Vincentini, J., Weiner, H. L., Ginsberg, S. D., et al. (2019). Calorie restriction slows age-related microbiota changes in an Alzheimer's disease model in female mice. *Sci. Rep.* 9:17904. doi: 10.1038/s41598-019-54187-x

Crane, J. D., Palanivel, R., Mottillo, E. P., Bujak, A. L., Wang, H., Ford, R. J., et al. (2015). Inhibiting peripheral serotonin synthesis reduces obesity and metabolic dysfunction by promoting brown adipose tissue thermogenesis. *Nat. Med.* 21, 166–172. doi: 10.1038/nm.3766

Cryan, J. F., O'Riordan, K. J., Sandhu, K., Peterson, V., and Dinan, T. G. (2020). The gut microbiome in neurological disorders. *Lancet Neurol.* 19, 179–194. doi: 10.1016/S1474-4422(19)30356-4

Daniels, M. J. D., Rivers-Auty, J., Schilling, T., Spencer, N. G., Watremez, W., Fasolino, V., et al. (2016). Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models. *Nat. Commun.* 7:12504. doi: 10.1038/ncomms12504

DeFilipp, Z., Bloom, P. P., Torres Soto, M., Mansour, M. K., Sater, M. R. A., Huntley, M. H., et al. (2019). Drug-resistant *E. coli* bacteremia transmitted by fecal microbiota transplant. *N. Engl. J. Med.* 381, 2043–2050. doi: 10.1056/NEJMoa1910437

Del Tredici, K., Rüb, U., De Vos, R. A. I., Bohl, J. R. E., and Braak, H. (2002). Where does parkinson disease pathology begin in the brain? *J. Neuropathol. Exp. Neurol.* 61, 413–426. doi: 10.1093/jnen/61.5.413

Dinan, T. G., and Cryan, J. F. (2017). The microbiome-gut-brain axis in health and disease. *Gastroenterol. Clin. North Am.* 46, 77–89. doi: 10.1016/j.gtc.2016.09.007

DiSabato, D. J., Quan, N., and Godbout, J. P. (2016). Neuroinflammation: the devil is in the details. *J. Neurochem.* 139, 136–153. doi: 10.1111/jnc.13607

Dubbelaar, M., Kracht, L., Eggen, B. J., and Boddeke, E. W. G. M. (2018). The kaleidoscope of microglial phenotypes. *Front. Immunol.* 9:1753. doi: 10.3389/fimmu.2018.01753

Duffy, S. S., Lees, J. G., and Moalem-Taylor, G. (2014). The contribution of immune and glial cell types in experimental autoimmune encephalomyelitis and multiple sclerosis. *Mult. Scler. Int.* 2014:285245. doi: 10.1155/2014/285245

Dumitrescu, L., Popescu-Olaru, I., Cozma, L., Tulba, D., Hinescu, M. E., Ceafalan, L. C., et al. (2018). Oxidative stress and the microbiota-gut-brain axis. *Oxid. Med. Cell. Longev.* 2018:2406594. doi: 10.1155/2018/2406594

Dutta, S. K., Verma, S., Jain, V., Surapaneni, B. K., Vinayek, R., Phillips, L., et al. (2019). Parkinson's disease: the emerging role of gut dysbiosis, antibiotics, probiotics and fecal microbiota transplantation. *J. Neurogastroenterol. Motil.* 25, 363–376. doi: 10.5056/jnm19044

Erny, D., Hrabě de Angelis, A. L., and Prinz, M. (2017). Communicating systems in the body: how microbiota and microglia cooperate. *Immunology* 150, 7–15. doi: 10.1111/imm.12645

Esquerda-Canals, G., Montoliu-Gaya, L., Güell-Bosch, J., and Villegas, S. (2017). Mouse models of Alzheimer's disease. *J. Alzheimers Dis.* 57, 1171–1183. doi: 10.3233/JAD-170045

Fakhoury, M. (2018). Microglia and astrocytes in Alzheimer's disease: implications for therapy. *Curr. Neuropharmacol.* 16, 508–518. doi: 10.2174/1570159x15666170720095240

Fang, X. (2019). Microbial treatment: the potential application for Parkinson's disease. *Neurol. Sci.* 40, 51–58. doi: 10.1007/s10072-018-3641-6

Fang, X., Wang, X., Yang, S., Meng, F., Wang, X., Wei, H., et al. (2016). Evaluation of the microbial diversity in amyotrophic lateral sclerosis using high-throughput sequencing. *Front. Microbiol.* 7:1479. doi: 10.3389/fmicb.2016.01479

Fasano, A., Visanji, N. P., Liu, L. W., Lang, A. E., and Pfeiffer, R. F. (2015). Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* 14, 625–639. doi: 10.1016/S1474-4422(15)00007-1

Feng, J., Dong, L., Zhang, J., Han, X., Tang, S., Song, L., et al. (2018). Unique expression pattern of KIBRA in the enteric nervous system of APP/PS1 mice. *Neurosci. Lett.* 675, 41–47. doi: 10.1016/j.neulet.2018.03.014

Foster, J. A., Rinaman, L., and Cryan, J. F. (2017). Stress and the gut-brain axis: regulation by the microbiome. *Neurobiol. Stress* 7, 124–136. doi: 10.1016/j.ynstr.2017.03.001

Franceschi, C., Bonafè, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., et al. (2000). Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann. N Y Acad. Sci.* 908, 244–254. doi: 10.1111/j.1749-6632.2000.tb06651.x

Fröhlich, E. E., Farzi, A., Mayerhofer, R., Reichmann, F., Jačan, A., Wagner, B., et al. (2016). Cognitive impairment by antibiotic-induced gut dysbiosis: analysis of gut microbiota-brain communication. *Brain Behav. Immun.* 56, 140–155. doi: 10.1016/j.bbi.2016.02.020

Fung, T. C. (2020). The microbiota-immune axis as a central mediator of gut-brain communication. *Neurobiol. Dis.* 136:104714. doi: 10.1016/j.nbd.2019.104714

Fung, T. C., Olson, C. A., and Hsiao, E. Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nat. Neurosci.* 20, 145–155. doi: 10.1038/nn.4476

Galland, L. (2014). The gut microbiome and the brain. *J. Med. Food* 17, 1261–1272. doi: 10.1089/jmf.2014.7000

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., et al. (1995). Alzheimer-type neuropathology in transgenic mice overexpressing V717F β-amyloid precursor protein. *Nature* 373, 523–527. doi: 10.1038/373523a0

Gareau, M. G., Wine, E., Rodrigues, D. M., Cho, J. H., Whary, M. T., Philpott, D. J., et al. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut* 60, 307–317. doi: 10.1136/gut.2009.202515

Gershon, M. D. (1999). The enteric nervous system: a second brain. *Hosp. Pract.* 34, 31–32, 35–38, 41–32passim. doi: 10.3810/hp.1999.07.153

Haghikia, A., Jörg, S., Duscha, A., Berg, J., Manzel, A., Waschbisch, A., et al. (2016). Dietary fatty acids directly impact central nervous system autoimmunity *via* the small intestine. *Immunity* 44, 951–953. doi: 10.1016/j.immuni.2015.09.007

Harach, T., Marungruang, N., Duthilleul, N., Cheatham, V., Mc Coy, K. D., Frisoni, G., et al. (2017). Reduction of Aβ amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. *Sci. Rep.* 7:41802. doi: 10.1038/srep46856

Haran, J. P., Bhattarai, S. K., Foley, S. E., Dutta, P., Ward, D. V., Bucci, V., et al. (2019). Alzheimer's disease microbiome is associated with dysregulation of the anti-inflammatory P-glycoprotein pathway. *mBio* 10:e00632-19. doi: 10.1128/mbio.00632-19

Harris, S. A., and Harris, E. A. (2015). Herpes simplex virus type 1 and other pathogens are key causative factors in sporadic Alzheimer's disease. *J. Alzheimers Dis.* 48, 319–353. doi: 10.3233/jad-142853

Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., et al. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493, 674–678. doi: 10.1038/nature11729

Hu, X., Wang, T., and Jin, F. (2016). Alzheimer's disease and gut microbiota. *Sci. China Life Sci.* 59, 1006–1023. doi: 10.1007/s11427-016-5083-9

Huang, H., Xu, H., Luo, Q., He, J., Li, M., Chen, H., et al. (2019). Fecal microbiota transplantation to treat Parkinson's disease with constipation: a case report. *Medicine* 98:e16163. doi: 10.1097/md.0000000000016163

Hueston, C. M., and Deak, T. (2014). The inflamed axis: the interaction between stress, hormones and the expression of inflammatory-related genes within key

structures comprising the hypothalamic-pituitary-adrenal axis. *Physiol. Behav.* 124, 77–91. doi: 10.1016/j.physbeh.2013.10.035

Hugon, P., Dufour, J.-C., Colson, P., Fournier, P.-E., Sallah, K., and Raoult, D. (2015). A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect. Dis.* 15, 1211–1219. doi: 10.1016/s1473-3099(15)00293-5

Ianiro, G., Tilg, H., and Gasbarrini, A. (2016). Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut* 65, 1906–1915. doi: 10.1136/gutjnl-2016-312297

Ising, C., Venegas, C., Zhang, S., Scheiblich, H., Schmidt, S. V., Vieira-Saecker, A., et al. (2019). NLRP3 inflammasome activation drives tau pathology. *Nature* 575, 669–673. doi: 10.1038/s41586-019-1769-z

Jangi, S., Gandhi, R., Cox, L. M., Li, N., von Glehn, F., Yan, R., et al. (2016). Alterations of the human gut microbiome in multiple sclerosis. *Nat. Commun.* 7:12015. doi: 10.1038/ncomms12015

Jernberg, C., Löfmark, S., Edlund, C., and Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J.* 1, 56–66. doi: 10.1038/ismej.2007.3

Jiang, C., Li, G., Huang, P., Liu, Z., and Zhao, B. (2017). The gut microbiota and Alzheimer's disease. *J. Alzheimers Dis.* 58, 1–15. doi: 10.3233/JAD-161141

Joachim, C. L., Mori, H., and Selkoe, D. J. (1989). Amyloid β-protein deposition in tissues other than brain in Alzheimer's disease. *Nature* 341, 226–230. doi: 10.1038/341226a0

Johnson, K. V. A., and Foster, K. R. (2018). Why does the microbiome affect behaviour? *Nat. Rev. Microbiol.* 16, 647–655. doi: 10.1038/s41579-018-0014-3

Jones, R. M., Mercante, J. W., and Neish, A. S. (2012). Reactive oxygen production induced by the gut microbiota: pharmacotherapeutic implications. *Curr. Med. Chem.* 19, 1519–1529. doi: 10.2174/092986712799828283

Kamphuis, W., Kooijman, L., Schetters, S., Orre, M., and Hol, E. M. (2016). Transcriptional profiling of CD11c-positive microglia accumulating around amyloid plaques in a mouse model for Alzheimer's disease. *Biochim. Biophys. Acta* 1862, 1847–1860. doi: 10.1016/j.bbadis.2016.07.007

Kowalski, K., and Mulak, A. (2019). Brain-gut-microbiota axis in Alzheimer's disease. *J. Neurogastroenterol. Motil.* 25, 48–60. doi: 10.5056/jnm18087

Kim, M.-S., Kim, Y., Choi, H., Kim, W., Park, S., Lee, D., et al. (2020). Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model. *Gut* 69, 283–294. doi: 10.1136/gutjnl-2018-317431

Kobayashi, Y., Sugahara, H., Shimada, K., Mitsuyama, E., Kuhara, T., Yasuoka, A., et al. (2017). Therapeutic potential of *Bifidobacterium* breve strain A1 for preventing cognitive impairment in Alzheimer's disease. *Sci. Rep.* 7:13510. doi: 10.1038/s41598-017-13368-2

Kong, G., Cao, K.-A. L., Judd, L. M., Li, S., Renoir, T., and Hannan, A. J. (2020). Microbiome profiling reveals gut dysbiosis in a transgenic mouse model of Huntington's disease. *Neurobiol. Dis.* 135:104268. doi: 10.1016/j.nbd.2018.09.001

Kountouras, J., Boziki, M., Gavalas, E., Zavos, C., Grigoriadis, N., Deretzi, G., et al. (2009). Eradication of *Helicobacter pylori* may be beneficial in the management of Alzheimer's disease. *J. Neurol.* 256, 758–767. doi: 10.1007/s00415-009-5011-z

Lach, G., Schellekens, H., Dinan, T. G., and Cryan, J. F. (2018). Anxiety, depression, and the microbiome: a role for gut peptides. *Neurotherapeutics* 15, 36–59. doi: 10.1007/s13311-017-0585-0

Lambert, J.-C., Heath, S., Even, G., Campion, D., Sleegers, K., Hiltunen, M., et al. (2009). Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat. Genet.* 41, 1094–1099. doi: 10.1038/ng.439

Leblhuber, F., Geisler, S., Steiner, K., Fuchs, D., and Schütz, B. (2015). Elevated fecal calprotectin in patients with Alzheimer's dementia indicates leaky gut. *J. Neural Transm.* 122, 1319–1322. doi: 10.1007/s00702-015-1381-9

Leblhuber, F., Steiner, K., Schuetz, B., Fuchs, D., and Gostner, J. M. (2018). Probiotic supplementation in patients with Alzheimer's dementia—an explorative intervention study. *Curr. Alzheimer Res.* 15, 1106–1113. doi: 10.2174/1389200219666180813144834

Levy, M., Kolodziejczyk, A. A., Thaiss, C. A., and Elinav, E. (2017). Dysbiosis and the immune system. *Nat. Rev. Immunol.* 17, 219–232. doi: 10.1038/nri.2017.7

Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., et al. (2014). An integrated catalog of reference genes in the human gut microbiome. *Nat. Biotechnol.* 32, 834–841. doi: 10.1038/nbt.2942

Liu, L., Gong, T., Tao, W., Lin, B., Li, C., Zheng, X., et al. (2019). Commensal viruses maintain intestinal intraepithelial lymphocytes *via* noncanonical RIG-I signaling. *Nat. Immunol.* 20, 1681–1691. doi: 10.1038/s41590-019-0513-z

Liu, J., He, Z., Ma, N., and Chen, Z.-Y. (2020). Beneficial effects of dietary polyphenols on high-fat diet-induced obesity linking with modulation of gut microbiota. *J. Agric. Food Chem.* 68, 33–47. doi: 10.1021/acs.jafc.9b06817

Liu, P., Wu, L., Peng, G., Han, Y., Tang, R., Ge, J., et al. (2019). Altered microbiomes distinguish Alzheimer's disease from amnestic mild cognitive impairment and health in a Chinese cohort. *Brain Behav. Immun.* 80, 633–643. doi: 10.1016/j.bbi.2019.05.008

Loeb, M. B., Molloy, D. W., Smieja, M., Standish, T., Goldsmith, C. H., Mahony, J., et al. (2004). A randomized, controlled trial of doxycycline and rifampin for patients with Alzheimer's disease. *J. Am. Geriatr. Soc.* 52, 381–387. doi: 10.1111/j.1532-5415.2004.52109.x

Logsdon, A. F., Erickson, M. A., Rhea, E. M., Salameh, T. S., and Banks, W. A. (2018). Gut reactions: how the blood-brain barrier connects the microbiome and the brain. *Exp. Biol. Med.* 243, 159–165. doi: 10.1177/1535370217743766

Long-Smith, C., O'Riordan, K. J., Clarke, G., Stanton, C., Dinan, T. G., and Cryan, J. F. (2020). Microbiota-gut-brain axis: new therapeutic opportunities. *Annu. Rev. Pharmacol. Toxicol.* 60, 477–502. doi: 10.1146/annurev-pharmtox-010919-023628

Luczynski, P., McVey Neufeld, K.-A., Oriach, C. S., Clarke, G., Dinan, T. G., and Cryan, J. F. (2016). Growing up in a bubble: using germ-free animals to assess the influence of the gut microbiota on brain and behavior. *Int. J. Neuropsychopharmacol.* 19:pyw020. doi: 10.1093/ijnp/pyw020

Lyte, M. (2013). Microbial endocrinology in the microbiome-gut-brain axis: how bacterial production and utilization of neurochemicals influence behavior. *PLoS Pathog.* 9:e1003726. doi: 10.1371/journal.ppat.1003726

Ma, S., Santhosh, D., Kumar, T. P., and Huang, Z. (2017). A brain-region-specific neural pathway regulating germinal matrix angiogenesis. *Dev. Cell* 41, 366.e4–381.e4. doi: 10.1016/j.devcel.2017.04.014

MahmoudianDehkordi, S., Arnold, M., Nho, K., Ahmad, S., Jia, W., Xie, G., et al. (2019). Altered bile acid profile associates with cognitive impairment in Alzheimer's disease-An emerging role for gut microbiome. *Alzheimers Dement.* 15, 76–92. doi: 10.1016/j.jalz.2019.03.002

Maslowski, K. M., Vieira, A. T., Ng, A., Kranich, J., Sierro, F., Yu, D., et al. (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 461, 1282–1286. doi: 10.1038/nature08530

Mayer, E. A., Tillisch, K., and Gupta, A. (2015). Gut/brain axis and the microbiota. *J. Clin. Invest.* 125, 926–938. doi: 10.1172/JCI76304

Mazzini, L., Mogna, L., De Marchi, F., Amoruso, A., Pane, M., et al. (2018). "Potential role of gut microbiota in als pathogenesis and possible novel therapeutic strategies. *J. Clin. Gastroenterol* 52 Suppl 1," in *Proceedings from the 9th Probiotics, Prebiotics and New Foods, Nutraceuticals and Botanicals for Nutrition and Human and Microbiota Health Meeting, Held in Rome, Italy from September 10 to 12, 2017*, S68–S70.

Merino, J., Aller, M. A., Rubio, S., Arias, N., Nava, M. P., Loscertales, M., et al. (2011). Gut-brain chemokine changes in portal hypertensive rats. *Dig. Dis. Sci.* 56, 2309–2317. doi: 10.1007/s10620-011-1625-y

Minato, T., Maeda, T., Fujisawa, Y., Tsuji, H., Nomoto, K., Ohno, K., et al. (2017). Progression of Parkinson's disease is associated with gut dysbiosis: two-year follow-up study. *PLoS One* 12:e0187307. doi: 10.1371/journal.pone.0187307

Minter, M. R., Hinterleitner, R., Meisel, M., Zhang, C., Leone, V., Zhang, X., et al. (2017). Antibiotic-induced perturbations in microbial diversity during post-natal development alters amyloid pathology in an aged APP/PS1 murine model of Alzheimer's disease. *Sci. Rep.* 7:10411. doi: 10.1038/s41598-017-11047-w

Minter, M. R., Zhang, C., Leone, V., Ringus, D. L., Zhang, X., Oyler-Castrillo, P., et al. (2016). Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. *Sci. Rep.* 6:30028. doi: 10.1038/srep30028

Miyake, S., Kim, S., Suda, W., Oshima, K., Nakamura, M., Matsuoka, T., et al. (2015). Dysbiosis in the gut microbiota of patients with multiple sclerosis, with a striking depletion of species belonging to clostridia XIVa and IV clusters. *PLoS One* 10:e0137429. doi: 10.1371/journal.pone.0137429

Molloy, D. W., Standish, T. I., Zhou, Q., and Guyatt, G. (2013). A multicenter, blinded, randomized, factorial controlled trial of doxycycline and rifampin for treatment of Alzheimer's disease: the DARAD trial. *Int. J. Geriatr. Psychiatry* 28, 463–470. doi: 10.1002/gps.3846

Monsonego, A., Zota, V., Karni, A., Krieger, J. I., Bar-Or, A., Bitan, G., et al. (2003). Increased T cell reactivity to amyloid β protein in older humans and patients with Alzheimer disease. *J. Clin. Invest.* 112, 415–422. doi: 10.1172/jci200318104

Mowry, E. M., and Glenn, J. D. (2018). The dynamics of the gut microbiome in multiple sclerosis in relation to disease. *Neurol. Clin.* 36, 185–196. doi: 10.1016/j.ncl.2017.08.008

Mukherjee, S., Joardar, N., Sengupta, S., and Sinha Babu, S. P. (2018). Gut microbes as future therapeutics in treating inflammatory and infectious diseases: lessons from recent findings. *J. Nutr. Biochem.* 61, 111–128. doi: 10.1016/j.jnutbio.2018.07.010

Murono, S., Hamaguchi, T., Yoshida, H., Nakanishi, Y., Tsuji, A., Endo, K., et al. (2015). Evaluation of dysphagia at the initial diagnosis of amyotrophic lateral sclerosis. *Auris Nasus Larynx* 42, 213–217. doi: 10.1016/j.anl.2014.10.012

Neufeld, N. H., Mohamed, N. S., Grujich, N., and Shulman, K. (2017). Acute neuropsychiatric symptoms associated with antibiotic treatment of *Helicobacter pylori* infections: a review. *J. Psychiatr. Pract.* 23, 25–35. doi: 10.1097/pra.0000000000000205

Niina, A., Kibe, R., Suzuki, R., Yuchi, Y., Teshima, T., Matsumoto, H., et al. (2019). Improvement in clinical symptoms and fecal microbiome after fecal microbiota transplantation in a dog with inflammatory bowel disease. *Vet. Med.* 10, 197–201. doi: 10.2147/vmrr.s230862

Nimgampalle, M., and Kuna, Y. (2017). Anti-alzheimer properties of probiotic, lactobacillus plantarum MTCC 1325 in Alzheimer's disease induced albino rats. *J. Clin. Diagn. Res.* 11, KC01–KC05. doi: 10.7860/jcdr/2017/26106.10428

Olszak, T., An, D., Zeissig, S., Vera, M. P., Richter, J., Franke, A., et al. (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336, 489–493. doi: 10.1126/science.1219328

Panza, F., Lozupone, M., Solfrizzi, V., Watling, M., and Imbimbo, B. P. (2019). Time to test antibacterial therapy in Alzheimer's disease. *Brain* 142, 2905–2929. doi: 10.1093/brain/awz244

Park, A. J., Collins, J., Blennerhassett, P. A., Ghia, J. E., Verdu, E. F., Bercik, P., et al. (2013). Altered colonic function and microbiota profile in a mouse model of chronic depression. *Neurogastroenterol. Motil.* 25:733-e575. doi: 10.1111/nmo.12153

Pellegrini, C., Antonioli, L., Colucci, R., Blandizzi, C., and Fornai, M. (2018). Interplay among gut microbiota, intestinal mucosal barrier and enteric neuro-immune system: a common path to neurodegenerative diseases? *Acta Neuropathol.* 136, 345–361. doi: 10.1007/s00401-018-1856-5

Pellegrini, C., Fornai, M., Antonioli, L., Blandizzi, C., and Calderone, V. (2019). Phytochemicals as novel therapeutic strategies for NLRP3 inflammasome-related neurological, metabolic, and inflammatory diseases. *Int. J. Mol. Sci.* 20:2876. doi: 10.3390/ijms20122876

Picca, A., Fanelli, F., Calvani, R., Mulè, G., Pesce, V., Sisto, A., et al. (2018). Gut dysbiosis and muscle aging: searching for novel targets against sarcopenia. *Mediators Inflamm.* 2018:7026198. doi: 10.1155/2018/7026198

Pistollato, F., Sumalla Cano, S., Elio, I., Masias Vergara, M., Giampieri, F., and Battino, M. (2016). Role of gut microbiota and nutrients in amyloid formation and pathogenesis of Alzheimer disease. *Nutr. Rev.* 74, 624–634. doi: 10.1093/nutrit/nuw023

Puig, K. L., Lutz, B. M., Urquhart, S. A., Rebel, A. A., Zhou, X., Manocha, G. D., et al. (2015). Overexpression of mutant amyloid-β protein precursor and presenilin 1 modulates enteric nervous system. *J. Alzheimers Dis.* 44, 1263–1278. doi: 10.3233/JAD-142259

Rogers, J., Luber-Narod, J., Styren, S. D., and Civin, W. H. (1988). Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol. Aging* 9, 339–349. doi: 10.1016/s0197-4580(88)80079-4

Roubaud-Baudron, C., Krolak-Salmon, P., Quadrio, I., Mégraud, F., and Salles, N. (2012). Impact of chronic *Helicobacter pylori* infection on Alzheimer's disease: preliminary results. *Neurobiol. Aging* 33, 1009.e11–1009.e19. doi: 10.1016/j.neurobiolaging.2011.10.021

Rumah, K. R., Vartanian, T. K., and Fischetti, V. A. (2017). Oral multiple sclerosis drugs inhibit the *in vitro* growth of epsilon toxin producing gut bacterium, *Clostridium perfringens*. *Front. Cell. Infect. Microbiol.* 7:11. doi: 10.3389/fcimb.2017.00011

Salter, M. W., and Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nat. Med.* 23, 1018–1027. doi: 10.1038/nm.4397

Scheperjans, F., Aho, V., Pereira, P. A., Koskinen, K., Paulin, L., Pekkonen, E., et al. (2015). Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord.* 30, 350–358. doi: 10.1002/mds.26069

Schneider, S., Wright, C. M., and Heuckeroth, R. O. (2019). Unexpected roles for the second brain: enteric nervous system as master regulator of bowel function. *Annu. Rev. Physiol.* 81, 235–259. doi: 10.1146/annurev-physiol-021317-121515

Scott, K. A., Ida, M., Peterson, V. L., Prenderville, J. A., Moloney, G. M., Izumo, T., et al. (2017). Revisiting Metchnikoff: age-related alterations in microbiota-gut-brain axis in the mouse. *Brain Behav. Immun.* 65, 20–32. doi: 10.1016/j.bbi.2017.02.004

Semar, S., Klotz, M., Letiembre, M., Van Ginneken, C., Braun, A., Jost, V., et al. (2013). Changes of the enteric nervous system in amyloid-β protein precursor transgenic mice correlate with disease progression. *J. Alzheimers Dis.* 36, 7–20. doi: 10.1002/9780470988756.ch1

Shahi, S. K., Freedman, S. N., and Mangalam, A. K. (2017). Gut microbiome in multiple sclerosis: the players involved and the roles they play. *Gut Microbes* 8, 607–615. doi: 10.1080/19490976.2017.1349041

Shapiro, H., Thaiss, C. A., Levy, M., and Elinav, E. (2014). The cross talk between microbiota and the immune system: metabolites take center stage. *Curr. Opin. Immunol.* 30, 54–62. doi: 10.1016/j.coi.2014.07.003

Shen, L., Liu, L., and Ji, H.-F. (2017). Alzheimer's disease histological and behavioral manifestations in transgenic mice correlate with specific gut microbiome state. *J. Alzheimers Dis.* 56, 385–390. doi: 10.3233/jad-160884

Silverman, M. N., and Sternberg, E. M. (2012). Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. *Ann. N Y Acad. Sci.* 1261, 55–63. doi: 10.1111/j.1749-6632.2012.06633.x

Sochocka, M., Donskow-Lysoniewska, K., Diniz, B. S., Kurpas, D., Brzozowska, E., and Leszek, J. (2019). The gut microbiome alterations and inflammation-driven pathogenesis of Alzheimer's disease-a critical review. *Mol. Neurobiol.* 56, 1841–1851. doi: 10.1007/s12035-018-1188-4

Stenman, L. K., Holma, R., Eggert, A., and Korpela, R. (2013). A novel mechanism for gut barrier dysfunction by dietary fat: epithelial disruption by hydrophobic bile acids. *Am. J. Physiol. Gastrointest. Liver Physiol.* 304, G227–G234. doi: 10.1152/ajpgi.00267.2012

Sun, J., Liu, S., Ling, Z., Wang, F., Ling, Y., Gong, T., et al. (2019a). Fructooligosaccharides ameliorating cognitive deficits and neurodegeneration in APP/PS1 transgenic mice through modulating gut microbiota. *J. Agric. Food Chem.* 67, 3006–3017. doi: 10.1021/acs.jafc.8b07313

Sun, J., Xu, J., Ling, Y., Wang, F., Gong, T., Yang, C., et al. (2019b). Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Transl. Psychiatry* 9:189. doi: 10.1038/s41398-019-0525-3

Sun, M.-F., Zhu, Y.-L., Zhou, Z.-L., Jia, X.-B., Xu, Y.-D., Yang, Q., et al. (2018). Neuroprotective effects of fecal microbiota transplantation on MPTP-induced Parkinson's disease mice: gut microbiota, glial reaction and TLR4/TNF-α signaling pathway. *Brain Behav. Immun.* 70, 48–60. doi: 10.1016/j.bbi.2018.02.005

Swidsinski, A., Loening-Baucke, V., Lochs, H., and Hale, L.-P. (2005). Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence *in situ* hybridization study in mice. *World J. Gastroenterol.* 11, 1131–1140. doi: 10.3748/wjg.v11.i8.1131

Tamtaji, O. R., Heidari-Soureshjani, R., Mirhosseini, N., Kouchaki, E., Bahmani, F., Aghadavod, E., et al. (2019a). Probiotic and selenium co-supplementation and the effects on clinical, metabolic and genetic status in Alzheimer's disease: a randomized, double-blind, controlled trial. *Clin. Nutr.* 38, 2569–2575. doi: 10.1016/j.clnu.2018.11.034

Tamtaji, O. R., Taghizadeh, M., Daneshvar Kakhaki, R., Kouchaki, E., Bahmani, F., Borzabadi, S., et al. (2019b). Clinical and metabolic response to probiotic administration in people with Parkinson's disease: a randomized, double-blind, placebo-controlled trial. *Clin. Nutr.* 38, 1031–1035. doi: 10.1016/j.clnu.2018.05.018

Tang, Y., and Le, W. (2016). Differential roles of M1 and M2 microglia in neurodegenerative diseases. *Mol. Neurobiol.* 53, 1181–1194. doi: 10.1007/s12035-014-9070-5

Tejera, D., Mercan, D., Sanchez-Caro, J. M., Hanan, M., Greenberg, D., Soreq, H., et al. (2019). Systemic inflammation impairs microglial Aβ clearance through NLRP3 inflammasome. *EMBO J.* 38:e101064. doi: 10.15252/embj.2018101064

Thaiss, C. A., Levy, M., Korem, T., Dohnalová, L., Shapiro, H., Jaitin, D. A., et al. (2016). Microbiota diurnal rhythmicity programs host transcriptome oscillations. *Cell* 167, 1495.e12–1510.e12. doi: 10.1016/j.cell.2016.11.003

Thomson, P., Medina, D. A., and Garrido, D. (2018). Human milk oligosaccharides and infant gut bifidobacteria: molecular strategies for their utilization. *Food Microbiol.* 75, 37–46. doi: 10.1016/j.fm.2017.09.001

Ticinesi, A., Nouvenne, A., Tana, C., Prati, B., and Meschi, T. (2019). Gut microbiota and microbiota-related metabolites as possible biomarkers of cognitive aging. *Adv. Exp. Med. Biol.* 1178, 129–154. doi: 10.1007/978-3-030-25650-0_8

Tognini, P. (2017). Gut microbiota: a potential regulator of neurodevelopment. *Front. Cell. Neurosci.* 11:25. doi: 10.3389/fncel.2017.00025

Togo, T., Akiyama, H., Iseki, E., Kondo, H., Ikeda, K., Kato, M., et al. (2002). Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. *J. Neuroimmunol.* 124, 83–92. doi: 10.1016/s0165-5728(01)00496-9

Tremlett, H., Fadrosh, D. W., Faruqi, A. A., Hart, J., Roalstad, S., Graves, J., et al. (2016). Gut microbiota composition and relapse risk in pediatric MS: a pilot study. *J. Neurol. Sci.* 363, 153–157. doi: 10.1016/j.jns.2016.02.042

Tremlett, H., Bauer, K. C., Appel-Cresswell, S., Finlay, B. B., and Waubant, E. (2017). The gut microbiome in human neurological disease: a review. *Ann. Neurol.* 81, 369–382. doi: 10.1002/ana.24901

Vogt, N. M., Kerby, R. L., Dill-McFarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., et al. (2017). Gut microbiome alterations in Alzheimer's disease. *Sci. Rep.* 7:13537. doi: 10.1038/s41598-017-13601-y

Vogt, N. M., Romano, K. A., Darst, B. F., Engelman, C. D., Johnson, S. C., Carlsson, C. M., et al. (2018). The gut microbiota-derived metabolite trimethylamine N-oxide is elevated in Alzheimer's disease. *Alzheimers Res. Ther.* 10:124. doi: 10.1186/s13195-018-0451-2

Wang, T., Hu, X., Liang, S., Li, W., Wu, X., Wang, L., et al. (2015). Lactobacillus fermentum NS9 restores the antibiotic induced physiological and psychological abnormalities in rats. *Benef. Microbes* 6, 707–717. doi: 10.3920/bm2014.0177

Wang, X., Sun, G., Feng, T., Zhang, J., Huang, X., Wang, T., et al. (2019). Sodium oligomannate therapeutically remodels gut microbiota and suppresses gut bacterial amino acids-shaped neuroinflammation to inhibit Alzheimer's disease progression. *Cell Res.* 29, 787–803. doi: 10.1038/s41422-019-0216-x

Wang, H.-X., and Wang, Y.-P. (2016). Gut microbiota-brain Axis. *Chin. Med. J.* 129, 2373–2380. doi: 10.4103/0366-6999.190667

Wang, X.-L., Zeng, J., Yang, Y., Xiong, Y., Zhang, Z.-H., Qiu, M., et al. (2015). *Helicobacter pylori* filtrate induces Alzheimer-like tau hyperphosphorylation by activating glycogen synthase kinase-3β. *J. Alzheimers Dis.* 43, 153–165. doi: 10.3233/jad-140198

Weinstein, L. I., Revuelta, A., and Pando, R. H. (2015). Catecholamines and acetylcholine are key regulators of the interaction between microbes and the immune system. *Ann. N Y Acad. Sci.* 1351, 39–51. doi: 10.1111/nyas.12792

Wekerle, H. (2016). The gut-brain connection: triggering of brain autoimmune disease by commensal gut bacteria. *Rheumatology* 55, ii68–ii75. doi: 10.1093/rheumatology/kew353

Xin, Y., Diling, C., Jian, Y., Ting, L., Guoyan, H., Hualun, L., et al. (2018). Effects of oligosaccharides from morinda officinalis on gut microbiota and metabolome of APP/PS1 transgenic mice. *Front. Neurol.* 9:412. doi: 10.3389/fneur.2018.00412

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., et al. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* 161, 264–276. doi: 10.1016/j.cell.2015.02.047

Yeh, F. L., Hansen, D. V., and Sheng, M. (2017). TREM2, microglia and neurodegenerative diseases. *Trends Mol. Med.* 23, 512–533. doi: 10.1016/j.molmed.2017.03.008

Yu, T., Hong, Y., Bo, Z., Dan, W., Bo, L., Jie, Z., et al. (2019). Promising neuroprotective function for M2 microglia in Kainic Acid-induced neurotoxicity *via* the down-regulation of NF-κB and caspase 3 signaling pathways. *Neuroscience* 406, 86–96. doi: 10.1016/j.neuroscience.2019.03.002

Zac-Varghese, S., Tan, T., and Bloom, S. R. (2010). Hormonal interactions between gut and brain. *Discov. Med.* 10, 543–552.

Zarrinpar, A., Chaix, A., Xu, Z. Z., Chang, M. W., Marotz, C. A., Saghatelian, A., et al. (2018). Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. *Nat. Commun.* 9:2872. doi: 10.1038/s41467-018-05336-9

Zhang, X., Dan, W., Bo, Z., Jie, Z., Zhulin, Z., and Li, C. (2020). Regulation of microglia by glutamate and its signal pathway in the neurodegenerative diseases. *Drug Discov. Today* 6, 1074–1085. doi: 10.1016/j.drudis.2020.04.001

Zhang, Y., Huang, R., Cheng, M., Wang, L., Chao, J., Li, J., et al. (2019). Gut microbiota from NLRP3-deficient mice ameliorates depressive-like behaviors by regulating astrocyte dysfunction *via* circHIPK2. *Microbiome* 7:116. doi: 10.1186/s40168-019-0733-3

Zhao, Y., and Lukiw, W. J. (2015). Microbiome-generated amyloid and potential impact on amyloidogenesis in Alzheimer's disease (AD). *J. Nat. Sci.* 1:e138.

Zhu, S., Jiang, Y., Xu, K., Cui, M., Ye, W., Zhao, G., et al. (2020). The progress of gut microbiome research related to brain disorders. *J. Neuroinflammation* 17:25. doi: 10.1186/s12974-020-1705-z

Zhuang, Z.-Q., Shen, L.-L., Li, W.-W., Fu, X., Zeng, F., Gui, L., et al. (2018). Gut microbiota is altered in patients with Alzheimer's disease. *J. Alzheimers Dis.* 63, 1337–1346. doi: 10.3233/JAD-180176

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Glycyrrhizic Acid Improves Cognitive Levels of Aging Mice by Regulating T/B Cell Proliferation

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Glycyrrhizic acid (GA) is the substance with the highest content of triterpenoid saponins that can be extracted from licorice, and has anti-inflammatory, neuroprotective, and anticancer functions, among others. The aim of this study was to investigate the protective effect of GA on cognitive decline in middle-aged mice and explore its mechanisms. We injected GA by the tail vein of C57BL/6 mice and measured their cognitive levels using the Morris water maze. The Morris water maze results demonstrated that GA improved learning and memory abilities in middle-aged mice. Furthermore, the RNA-sequencing and flow cytometric analyses revealed that GA could increase T and B cells. We then confirmed the relationship between cognition and the immune system in the immune-deficient B-NDG mouse model. Our results suggest that GA improves cognition in aging mice by regulating T/B cell proliferation.

Keywords: glycyrrhizic acid, learning and memory, cognition, T cells, B cells

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INTRODUCTION

Cognitive decline is a characteristic of human aging, and age-related deterioration of learning and memory also occurs in rats (Frick et al., 2000). Therefore, age-related decline in spatial memory has been extensively studied in rats. These studies have shown that deteriorations in spatial learning and memory functions can be observed after 4–5 months of age (Shoji et al., 2016), and these functions decline from 12 months of age in C57BL/6J mice (Bach et al., 1999). Therefore, preventing age-related decline in middle-aged mice has important applications for humans.

Licorice is the root of *Glycyrrhiza glabra* L. (Leguminosae), which grows in various warm climates such as the Middle East, Asia, and Southern Europe. It is one of the oldest known medicinal herbs and is referred to as "the father of herbal medicine." Glycyrrhizic acid (GA) (**Figure 1A**), a triterpenoid saponin, is a major component of licorice. It has a variety of pharmacological activities such as anti-inflammatory, antioxidant, anticancer, neuroprotective, and immune-regulatory effects, among others (Wang et al., 2020). Previous studies indicated that GA produces robust neuroprotection via the modulation of anti-apoptotic and pro-apoptotic factors, primarily through the ERK signaling pathway and its anti-inflammatory properties against high-mobility group box 1 phosphorylation and the suppression of inflammatory cytokine induction

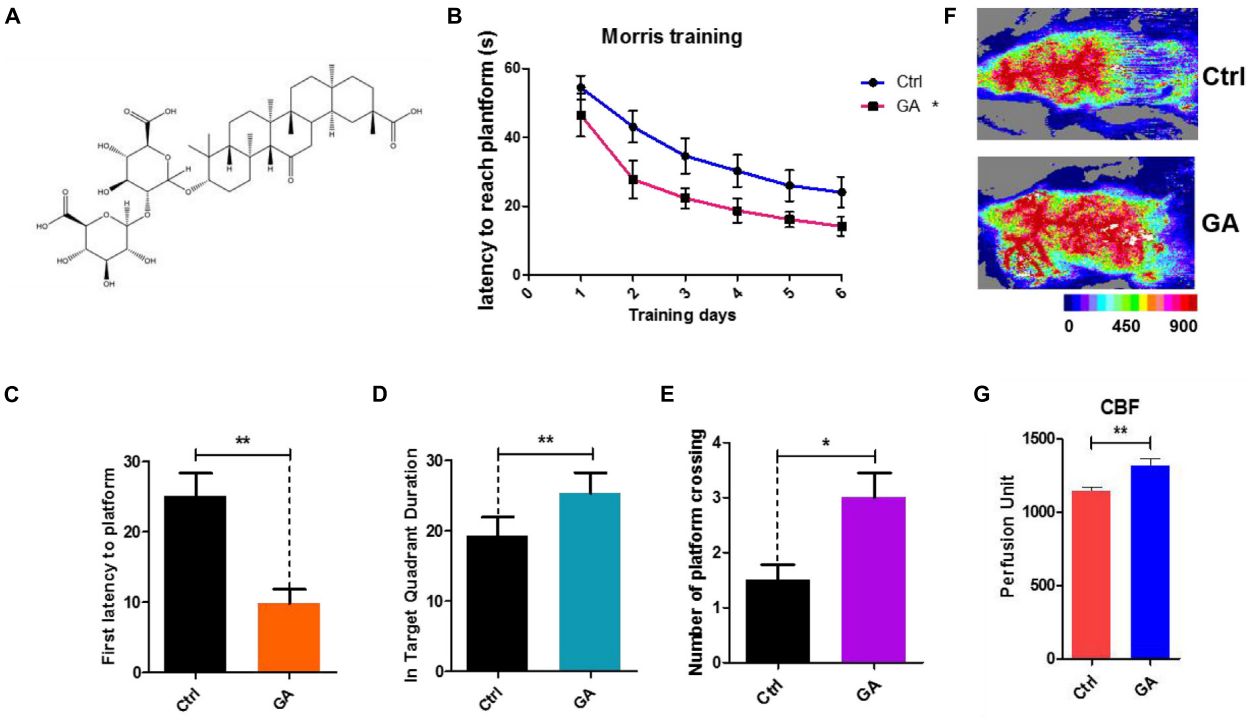

FIGURE 1 | Glycyrrhizic acid ameliorated cognitive declines in middle-aged mice, improved cerebral blood flow. **(A)** The chemical structure of Glycyrrhizic acid (GA). **(B)** Escape latency of six consecutive daily tests. Statistical comparisons used the repeated measures ANOVA. **(C)** First latency to the platform in the probe trial. **(D)** Time spent in the target quadrant in the probe trial. **(E)** Crossing times of the target platform in the probe trial. **(F)** Images of CBF in C57BL/6. **(G)** Quantification of blood perfusion in C57BL/6. GA (5 mg/kg, once every other day); Data represent mean ± SEM (n = 8 per group). $^*P < 0.05$, $^{**}P < 0.01$, vs. Ctrl.

(Gong et al., 2012; Kim et al., 2012; Teng et al., 2014). These results were based on pathological models. Although numerous pathways have been implicated in the neuroprotective effects of GA, the molecular mechanisms are not yet completely understood. In this study, we aimed to investigate the effects of GA in preventing age-related cognitive disorders, and the underlying molecular mechanisms.

MATERIALS AND METHODS

Reagents

Glycyrrhizic acid (GA) was obtained from TAUTO (Sichuan, China). 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), lysis buffer, and all antibodies used for flow cytometry were purchased from BD Pharmingen (San Diego, CA). Information regarding the antibodies used in this study is listed in the **Table 1**.

Animals

Both 8-week-old and 8-month-old female SPF C57BL/6 mice were obtained from Shanghai SLACCAS Co., Ltd. (Shanghai, China). 8-week-old female SPF B-NDG mice were obtained from Jiangsu Biocytogen Co., Ltd. (Nantong, China). All mice were housed five per cage and maintained on a 12 h light/dark schedule and allowed free access to food and water following a protocol approved by the Animal Research Committee of Tongji University School of Medicine, China. GA was administrated by tail vein every other day for 42 days. Control mice were tail vein administrated the same volume of PBS.

qRT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, United States), and cDNA was prepared using the Prime Script™ RT Master Mix (Perfect Real Time) (Takara, Dalian, China) according to the manufacturer's protocols. The qRT-PCR reactions were performed using SYBR green fluorescent dye (BioRad). Primer sequences are listed in the **Table 1**.

The Morris Water Maze Task

The Morris water maze consists of a round, black pool of 120 cm in diameter and 31 cm deep containing water at 23 ± 1°C. The escape platform (11 cm in diameter, adjustable height) was placed in the center of one quadrant of the pool and hidden below the water surface at 1.5 cm deep. Various prominent visual cues were placed around the pool and remained in the same position during training and testing periods. Each group was trained for 6 consecutive days from four locations and then tested on day 6 with a new direction, removing the hidden platform and allowing free swimming for 60 s. The escape latencies from the water, and the distance

TABLE 1 | Resources information used in this study.

Reagent or resource	Source	Identifier
Antibodies		
CD3e	BD Biosciences	Cat# 553063
CD45R/B220	BD Biosciences	Cat# 553087
CD49b	BD Biosciences	Cat# 558295
CD4	BD Biosciences	Cat# 550954
CD8a	BD Biosciences	Cat# 563152
CD45	BD Biosciences	Cat# 566439
CD3	BD Biosciences	Cat# 560590
CD44	BD Biosciences	Cat# 553134
CD62l	BD Biosciences	Cat# 560516
CD45R/B220	BD Biosciences	Cat# 553087
CD138	BD Biosciences	Cat# 558626
CD27	BD Biosciences	Cat# 558754
Chemicals		
Glycyrrhizic acid	TAUTO	Cat# 1405-86-3
Lysing Buffer	BD Biosciences	Cat# 555899
DAPI	BD Biosciences	Cat# 564907
TRIzol	Gibco	Cat# 15596018
the Prime Script™ RT Master Mix (Perfect Real Time)	Takara	Cat# RR036A
SYBR Green fluorescent dye	BioRad	Cat# 172-5120
PBS	Gibco	Cat# 10010049
PFA	Sigma	Cat# 158127
Nestin	Millipore	Cat# MAB5326
SOX2	Abcam	Cat# ab93689
Experimental model		
B-NDG	Biocytogen	B-CM-002
C57BL/6	SLACCAS	
Oligonucleotides		
Gene	**Forward primer**	**Reverse primer**
CD3e	CTGCTACACACCAG CCTCAA	GTAATAAATGACCATCA GCAAGC
CD45R/B220	CCAGTGATGCTACCA CAACG	CAATCCTCATTTCCACAC TTAGC
β-actin	TATTGGCAACGAGC GGTTC	ATGCCACAGGATTCCA TACCC

traveled to find the platform was recorded using video-animal tracking software.

Novel Object Recognition

Each mouse was habituated to an empty novel object recognition (NOR) open-field box for two 10 min test sessions 24 h apart. Twenty-four hours after the last habituation session, mice were subjected to training during a 10 min exposure session of two identical, non-toxic objects (metal or hard plastic items) in the open-field box. The time spent exploring each object was recorded using ObjectScan software (Clever Sys. Inc., Reston, VA); an area 2 cm^2 surrounding the object was defined, so that nose entries within 2 cm of the object was recorded as time exploring the object. After the training session, the animal was returned to its home cage. After a retention interval of 1 h (Zhang et al., 2016), the animal was returned to the arena in which two objects, one identical to the familiar object but previously unused (to prevent olfactory cues and prevent the necessity of washing objects during experimentation) and one novel object. The animal was allowed to explore for 10 min, during which the amount of time exploring each object was recorded. Objects were randomized and counterbalanced across animals. Animals that spent <7 s exploring the objects during the 10 min test session were omitted from the analysis. Objects and arenas were thoroughly cleaned with 70% isopropanol between trials. For the novel object recognition tests, the time spent exploring the novel object (familiar vs. novel) was reported as the recognition index, and was calculated using the following formula: [(time exploring specified object)/(time exploring novel object + time exploring familiar object)] × 100 (Taglialatela et al., 2009). Statistical analysis of NOR data is done by first performing an one-sample *t*-test to determine if the mean percentage exploration time is significantly different from a theoretical mean of 0.5000. This is followed by one-way ANOVA to determine group differences (Hernandez et al., 2010).

Open Field Test

The device is based on a square field (50 × 50 × 30 cm). A lamp was located 150 cm above the field, and the illumination of the central area was approximately 100 lux. At the beginning of each experiment, a mouse was placed in a 15 × 15 cm central area. At the beginning of the experiment, each mouse was placed in the center of the field, and the time spent in the central area within 10 min was recorded.

Immune Reconstitution (IR)

Harvested mouse spleens were digested and washed with phosphate buffered saline (Thermo Fisher Scientific, Waltham, MA, United States). Then, splenocytes were obtained by removing the red blood cells with lysis buffer (BD Biosciences, San Jose, CA, United States) and filtering through a 70 μm cell strainer (Jet Biofil, Guangzhou, China). Then, 3×10^5 splenocytes were administered via the tail vein (McDermott et al., 2010).

Cortical Cerebral Blood Flow Measurements

Images were acquired with a laser speckle contrast imager (PeriCam PSI System, Perimed, Stockholm, Sweden). We used the PeriCam PSI HD system to calculate an arbitrary index of cerebral blood flow (perfusion units) in the ipsilateral hemisphere.

Cell Preparation and Flow Cytometric Analysis

Harvested mouse spleens were macerated and washed with phosphate buffer saline (Thermo Fisher Scientific, Waltham, MA, United States). Then, splenocytes were obtained by removing the red blood cells with lysis buffer (BD Biosciences, San

Jose, CA, United States) and filtering through a 70 μm cell strainer (Jet Biofil, Guangzhou, China). Additionally, fresh blood samples were collected in heparinized tubes. T ($CD3^+$) cells, B ($CD3^-CD45R/B220^+$) cells, and NK ($CD3^-CD49b^+$) cells in the spleen and blood were directly quantified using flow cytometry (Beckman FC-500, Miami, FL, United States).

Some splenocytes were cultured with added GA in 6-well plates for 36 h. Then effector T ($CD45^+CD3^+CD44^+CD62l^-$) cells, and effector B ($CD45^+CD3^-CD45R/B220^+CD138^+CD27^+$) cells were directly quantified using flow cytometry. All antibodies were used at an optimized working concentration of 1 μg/ml.

Immunofluorescence Images

Brain tissue was collected from mice following treatment with GA. Brain tissue was fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) overnight, followed by a 15–30% sucrose gradient dehydration for 1 day until the brain had completely sunk to the bottom of the sucrose solution. An optimal cutting temperature compound was used to embed tissue samples. Successive coronal sections of 10 μm were cut using a freezing microtome. Tissue slices were washed with PBS and blocked for 1 h (in 10% bovine serum albumin, 3% normal donkey serum, and 1% Triton X-100 in PBS) and mounted on tissue slides. Antibodies against Nestin and SOX2 (Abcam, Cambridge, MA, United States), which can be used to label quiescent radial-glia-like type I neural progenitor cells (Encinas et al., 2011), were used. The next day, the slides were washed three times and incubated with the appropriate Alexa 488- and Alexa 568- secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature (1:1,000 dilution). DAPI staining was used to label nuclei. Slides were examined using an OLYMPUS BX53 microscope (Olympus, Madison, WI).

RNA Sequencing Analysis

RNA sequencing was performed independently and uniformly for each sample. GA- and control-treated mice were anesthetized and euthanized, and two blood samples were removed for RNA-seq following extraction of total RNA. Clean reads were aligned to the reference gene sequence using bowtie-2, and the gene expression levels of each sample were calculated. DEG detection was conducted using the DEGseq method. The statistical results were based on the ma-plot method. The number of reads of specific genes obtained from the sample was sampled randomly, and then *P*-values were calculated according to the normal distribution and corrected to *q*-values. To improve the accuracy of DEG detection, genes with a difference multiple of more than twice, and a $q \leq 0.001$ were screened and defined as significantly differentially expressed genes.

Statistical Analysis

Statistical analysis of data was conducted using Graphpad Prism 5.0 and expressed as the mean ± standard error of the mean (SEM). Statistical comparisons of two groups were made using the unpaired *t*-test. Escape latency of six consecutive daily tests used the repeated measures ANOVA. Probability values less than 0.05 were considered statistically significant.

RESULTS

GA Prevented Impairments of Learning and Memory Displayed and Improved CBF in Middle-Aged C57BL/6 Mice

Twelve-month-old female C57BL/6 mice were injected with GA (5 mg/kg, once every other day, tail intravenous injection, i.v.) for 6 weeks. Then, we tested the behavioral effects of GA using the Morris water maze (MWM) task to evaluate spatial learning and memory. The MWM showed that the GA-treated mouse latencies to the platform gradually decreased compared with the control after 6 days of training (**Figure 1B**). In the training test, the test results for the main effect of time, age are respectively, $F = 45.34$, $P < 0.001$ and $F = 4.569$, $P = 0.0451$. The interaction effects were not statistically significant ($P = 0.6212$). The impairment in learning and memory displayed in middle-aged mice can be prevented by GA treatment as indicated by decreased escape latencies (**Figure 1C**), more time in the target quadrant (**Figure 1D**), and increased platform crossings (**Figure 1E**). This effect was accompanied by improved cerebral blood flow (CBF) (**Figures 1F,G**). Both the control and GA groups exhibited similar swim speeds to the virtual platform (**Supplementary Figure S1**). These data suggested that GA improved CBF and prevented impairments of learning and memory displayed in middle-aged C57BL/6 mice.

GA-Treatment Actived T/B Genes Expression in the Blood RNA-Seq

We analyzed the transcriptome of C57BL/6 mice blood using RNA-seq. There were 940 differentially expressed genes between the GA and control groups, of which 81 were upregulated and 859 were downregulated (**Figure 2A**). KEGG pathway analysis also demonstrated that GA significantly influenced the hematopoietic cell lineage (**Figure 2B**). Furthermore, gene set enrichment analysis of these differentially expressed genes revealed that GA treatment activated the hematopoietic cell lineage (**Figure 2C**). The differential genes in the pathway were clustered using heat mapping (**Figure 2D**). The RNA-seq results showed that GA activated CD8a, Fcer2a (CD23), and Cr2 (CD21) expression and that it inhibits the expressions of macrophage and neutrophil-related genes.

GA-Treatment Increased T/B Cell Numbers in the Blood and Spleen

To further verify the relationships of the active genes to cognitive ability, we analyzed the levels of $CD3e^+$ T cells, $CD45R/B220^+$ B cells, and $CD49b^+$ NK cells in the blood and spleen after 42 days of GA treatment. Flow cytometry results showed that GA could increase T and B cell numbers in the blood and spleen (**Figures 3A,B**), whereas NK cell numbers were unchanged in the spleen (**Figure 3C**).

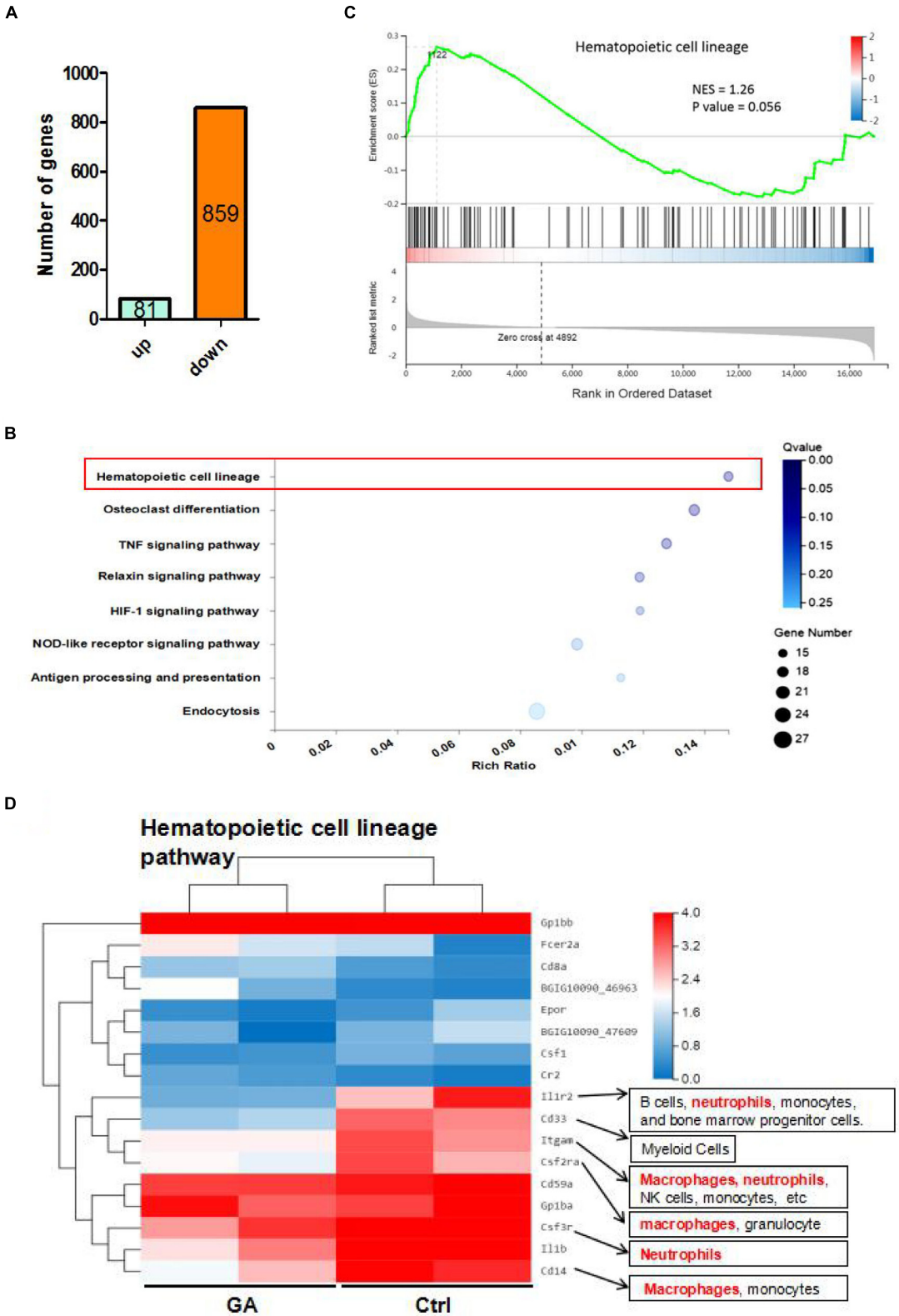

FIGURE 2 | The RNA-seq results of GA and control mice. **(A)** The differential expressed genes between GA and control group. **(B)** Enrichment plots of gene expression signatures for hematopoietic cell lineage. Barcode plot indicates positions of genes in each gene-set. NES, normalized enrichment score. **(C)** Bubble diagram showing GA treatment influence the hematopoietic cell lineage. **(D)** Heatmap showing differentially expressed genes (DEGs) in hematopoietic cell lineage of control and GA-treated C57BL/6 mice. There were two different representative mice from each treatment group.

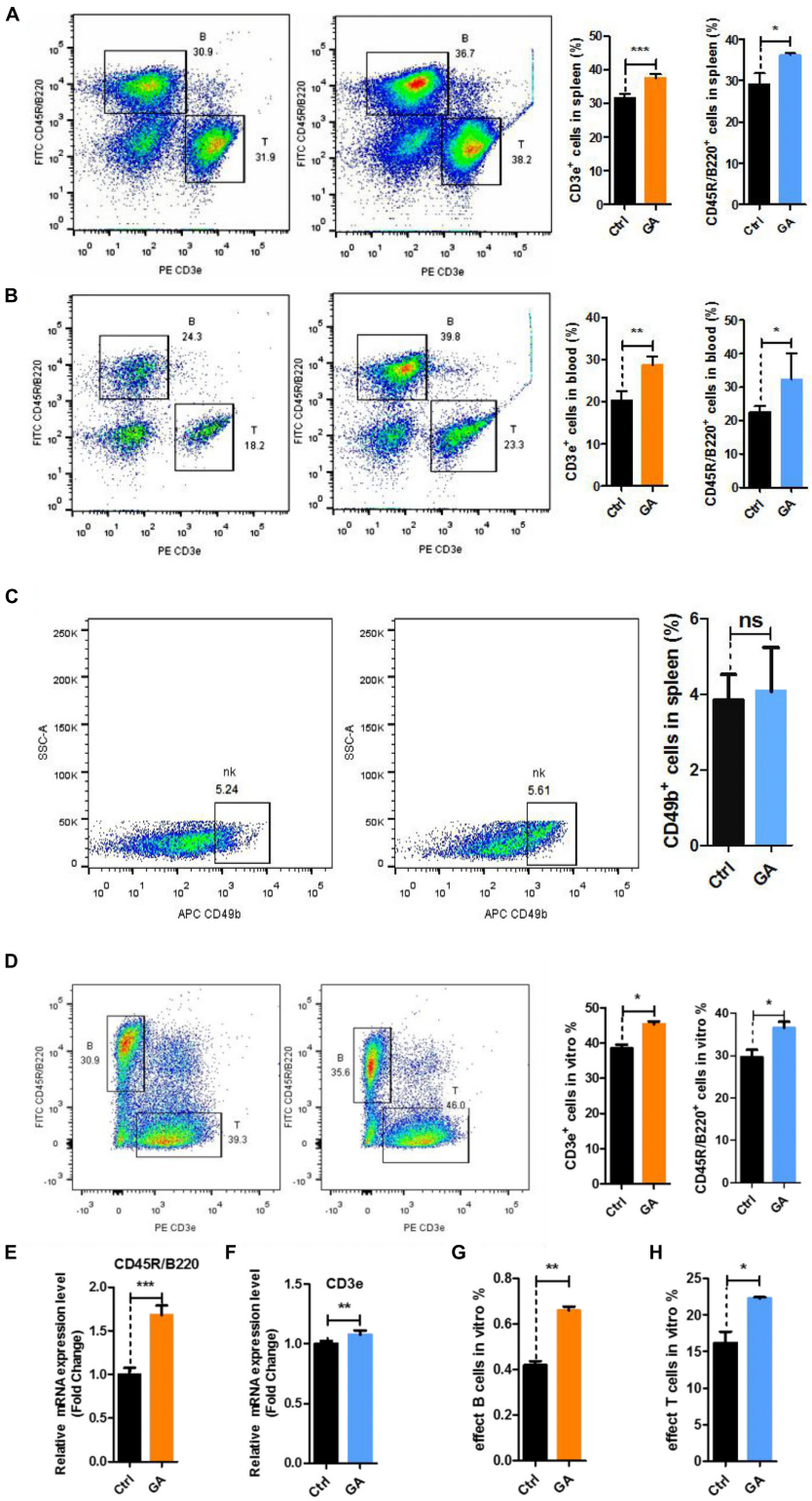

FIGURE 3 | GA increased the proliferation of T and B cell subsets *in vitro* and *vivo*. **(A)** Representative FACS plots showing CD3e$^+$ T and CD45R/B220$^+$ B cells in the spleen of C57BL/6 mice; Bar graphs for statistical results of T and B cells in spleen of C57BL/6 mice. **(B)** Representative FACS plots showing CD3e$^+$ T and CD45R/B220$^+$ B cells in the blood of C57BL/6 mice. **(C)** Representative FACS plots showing CD49b$^+$ NK cells in the spleen of C57BL/6 mice. **(D)** Representative FACS plots showing CD3e$^+$ T and CD45R/B220$^+$ B cells *in vitro*. **(E)** CD3e mRNA expression in spleen of C57BL/6 mice. **(F)** CD45R/B220 mRNA expression in spleen of C57BL/6 mice. **(G)** Bar graphs for statistical results of effect B cells after flow cytometry sorting. **(H)** Bar graphs for statistical results of effect T cells after flow cytometry sorting. Data represent mean ± SEM (n = 8 per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant, vs. Ctrl.

To demonstrate that GA produces the similar effects *in vitro*, we used GA-treated single cells obtained from 8-week-old C57BL/6 mouse spleens for 36 h and measured the changes of T and B cells. Flow cytometry showed that GA could significantly increase the numbers of T and B cells *in vitro* (**Figure 3D**). Quantitative real-time PCR showed that *CD3e* and *CD45R/B220* genes are overexpressed (**Figures 3E,F**). We treated T cells and B cells obtained from 8-week-old C57BL/6 mouse spleens using flow cytometry sorting with 50 μM GA and respectively measured effector T cells ($CD45^+CD3^-CD45R/B220^+CD138^+CD27^+$) and effector B cells ($CD45^+CD3^+CD44^+CD62l^-$). The results showed that GA could increase effector cells *in vitro* (**Figures 3G,H**). These results showed that GA increased T and B cells *in vivo* and *in vitro*.

GA Improved Cognitive Levels by Regulating T/B Cell Proliferation

To further investigate whether the neuroprotective effects of GA were related to the T and B cells, we used B-NDG (NOD-*Prkd*^{scid} *IL2rg*^{tm1}/Bcgen) mice (**Supplementary Figure S2**), which lack T, B, and NK cells. We tested behavioral effects after immune reconstitution (IR) and IR + GA treatment by performing a novel object recognition task to measure the attention and non-spatial declarative memory in B-NDG mice. Before the novel object recognition task, we performed the open field test to evaluate the anxiety behavior of these mice. Anxiety levels can be determined based upon the time the mice remain in the corner of the enclosure. We found that the ratio of the time the IR + PBS and IR + GA mice spent in the center of the enclosure to the time spent in a corner over a 10 min test session was not significantly different compared with the control group (**Figure 4A**). The novel object recognition task showed that IR + PBS mice exhibited a significant preference for object exploration (**Figure 4B**). After IR, the GA treatment showed a more significant preference when contrasted with PBS treatment (**Figure 4B**).

We analyzed the levels of $CD3e^+$ positive T cells, $CD45R/B220^+$ positive B cells, and $CD49b^+$ positive NK cells in the blood and spleen (**Supplementary Figure S3**). Flow cytometry results showed that the IRs of B-NDG mice were successful given that T, B, and NK cells in IR + PBS mice were significantly increased compared with controls (**Figures 4C,E** and **Supplementary Figure S4**). Thus, GA treatment after IR can increase the number of T and B cells compared with PBS treatment after IR (**Figures 4C,E**), whereas NK cell numbers were unchanged (**Supplementary Figure S4**). This result was consistent with those above from C57BL/6 mice. Furthermore, we measured the numbers of CD4 and CD8 cells in the spleen and found they were increased (**Figure 4D**), which was consistent with our RNA-sequencing results.

GA-Treatment Increased Neural Stem Cells in the Dentate Gyrus

To further investigate various mouse brain tissue characteristics, we examined the neural stem cell markers Nestin and SOX2 in the dentate gyrus (DG) of the hippocampus in IR + PBS, or IR + GA treated mice and control mice. The results showed that the numbers of Nestin + SOX2 + double-positive cells were significantly increased in the DG of immunologically reconstituted mice compared with the control and GA treatment, or compared with PBS injection after IR (**Figures 5A,B**).

DISCUSSION

Our work has thus revealed that GA treatment can revitalize the aging brain and alleviate aging-associated cognitive declines and that these effects may be related to the immune system. RNA-seq data demonstrated the effect of GA on hematopoietic cell lineage, and we posited that GA could enhance cognition through immune system modulation. We observed lymphocyte changes in C57BL/6 mice, then used B-NDG mice to verify further the importance of the immune system in cognitive ability. The B-NDG mice exhibit a severe immunodeficiency phenotype with no mature T cells, B cells, or functional NK cells and a lack of cytokine signaling ability. The results of the novel object recognition task confirmed our hypothesis; after IR, cognitive ability was improved (**Figure 4B**).

A marked increase in the number of cytotoxic CD4 T cells (CD4 cytotoxic T lymphocytes) is a signature characteristic of supercentenarians (Hashimoto et al., 2019). We observed that $CD4^+$ T cells were significantly expanded in number (**Figure 4D**), but we cannot confirm whether these represent cytotoxic CD4 T cells. Although we did not investigate immune responses in the brain, many studies have demonstrated the role of CD4 T cells in neuroinflammatory and neurodegenerative processes (Brochard et al., 2009; Baruch et al., 2015; Dansokho et al., 2016). Pasciuto showed that absence of the CD4 T cell population resulted in microglia remaining suspended between a fetal and adult developmental state, resulting in defects in synaptic pruning function and normal mouse behavior (Pasciuto et al., 2020). Additionally, microglial regeneration was found to improve spatial learning ability and promote hippocampal nerve regeneration (Willis et al., 2020). We hypothesized that GA affects cognition by acting on immune cells in the blood and brain, further influencing microglia in the mouse brain, which requires further study.

The brain is particularly susceptible to the effects of aging, and aging-associated inflammation is a major risk factor for a variety of neurocognitive and neurodegenerative diseases (Fung et al., 2020). There are abnormal neural stem cells, neurons, and microbes in the brain, and their clearance by immune cells can preserve cognitive function (Bussian et al., 2018). Further efforts to understand the effector cells in this process and how these events occur would be worthwhile in understanding the roles of immune cells in brain aging.

During cytokine storms, cytokine levels are abnormally high, which can lead to fever, low blood pressure, and heart problems and, in some cases, organ failure and death (RIddell, 2018). Bacterial infection and viruses such as SARS (severe acute respiratory syndrome) and MERS (Middle East

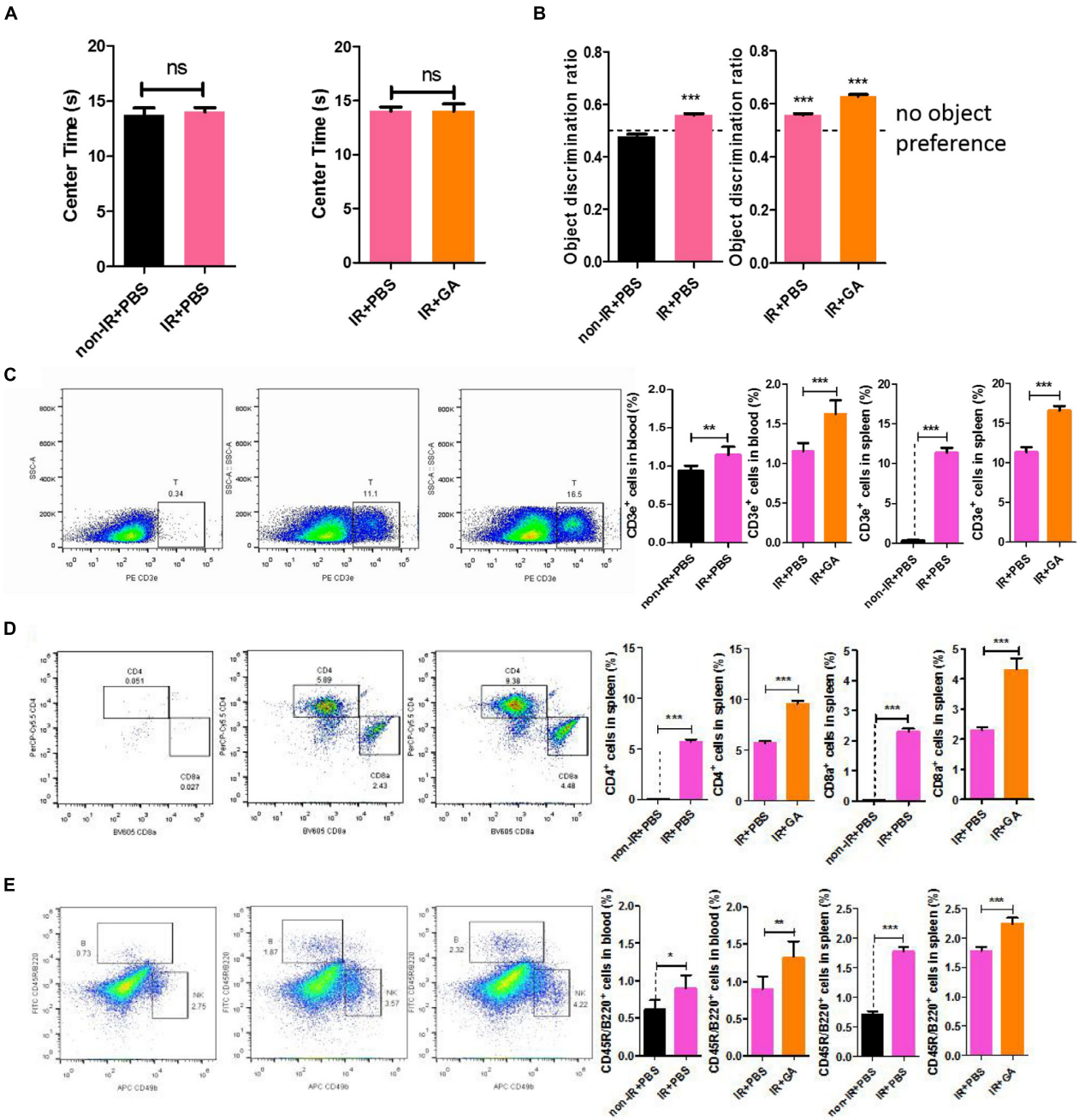

FIGURE 4 | GA increased the speed of immunological reconstitution in B-NDG mice and enhanced the cognitive ability. **(A)** Detection of anxiety using open-field test. Results are expressed the time spent in the center area. **(B)** IR + PBS and IR + GA mice showed a preference for the novel object after a 1 h delay between identical object exploration during training and the introduction of a novel object during the test phase. *T*-test against a theoretical mean of 0.5000 yielded *p*-values of 0.0005 and <0.0001 for IR + PBS and IR + GA, respectively. Non-IR + PBS not exhibits any preference for object exploration ($p = 0.051$). *** indicates significant difference at $p < 0.0001$ from theoretical mean of 0.50. **(C)** Representative FACS plots showing $CD3e^+$ T cells in the spleen of B-NDG mice; Bar graphs for statistical results of T cells in blood and spleen of B-NDG mice. **(D)** Representative FACS plots showing CD4 and CD8 T cells in the spleen of B-NDG mice; Bar graphs for statistical results of CD4 and CD8 T cells in spleen of B-NDG mice. **(E)** Representative FACS plots showing $CD45R/B220^+$ B and $CD49b^+$ NK cells in the spleen of B-NDG mice; Bar graphs for statistical results of B cells in blood and spleen of B-NDG mice. GA (5 mg/kg/2 days); Data represent mean ± SEM ($n = 8$ per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant, vs. Ctrl.

respiratory syndrome) can cause cytokine storms, which attack host cells, mainly the patient's immune cells (Tisoncik et al., 2012). Macrophages and neutrophils are known to produce catecholamines in response to inflammatory stimuli such as lipopolysaccharide (LPS), which is a hallmark of many types of bacterial infection (Flierl et al., 2007). Our RNA-seq

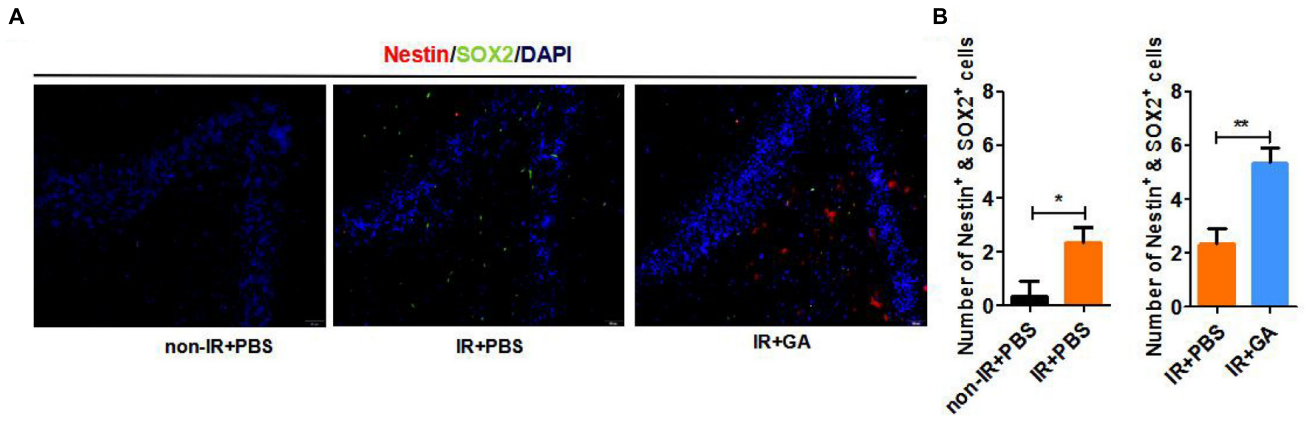

FIGURE 5 | GA-treatment increased neural stem cells in the dentate gyrus. **(A)** Expression of Nestin and SOX2 neural stem cell marker in B-NDG mice dentate gyrus. **(B)** Quantitative analysis of the numbers of Nestin + SOX2 + double-positive cells in dentate gyrus. GA (5 mg/kg/2 days); Data represent mean ± SEM (n = 8 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. Ctrl.

results showed that GA treatment could inhibit several genes related to macrophages, neutrophils, and IL1β (**Supplementary Figure S1**). This finding indicates that GA might inhibit the proliferation of macrophages and neutrophils from preventing cytokine storms. Treatment with GA can also alleviate β-amyloid (Ahn et al., 2006; Zhao et al., 2013) or systemic LPS-induced (Song et al., 2013) cognitive impairment via inhibition of neuroinflammation. Some COVID-19 patients have been alleviated symptoms using diammonium glycyrrhizinate, an ammonium salt preparation of 18-alpha-GA (Ding et al., 2020; Li et al., 2020). This phenomenon could be explained by the anti-inflammatory properties of GA. In conclusion, the potential effects of GA treatments on memory and behavior, longevity, and anti-viral activity may be worthy of future investigation.

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 below: https://www.ncbi.nlm.nih.gov/, GSE146239.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research Committee of Tongji University School of Medicine, China.

AUTHOR CONTRIBUTIONS

RJ, JG, JS, XZ, HW, SF, and CH performed the experiments. RJ and HL analyzed the data and prepared the manuscript. HL and HS supervised the project. HL designed the project. All authors contributed to the article and approved the submitted version.

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This manuscript has been released as a pre-print at bioRxiv (Jiang et al., 2020).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.570116/full#supplementary-material

Supplementary Figure 1 | Mean speed was also not different between the two groups. Ctrl, Control; GA, Glycyrrhizic acid. The overall significance between two groups was determined by Student's t-test. ns, not significant.

Supplementary Figure 2 | The animal graphical abstract in B-NDG mice.

Supplementary Figure 3 | Gating strategies for identifying T (including CD4 and CD8a cells), B and NK cells in B-NDG mice.

Supplementary Figure 4 | Related to **Figure 4E**. Bar graphs for statistical results of NK cells in blood and spleen of B-NDG mice. $^{***}P < 0.001$; ns, not significant.

REFERENCES

Ahn, J., Um, M., Choi, W., Kim, S., and Ha, T. (2006). Protective effects of *Glycyrrhiza uralensis* Fisch. on the cognitive deficits caused by beta-amyloid peptide 25-35 in young mice. *Biogerontology* 7, 239–247. doi: 10.1007/s10522-006-9023-0

Bach, M. E., Barad, M., Son, H., Zhuo, M., Lu, Y. F., Shih, R., et al. (1999). Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5280–5285. doi: 10.1073/pnas.96.9.5280

Baruch, K., Rosenzweig, N., Kertser, A., Deczkowska, A., Sharif, A. M., Spinrad, A., et al. (2015). Breaking immune tolerance by targeting Foxp3(+) regulatory T cells mitigates Alzheimer's disease pathology. *Nat. Commun.* 6:7967. doi: 10.1038/ncomms8967

Brochard, V., Combadière, B., Prigent, A., Laouar, Y., Perrin, A., Beray-Berthat, V., et al. (2009). Infiltration of CD4+ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease. *J. Clin. Invest.* 119, 182–192. doi: 10.1172/JCI36470

Bussian, T. J., Aziz, A., Meyer, C. F., Swenson, B. L., van Deursen, J. M., and Baker, D. J. (2018). Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. *Nature* 562, 578–582. doi: 10.1038/s41586-018-0543-y

Dansokho, C., Ait Ahmed, D., Aid, S., Toly-Ndour, C., Chaigneau, T., Calle, V., et al. (2016). Regulatory T cells delay disease progression in Alzheimer-like pathology. *Brain J. Neurol.* 139, 1237–1251. doi: 10.1093/brain/awv408

Ding, H., Deng, W., Ding, L., Ye, X., Yin, S., and Huang, W. (2020). Glycyrrhetinic acid and its derivatives as potential alternative medicine to relieve symptoms in nonhospitalized COVID-19 patients. *J. Med. Virol.* 92, 2200–2204. doi: 10.1002/jmv.26064

Encinas, J. M., Michurina, T. V., Peunova, N., Park, J. H., Tordo, J., Peterson, D. A., et al. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell* 8, 566–579. doi: 10.1016/j.stem.2011.03.010

Flierl, M. A., Rittirsch, D., Nadeau, B. A., Chen, A. J., Sarma, J. V., Zetoune, F. S., et al. (2007). Phagocyte-derived catecholamines enhance acute inflammatory injury. *Nature* 449, 721–725. doi: 10.1038/nature06185

Frick, K. M., Burlingame, L. A., Arters, J. A., and Berger-Sweeney, J. (2000). Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. *Neuroscience* 95, 293–307. doi: 10.1016/s0306-4522(99)00418-2

Fung, I. T. H., Sankar, P., Zhang, Y., Robison, L. S., Zhao, X., D'Souza, S. S., et al. (2020). Activation of group 2 innate lymphoid cells alleviates aging-associated cognitive decline. *J. Exp. Med.* 217:e20190915. doi: 10.1084/jem.20190915

Gong, G., Yuan, L. B., Hu, L., Wu, W., Yin, L., Hou, J. L., et al. (2012). Glycyrrhizin attenuates rat ischemic spinal cord injury by suppressing inflammatory cytokines and HMGB1. *Acta Pharmacol. Sin.* 33, 11–18. doi: 10.1038/aps.2011.151

Hashimoto, K., Kouno, T., Ikawa, T., Hayatsu, N., Miyajima, Y., Yabukami, H., et al. (2019). Single-cell transcriptomics reveals expansion of cytotoxic CD4 T cells in supercentenarians. *Proc. Natl. Acad. Sci. U.S.A.* 116, 24242–24251. doi: 10.1073/pnas.1907883116

Hernandez, C. M., Kayed, R., Zheng, H., Sweatt, J. D., and Dineley, K. T. (2010). Loss of alpha7 nicotinic receptors enhances beta-amyloid oligomer accumulation, exacerbating early-stage cognitive decline and septohippocampal pathology in a mouse model of Alzheimer's disease. *J. Neurosci.* 30, 2442–2453. doi: 10.1523/JNEUROSCI.5038-09.2010

Jiang, R., Gao, J., Shen, J., Zhu, X., Wang, H., Feng, S., et al. (2020). Glycyrrhizic acid improves cognitive levels of aging mice by regulating T/B cell proliferation. *bioRxiv* [Preprint]. doi: 10.1101/2020.03.25.008821

Kim, S. W., Jin, Y., Shin, J. H., Kim, I. D., Lee, H. K., Park, S., et al. (2012). Glycyrrhizic acid affords robust neuroprotection in the postischemic brain via anti-inflammatory effect by inhibiting HMGB1 phosphorylation and secretion. *Neurobiol. Dis.* 46, 147–156. doi: 10.1016/j.nbd.2011.12.056

Li, R., Wu, K., Li, Y., Liang, X., Lai, K. P., and Chen, J. (2020). Integrative pharmacological mechanism of vitamin C combined with glycyrrhizic acid against COVID-19: findings of bioinformatics analyses. *Brief. Bioinform.* bbaa141. doi: 10.1093/bib/bbaa141

McDermott, S. P., Eppert, K., Lechman, E. R., Doedens, M., and Dick, J. E. (2010). Comparison of human cord blood engraftment between immunocompromised mouse strains. *Blood* 116, 193–200. doi: 10.1182/blood-2010-02-271841

Pasciuto, E., Burton, O. T., Roca, C. P., Lagou, V., Rajan, W. D., Theys, T., et al. (2020). Microglia require CD4 T cells to complete the fetal-to-adult transition. *Cell* 182, 625–640.e24. doi: 10.1016/j.cell.2020.06.026

RIddell, S. R. (2018). Adrenaline fuels a cytokine storm during immunotherapy. *Nature* 564, 194–196. doi: 10.1038/d41586-018-07581-w

Shoji, H., Takao, K., Hattori, S., and Miyakawa, T. (2016). Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. *Mol. Brain* 9:11. doi: 10.1186/s13041-016-0191-9

Song, J. H., Lee, J. W., Shim, B., Lee, C. Y., Choi, S., Kang, C., et al. (2013). Glycyrrhizin alleviates neuroinflammation and memory deficit induced by systemic lipopolysaccharide treatment in mice. *Molecules* 18, 15788–15803. doi: 10.3390/molecules181215788

Taglialatela, G., Hogan, D., Zhang, W. R., and Dineley, K. T. (2009). Intermediate- and long-term recognition memory deficits in Tg2576 mice are reversed with acute calcineurin inhibition. *Behav. Brain Res.* 200, 95–99. doi: 10.1016/j.bbr.2008.12.034

Teng, L., Kou, C., Lu, C., Xu, J., Xie, J., Lu, J., et al. (2014). Involvement of the ERK pathway in the protective effects of glycyrrhizic acid against the MPP+-induced apoptosis of dopaminergic neuronal cells. *Int. J. Mol. Med.* 34, 742–748. doi: 10.3892/ijmm.2014.1830

Tisoncik, J. R., Korth, M. J., Simmons, C. P., Farrar, J., Martin, T. R., and Katze, M. G. (2012). Into the eye of the cytokine storm. *Microbiol. Mol. Biol. Rev.* 76, 16–32. doi: 10.1128/MMBR.05015-11

Wang, C., Chen, L., Xu, C., Shi, J., Chen, S., Tan, M., et al. (2020). A comprehensive review for phytochemical, pharmacological, and biosynthesis studies on *Glycyrrhiza* spp. *Am. J. Chin. Med.* 48, 17–45. doi: 10.1142/S0192415X20500020

Willis, E. F., MacDonald, K., Nguyen, Q. H., Garrido, A. L., Gillespie, E. R., Harley, S., et al. (2020). Repopulating microglia promote brain repair in an IL-6-dependent manner. *Cell* 180, 833–846.e16. doi: 10.1016/j.cell.2020.02.013

Zhang, J. B., Chen, L., Lv, Z. M., Niu, X. Y., Shao, C. C., Zhang, C., et al. (2016). Oxytocin is implicated in social memory deficits induced by early sensory deprivation in mice. *Mol. Brain* 9:98. doi: 10.1186/s13041-016-0278-3

Zhao, H., Wang, S. L., Qian, L., Jin, J. L., Li, H., Xu, Y., et al. (2013). Diammonium glycyrrhizinate attenuates Abeta(1-42) -induced neuroinflammation and regulates MAPK and NF-kappaB pathways in vitro and in vivo. *CNS Neurosci. Ther.* 19, 117–124. doi: 10.1111/cns.12043

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frontiers in Aging Neuroscience

REVIEW
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The Neuroimmunology of Guillain-Barré Syndrome and the Potential Role of an Aging Immune System

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Guillain-Barré syndrome (GBS) is a paralyzing autoimmune condition affecting the peripheral nervous system (PNS). Within GBS there are several variants affecting different aspects of the peripheral nerve. In general, there appears to be a role for T cells, macrophages, B cells, and complement in initiating and perpetuating attacks on gangliosides of Schwann cells and axons. Of note, GBS has an increased prevalence and severity with increasing age. In addition, there are alterations in immune cell functioning that may play a role in differences in GBS with age alongside general age-related declines in reparative processes (e.g., delayed de-differentiation of Schwann cells and decline in phagocytic ability of macrophages). The present review will explore the immune response in GBS as well as in animal models of several variants of the disorder. In addition, the potential involvement of an aging immune system in contributing to the increased prevalence and severity of GBS with age will be theorized.

Keywords: peripheral nervous system, PNS, guillain-barré syndrome, GBS, immune system, aging, gangliosides

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OVERVIEW OF THE IMMUNOLOGY OF THE PERIPHERAL NERVOUS SYSTEM

In the intact peripheral nerve, there are many cell types that reside. Among them are neurons, Schwann cells (SCs), fibroblasts, pericytes, endothelial cells, and resident macrophages. After sufficient injury to peripheral nerves, other immune cells such as infiltrating macrophages, neutrophils, mast cells, dendritic cells, and T cells contribute to degenerative and regenerative processes. For example, myelinating and non-myelinating SCs de-differentiate into a repair phenotype that secretes cytokines, chemokines, and neurotrophic factors. In addition, several beneficial processes occur: bands of Büngner are formed by repair SCs; neutrophils and macrophages are recruited for debris clearance alongside repair SCs (Gaudet et al., 2011); and activation of mast cells increase blood-nerve barrier permeability (Olsson, 1967; Esposito et al., 2002). Other responses are also noted including recruitment of various pro- and anti-inflammatory T cell types (Xin et al., 2008; Austin et al., 2012), modulation of SC sorting and cytokine production by fibroblasts (Reichert et al., 1996; Parrinello et al., 2010), and the secretion of neurotrophic factors and axon guidance molecules by endothelial cells (Cattin et al., 2015; Grasman and Kaplan, 2017).

Of note, there is augmented severity and/or prevalence of immune cells within the PNS with increasing age. For instance, there is an increased presence of mast cells and macrophages not only

in the uninjured nerve (Ceballos et al., 1999), but also after injury (Büttner et al., 2018; Stratton et al., 2020). As reviewed by Verdú et al. (2000), this heightened immune response may contribute to the decreased regenerative ability of nerves with age. That is, it does not appear to be an inability of the axons themselves to regrow with increasing age, but rather it appears to be deficits in the aged nerve environment that are involved (Kang and Lichtman, 2013). For example, there is an age-related decline in SC de-differentiation that may delay the clearance of debris following degeneration and thus impair the growth of regenerating axons (Painter et al., 2014). In addition, with age, there is a delay in macrophage recruitment and a defect in the ability of macrophages and SCs to phagocytose debris (Scheib and Höke, 2015).

Since injury-related immunological responses in the injured PNS have been reviewed in detail in the past (e.g., Gaudet et al., 2011; DeFrancesco-Lisowitz et al., 2014; Mietto et al., 2015), we will focus here on the immunological aspects of GBS, a disorder that involves non-mechanical damage of peripheral nerves, by discussing studies involving the immune system in the disease. This will be accompanied by the authors' views on age-mediated changes in the immune system in addition to the aforementioned alterations to macrophages and SCs that could account for the particular severity of the disease seen in older GBS humans.

GUILLAIN-BARRÉ SYNDROME (GBS)

Incidence Rate

Guillain-Barré syndrome (GBS) has a general incidence rate of between 1.1 and 1.8/100,000 per year (McGrogan et al., 2009). It typically affects more men than women in a 1.25:1 ratio (Hughes and Rees, 1997). When broken down by age in a meta-analysis study that examined incidence rates from North American and European epidemiological reports, the prevalence of GBS was found to change dramatically (Sejvar et al., 2011). In those aged 0–9-years-old, the incidence rate was found to be 0.62/100,000 per year, specifically 0.8 in males and 0.45 in females. However, the rate increases by 20% per decade of life leading to a prevalence of 2.66/100,000 per year in those aged 80–89 years (specifically 3.72 in males and 2.09 in females). It should be noted that older studies examining the prevalence of GBS have reported that there is a decrease in the incidence rate over the age of 75 years of age (George and Twomey, 1985); this difference in findings could be due to improvements in diagnostic methods. Of further note and pertinent to the subject of this review, Hughes and Rees speculated early on that the higher incidence rate in older populations may reflect a failure in the typical suppression of the immune system with increasing age (Hughes and Rees, 1997).

Severity

In addition to higher incidence rates of GBS in the elderly, one study found greater severity of the disorder at its peak as well as a higher mortality rate in older individuals compared to younger patients (Peric et al., 2016). In the aforementioned study, the authors striated their patients into young (<60-years-old), young-old (60–80-years-old), and old-old (>80-years-old). While the severity of disease at peak was not significantly different between young-old and old-old patients, there was more disability at discharge, more frequent involvement of bulbar symptoms, and higher rates of comorbidity in the old-old group compared to the young-old patients. Furthermore, in a study of clinical predictors of plasmapheresis treatment, age was the only predictor, with young patients responding better than older patients (Gruener et al., 1987).

Symptoms and Fluid Bio-factors

GBS is broadly characterized by areflexia and symmetrical limb weakness which typically begins proximally and can involve motor, sensory, and/or cranial nerves (George and Twomey, 1985; van Doorn et al., 2008). GBS symptoms usually progress, typically lasting around 4 weeks from onset (Ho et al., 1997) and can present with autonomic dysfunction that can lead to changes in mental state (Lehmann et al., 2010), fatigue, and pain (van Doorn et al., 2008). The disease has also been associated with increases in cerebrospinal fluid (CSF) levels of protein, albumin (Li et al., 2012), neuroactive steroids such as dehydroepiandrosterone sulfate (Azuma et al., 1993), malondialdehyde and antioxidant activity (Ghabaee et al., 2010), haptoglobin, α-1-antitrypsin, apolipoprotein A-IV, and neurofilament (Yang et al., 2008), and complement products C3a and C5a (Hartung et al., 1987). On the other hand, decreases in Cystatin C, transthyretin, apolipoprotein E, and heat shock protein 70 (Yang et al., 2008) in the CSF have been noted.

Molecular alterations are also observed in the serum of GBS patients. For instance, Ghabaee et al. found decreases in serum malondialdehyde and antioxidant activity (Ghabaee et al., 2010). Other groups have also examined lipophilic antioxidants in the context of GBS, however they did not find a significant difference in the concentrations of malondialdehyde in patient plasma as in the previously mentioned study, nor did they find a difference in myeloperoxidase concentrations (Tang et al., 2017a). However, they did find a significant decrease in the concentrations of γ-tocopherol and δ-tocopherol and an increase in α-carotene concentrations in GBS patients compared to controls. The latter observation may be indicative of increased risk to oxidative stress-induced damage. In addition, it was noted that GBS patients had a lower concentration of cholesterol compared to controls, although this may reflect demyelination. In a metabolomics analysis in GBS patients, it was found that patient plasma had lower levels of aspartate, creatinine, serotonin, taurine, phosphatidylcholines, lysophosphatidylcholines, sphingomyelins, and acylcarnitines, and higher levels of isoleucine compared to controls (Tang et al., 2017b). Thus, there are alterations in a wide variety of metabolic factors and the question is whether any are the primary mediators of disease or simply markers of damage.

Etiology

GBS has been associated with preceding infections in approximately two-thirds of patients (Hughes and Rees, 1997); these infections typically involve the digestive or respiratory systems and are the basis for an underlying "molecular mimicry" causation (reviewed in Jasti et al., 2016).

Some examples of common infections associated with GBS include *Cytomegalovirus*, *Epstein–Barr virus*, *Herpes*, *Hepatitis A and E*, *Human Immunodeficiency Virus*, *Hemophilus influenza*, *Mycoplasma pneumoniae*, and *Campylobacter jejuni* (Dalakas, 2015).

The Immunology of GBS

First identified in 1969 by Asbury and colleagues, a common characteristic of many GBS patients is a prominent lymphocyte infiltration into the nerves (Asbury et al., 1969). The authors also found that consistent inflammation was seen even in patients who had recovered and therefore speculated that this could be the underlying basis of a relapse. Since then, significant work has gone into identifying what immune cell populations are involved in the inflammation. For example, T cells have been identified in the endoneurium and the epineurial space near venules in sural nerve biopsies of GBS patients, and of these infiltrating T cells, both $CD4^+$ and $CD8^+$ phenotypes were seen (Schmidt et al., 1996). Along with T cells, there is an increase in macrophages in the endoneurium and epineurium of these nerves. Further, Yoshii and Shinohara noted that natural killer cell activity was found to be decreased in GBS patients compared to controls; the authors speculated that deficits in natural killer cell function could leave individuals at a predisposition to acquire GBS because of the immune suppressing ability of these cells (Yoshii and Shinohara, 1998). The majority of work in GBS research has however, focused on T cells. Interestingly, it is known that clonal expansion occurs when T cells are activated, and when this expansion happens, it can lead to random mutations in their genome. It has been shown that there is a higher frequency of hypoxanthine-guanine phosphoribosyltransferase mutant T cells in patients with GBS compared to healthy controls with the frequency of the mutations lessening during the recovery phase (Van den Berg et al., 1995).

Gamma Delta (γδ) T Cells

Of the various types of T cells, Borsellino and colleagues concluded in a review of the literature, that the Vδ1 subset of γδ T cells was the most prevalent in GBS as it has been found to be at three times its normal numbers in patients (Borsellino et al., 2000). Also, in some patients that had become infected with *C. jejuni*, there is an enrichment in the γδ T cell population, specifically of the Vγ5/Vδ1 subset (Ben-Smith et al., 1997). In addition, γδ T cells have been seen in nerve biopsies of acute inflammatory demyelinating polyradiculoneuropathy (AIDP) patients, a variant of GBS (Khalili-Shirazi et al., 1998). γδ T cells have the ability to recognize non-protein epitopes which means they may be able to recognize carbohydrates in gangliosides and ganglioside complexes and contribute to the immune response against *C. jejuni*, thus promoting molecular mimicry (Winer et al., 2002).

Aging γδ T Cells and GBS

With age in the typical immune system, the number of γδ T cells decreases which contrasts with what is seen in GBS where there is an increase in the number of γδ T cells; however, the age-related reduction in γδ T cells has been associated with the Vδ2 subset rather than the Vδ1 subset (Michishita et al., 2011). Furthermore, when the total number of γδ T cells and the Vδ2 subset were quantified in age-matched pairs, it was discovered that although there were significantly higher numbers of these populations in males than in females, no differences in the Vδ1 subset were evident. Interestingly, when age was considered both males and females had a significant negative correlation in Vδ2 T cell numbers (i.e., $R = -0.462$ in males, $R = -0.330$ in females). Thus, alteration in the number of Vδ1 cells in GBS may not be a major contributor to possible age-related immunological drivers of the disease.

However, in terms of the activation profile of γδ T cells during aging, De Rosa et al. found that within 1 year of life, the majority of γδ T cells, and specifically Vδ2 T cells, showed signs of previous activation (i.e., memory T cells) (De Rosa et al., 2004). The authors hypothesized that a portion of γδ T cells may be responsive to self antigens because after an examination of the umbilical cord blood of newborn infants, they found that a portion of both Vδ1 and Vδ2 T cells were non-naïve which could only have occurred prior to exposure to external antigens. This is possible since γδ T cell populations are found in barrier tissues throughout the body (Khairallah et al., 2018). Also, because of their location and exposure to antigens, they appear to be able to form a memory population. In the context of GBS, the question is whether the observed increase in γδ T cells (Ben-Smith et al., 1997; Khalili-Shirazi et al., 1998) is due to tissue-resident γδ T cells responding to gangliosides that resemble previously encountered antigens. Some evidence to support this possibility was shown by Spada et al. who found that Vδ1 γδ T cells have the ability to recognize and attack $CD1c^+$ antigen presenting cells (APCs) (Spada et al., 2000). Further, the γδ T cells that were reactive to $CD1c^+$ APCs produced Th1-like cytokines. The authors concluded that this was a method for the immune system to target these cells in the absence of foreign antigen via both perforin- and Fas-dependent cytotoxicity. It is plausible that the increase in γδ T cells seen in GBS may be an attempt to regulate the immune response thereby removing $CD1c^+$ APCs (e.g., B cells, activated macrophages). Indeed, there is data that this may be occurring in humans. For instance, Vasudev et al. showed in elderly individuals, there was a decrease in the percentage of $CD8^+$ and γδ T cells compared to the young, while there was an increased percentage of $CD4^+$ T cells in the elderly compared to the young (Vasudev et al., 2014). It has also been found that γδ T cells from old (66–96 years of age) and very old (99–103 years of age) individuals are pre-primed in that they have an increased expression of CD69 and increased basal TNF-α secretion compared to young individuals (20–48 years of age) (Colonna-Romano et al., 2002). However, upon stimulation, these aged subjects' cells produced less TNF-α compared to the young. Furthermore, those over 100 years of age had an increased percentage of apoptotic γδ T cells compared to old (aged 75–94) and young (aged 24–53) individuals (Colonna-Romano et al., 2004). Because of the proneness of these cells to undergo apoptosis with increasing age, perhaps there is less of a "brake" on the immune response to preceding infections, a

speculation that would favor the molecular mimicry hypothesis that is linked to GBS.

$CD4^+$ $CD25^+$ T Cells

In a study by Pritchard et al., it was found that there was a reduction in the number of $CD4^+$ $CD25^+$ T cells in GBS patients in the first 1–2 weeks of the disease compared with healthy controls; no differences were seen in the numbers of total B cells, $CD5^+$ B cells, memory B cells, γδ T cells, natural killer cells, or $CD16^+$ natural killer T cells (Pritchard et al., 2007). There were also less $CD4^+$ $CD25^+$ T cells expressing HLA-DP, DQ, DR in the GBS patients compared to healthy controls. Among the patients with the lowest numbers of $CD4^+$ $CD25^+$ T cells, 4 of the 6 patients had antibodies against gangliosides while only 3 out of 14 patients with slightly higher numbers of $CD4^+$ $CD25^+$ T cells tested positive for those antibodies. It is worth noting that after the first 1–2 weeks of the disease, the majority of patients in the study had received intravenous immunoglobulin (IVIg) therapy which may have contributed to the return of $CD4^+$ $CD25^+$ T cell numbers to control levels. In keeping with these findings, Chi et al. also reported reductions in $CD4^+$ $CD25^+$ T cells in the initial stages of GBS and an increase in their numbers following IVIg therapy (Chi et al., 2007). The authors also examined the functionality of these T cells between patients and controls and found that both groups' cells showed equivalent expression of FoxP3 mRNA and suppressor functions (i.e., reduced proliferation and cytokine secretion in responder cells) when co-cultured with $CD4^+$ $CD25^+$ effector T cells. From this, the authors concluded that the initiation of GBS could be due to a short-term reduction of this $CD4^+$ $CD25^+$ regulatory T cell population and the reversibility of this effect may contribute to the monophasic course of the disease. Harness and McCombe also examined T cell populations in GBS peripheral blood mononuclear cells (PBMCs) and compared them with other neuropathies and healthy controls (Harness and McCombe, 2008). In the GBS patients, they found a reduction in the percentage of $CD4^+$ $CD25^+$, $CD3^+$, and $CD8^+$ T cells and total cell population expression of CD95 (Fas), while an increase in Bcl-2 expression within the $CD4^+$ $CD25^+$ T cell population was noted. The reduced expression of $CD3^+$ T cells was explained by a reduction in $CD8^+$ T cells.

Aging Regulatory T Cells and GBS

In terms of age and regulatory T cells (Tregs), Hwang et al. have examined the frequency of regulatory T cells in peripheral blood and found that there was no significant difference between young and old individuals; however within regulatory T cells from older individuals there was an increase in non-naïve regulatory T cells compared to young individuals (Hwang et al., 2009). In addition, their main functional change with age appeared to lie in their ability to suppress interleukin (IL)-10. That is, in an experiment comparing co-cultures of regulatory T cells with $CD4^+$ effector cells, the aged cells (samples from patients >65 years of age) were better at suppressing IL-10 production compared to T cells from younger (<40 years of age). However, the aforementioned effect was only seen when T cells were cultured in a one-to-one ratio, which would not be the case in the context of GBS in which there is a decrease in regulatory T cells.

While Hwang et al. reported no difference in frequency of regulatory T cells between ages in peripheral blood in humans, others have reported that there is actually an increase in regulatory T cells in aged individuals (Gregg et al., 2005; Rosenkranz et al., 2007; Raynor et al., 2012). This increase in Tregs appears to be caused by a down-regulation of Bim which is an apoptotic factor (Chougnet et al., 2011). There are no studies to date that have assessed the number of regulatory T cells or the function of these cells in GBS in the context of aging. Thus, it is unknown whether there is an increase in regulatory T cells like that seen with normal aging. One can speculate that even if there was an increase, it would be below that of normal aging because of the fewer numbers of these cells in GBS individuals. Thus, one can further hypothesize that fewer regulatory cells in elderly people could contribute to the increased severity of the disease in this population.

T Cell Cytokines

T Helper (Th)1 and Th2

When analyzing GBS patient lymphocytes stimulated with *C. jejuni*, it was found that in the initial stages of the disease there is an upregulation of Th1 pro-inflammatory cytokine mRNA [i.e., interferon-γ (IFN-γ), IL-1β, tumor necrosis factor (TNF), IL-6, IL-10, and IFN-γ:IL-4 ratio] and a decrease in Th2 anti-inflammatory cytokine mRNA (i.e., transforming growth factor (TGF)-β and IL-4) (Nyati et al., 2011). However, in the recovery phase, there is a predominant upregulation of Th2 cytokines and a decrease in the expression of Th1 cytokines. IFN-γ is not the only Th1 cytokine dysregulated in people with GBS. GBS PBMCs produce less IL-2 and have a lessened response to IL-2 compared to healthy controls, however this effect dissipated after treatment with plasma exchange (Yoshii and Shinohara, 2000). There was also an increase in soluble IL-2 receptors which decreased over the disease course. The authors postulated that this could be due to a functional depression in T cells due to a previous reaction to a preceding infection and therefore may be allowing the body to recover from the previous immune response.

Th17

There is a growing body of evidence supporting the role of Th17 cells and their pro-inflammatory cytokines in the pathology of GBS (Wu et al., 2016). The levels of IL-17 and IL-22 are elevated in the plasma and CSF of patients with GBS compared to healthy controls, and further, the CSF levels of the two cytokines correlate with disease score severity (Li et al., 2012). It is speculated that the presence of the cytokines in CSF may reflect a disruption in the blood-nerve barrier (BNB). In terms of mechanisms, Liang et al. isolated PBMCs from GBS patients and controls and examined mRNA expression of T cell immunoglobulin and mucin-3 (TIM-3), which is highly expressed on Th1 cells and is thought to decrease IFN-γ secretion when bound (Liang et al., 2012). They also examined the presence of Th17 cells. In $CD4^+$ T cells, the group found a decrease in the expression TIM-3 and an increase in the expression of RORγt. When anti-TIM-3 antibodies were given, secretion of IFN-γ and IL-17 increased in GBS compared

to controls. Further, there was an increase in the number of Th17 cells compared to Th1 and Th2 cells in the circulation of GBS patients. The authors concluded from these findings that TIM-3 may play an inhibitory role in Th17 activation. In alignment with this study, other groups have also reported increased levels of Th17 cells, increased mRNA expression of RORγt, and increased IL-17 concentrations in the plasma and CSF of GBS patients (Han et al., 2014). For example, Li et al. studied the prevalence of Th1, Th17, and Th22 cells in GBS compared to controls and other conditions and found that GBS patients had increased frequency of Th1, Th17, and Th22 cells in their circulation along with elevated IL-17 and IL-22 in their serum (Li et al., 2014). Of further note, IVIg therapy led to a decrease in serum levels of IL-17 and IL-22 and a general decrease in Th17 and Th22 cells.

Aging T Helper Cells, Their Cytokines, and GBS

Because of thymic involution and the decline in peripheral naïve T cell proliferation with age, there is a significant disruption in the production of naïve T cells as one grows older (Haynes et al., 2000; den Braber et al., 2012). This would impact Tregs and Th17 cells that are both derived from naïve T cells (Lynch et al., 2009). During typical aging there is an increase in Tregs, however in GBS patients there is a decrease in Tregs and an increase in Th17. Perhaps because there are so few naïve T cells with age, there is an imbalance in the differentiation of Th17 and Tregs once a concurrent infection triggers the immune response via γδ T cells in GBS patients. Indeed, Schmitt et al. have shown that the balance between Th17 cells and Tregs changes with age (Schmitt et al., 2013). Specifically, they noted that in unstimulated cells, there was an increase in the Th17/Treg ratio with increasing age, but when stimulated, there was a decrease in the Th17/Treg ratio with increasing age. Interestingly, Lim et al. have shown that in aged mouse splenocytes, there is an increased tendency to produce Th17 cells compared to cells from young mice (Lim et al., 2014). In addition, increased IL-1β and decreased IL-2 signaling associated with age are important factors in the upregulation of Th17 cells.

IL-2 is another cytokine that is important for the expansion of regulatory T cells (Nelson, 2004). As previously mentioned, Yoshii and Shinohara had demonstrated that PBMCs from GBS patients produce less IL-2 and have a lessened response to IL-2 compared to healthy controls (Yoshii and Shinohara, 2000). In addition, with age, there is reduced production and response to IL-2 (Rabinowich et al., 1985; Pahlavani and Richardson, 1996). Altogether, this could indicate a deficiency in IL-2 impairing normal age-related accumulation of regulatory T cells and thus leading to an increase in the susceptibility of acquiring GBS with age.

In addition to a decline in naïve T cells via thymic involution and decreases in peripheral T cell proliferation, naïve T cells from older individuals produce less IL-2 which would negatively affect the generation of effector T cells (Haynes et al., 2000; den Braber et al., 2012). When IL-2 was exogenously given, it rescued effector cell expansion and defective functioning in aged naïve T cells. However, while in the effector state, aged $CD4^+$ T cells can produce equivalent IL-2 levels to young $CD4^+$ T cells when exposed to exogenous IL-2. Interestingly, when these exogenously IL-2-exposed effector T cells from aged animals convert to memory T cells, they no longer produce equivalent IL-2 levels, instead they revert back to low IL-2 production when re-stimulated. This may be related to the increase in Th1 cytokines and a decrease in Th2 cytokines seen early in the disease course of GBS; this effect is reversed during the recovery phase of GBS (Nyati et al., 2011). Because IL-2 is mainly produced by Th1 cells (Pahlavani and Richardson, 1996) and is needed for effector T cell expansion (Nelson, 2004), this could explain the increase in helper T cells seen in GBS. While there is an increase in helper T cells in GBS, with age there is a decrease in IL-2 production and responsiveness and thereby a decline in Treg expansion as previously mentioned (Rabinowich et al., 1985; Pahlavani and Richardson, 1996), thus allowing helper T cells to function unregulated in the context of GBS.

The cytokines produced by Th1 and Th17 cells could lead to defects in APCs such as macrophages. There are more macrophages present in the intact nerves of older mice relative to that of a mature adult mouse (Büttner et al., 2018). In addition, there is an upregulation of IL-4 in sciatic nerves from adult animals, however in older animals, this upregulation is no longer seen. IL-4 is needed to induce an anti-inflammatory phenotype in macrophages and with age, macrophage responses to IL-4 are altered (Mahbub et al., 2012; Albright et al., 2016). As GBS progresses and the inflammatory landscape resolves, the upregulation of Th2 cytokines, specifically IL-4, may contribute the recovery of the disease via induction of phenotypic shifts in macrophages from pro-inflammatory to anti-inflammatory.

Other Immune-Contributing Factors

BNB Permeability

Another potential mechanism underlying nerve damage in GBS could be "bystander" inflammation (Harvey et al., 1995). In one study, T cells sensitized to ovalbumin were transferred into Lewis rats followed by an intraneural injection of ovalbumin or casein. In the ovalbumin-injected group, there was a rapid influx of immune cells into the nerve, specifically αβ T cells and macrophages. The increase in cells into the nerve was consistent with increased BNB permeability which is notable since increased permeability has the added detrimental effect of opening up the nerve environment to circulating neural-specific antibodies (Pollard et al., 1995).

CD1 Polymorphisms

Polymorphisms on the CD1 gene in humans have been associated with increased risk of developing GBS. Individuals with CD1E*01/01 genotype have an increased likelihood of developing GBS while those with a CD1A*01/02, CD1E*01/02, or both have a decreased chance in developing GBS (Caporale et al., 2006a).

Integrins

In GBS patients, differences in integrin expression have been found relative to controls (Previtali et al., 1998). Specifically, the expression of β4 integrins was absent from both myelinating and

non-myelinating SCs, and in sural nerves with axonal loss. SCs were also found to express α5 integrins, but it is unknown what role these integrins play.

Antigen(s)

Many studies have sought to identify the target antigen of the immunological response in GBS. Work by Terryberry et al. found a strong correlation between the presence of autoantibodies against tubulin and neurofilament-H in GBS patient serum (Terryberry et al., 1998). Further, Notturno et al. found that 71% of GBS patients possessed antibodies against single gangliosides or ganglioside complexes (Notturno et al., 2009). These results have been strengthened with others finding an association between antibodies against ganglioside complexes and preceding infections. For example, Kaida et al. showed that 17% of the GBS patients in their study had antibodies against a ganglioside complex and that those who had anti-monosialotetrahexosylganglioside (GD)1a/GD1b and/or anti-GD1b/polysialoganglioside (GT)1b antibodies had an increased association with the need for mechanical ventilation (Kaida et al., 2007). Interestingly, patients with GD1a/GD1b and/or anti-GD1b/GT1b antibodies had sera that reacted with GM1/GD1a and GM1/GT1b. Gangliosides serve many purposes, but among them is protection from complement activation via the binding of complement regulatory protein factor H (Cutillo et al., 2020). Given that GBS is often associated with anti-ganglioside antibodies, it could be that these antibodies are allowing for the activation of the complement system by attacking gangliosides and thus leading to further recruitment of immune cells. In addition, anti-GM1 antibodies have been shown *in vitro* to facilitate leakage of the BNB thus allowing for penetration into the nerve environment (Kanda et al., 2000).

Because of the known complement activation see in GBS patients, recent clinical trials have examined the role of eculizumab (a humanized monoclonal antibody against C5 that prevents cleavage into C5a and C5b components) in patients with GBS (Davidson et al., 2017; Misawa et al., 2018). Results of a Japanese, multicenter, Phase 2 clinical trial showed that 14 of 23 patients were able to walk independently at the conclusion of the trial in the eculizumab group compared to 5 of 11 patients in the placebo/IVIg group. However, of the 34 GBS patients enrolled in the study, three experienced serious adverse effects (Misawa et al., 2018). Also, due to the small sample size, statistical comparisons between the eculizumab and placebo groups could not be performed. Earlier examination of eculizumab in humans had been performed in the United Kingdom, however again, sample size was very small due to low recruitment levels (Davidson et al., 2017). In this study, patients were randomly assigned to the IVIg with eculizumab or IVIg with placebo groups. After pre-screening and informed consent of GBS patients, only seven patients were eligible with five being assigned to the eculizumab group and two patients being given placebo. In this trial, four serious adverse events were reported. In terms of primary outcomes, both placebo controls and two of the five eculizumab-receiving patients experienced a decrease in GBS disability score by at least one grade. Taking both studies together, it appears that eculizumab may be effective in severe cases although given the sample sizes of the aforementioned studies, there is need for more studies on the effectiveness and possible adverse consequences of the treatment (Misawa and Suichi, 2020).

Gangliosides With Age

The composition of gangliosides within the CNS undergoes dramatic changes with age (Kracun et al., 1991), and although the composition of gangliosides differs between the CNS and PNS, it was hypothesized that perhaps similar age-related alterations in ganglioside composition are seen in the PNS as in the CNS. Ohsawa tested this idea by examining the ganglioside composition in dorsal root ganglia (DRG) of rats across their lifespan up to 24 months of age (Ohsawa, 1990). From birth to 12 months of age, there was an increase in total gangliosides within the DRG, but this coincided with an increase in the weight of the DRG. From 12- to 24-months of age, the total gangliosides continued to increase, but much less rapidly. This latter observation also occurred in parallel with an increase in DRG weight. The question then is whether the increase in ganglioside content is simply related to the increased size of the animal. Importantly also, in terms of composition, Ohsawa found that after the first few postnatal months, there was relatively little alteration in the composition of gangliosides within the DRG. However, it is unknown whether ganglioside content is changed with age in humans or in GBS.

GBS VARIANTS

Currently, GBS is viewed as an autoimmune condition with a diverse spectrum of presentation. The unique variants of GBS, namely AIDP, acute motor axonal neuropathy (AMAN), acute motor-sensory neuropathy (AMSAN), Miller Fisher syndrome/Bickerstaff brainstem encephalitis (MFS/BBE), and ataxic GBS/acute sensory ataxic neuropathy have differing immunological causes and will be discussed further in the present review.

ACUTE INFLAMMATORY DEMYELINATING POLYRADICULONEUROPATHY (AIDP)

The most common variant of GBS in North America and Europe is AIDP (Suzuki, 2013). AIDP was also the most common variant in a study of GBS in the elderly (Peric et al., 2016). As its name implies, it is characterized by inflammatory demyelination and when it is very severe, it can also lead to secondary axonal degeneration (Griffin et al., 1996; Ho et al., 1998; Jasti et al., 2016).

Molecular Mimicry

In a study examining the prevalence of *C. jejuni* infections in GBS cases in rural China, it was found that of those diagnosed with AIDP, 42% of their sample was seropositive for a previous *C. jejuni* infection (Ho et al., 1995). Given that γδ T cells may be underlying the response to *C. jejuni* due to their ability to recognize non-protein epitopes, Cooper et al. performed a

preliminary examination of the reactivity of PBMCs isolated from GBS patients to a *C. jejuni* strain cultured from a single patient with GBS (Cooper et al., 2002). They found that there was an increase in proliferation in PBMCs from two of the GBS patients. In subsequent sural nerve biopsies from eight of the patients who had *C. jejuni* infections themselves, there was an increase in the expression of CD1b (necessary for the presentation of non-protein antigens to γδ T cells). This was in keeping with findings by Khalili-Shirazi et al. who found CD1b expression on endoneurial macrophages in two AIDP patients (Khalili-Shirazi et al., 1998). Along with CD1b expression there was also an infiltration of γδ T cells, $CD8^+$ T cells, and macrophages and an increase in the expression of HLA-DR.

C. jejuni is not the only bacteria associated with AIDP. A preceding infection with *M. pneumoniae* has been associated with the presence of galactocerebroside antibodies (Kusunoki et al., 2001). Moreover, there is a galactocerebroside-like structure on *M. pneumoniae* which could be leading to molecular mimicry in AIDP. Definitive data that *C. jejuni* or *M. pneumoniae* has a causative role in AIDP is still however outstanding.

Immunological Mechanism

In early studies examining the pathogenesis of GBS, it was thought that the main immunological players were macrophages and lymphocytes, since the majority of autopsy and biopsy studies found marked infiltration of macrophages in the basement membrane that appeared to be stripping myelin from axons (Prineas, 1981). The current thinking regarding the mechanism underlying the demyelinating aspect of AIDP is that complement activation is aimed at the surface of SCs (Hafer-Macko C. E. et al., 1996) that would subsequently lead to breakdown of myelin and thus recruitment of macrophages (Jasti et al., 2016). Hafer-Macko et al. have indeed shown in an autopsy study that individuals with AIDP did display C3d and membrane attack complex (MAC) deposition on the surface of their SCs (Hafer-Macko C. E. et al., 1996). This study suggested that complement was involved in the pathology of AIDP and the authors speculated that complement activation could be caused by antibody binding that leads to myelin breakdown and macrophage recruitment. As reported, these macrophages could penetrate the basal lamina of the SCs, dissociate the SCs, and then begin to strip away myelin (Griffin et al., 1996).

As T lymphocytes were also observed in autopsy and biopsy studies, Wanschitz et al. aimed to dissociate the role of complement and T cells in AIDP by using autopsy material to examine complement components and their regulators (i.e., CD59) as well as infiltrating T cells (Wanschitz et al., 2003). Like Hafer-Macko et al., the investigators found an increase in complement deposition along myelinated fibers as well as an increase in C9neo antigen (a component of MAC) in areas of demyelination. Interestingly, CD59 was upregulated where demyelination was taking place.

Within the endoneurium, there was a further correlation with the degree of demyelination and the number of $CD3^+$ T cells. There is some evidence that these T cells may recognize self antigens. In one study, lymphocytes from AIDP patients and other disease controls were exposed to Protein (P) 2 protein, crude human peripheral nerve, or CNS myelin basic protein (MBP) and assayed for macrophage migration inhibitory factor (MIP) (Sheremata et al., 1975). The authors found that only AIDP patient lymphocytes showed sensitization to P2 or crude peripheral nerve. Others have also found that PBMCs from some GBS patients show reactivity to myelin protein zero (MPZ/P0) (Khalili-Shirazi et al., 1992). Further, when sera from patients with AIDP was applied to teased mouse sciatic nerves, 44% showed IgG bound to the nodes of Ranvier or the paranodes. Others have also noted that patient sera significantly contained anti-gliomedin antibodies (Devaux et al., 2012) and that anti-LM1 antibodies associate with demyelinating GBS (Kuwahara et al., 2011).

In addition to immune cells, SCs have been found to possess the necessary components to process and present antigens when cultured *in vitro* with IFN-γ (Meyer Zu Horste et al., 2010). These include proteasome subunit delta (Y), TAP2, HLA Class I, and HLA-DP, DQ, DR. Some evidence that this may occur *in vivo* was the discovery in sural nerve biopsies from AIDP patients that SCs were found to be co-localized strongly with proteasome subunit delta (Y), TAP2, HLA Class I, and HLA-DP, DQ, DR compared to control nerves.

Discussion: Possible AIDP Mechanism(s) Including the Plausible Role of Aging

As mentioned previously, γδ T cells were identified in nerve biopsies from two of two AIDP patients and may represent a pathological population in AIDP due to their ability to recognize non-protein epitopes. While this represents a very small sample size, γδ T cell recognition of *C. jejuni* would strengthen the molecular mimicry hypothesis. This possibility is strengthened by the observation that CD1b expression, which is necessary for antigen presentation to γδ T cells, is increased in patients who had previous *C. jejuni* infections (Cooper et al., 2002). In addition to a possible involvement of γδ T cells, several groups have identified complement activation in areas with active demyelination in AIDP patients (Hafer-Macko C. E. et al., 1996; Wanschitz et al., 2003); specifically, complement products have been localized to the surface of SCs (Hafer-Macko C. E. et al., 1996). This complement activation is believed to have been triggered by antibody binding to glycoconjugates on the surface of SCs. It is plausible that this antibody-driven activation could explain the increased presence of macrophages seen in AIDP autopsy and biopsy samples (Prineas, 1981). Furthermore, given that previous studies have shown that T cells from AIDP patients have a sensitization to crude peripheral nerve and P2 protein, it stands to reason that these sensitized T cell could be initiating B cell activation and antibody production.

With respect to aging, Márquez et al. recently assessed for sex differences and age-related changes of the immune system in humans (Márquez et al., 2020). In regard to B cells, the authors found that older males display a decrease in the percentage of B cells compared to young males and older females. Further, with age, there were alterations in the B cell repertoire (Weksler and Szabo, 2000), and antibody production in response to vaccination was lessened in the elderly relative to the young (Stiasny et al.,

2012). In the latter however, the responsiveness and functionality of antibodies produced did not appear to be affected by age (Stiasny et al., 2012). In keeping with this, Pritchard et al. found no change in B cell populations in GBS relative to controls (Pritchard et al., 2007). Aside from a decline in B cells, Marquez et al. also found that males appear to have accelerated age-related transcriptomic changes to their PBMCs compared to females (Márquez et al., 2020). Given that GBS affects more males than females, this could imply that the immune changes seen with aging in males may be a contributing factor to the pathogenesis of GBS via alterations in B cells. In addition, there have been some studies suggesting that γδ T cells and B cells interact (Häcker et al., 1995; Rampoldi et al., 2020). More specifically, Häcker et al. have found that when autologous B cells were used to stimulate γδ T cells in culture, it led to an increase in the percentage of $V\delta1^+$ cells (Häcker et al., 1995). This is of particular interest given that Borsellino et al. noted an increased prevalence of the Vδ1 subset in GBS patients (Borsellino et al., 2000).

Experimental Allergic Neuritis (EAN) Model

The majority of the mechanistic information gleaned about AIDP has come from work in an animal model known as EAN. EAN was first described in 1955 by Waksman and Adams (Waksman and Adams, 1955) and consists of immunizing rodents with whole myelin or specific myelin proteins, or through the adoptive transfer of T cells sensitized to the P2 myelin antigen, similar to the CNS equivalent model experimental autoimmune encephalomyelitis (EAE) (Izumo et al., 1985). As the disease progresses, the rodents experience weight loss and increasing weakness and paralysis until a peak is reached, followed by a gradual recovery phase. Support that EAN was a useful model for AIDP was provided by Asbury et al. who drew many similarities between the inflammatory infiltrate seen in autopsied AIDP tissue and that in EAN (Asbury et al., 1969). More specifically, during the clinical course of EAN, $CD4^+$ T cells can be seen in the sciatic nerve in detectable numbers prior to the onset of clinical symptoms (Zhu et al., 1997). At the peak of disease, there is a steep increase in the number of $CD4^+$ cells that then eventually decreases to slightly above baseline levels around the beginning of the recovery phase. $CD8^+$ T cells, albeit much less than $CD4^+$ T cells, can also be seen in EAN. However, they are not detected until the peak of the disease and they themselves do not peak until the initial phases of recovery.

In terms of disease resolution, it has been found that apoptosis of T cells accounts for the eventual reduction of approximately 19% of the T cell infiltrate seen at peak EAN (Zettl et al., 1994). Using the adoptive transfer model of EAN, Mausberg et al. developed a P2-sensitized T cell line expressing green fluorescent protein (GFP) so as to track the kinetics of these cells in EAN (Mausberg et al., 2018). Following transfer, the authors found that T cells enter the PNS in two distinct waves. The first wave occurs from day 1 to day 3 and is composed of endogenous T cells close to but not within the nerve environment. Around day four, there is another wave made up of both transferred and endogenous T cells found close to the nerves, however in this instance, only the transferred T cells were able to cross into the endoneurium from the epineurium.

In addition to mice, EAN has been modeled in rats. Using adoptive transfer, Hahn et al. injected T cells sensitized to P2 peptide and antibodies against galactocerebroside into naïve Lewis rats (Hahn et al., 1993). They found extensive immune infiltration and demyelination and from this concluded that when T cells enter the peripheral nerve environment, they alter the BNB. The alterations to the BNB open up the endoneurium to an attack via circulating antibodies to nerve antigens. Of note, EAN in rats also involves a very strong B cell response with an increase in antibody-producing cells prior to disease onset and which peaked at peak disease (Zhu et al., 1994).

In terms of the molecular factors underlying EAN etiology in rats, MAC (C5b-9) has been found to be deposited on the surface of SCs and myelin sheaths prior to the onset of symptoms in EAN (Stoll et al., 1991). Clusterin, a protein involved in lipid transport, membrane recycling, and regulation of injury-induced attack of the complement on membranes, has been found to reduce the severity of the disease course of EAN but did not change the initial onset of the disease (Dati et al., 2007). In addition, when the nerves were examined histologically, it was found that the clusterin-treated nerves showed significantly more remyelinated fibers compared to controls showing that its administration was more effective in the recovery phase compared to the induction phase of EAN.

In addition to prominent infiltration of T cells during the disease course of EAN, macrophages are also involved. Hartung et al. have demonstrated that when the production of eicosanoids by macrophages is inhibited, the disease course of EAN is attenuated (Hartung et al., 1988). Macrophages may also contribute to the regulation and apoptosis of T cells via the secretion of TNF-α, reactive oxygen and nitrogen species, or direct contact (Wu et al., 1995; Aliprantis et al., 1996; Kiefer et al., 2001). During recovery, macrophages may also contribute via functions seen in Wallerian and Wallerian-like degeneration (e.g., myelin debris clearance) (Kiefer et al., 2001).

Several studies have also identified a role for mast cells in the course of EAN. Brosman et al. examined the presence of mast cells over the course of EAN in Lewis rats and found that at 8 days post EAN-induction, there was a decrease in the number of mast cells present compared to control animals (Brosman et al., 1985). In addition to number of mast cells, the authors also examined the percentage of degranulated mast cells and found a significant increase from controls at 9 days post EAN-induction. The decrease in mast cell counts and the increase in the percentage of degranulated mast cells occurred prior to symptom onset (10 days post-induction). Later in the disease course (∼3 weeks post-induction), there was an increase above control levels in the number of mast cells as well as the percentage of degranulated mast cells that persisted until 16 weeks post-induction; although the authors pointed out that these cells could have actually been a combination of mast cells and basophils. Interestingly, Seeldrayers et al. attempted to hinder the functioning of mast cells by giving an anti-inflammatory drug called nedocromil sodium which is known to stabilize mast cells starting at 7 days post-EAN induction (Seeldrayers et al., 1989). When treated with the drug, there was a 50%

reduction in the incidence of EAN. Further, at 15 days post-induction, the average clinical score was significantly lower in the treated group (average score of 1.02) compared to the saline-treated group (average score of 1.99). Therefore, there appears to be a role for mast cells in the initiation of EAN, possibly in alterations to the BNB that allow for their penetration into the nerve environment.

Integrins in EAN

The role of integrins has been examined in EAN. At peak disease, it was found that myelinating SC units were abnormal in that staining revealed many broken myelin rings with uniform staining. Further, these glia lost α2 and β4 integrins unlike in GBS where only β4 integrins were absent (Previtali et al., 1998). Similar absence of α2 and β4 integrins was seen in non-myelinating SCs as well. Following peak disease, β4 integrins were still not present while α2 integrins were re-expressed on SCs again. In the aforementioned study, the authors also induced a more severe form of EAN through a higher dose of myelin and found extensive myelin degradation and axonal loss as well as the appearance of α5 expression on SCs.

In addition to these integrins, others have looked at very late antigen-4 (VLA-4 or α4/β1 integrin) and vascular cell adhesion molecule-1 (VCAM-1) in EAN (Enders et al., 1998). At the onset of clinical symptoms, VCAM-1 expression was present and increased indicating a role in disease progression. Regarding VLA-4, it was found that when VLA-4 antibodies were given in an adoptive transfer EAN model, the treatment significantly weakened the disease severity and was associated with minimal T cell or macrophage infiltration. This was also the case when VCAM-1 antibodies were given but less strongly than VLA-4. Intercellular adhesion molecule-1 (ICAM-1) has also been implicated in EAN (Stoll et al., 1993). It was found to be expressed on endothelial cells and on infiltrating macrophages at or just before the commencement of clinical symptoms. Endothelial-leukocyte adhesion molecule-1 (ELAM-1) has also been found to be increased in GBS patient sera (Hartung et al., 1994).

Matrix Metalloproteinases (MMPs)

Changes in MMPs have been reported in EAN. MMPs are endopeptidases that contain zinc and can be secreted by macrophages and T cells (Goetzl et al., 1996). Among many actions, MMP effector functions in the context of the immune system can include facilitating T cell migration across the basement membrane and activation of TNF-α from its membrane-bound form via cleavage. In EAN, the mRNA for a 92 kD gelatinase (MMP9) was found to peak at peak disease and then decrease through recovery (Hughes et al., 1998; Kieseier et al., 1998). Matrilysin (MMP7) also peaked at peak disease but remained elevated thereafter unlike MMP9. Macrophage metalloelastase followed a similar expression pattern as MMP7, however the maximum of macrophage metalloelastase was drastically lower than MMP7 (Hughes et al., 1998). Alterations in stromelysin-1 mRNA levels have also been reported where it began increasing with symptom onset, peaked at peak disease and then decreased sharply as recovery begins.

Cytokines

Not surprisingly because of the robust immune response, there are changes in cytokine levels in EAN. In an examination of mRNA levels of IFN-γ, IL-1β, IL-12, and IL-10 over the time course of EAN, Gillen et al. found that at symptom onset, there was an increase in IFN-γ, IL-1β, and IL-10 mRNA (Gillen et al., 1998). IFN-γ mRNA expression decreased in the following days but persisted at low levels for the duration of the disease. IL-10 mRNA was above control levels for most of the disease but decreased in the early recovery phase while IL-1β was at its highest at symptom onset and decreased across the disease course. Lastly, IL-12 did not show an increase until peak disease and persisted until the recovery phase. Based on these findings, the authors made several conclusions. Because IFN-γ and IL-1β mRNA expression was increased at the onset of symptoms, this could mean that the two pro-inflammatory cytokines may be playing a role in the amplification of the immune response during EAN. IL-12 is typically an inducer of IFN-γ, however in this study, it did not reach its peak mRNA expression until after IL-12, therefore more experiments need to be performed to elucidate its function in the context of EAN.

In contrast to the pro-inflammatory cytokines, IL-10 may be playing a role in disease recovery as is TGF-β1 which are upregulated by macrophages and may act as an immunosuppressant in EAN (Kiefer et al., 1996). Some direct evidence that IL-10 has an immunosuppressive effect in EAN is the reduction in symptom severity seen when IL-10 is administered at immunization or after disease onset (Bai et al., 1997). In a similar study examining the number of immune cells expressing cytokine mRNA during EAN, Zhu and colleagues corroborated the previously mentioned findings in regard to IL-1β and IL-12 (Zhu et al., 1997). For IL-10 however, they reported that the number of IL-10 mRNA-expressing cells began increasing at peak disease and increased through recovery to reach its peak at the end of the disease course. Unlike the aforementioned studies, Zhu et al. also examined IL-6, TNF-α, TNF-β, and cytolysin mRNA expression. The number of IL-6 mRNA expressing cells followed a similar pattern as IL-1β in that it peaked around the time of disease onset and gradually declined over the course of the disease. However, when examining the number of IL-6-producing cells, the peak occurred at peak disease. Similarly, the number of TNF-α mRNA expressing cells began increasing prior to symptom onset and peaked at peak disease. TNF-β expression also followed a similar trajectory as TNF-α, but with a smaller number of expressing cells. Lastly for the number of cytolysin-expressing cells, there was a peak at the onset of recovery with a slight decrease as recovery continued. An important caveat to all of these studies however is that they were examining the expression strictly in EAN and were not comparing relative to controls. This is important to note because many of these cytokines are expressed in Wallerian degeneration and may simply be an indication of that process (Kiefer et al., 2001).

IL-23p19 is another cytokine observed in EAN (Hu et al., 2006). Its mRNA first appeared prior to symptom onset, peaked at the onset of symptoms and decreased through the beginning

phases of disease recovery. The authors then examined sural nerve biopsies of AIDP patients and found that the cellular source of IL-23p19 was endoneurial macrophages. Because the expression of IL-23p19 was detected prior to the infiltration of immune cells associated with symptom onset, the authors concluded that the resident macrophages are the likely cellular source of this cytokine.

While it has been found that there is an increase in the expression of IFN-γ mRNA in the initial stages of EAN, others have shown that if IFN-γ is knocked out, there is significantly worse disease following the acute stage (Zhang et al., 2012). The authors of this study were attempting to elucidate the role of Th17 cells in EAN by knocking out the potent Th1 cytokine, IFN-γ. Indeed, cultured macrophages from the null animals displayed reduced production of nitric oxide and Th1 cytokines (IL-6 and IL-12) which implied a reduced Th1 response. As such, it was surmised that the worse disease in the knockout mice was likely due to an increase in IL-17A-expressing $CD4^+$ cells infiltrating the cauda equina compared to controls. Further, lymphocytes isolated from the knockout mice also had increased proliferative responses to P0 which was used to induce EAN in this study. This study highlighted a potential role for Th17 cells in EAN. To further distinguish the role of Th17 cells in EAN, Wang et al. examined cytokine expression in EAN (Wang et al., 2014). Compared to symptom onset, there was a greater proportion of both $CD4^+$ $IL\text{-}17A^+$ and $CD4^+$ $IL\text{-}22^+$ cells at peak disease. In addition, they found the expression of cytokines IL-17A and IL-22, and transcription factor RORγt to be increased at peak compared to onset. Opposite to this and in alignment with disease progression, there was a decrease in the proportion of Tregs at peak compared to onset.

Chemokines

Chemokines play critical role in immune cell chemotaxis. To identify if specific chemokines are involved in EAN pathogenesis, Zou et al. examined expression of macrophage inflammatory protein-1α (MIP-1α), MIP-2, and monocyte chemotactic protein-1 (MCP-1) in EAN (Zou et al., 1999). MIP-1α peaked at the peak of disease and then steadily decreased, while MCP-1 peaked just prior to symptom onset and then decreased thereafter until the beginning of the recovery phase. MIP-2 began increasing at peak disease and then peaked in the beginning of the recovery and then decreased into full recovery. In this study, the authors also gave anti-chemokine antibodies and examined the effect on EAN progression. Anti-MIP-1α delayed the onset of the disease and significantly weakened the severity. Similarly, MCP-1 antibodies also delayed disease onset and minimized the severity, but much less so than the anti-MIP-1α treatment. Lastly, MIP-2 did not have a significant effect on either the onset or the severity of EAN.

Expanding on this work, Xia et al. examined the prevalence of pro-inflammatory chemokine ligand and receptor pairs in a severe model of EAN (Xia et al., 2010). Using qPCR, the authors found significant increases in the level of CCL2/CCR2, CXCL10/CXCR3 and CCL5/CCR1, and CCR5 in EAN mice compared to controls. When looking at the cellular source, the authors found that CXCL10 co-localized with endoneurial microvessels while its receptor co-localized with $CD3^+$ T cells. They also noted that CCL2 was expressed on SCs with its receptor CCR2 co-localizing with macrophages and occasionally $CD3^+$ T cells. Lastly, CCL5 was expressed on axons while macrophages and $CD3^+$ T cells also occasionally co-localized with the receptors, CCR1 and CCR5.

Discussion: Possible EAN Mechanisms and the Plausible Involvement of Aging

Finally, age may be important player in EAN. In a study examining the pattern of EAN in different ages of rats, the authors found that younger rats (i.e., 4 weeks old) had less severe disease with more frequent relapses compared to older rats (i.e., 25 weeks old) (Brosnan et al., 1988). In the older rats, their disease was acutely severe, but monophasic in disease course. When the animals were challenged a second time 44 days after the initial disease induction, the older rats showed a major effect while the younger rats had a mild reaction to the re-challenge with some instances of relapse. It is possible that the decreased responsiveness of macrophages to IL-4 with increasing age, may explain the increased severity of EAN in older animals (Brosnan et al., 1988). The authors however cautioned that due to the differences in disease patterns in EAN compared to GBS, their findings should be accepted with caution.

Differences in EAN between newborn and adult Lewis rats were also examined by Pilartz et al. (2002). They found that adoptive transfer of EAN in newborn rats led to disease induction characterized by immune infiltration and mast cell degranulation. Although this immune infiltration was present, in the newborn rats they were unable to identify any instances of demyelination possibly due to the relative size differences of myelinated fibers between adult and newborn animals.

In general, the mechanism underlying EAN is very similar to AIDP, as one would hope an animal model would be. In EAN, it appears that T cells and mast cells play a role in the opening of the BNB since sensitized T cells are able to enter into the nerve (Mausberg et al., 2018). Because adoptive transfer studies of T cells sensitized to P2 protein have shown effective transfer of EAN, it stands to reason that T cell-mediated attack on peripheral nerve proteins would underlie the model. Following the contributions of T cells (Hahn et al., 1993) and mast cells in opening the BNB (Olsson, 1967; Esposito et al., 2002), this would then allow for the infiltration of antibodies and complement products that would further contribute to the demyelination by attacking the SC surface (Stoll et al., 1991). As noted by Zhu et al., EAN in rats has been associated with a robust B cell response and increased antibody production in the early phases of the model (Zhu et al., 1994). Because it has been shown that with increasing age there is an increased presence of macrophages and mast cells within the peripheral nerve (Ceballos et al., 1999), it is plausible that these cells could contribute to the increased severity seen with age in EAN. Notably, if there is an increased presence of these cells already within the nerve then the initial attack by T cells may trigger a very robust and acute response due to the immediate proximity of these other cell types.

ACUTE MOTOR AXONAL NEUROPATHY (AMAN) AND ACUTE MOTOR-SENSORY NEUROPATHY (AMSAN)

AMAN and AMSAN are referred to as the axonal variants of GBS and were not properly defined until the early 1990s (Feasby, 1994). AMAN is the most common variant in Asia and South America (Kuwabara and Yuki, 2013). Interestingly, there are seasonal variations in the prevalence of AMAN; for example in China, acquisition of the disease coincides with increases in *C. jejuni* infections (Ho et al., 1995; Hafer-Macko C. et al., 1996). In AMAN, the target of the immune attack is motor fibers, while in AMSAN the target is both motor and sensory fibers (Griffin et al., 1995, 1996). AMAN has been found to have a more rapid onset compared to AIDP (Kuwabara and Yuki, 2013), and further, it is believed that AMSAN is a more severe form of AMAN (Griffin et al., 1996). Interestingly, in AMAN there is minimal demyelination and lymphocyte activity, and as such it is speculated to be an antibody- and complement-mediated attack on peripheral axons (Hafer-Macko C. et al., 1996). Patients with AMAN tend to recover rapidly, and it has been speculated that the motor deficits seen may be caused by antibody binding at the nodes of Ranvier (Ho et al., 1997). Of note, AMSAN is usually seen in older patients as compared to AMAN (Webb et al., 2015). That is, the rate of incidence of AMSAN in an elderly group of patients was twice that of a young cohort (12% vs. 6%) (Peric et al., 2016). However, in another group of AMAN patients, it was found that 58% of those presenting with antibodies to nodal adhesion molecules were over the age of 60 (Devaux et al., 2012).

Immunoglobulins

A current theory as to the cause of AMAN and AMSAN is molecular mimicry. The strong association between *C. jejuni* infections and AMAN has been shown to be caused by the presence of Gal(β1-3)GalNAc in the lipopolysaccharides of some strains of *C. jejuni*. This particular epitope is also found in GM1 gangliosides that are localized at nodes of Ranvier (Hafer-Macko C. et al., 1996). In conjunction with this, those with AMAN have been found to have IgG antibodies against GM1, although not in every case. To demonstrate that the antibodies and complement were involved in the pathogenesis of AMAN, Hafer-Macko et al. performed post-mortem examinations of AMAN patients and found the presence of IgG antibodies and the complement product C3d present at the nodes of Ranvier, specifically bound to the axolemma (Hafer-Macko C. et al., 1996). They postulated that the activation of complement leads to the recruitment of macrophages that target axons whereupon they enter the periaxonal space, displace axons, and separate them from their myelin sheaths. In some cases, this led to axonal degeneration. Findings of macrophages entering the periaxonal space and displacing axons has been found by other groups as well and likely explains the deficits in nerve conduction studies see in AMAN patients (Griffin et al., 1995, 1996).

In keeping with these findings and similar to that seen in AIDP, when AMAN patient serum was bathed onto teased mouse sciatic nerves, 42% showed IgG deposition at nodes of Ranvier and paranodal regions with a significant percentage of patient sera containing antibodies directed against NF186 (Devaux et al., 2012). GM1 gangliosides have also been found to increase TrkA autophosphorylation induced by nerve growth factor (Tanaka et al., 2007). When IgG from the sera of four AMAN patients was added to a PC12 sympathetic nerve cell line pre-incubated with GM1, neurite outgrowth was arrested. The same effect was seen in cells treated with nerve growth factor prior to the addition of IgG. Further, when patient IgG was depleted of anti-GM1 antibodies, the effect on neurite outgrowth was no longer seen. Other antibodies associated with AMAN include GD1a, GM1b, and GalNAc-GD1a (Willison and Yuki, 2002; Suzuki, 2013) whereas they are minimally present in AIDP (Ho et al., 1999).

Role of Gangliosides in AMAN, AMSAN, and Animal Models

Greenshields et al. went further to show that the antibody-binding domain of GM1 gangliosides can be shielded by the presence of other gangliosides such as GD1a in ganglioside complexes in cell membranes (Greenshields et al., 2009). In their study, they found that the ability of antibodies to bind to GM1 and other ganglioside complexes was inhibited to varying degrees depending on the antibody and the second ganglioside in the complex. The masking effect prevented binding and complement activation and this effect was reversed when treated with enzymes that exposed the domain as evidenced by increased MAC and C3 deposits.

Because there have been associations with the gangliosides, GM1 and GD1a, in AMAN, Susuki et al. examined the nodes of Ranvier of mice lacking these gangliosides and found many structural changes in the nodal region including changes in ion channel localization (Susuki et al., 2007a). For instance, potassium channels were located in the paranodes rather than juxtaparanode and there were broader clusters of sodium channels. Other structural deficits were also noted such as unattached paranodal loops and weakened staining of Caspr and NF155 compared to control mice. Interestingly, the authors found that as the knockout mice aged, there were increased abnormalities in nodal structure implying that GM1 and GD1a must be involved in the maintenance of the nodal region with age. A similar study examining mice with an ablation for GalNAc-transferase specifically from SCs, showed that although development was normal, the animals displayed reduced myelin and an increase in axon degeneration with increasing age (Yao et al., 2014). Thus, antibody-mediated insults on peripheral nerve gangliosides may carry with them greater implication for the severity of GBS because of their importance in nerve maintenance with age.

Recently, a transgenic mouse model has been created for AMAN by knocking out complex gangliosides everywhere but on neurons (McGonigal et al., 2016); this allows for the specific targeting of antibodies against gangliosides. When

given anti-GM1 antibodies and normal human serum, the transgenic mice developed a phenotype similar to that seen in AMAN. Interestingly, when anti-C1q antibodies were given to block the classical complement cascade, there was no detectable complement activation products which coincided with increased axonal integrity compared to untreated mice. However, this did not impact immune cell recruitment between treated and untreated mice as there were no significant differences in the number of neutrophils or $CD11b^+$ leukocytes. This transgenic mouse line has also been used to demonstrate that neurons are capable of greatly reducing the levels of circulating anti-ganglioside antibodies via endocytosis (Cunningham et al., 2016). Specifically, when given injections of two different monoclonal antibodies against GM1 (one that is inhibited from binding due to shielding by neighboring gangliosides, and one with a strong binding ability regardless of neighboring gangliosides), only the strongly binding antibody was reduced from circulation indicating that the antibody must be bound to the membrane in order to be endocytosed by the cell. It was further found that this occurs most prominently at the presynaptic terminal of the neuromuscular junction (NMJ). Altogether, these findings could account for the variation in antibody presence in GBS patient sera.

In animal models of AMAN, IgG antibodies have been found to bind to both the nodal and paranodal regions of the axon (Shahrizaila and Yuki, 2013). This led to alterations in sodium channel presence in addition to a detachment of myelin. Others have shown that anti-GM1 antibodies are able to disrupt sodium channels at nodes of Ranvier through the activation of complement, and as recovery progresses, complement depositions decrease and sodium channels normalize (Susuki et al., 2007b). Furthermore, when inhibitors of the complement are given, sodium channels are unaltered after anti-GM1 sensitization (Phongsisay et al., 2008).

Yuki et al. have developed a model of the axonal variants of GBS (AMAN and AMSAM) in rabbits through the immunization of a mixture of bovine brain gangliosides or GM1, Freund's adjuvant, and keyhole limpet hemocyanin (KLH) (Yuki et al., 2001). The rabbits experienced axonal degeneration with no demyelination or lymphocytic infiltration similar to that seen in axonal GBS. In order to determine the role of KLH in the induction of this model, Caporale et al. immunized New Zealand white rabbits with lipopolysaccharide from the isolated *C. jejuni* of GBS patients with GM1 and GD1a epitope expression and Freund's adjuvant with or without KLH (Caporale et al., 2006b). They found that in the KLH group, 6 out of 7 rabbits developed disease while only 1 out of 11 from the non-KLH group developed disease. The authors also noted that in both groups, the rabbits developed high volumes of antibodies against lipopolysaccharide and GM1 and low volumes of antibodies against GD1b and GD1a. While they were unable to determine the exact function of KLH in this model, they speculated that it may play a role in T cell recruitment and activation.

In another model of AMAN, Yuki et al. have demonstrated that immunization of rabbits with lipo-oligosaccharides derived from *C. jejuni* led to the development of anti-GM1 antibodies and clinical symptoms similar to those seen in human axonal GBS (Yuki et al., 2004). Furthermore, in their study, the authors immunized mice lacking the enzymes necessary to develop complex gangliosides with lipo-oligosaccharides derived from *C. jejuni* and found that the mice developed monoclonal antibodies against GM1. The specificity and purity of the lipo-oligosaccharides is critical for the proper induction of the model (Notturno et al., 2010). When two lipo-oligosaccharides were derived from the same strain of *C. jejuni*, only the purer of the two induced the symptoms. Both groups developed antibodies against lipo-oligosaccharides, however significantly higher levels were seen in the disease-induced group. Both cohorts also developed IgM and IgG anti-GM1, anti-GD1a, and anti-GD1b, however no significant differences were seen between the two groups although there were significant differences in the neuropathy developing group which possessed high levels of monospecific and high-affinity anti-GM1 antibodies not seen in the other rabbits. In addition, the sera of rabbits which developed the neuropathy were able to activate complement as assessed by higher deposition of C3b/c.

Aside from anti-GM1 antibodies, anti-GD1a antibodies have also been associated with AMAN. In a mouse model, it has been found that there is a preference for anti-GD1a antibody binding in more distal nodes of Ranvier on motor nerve bundles due to the added protection of the BNB at more proximal sites (McGonigal et al., 2010). When antibody binding occurs at these sites, it leads to complement activation and MAC pore deposition which in turn causes calpain activation. This results in a loss of Nav1.6 channels and thus loss of action potentials. However, when axonal structures are preserved through the inhibition of calpains, there is still a loss of action potentials indicating that the deposition of MAC pores are causing ionic imbalances along the axon and thus preventing signal condition. Aside from complement, there is also immune cell recruitment in animal models of axonal GBS. Finally, in a study where anti-GD1b antibodies and normal human serum were used to induce neuropathy, it was found that neutrophils and macrophages were not recruited while perisynaptic SCs functioned as the main phagocyte to clear debris in distal nerve injuries induced by antibody-mediated attacks on gangliosides (Cunningham et al., 2020).

Discussion: Mechanisms in AMAN, AMSAN, and Animal Models and Implications of Aging

Gangliosides are important for nerve health with age and an attack on these structures in older subjects may lead to even worse outcomes. This suggestion is in keeping with the incidence rates of AMAN and AMSAN with age. As noted above, AMSAN is associated with older patients relative to AMAN (Webb et al., 2015), however others have shown that 58% of AMAN patients with detectable antibodies against nodal adhesion molecules were over 60 years old (Devaux et al., 2012). Particularly in the axonal variants, it appears that antibodies against gangliosides and

ganglioside complexes located at the nodes of Ranvier are critical to disease development. This leads to complement activation and further recruitment of macrophages which can further displace myelin sheaths and cause secondary axonal degeneration (Hafer-Macko C. et al., 1996). Similar to AIDP, with increasing age, there is an increase in the presence of macrophages within the peripheral nerve (Ceballos et al., 1999) which may lead to increased disease severity in the elderly in concert with decreased regenerative abilities with age.

ANTI-GQ1B ANTIBODY SYNDROME: MILLER FISHER SYNDROME/ BICKERSTAFF BRAINSTEM ENCEPHALITIS (MFS/BBE)

Miller Fisher Syndrome (MFS) was first described in 1956 by Miller Fisher (Fisher, 1956). It is classically characterized by ataxia, ophthalmoplegia, and areflexia. Recently, it has been found that MFS is itself part of a spectrum that includes Bickerstaff brainstem encephalitis (BBE) (Shahrizaila and Yuki, 2013). Symptoms of BBE include ataxia, ophthalmoplegia, and differing from MFS, impairments in consciousness or hyperreflexia. What led to this discovery was the common presence of anti-GQ1b IgG antibodies in both conditions. Other anti-ganglioside antibodies associated with this spectrum include GD3 and GT1a (van Doorn et al., 2008). Similar to AMAN, there is a strong association with preceding infections (e.g., *C. jejuni*) and when lipo-oligosaccharides were isolated and cultured from patients who had a preceding *C. jejuni* infection, it was found that anti-GQ1b antibodies bound to them (Koga et al., 2005; Shahrizaila and Yuki, 2013). In the first paper to describe IgG anti-GQ1b antibodies in MFS, the authors explained that it could be a new diagnostic marker for the variant (Chiba et al., 1992) since antibodies against GQ1b have been shown to be localized to the human oculomotor, trochlear, abducens, glossopharyngeal, and vagal nerves, as well as muscle spindles which may contribute to the symptomology (Chiba et al., 1993; Kusunoki et al., 1999; Shahrizaila and Yuki, 2013). In BBE, due to the CNS involvement (i.e., altered consciousness), it is likely that the reticular formation is implicated as well (Shahrizaila and Yuki, 2013). However, others have looked at the presence of antibodies against anti-ganglioside complexes in MFS and found that some patients have serum antibodies to GQ1b-GM1, GT1a/GM1, GQ1b/GD1a, and/or GQ1b/GT1b complexes (Kaida et al., 2006) and thus it may be best to assay for a spectrum of antibodies for diagnostic purposes.

The NMJ is a vulnerable area for anti-ganglioside antibody attack due to its high concentration of gangliosides and its location outside of the BNB. Anti-GQ1b antibodies from MFS patients have been found to decrease neurotransmitter release from the presynaptic terminals (Buchwald et al., 1995). In an *ex vivo* model, anti-GQ1b antibodies have also been shown to activate complement and therefore the degeneration of the motor nerve terminals (O'Hanlon et al., 2001). In a mouse model of MFS, when given an experimental complement inhibitor, there was a lack of membrane attack complex formation on perisynaptic SCs and preserved axonal integrity (Halstead et al., 2005). This was also seen in another model when anti-GQ1b/GD3 IgM antibodies followed by normal human serum were given to wild-type mice or when anti-C1q antibodies were given to block the classical complement cascade. There was reduced activation of the complement system and deposition of complement products as well as less immune cell presence and injury to the axons (Cunningham et al., 2016). Inhibitors of complement further downstream (i.e., C5 inhibitor rEV576) were also able to protect from complement-mediated degeneration of NMJs and perisynaptic SC injury (Halstead et al., 2008a). Furthermore, as seen in the human clinical trials mentioned earlier, eculizumab (humanized monoclonal antibody against C5) was also able to protect the NMJ in the MFS mouse model (Halstead et al., 2008b).

Because complement activation leads to MAC formation, there is an influx of calcium associated with the activation of calpains within the neuron leading to degeneration of the NMJ. When given calpain inhibitors or when calcium is depleted, there is a lack of degeneration of the NMJ in this MFS model (O'Hanlon et al., 2003). Altogether, because there are some overlap of MFS with other forms of GBS such as AMAN and AMSAN in terms of types of antibodies deposited, therapies targeting such antibodies or other overlapping factors would benefit more than one disease.

Acute Ataxic Neuropathy Without Ophthalmoplegia: Ataxic Gbs/Acute Sensory Ataxic Neuropathy

Another subgroup within the anti-GQ1b antibody syndrome GBS variant is acute ataxic neuropathy without ophthalmoplegia (Ito et al., 2011; Shahrizaila and Yuki, 2013). This subgroup is a clinical spectrum comprised of ataxic GBS and acute sensory ataxic neuropathy (Ito et al., 2011). Acute sensory ataxic neuropathy patients present with sensory ataxia without ophthalmoplegia and have a high prevalence of recent *C. jejuni* infections (Notturno et al., 2008). In addition, these patients have antibodies to GD1b and GQ1b. GD1b has been found on primary sensory neurons and in the paranodal regions of both motor and sensory nerves (Kusunoki et al., 1993), and therefore antibodies against GD1b could be targeting these sensory nerves (Pan et al., 2001). In one study, it was found that when another anti-ganglioside along with GD1b was found in the sera of GBS patients, the patients were less likely to have ataxia as a symptom (Kaida et al., 2008). From this, the authors suggested that very specific IgG antibodies against GD1b could be involved in the ataxia in GBS. In addition to having antibodies against GD1b and GQ1b as in acute sensory ataxic neuropathy, ataxic GBS involves acute ataxia with a negative Romberg sign and no ophthalmoplegia (Richter, 1962). Relative to MFS, patients in this spectrum more often have antibodies against GD1b and less often against GQ1b (Ito et al., 2011).

Discussion: Mechanisms in Anti-GQ1b Antibody Syndrome

As mentioned above, antibodies against GQ1b are a hallmark feature of this variant and furthermore, anti-GD1b antibodies appear to be critical for the development of the anti-GQ1b antibody syndrome subtype acute ataxic neuropathy without ophthalmoplegia. GQ1b gangliosides and complexes containing it appear to localize clearly to a variety of nerves (Chiba et al., 1993; Kusunoki et al., 1999; Shahrizaila and Yuki, 2013) and the NMJ (O'Hanlon et al., 2001) that would lead to the three main symptoms ataxia, ophthalmoplegia, and areflexia. Similar to other GBS variants, antibody and complement-mediated attacks appear to be critical to the development of this variant. Interestingly, eculizumab and other complement inhibitors showed promising results in mouse models of MFS.

CONCLUSION

As has been discussed above, the pathology of GBS can be characterized by abhorrent immune cell functioning wherein the host becomes the victim of its own immune response. Broadly, it appears that the attack against peripheral nerves and specific gangliosides is the product of interactions between T cells, B cells, complement products, and macrophages. In addition, the immune system and the ability of the PNS to regenerate changes over the course of the lifespan. Altogether, the combined impact of an aging immune system and a poor response to a prior infection may push the body into an altered and self-attacking state contributing to the increased frequency and severity of GBS with age.

AUTHOR CONTRIBUTIONS

KH and SO wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Albright, J. M., Dunn, R. C., Shults, J. A., Boe, D. M., Afshar, M., and Kovacs, E. J. (2016). Advanced age alters monocyte and macrophage responses. *Antioxid. Redox. Signal.* 25, 805–815. doi: 10.1089/ars.2016.6691

Aliprantis, A. O., Diez-Roux, G., Mulder, L. C., Zychlinsky, A., and Lang, R. A. (1996). Do macrophages kill through apoptosis? *Immunol. Today* 17, 573–576. doi: 10.1016/s0167-5699(96)10071-2

Asbury, A. K., Arnason, B. G., and Adams, R. D. (1969). The inflammatory lesion in idiopathic polyneuritis. Its role in pathogenesis. *Medicine* 48, 173–215. doi: 10.1097/00005792-196905000-00001

Austin, P. J., Kim, C. F., Perera, C. J., and Moalem-Taylor, G. (2012). Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis. *Pain* 153, 1916–1931. doi: 10.1016/j.pain.2012.06.005

Azuma, T., Matsubara, T., Shima, Y., Haeno, S., Fujimoto, T., Tone, K., et al. (1993). Neurosteroids in cerebrospinal fluid in neurologic disorders. *J. Neurol. Sci.* 120, 87–92. doi: 10.1016/0022-510x(93)90030-3

Bai, X. F., Zhu, J., Zhang, G. X., Kaponides, G., Höjeberg, B., van der Meide, P. H., et al. (1997). IL-10 suppresses experimental autoimmune neuritis and down-regulates TH1-type immune responses. *Clin. Immunol. Immunopathol.* 83, 117–126. doi: 10.1006/clin.1997.4331

Ben-Smith, A., Gaston, J. S., Barber, P. C., and Winer, J. B. (1997). Stimulation of peripheral blood lymphocytes with *Campylobacter jejuni* generates a gammadelta T cell response in patients with Guillain-Barré syndrome. *Clin. Exp. Immunol.* 109, 121–126. doi: 10.1046/j.1365-2249.1997.4221318.x

Borsellino, G., Koul, O., Placido, R., Tramonti, D., Luchetti, S., Galgani, S., et al. (2000). Evidence for a role of gammadelta T cells in demyelinating diseases as determined by activation states and responses to lipid antigens. *J. Neuroimmunol.* 107, 124–129. doi: 10.1016/s0165-5728(00)00220-4

Brosman, C. F., Lyman, W. D., Tansey, F. A., and Carter, T. H. (1985). Quantitation of mast cells in experimental allergic neuritis. *J. Neuropathol. Exp. Neurol.* 44, 196–203. doi: 10.1097/00005072-198503000-00008

Brosnan, J. V., King, R. H., Thomas, P. K., and Craggs, R. I. (1988). Disease patterns in experimental allergic neuritis (EAN) in the Lewis rat. Is EAN a good model for the Guillain-Barré syndrome? *J. Neurol. Sci.* 88, 261–276. doi: 10.1016/0022-510x(88)90223-7

Buchwald, B., Weishaupt, A., Toyka, K. V., and Dudel, J. (1995). Immunoglobulin G from a patient with miller-fisher syndrome rapidly and reversibly depresses evoked quantal release at the neuromuscular junction of mice. *Neurosci. Lett.* 201, 163–166. doi: 10.1016/0304-3940(95)12155-2

Büttner, R., Schulz, A., Reuter, M., Akula, A. K., Mindos, T., Carlstedt, A., et al. (2018). Inflammaging impairs peripheral nerve maintenance and regeneration. *Aging Cell* 17:e12833. doi: 10.1111/acel.12833

Caporale, C. M., Capasso, M., Luciani, M., Prencipe, V., Creati, B., Gandolfi, P., et al. (2006b). Experimental axonopathy induced by immunization with *Campylobacter jejuni* lipopolysaccharide from a patient with Guillain-Barré syndrome. *J. Neuroimmunol.* 174, 12–20. doi: 10.1016/j.jneuroim.2005.12.005

Caporale, C. M., Papola, F., Fioroni, M. A., Aureli, A., Giovannini, A., Notturno, F., et al. (2006a). Susceptibility to Guillain-Barré syndrome is associated to polymorphisms of CD1 genes. *J. Neuroimmunol.* 177, 112–118. doi: 10.1016/j.jneuroim.2006.05.018

Cattin, A. L., Burden, J. J., Van Emmenis, L., Mackenzie, F. E., Hoving, J. J., Garcia Calavia, N., et al. (2015). Macrophage-induced blood vessels guide schwann cell-mediated regeneration of peripheral nerves. *Cell* 162, 1127–1139. doi: 10.1016/j.cell.2015.07.021

Ceballos, D., Cuadras, J., Verdú E., and Navarro, X. (1999). Morphometric and ultrastructural changes with ageing in mouse peripheral nerve. *J. Anat.* 195, 563–576. doi: 10.1046/j.1469-7580.1999.19540563.x

Chi, L. J., Wang, H. B., Zhang, Y., and Wang, W. Z. (2007). Abnormality of circulating CD4(+)CD25(+) regulatory T cell in patients with Guillain-Barré syndrome. *J. Neuroimmunol.* 192, 206–214. doi: 10.1016/j.jneuroim.2007.09.034

Chiba, A., Kusunoki, S., Obata, H., Machinami, R., and Kanazawa, I. (1993). Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in miller fisher syndrome and Guillain-Barré syndrome: clinical and immunohistochemical studies. *Neurology* 43, 1911–1917. doi: 10.1212/wnl.43.10.1911

Chiba, A., Kusunoki, S., Shimizu, T., and Kanazawa, I. (1992). Serum IgG antibody to ganglioside GQ1b is a possible marker of miller fisher syndrome. *Ann. Neurol.* 31, 677–679. doi: 10.1002/ana.410310619

Chougnet, C. A., Tripathi, P., Lages, C. S., Raynor, J., Sholl, A., Fink, P., et al. (2011). A major role for Bim in regulatory T cell homeostasis. *J. Immunol.* 186, 156–163. doi: 10.4049/jimmunol.1001505

Colonna-Romano, G., Aquino, A., Bulati, M., Lio, D., Candore, G., Oddo, G., et al. (2004). Impairment of gamma/delta T lymphocytes in elderly:

implications for immunosenescence. *Exp. Gerontol.* 39, 1439–1446. doi: 10.1016/j.exger.2004.07.005

Colonna-Romano, G., Potestio, M., Aquino, A., Candore, G., Lio, D., and Caruso, C. (2002). Gamma/delta T lymphocytes are affected in the elderly. *Exp. Gerontol.* 37, 205–211. doi: 10.1016/s0531-5565(01)00185-1

Cooper, J., Hughes, S., Ben-Smith, A., Savage, C., and Winer, J. (2002). T cell recognition of a non-protein antigen preparation of *Campylobacter jejuni* in patients with Guillain-Barré syndrome. *J. Neurol. Neurosurg. Psychiatr.* 72, 413–414. doi: 10.1136/jnnp.72.3.413

Cunningham, M. E., McGonigal, R., Meehan, G. R., Barrie, J. A., Yao, D., Halstead, S. K., et al. (2016). Anti-ganglioside antibodies are removed from circulation in mice by neuronal endocytosis. *Brain* 139, 1657–1665. doi: 10.1093/brain/aww056

Cunningham, M. E., Meehan, G. R., Robinson, S., Yao, D., McGonigal, R., and Willison, H. J. (2020). Perisynaptic schwann cells phagocytose nerve terminal debris in a mouse model of Guillain-Barré syndrome. *J. Peripher. Nerv. Syst.* 25, 143–151. doi: 10.1111/jns.12373

Cutillo, G., Saariaho, A. H., and Meri, S. (2020). Physiology of gangliosides and the role of antiganglioside antibodies in human diseases. *Cell. Mol. Immunol.* 17, 313–322. doi: 10.1038/s41423-020-0388-9

Dalakas, M. C. (2015). Pathogenesis of immune-mediated neuropathies. *Biochim. Biophys. Acta* 1852, 658–666. doi: 10.1016/j.bbadis.2014.06.013

Dati, G., Quattrini, A., Bernasconi, L., Malaguti, M. C., Antonsson, B., Nicoletti, F., et al. (2007). Beneficial effects of r-h-CLU on disease severity in different animal models of peripheral neuropathies. *J. Neuroimmunol* 190, 8–17. doi: 10.1016/j.jneuroim.2007.07.014

Davidson, A. I., Halstead, S. K., Goodfellow, J. A., Chavada, G., Mallik, A., Overell, J., et al. (2017). Inhibition of complement in Guillain-Barré syndrome: the ICA-GBS study. *J. Peripher. Nerv. Syst.* 22, 4–12. doi: 10.1111/jns.12194

De Rosa, S. C., Andrus, J. P., Perfetto, S. P., Mantovani, J. J., Herzenberg, L. A., Herzenberg, L. A., et al. (2004). Ontogeny of gamma delta T cells in humans. *J. Immunol.* 172, 1637–1645. doi: 10.4049/jimmunol.172.3.1637

DeFrancesco-Lisowitz, A., Lindborg, J. A., Niemi, J. P., and Zigmond, R. E. (2014). The neuroimmunology of degeneration and regeneration in the peripheral nervous system. *Neuroscience* 302, 174–203. doi: 10.1016/j.neuroscience.2014.09.027

den Braber, I., Mugwagwa, T., Vrisekoop, N., Westera, L., Mögling, R., de Boer, A. B., et al. (2012). Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity* 36, 288–297. doi: 10.1016/j.immuni.2012.02.006

Devaux, J. J., Odaka, M., and Yuki, N. (2012). Nodal proteins are target antigens in Guillain-Barré syndrome. *J. Peripher. Nerv. Syst.* 17, 62–71. doi: 10.1111/j.1529-8027.2012.00372.x

Enders, U., Lobb, R., Pepinsky, R. B., Hartung, H. P., Toyka, K. V., and Gold, R. (1998). The role of the very late antigen-4 and its counterligand vascular cell adhesion molecule-1 in the pathogenesis of experimental autoimmune neuritis of the Lewis rat. *Brain* 121, 1257–1266. doi: 10.1093/brain/121.7.1257

Esposito, B., De Santis, A., Monteforte, R., and Baccari, G. C. (2002). Mast cells in wallerian degeneration: morphologic and ultrastructural changes. *J. Comp. Neurol.* 445, 199–210. doi: 10.1002/cne.10169

Feasby, T. E. (1994). Axonal Guillain-Barré syndrome. *Muscle Nerve* 17, 678–679. doi: 10.1002/mus.880170618

Fisher, M. (1956). An unusual variant of acute idiopathic polyneuritis (syndrome of ophthalmoplegia, ataxia and areflexia). *N. Engl. J. Med.* 255, 57–65. doi: 10.1056/NEJM195607122550201

Gaudet, A. D., Popovich, P. G., and Ramer, M. S. (2011). Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *J. Neuroinflammation* 30:110. doi: 10.1186/1742-2094-8-110

George, J., and Twomey, J. A. (1985). The Guillain-Barré syndrome in the elderly: clinical and electrophysiological features of five cases. *Age Ageing* 14, 216–219. doi: 10.1093/ageing/14.4.216

Ghabaee, M., Jabedari, B., Al-E-Eshagh, N., Ghaffarpour, M., and Asadi, F. (2010). Serum and cerebrospinal fluid antioxidant activity and lipid peroxidation in Guillain-Barre syndrome and multiple sclerosis patients. *Int. J. Neurosci.* 120, 301–304. doi: 10.3109/00207451003695690

Gillen, C., Jander, S., and Stoll, G. (1998). Sequential expression of mRNA for proinflammatory cytokines and interleukin-10 in the rat peripheral nervous system: comparison between immune-mediated demyelination and wallerian degeneration. *J. Neurosci. Res.* 51, 489–496. doi: 10.1002/(SICI)1097-4547(19980215)51:4<489::AID-JNR8>3.0.CO;2-8

Goetzl, E. J., Banda, M. J., and Leppert, D. (1996). Matrix metalloproteinases in immunity. *J. Immunol.* 156, 1–4.

Grasman, J. M., and Kaplan, D. L. (2017). Human endothelial cells secrete neurotropic factors to direct axonal growth of peripheral nerves. *Sci. Rep.* 7:4092. doi: 10.1038/s41598-017-04460-8

Greenshields, K. N., Halstead, S. K., Zitman, F. M., Rinaldi, S., Brennan, K. M., O'Leary, C., et al. (2009). The neuropathic potential of anti-GM1 autoantibodies is regulated by the local glycolipid environment in mice. *J. Clin. Invest.* 119, 595–610. doi: 10.1172/JCI37338

Gregg, R., Smith, C. M., Clark, F. J., Dunnion, D., Khan, N., Chakraverty, R., et al. (2005). The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. *Clin. Exp. Immunol.* 140, 540–546. doi: 10.1111/j.1365-2249.2005.02798.x

Griffin, J. W., Li, C. Y., Ho, T. W., Tian, M., Gao, C. Y., Xue, P., et al. (1996). Pathology of the motor-sensory axonal Guillain-Barré syndrome. *Ann. Neurol.* 39, 17–28. doi: 10.1002/ana.410390105

Griffin, J. W., Li, C. Y., Ho, T. W., Xue, P., Macko, C., Gao, C. Y., et al. (1995). Guillain-Barré syndrome in northern China. the spectrum of neuropathological changes in clinically defined cases. *Brain* 118, 577–595. doi: 10.1093/brain/118.3.577

Gruener, G., Bosch, E. P., Strauss, R. G., Klugman, M., and Kimura, J. (1987). Prediction of early beneficial response to plasma exchange in Guillain-Barré syndrome. *Arch. Neurol.* 44, 295–298. doi: 10.1001/archneur.1987.00520150043019

Häcker, G., Adam, S., and Wagner, H. (1995). Interaction between gamma delta T cells and B cells regulating IgG production. *Immunology* 84, 105–110.

Hafer-Macko, C., Hsieh, S. T., Li, C. Y., Ho, T. W., Sheikh, K., Cornblath, D. R., et al. (1996). Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. *Ann. Neurol.* 40, 635–644. doi: 10.1002/ana.410400414

Hafer-Macko, C. E., Sheikh, K. A., Li, C. Y., Ho, T. W., Cornblath, D. R., McKhann, G. M., et al. (1996). Immune attack on the schwann cell surface in acute inflammatory demyelinating polyneuropathy. *Ann. Neurol.* 39, 625–635. doi: 10.1002/ana.410390512

Hahn, A. F., Feasby, T. E., Wilkie, L., and Lovgren, D. (1993). Antigalactocerebroside antibody increases demyelination in adoptive transfer experimental allergic neuritis. *Muscle Nerve* 16, 1174–1180. doi: 10.1002/mus.880161106

Halstead, S. K., Humphreys, P. D., Goodfellow, J. A., Wagner, E. R., Smith, R. A., and Willison, H. J. (2005). Complement inhibition abrogates nerve terminal injury in miller fisher syndrome. *Ann. Neurol.* 58, 203–210. doi: 10.1002/ana.20546

Halstead, S. K., Humphreys, P. D., Zitman, F. M., Hamer, J., Plomp, J. J., and Willison, H. J. (2008a). C5 inhibitor rEV576 protects against neural injury in an *in vitro* mouse model of miller fisher syndrome. *J. Peripher. Nerv. Syst.* 13, 228–235. doi: 10.1111/j.1529-8027.2008.00181.x

Halstead, S. K., Zitman, F. M., Humphreys, P. D., Greenshields, K., Verschuuren, J. J., Jacobs, B. C., et al. (2008b). Eculizumab prevents anti-ganglioside antibody-mediated neuropathy in a murine model. *Brain 131,* 1197–1208. doi: 10.1093/brain/awm316

Han, R. K., Cheng, Y. F., Zhou, S. S., Guo, H., He, R. D., Chi, L. J., et al. (2014). Increased circulating Th17 cell populations and elevated CSF osteopontin and IL-17 concentrations in patients with Guillain-Barré syndrome. *J. Clin. Immunol.* 34, 94–103. doi: 10.1007/s10875-013-9965-3

Harness, J., and McCombe, P. A. (2008). Increased levels of activated T-cells and reduced levels of CD4/CD25+ cells in peripheral blood of Guillain-Barré syndrome patients compared to controls. *J. Clin. Neurosci.* 15, 1031–1035. doi: 10.1016/j.jocn.2007.09.016

Hartung, H. P., Reiners, K., Michels, M., Hughes, R. A., Heidenreich, F., Zielasek, J., et al. (1994). Serum levels of soluble E-selectin (ELAM-1) in immune-mediated neuropathies. *Neurology* 44, 1153–1158. doi: 10.1212/wnl.44.6.1153

Hartung, H. P., Schäfer, B., Heininger, K., Stoll, G., and Toyka, K. V. (1988). The role of macrophages and eicosanoids in the pathogenesis of experimental allergic neuritis. Serial clinical, electrophysiological, biochemical and morphological observations. *Brain 111,* 1039–1059. doi: 10.1093/brain/111.5.1039

Hartung, H. P., Schwenke, C., Bitter-Suermann, D., and Toyka, K. V. (1987). Guillain-Barré syndrome: activated complement components C3a and C5a in CSF. *Neurology* 37, 1006–1009. doi: 10.1212/wnl.37.6.1006

Harvey, G. K., Gold, R., Hartung, H. P., and Toyka, K. V. (1995). Non-neural-specific T lymphocytes can orchestrate inflammatory peripheral neuropathy. *Brain 118,* 1263–1272. doi: 10.1093/brain/118.5.1263

Haynes, L., Eaton, S. M., and Swain, S. L. (2000). The defects in effector generation associated with aging can be reversed by addition of IL-2 but not other related gamma(c)-receptor binding cytokines. *Vaccine* 18, 1649–1653. doi: 10.1016/s0264-410x(99)00501-0

Ho, T. W., Hsieh, S. T., Nachamkin, I., Willison, H. J., Sheikh, K., Kiehlbauch, J., et al. (1997). Motor nerve terminal degeneration provides a potential mechanism for rapid recovery in acute motor axonal neuropathy after campylobacter infection. *Neurology* 48, 717–724. doi: 10.1212/wnl.48.3.717

Ho, T. W., McKhann, G. M., and Griffin, J. W. (1998). Human autoimmune neuropathies. *Annu. Rev. Neurosci.* 21, 187–226. doi: 10.1146/annurev.neuro.21.1.187

Ho, T. W., Mishu, B., Li, C. Y., Gao, C. Y., Cornblath, D. R., Griffin, J. W., et al. (1995). Guillain-Barre syndrome in northern China relationship to *Campylobacter jejuni* infection and anti-glycolipid antibodies. *Brain 118,* 597–605. doi: 10.1093/brain/118.3.597

Ho, T. W., Willison, H. J., Nachamkin, I., Li, C. Y., Veitch, J., Ung, H., et al. (1999). Anti-GD1a antibody is associated with axonal but not demyelinating forms of Guillain-Barré syndrome. *Ann. Neurol.* 45, 168–173. doi: 10.1002/1531-8249(199902)45:2<168::aid-ana6>3.0.co;2-6

Hu, W., Dehmel, T., Pirhonen, J., Hartung, H. P., and Kieseier, B. C. (2006). Interleukin 23 in acute inflammatory demyelination of the peripheral nerve. *Arch. Neurol.* 63, 858–864. doi: 10.1001/archneur.63.6.858

Hughes, P. M., Wells, G. M., Clements, J. M., Gearing, A. J., Redford, E. J., Davies, M., et al. (1998). Matrix metalloproteinase expression during experimental autoimmune neuritis. *Brain* 121, 481–494. doi: 10.1093/brain/121.3.481

Hughes, R. A., and Rees, J. H. (1997). Clinical and epidemiologic features of Guillain-Barré syndrome. *J. Infect. Dis.* 176(Suppl. 2), S92–S98. doi: 10.1086/513793

Hwang, K. A., Kim, H. R., and Kang, I. (2009). Aging and human CD4(+) regulatory T cells. *Mech. Ageing Dev.* 130, 509–517. doi: 10.1016/j.mad.2009.06.003

Ito, M., Matsuno, K., Sakumoto, Y., Hirata, K., and Yuki, N. (2011). Ataxic Guillain-Barré syndrome and acute sensory ataxic neuropathy form a continuous spectrum. *J. Neurol. Neurosurg. Psychiatr.* 82, 294–299. doi: 10.1136/jnnp.2010.222836

Izumo, S., Linington, C., Wekerle, H., and Meyermann, R. (1985). Morphologic study on experimental allergic neuritis mediated by T cell line specific for bovine P2 protein in Lewis rats. *Lab. Invest.* 53, 209–218.

Jasti, A. K., Selmi, C., Sarmiento-Monroy, J. C., Vega, D. A., Anaya, J. M., and Gershwin, M. E. (2016). Guillain-Barré syndrome: causes, immunopathogenic mechanisms and treatment. *Expert Rev. Clin. Immunol.* 12, 1175–1189. doi: 10.1080/1744666X.2016.1193006

Kaida, K., Kamakura, K., Ogawa, G., Ueda, M., Motoyoshi, K., Arita, M., et al. (2008). GD1b-specific antibody induces ataxia in Guillain-Barre syndrome. *Neurology* 71, 196–201. doi: 10.1212/01.wnl.0000317093.57106.33

Kaida, K., Kanzaki, M., Morita, D., Kamakura, K., Motoyoshi, K., Hirakawa, M., et al. (2006). Anti-ganglioside complex antibodies in miller fisher syndrome. *J. Neurol. Neurosurg. Psychiatr.* 77, 1043–1046. doi: 10.1136/jnnp.2006.087940

Kaida, K., Morita, D., Kanzaki, M., Kamakura, K., Motoyoshi, K., Hirakawa, M., et al. (2007). Anti-ganglioside complex antibodies associated with severe disability in GBS. *J. Neuroimmunol.* 182, 212–218. doi: 10.1016/j.jneuroim.2006.09.013

Kanda, T., Iwasaki, T., Yamawaki, M., Tai, T., and Mizusawa, H. (2000). Anti-GM1 antibody facilitates leakage in an *in vitro* blood-nerve barrier model. *Neurology* 55, 585–587. doi: 10.1212/wnl.55.4.585

Kang, H., and Lichtman, J. W. (2013). Motor axon regeneration and muscle reinnervation in young adult and aged animals. *J. Neurosci.* 33, 19480–19491. doi: 10.1523/JNEUROSCI.4067-13.2013

Khairallah, C., Chu, T. H., and Sheridan, B. S. (2018). Tissue adaptations of memory and tissue-resident gamma delta T cells. *Front. Immunol.* 9:2636. doi: 10.3389/fimmu.2018.02636

Khalili-Shirazi, A., Gregson, N. A., Londei, M., Summers, L., and Hughes, R. A. (1998). The distribution of CD1 molecules in inflammatory neuropathy. *J. Neurol. Sci.* 158, 154–163. doi: 10.1016/s0022-510x(98)00121-x

Khalili-Shirazi, A., Hughes, R. A., Brostoff, S. W., Linington, C., and Gregson, N. (1992). T cell responses to myelin proteins in Guillain-Barré syndrome. *J. Neurol. Sci.* 111, 200–203. doi: 10.1016/0022-510x(92)90069-w

Kiefer, R., Funa, K., Schweitzer, T., Jung, S., Bourde, O., Toyka, K. V., et al. (1996). Transforming growth factor-beta 1 in experimental autoimmune neuritis. Cellular localization and time course. *Am. J. Pathol.* 148, 211–223.

Kiefer, R., Kieseier, B. C., Stoll, G., and Hartung, H. P. (2001). The role of macrophages in immune-mediated damage to the peripheral nervous system. *Prog. Neurobiol.* 64, 109–127. doi: 10.1016/s0301-0082(00)00060-5

Kieseier, B. C., Clements, J. M., Pischel, H. B., Wells, G. M., Miller, K., Gearing, A. J., et al. (1998). Matrix metalloproteinases MMP-9 and MMP-7 are expressed in experimental autoimmune neuritis and the Guillain-Barré syndrome. *Ann. Neurol.* 43, 427–434. doi: 10.1002/ana.410430404

Koga, M., Gilbert, M., Li, J., Koike, S., Takahashi, M., Furukawa, K., et al. (2005). Antecedent infections in fisher syndrome: a common pathogenesis of molecular mimicry. *Neurology* 64, 1605–1611. doi: 10.1212/01.WNL.0000160399.08456.7C

Kracun, I., Rosner, H., Drnovsek, V., Heffer-Lauc, M., Cosović C., and Lauc, G. (1991). Human brain gangliosides in development, aging and disease. *Int. J. Dev. Biol.* 35, 289–295.

Kusunoki, S., Chiba, A., and Kanazawa, I. (1999). Anti-GQ1b IgG antibody is associated with ataxia as well as ophthalmoplegia. *Muscle Nerve* 22, 1071–1074. doi: 10.1002/(sici)1097-4598(199908)22:8<1071::aid-mus10>3.0.co;2-0

Kusunoki, S., Chiba, A., Tai, T., and Kanazawa, I. (1993). Localization of GM1 and GD1b antigens in the human peripheral nervous system. *Muscle Nerve* 16, 752–756. doi: 10.1002/mus.880160710

Kusunoki, S., Shiina, M., and Kanazawa, I. (2001). Anti-Gal-C antibodies in GBS subsequent to mycoplasma infection: evidence of molecular mimicry. *Neurology* 57, 736–738. doi: 10.1212/wnl.57.4.736

Kuwabara, S., and Yuki, N. (2013). Axonal Guillain-Barré syndrome: concepts and controversies. *Lancet Neurol.* 12, 1180–1188. doi: 10.1016/S1474-4422(13)70215-1

Kuwahara, M., Suzuki, S., Takada, K., and Kusunoki, S. (2011). Antibodies to LM1 and LM1-containing ganglioside complexes in Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy. *J. Neuroimmunol.* 239, 87–90. doi: 10.1016/j.jneuroim.2011.08.016

Lehmann, H. C., Jangouk, P., Kierysch, E. K., Meyer zu Hörste, G., Hartung, H. P., and Kieseier, B. C. (2010). Autoantibody-mediated dysfunction of sympathetic neurons in guillain-barre syndrome. *Arch. Neurol.* 67, 203–210. doi: 10.1001/archneurol.2009.331

Li, S., Jin, T., Zhang, H. L., Yu, H., Meng, F., Concha Quezada, H., et al. (2014). Circulating Th17, Th22, and Th1 cells are elevated in the Guillain-Barré syndrome and downregulated by IVIg treatments. *Mediators Inflamm.* 2014:740947. doi: 10.1155/2014/740947

Li, S., Yu, M., Li, H., Zhang, H., and Jiang, Y. (2012). IL-17 and IL-22 in cerebrospinal fluid and plasma are elevated in Guillain-Barré syndrome. *Mediators Inflamm.* 2012:260473. doi: 10.1155/2012/260473

Liang, S. L., Wang, W. Z., Huang, S., Wang, X. K., Zhang, S., and Wu, Y. (2012). Th17 helper cell and T-cell immunoglobulin and mucin domain 3 involvement in Guillain-Barré syndrome. *Immunopharmacol. Immunotoxicol.* 34, 1039–1046. doi: 10.3109/08923973.2012.697469

Lim, M. A., Lee, J., Park, J. S., Jhun, J. Y., Moon, Y. M., Cho, M. L., et al. (2014). Increased Th17 differentiation in aged mice is significantly associated with high IL-1β level and low IL-2 expression. *Exp. Gerontol.* 49, 55–62. doi: 10.1016/j.exger.2013.10.006

Lynch, H. E., Goldberg, G. L., Chidgey, A., Van den Brink, M. R., Boyd, R., and Sempowski, G. D. (2009). Thymic involution and immune reconstitution. *Trends Immunol.* 30, 366–373. doi: 10.1016/j.it.2009.04.003

Mahbub, S., Deburghgraeve, C. R., and Kovacs, E. J. (2012). Advanced age impairs macrophage polarization. *J. Interferon Cytokine Res.* 32, 18–26. doi: 10.1089/jir.2011.0058

Márquez, E. J., Chung, C. H., Marches, R., Rossi, R. J., Nehar-Belaid, D., Eroglu, A., et al. (2020). Sexual-dimorphism in human immune system aging. *Nat. Commun.* 11:751. doi: 10.1038/s41467-020-14396-9

Mausberg, A. K., Szepanowski, F., Odoardi, F., Flügel, A., Kleinschnitz, C., Stettner, M., et al. (2018). Trapped in the epineurium: early entry into the endoneurium is restricted to neuritogenic T cells in experimental autoimmune neuritis. *J. Neuroinflammation* 15:217. doi: 10.1186/s12974-018-1259-5

McGonigal, R., Cunningham, M. E., Yao, D., Barrie, J. A., Sankaranarayanan, S., Fewou, S. N., et al. (2016). C1q-targeted inhibition of the classical complement pathway prevents injury in a novel mouse model of acute motor axonal neuropathy. *Acta Neuropathol. Commun.* 4:23. doi: 10.1186/s40478-016-0291-x

McGonigal, R., Rowan, E. G., Greenshields, K. N., Halstead, S. K., Humphreys, P. D., Rother, R. P., et al. (2010). Anti-GD1a antibodies activate complement and calpain to injure distal motor nodes of ranvier in mice. *Brain 133,* 1944–1960. doi: 10.1093/brain/awq119

McGrogan, A., Madle, G. C., Seaman, H. E., and de Vries, C. S. (2009). The epidemiology of Guillain-Barré syndrome worldwide. a systematic literature review. *Neuroepidemiology* 32, 150–163. doi: 10.1159/000184748

Meyer Zu Horste, G., Heidenreich, H., Lehmann, H. C., Ferrone, S., Hartung, H. P., Wiendl, H., et al. (2010). Expression of antigen processing and presenting molecules by Schwann cells in nflammatory neuropathies. *Glia* 58:80–92. doi: 10.1002/glia.20903

Michishita, Y., Hirokawa, M., Guo, Y. M., Abe, Y., Liu, J., Ubukawa, K., et al. (2011). Age-associated alteration of γδ T-cell repertoire and different profiles of activation-induced death of Vδ1 and Vδ2 T cells. *Int. J. Hematol.* 94, 230–240. doi: 10.1007/s12185-011-0907-7

Mietto, B. S., Mostacada, K., and Martinez, A. M. (2015). Neurotrauma and inflammation: CNS and PNS responses. *Mediators Inflamm.* 2015:251204. doi: 10.1155/2015/251204

Misawa, S., Kuwabara, S., Sato, Y., Yamaguchi, N., Nagashima, K., Katayama, K., et al. (2018). Safety and efficacy of eculizumab in Guillain-Barré syndrome: a multicentre, double-blind, randomised phase 2 trial. *Lancet Neurol.* 17, 519–529. doi: 10.1016/S1474-4422(18)30114-5

Misawa, S., and Suichi, T. (2020). Guillain-Barré syndrome: novel treatment by complement inhibition. *Clin. Exp. Neuroimmunol.* 11, 90–93. doi: 10.1111/cen3.12587

Nelson, B. H. (2004). IL-2, regulatory T cells, and tolerance. *J. Immunol.* 172, 3983–3988. doi: 10.4049/jimmunol.172.7.3983

Notturno, F., Caporale, C. M., and Uncini, A. (2008). Acute sensory ataxic neuropathy with antibodies to GD1b and GQ1b gangliosides and prompt recovery. *Muscle Nerve* 37, 265–268. doi: 10.1002/mus.20875

Notturno, F., Del Boccio, P., Luciani, M., Caporale, C. M., Pieragostino, D., Prencipe, V., et al. (2010). Monospecific high-affinity and complement activating anti-GM1 antibodies are determinants in experimental axonal neuropathy. *J. Neurol. Sci.* 293, 76–81. doi: 10.1016/j.jns.2010.03.003

Notturno, F., Luciani, M., Caporale, C. M., Ciarelli, A., and Uncini, A. (2009). Antibodies to ganglioside complexes in Guillain-Barré syndrome: clinical correlates, fine specificity and complement activation. *Int. J. Immunopathol. Pharmacol.* 22, 437–445. doi: 10.1177/039463200902200220

Nyati, K. K., Prasad, K. N., Rizwan, A., Verma, A., and Paliwal, V. K. (2011). TH1 and TH2 response to *Campylobacter jejuni* antigen in guillain-barre syndrome. *Arch. Neurol.* 68, 445–452. doi: 10.1001/archneurol.2011.51

O'Hanlon, G. M., Humphreys, P. D., Goldman, R. S., Halstead, S. K., Bullens, R. W., Plomp, J. J., et al. (2003). Calpain inhibitors protect against axonal degeneration in a model of anti-ganglioside antibody-mediated motor nerve terminal injury. *Brain 126,* 2497–2509. doi: 10.1093/brain/awg254

O'Hanlon, G. M., Plomp, J. J., Chakrabarti, M., Morrison, I., Wagner, E. R., Goodyear, C. S., et al. (2001). Anti-GQ1b ganglioside antibodies mediate complement-dependent destruction of the motor nerve terminal. *Brain* 124, 893–906. doi: 10.1093/brain/124.5.893

Ohsawa, T. (1990). Rat dorsal root ganglion gangliosides during early development until senescence. *Mech. Ageing Dev.* 53, 259–266. doi: 10.1016/0047-6374(90)90043-f

Olsson, Y. (1967). Degranulation of mast cells in peripheral nerve injuries. *Acta Neurol. Scand.* 43, 365–374. doi: 10.1111/j.1600-0404.1967.tb05739.x

Pahlavani, M. A., and Richardson, A. (1996). The effect of age on the expression of interleukin-2. *Mech. Ageing Dev.* 89, 125–154. doi: 10.1016/0047-6374(96)01725-3

Painter, M. W., Brosius Lutz, A., Cheng, Y. C., Latremoliere, A., Duong, K., Miller, C. M., et al. (2014). Diminished schwann cell repair responses underlie age-associated impaired axonal regeneration. *Neuron* 83, 331–343. doi: 10.1016/j.neuron.2014.06.016

Pan, C. L., Yuki, N., Koga, M., Chiang, M. C., and Hsieh, S. T. (2001). Acute sensory ataxic neuropathy associated with monospecific anti-GD1b IgG antibody. *Neurology* 57, 1316–1318. doi: 10.1212/wnl.57.7.1316

Parrinello, S., Napoli, I., Ribeiro, S., Wingfield Digby, P., Fedorova, M., Parkinson, D. B., et al. (2010). EphB signaling directs peripheral nerve regeneration through Sox2-dependent schwann cell sorting. *Cell* 143, 145–155. doi: 10.1016/j.cell.2010.08.039

Peric, S., Berisavac, I., Stojiljkovic Tamas, O., Rajic, S., Babic, M., Cvijanovic, M., et al. (2016). Guillain-Barré syndrome in the elderly. *J. Peripher. Nerv. Syst.* 21, 105–110. doi: 10.1111/jns.12163

Phongsisay, V., Susuki, K., Matsuno, K., Yamahashi, T., Okamoto, S., Funakoshi, K., et al. (2008). Complement inhibitor prevents disruption of sodium channel clusters in a rabbit model of Guillain-Barré syndrome. *J. Neuroimmunol.* 205, 101–104. doi: 10.1016/j.jneuroim.2008.09.016

Pilartz, M., Jess, T., Indefrei, D., and Schröder, J. M. (2002). Adoptive transfer-experimental allergic neuritis in newborn Lewis rats results in inflammatory infiltrates, mast cell activation, and increased Ia expression with only minor nerve fiber degeneration. *Acta Neuropathol.* 104, 513–524. doi: 10.1007/s00401-002-0586-9

Pollard, J. D., Westland, K. W., Harvey, G. K., Jung, S., Bonner, J., Spies, J. M., et al. (1995). Activated T cells of nonneural specificity open the blood-nerve barrier to circulating antibody. *Ann. Neurol.* 37, 467–475. doi: 10.1002/ana.410370409

Previtali, S. C., Archelos, J. J., and Hartung, H. P. (1998). Expression of integrins in experimental autoimmune neuritis and Guillain-Barré syndrome. *Ann. Neurol.* 44, 611–621. doi: 10.1002/ana.410440406

Prineas, J. W. (1981). Pathology of the Guillain-Barré syndrome. *Ann. Neurol.* 9, 6–19. doi: 10.1002/ana.410090704

Pritchard, J., Makowska, A., Gregson, N. A., Hayday, A. C., and Hughes, R. A. (2007). Reduced circulating CD4+CD25+ cell populations in Guillain-Barré syndrome. *J. Neuroimmunol.* 183, 232–238. doi: 10.1016/j.jneuroim.2006.12.002

Rabinowich, H., Goses, Y., Reshef, T., and Klajman, A. (1985). Interleukin-2 production and activity in aged humans. *Mech. Ageing Dev.* 32, 213–226. doi: 10.1016/0047-6374(85)90081-8

Rampoldi, F., Ullrich, L., and Prinz, I. (2020). Revisiting the interaction of γδ T-cells and B-cells. *Cells* 9:743. doi: 10.3390/cells9030743

Raynor, J., Lages, C. S., Shehata, H., Hildeman, D. A., and Chougnet, C. A. (2012). Homeostasis and function of regulatory T cells in aging. *Curr. Opin. Immunol.* 24, 482–487. doi: 10.1016/j.coi.2012.04.005

Reichert, F., Levitzky, R., and Rotshenker, S. (1996). Interleukin 6 in intact and injured mouse peripheral nerves. *Eur. J. Neurosci.* 8, 530–535. doi: 10.1111/j.1460-9568.1996.tb01237.x

Richter, R. B. (1962). The ataxic form of polyradiculoneuritis (Landry-Guillain-Barre syndrome). Clinical and pathologic observations. *J. Neuropathol. Exp. Neurol.* 21, 171–184.

Rosenkranz, D., Weyer, S., Tolosa, E., Gaenslen, A., Berg, D., Leyhe, T., et al. (2007). Higher frequency of regulatory T cells in the elderly and increased suppressive activity in neurodegeneration. *J. Neuroimmunol.* 188, 117–127. doi: 10.1016/j.jneuroim.2007.05.011

Scheib, J. L., and Höke, A. (2015). An attenuated immune response by schwann cells and macrophages inhibits nerve regeneration in aged rats. *Neurobiol. Aging* 45, 1–9. doi: 10.1016/j.neurobiolaging.2016.05.004

Schmidt, B., Toyka, K. V., Kiefer, R., Full, J., Hartung, H. P., and Pollard, J. (1996). Inflammatory infiltrates in sural nerve biopsies in guillain-barré syndrome and chronic inflammatory demyelinating neuropathy. *Muscle Nerve* 19, 474–487. doi: 10.1002/(SICI)1097-4598(199604)19:4<474::AID-MUS8>3.0.CO;2-9

Schmitt, V., Rink, L., and Uciechowski, P. (2013). The Th17/Treg balance is disturbed during aging. *Exp. Gerontol.* 48, 1379–1386. doi: 10.1016/j.exger.2013.09.003

Seeldrayers, P. A., Yasui, D., Weiner, H. L., and Johnson, D. (1989). Treatment of experimental allergic neuritis with nedocromil sodium. *J. Neuroimmunol.* 25, 221–226. doi: 10.1016/0165-5728(89)90140-9

Sejvar, J. J., Baughman, A. L., Wise, M., and Morgan, O. W. (2011). Population incidence of Guillain-Barré syndrome: a systematic review and meta-analysis. *Neuroepidemiology* 36, 123–133. doi: 10.1159/000324710

Shahrizaila, N., and Yuki, N. (2013). Bickerstaff brainstem encephalitis and Fisher syndrome: anti-GQ1b antibody syndrome. *J. Neurol. Neurosurg. Psychiatr.* 84, 576–583. doi: 10.1136/jnnp-2012-302824

Sheremata, W., Colby, S., Karkhanis, Y., and Eylar, E. H. (1975). Cellular hypersensitivity to basic myelin (P2) protein in the Guillain-Barré syndrome. *Can. J. Neurol. Sci.* 2, 87–90. doi: 10.1017/s0317167100020059

Spada, F. M., Grant, E. P., Peters, P. J., Sugita, M., Melián, A., Leslie, D. S., et al. (2000). Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *J. Exp. Med.* 191, 937–948. doi: 10.1084/jem.191.6.937

Stiasny, K., Aberle, J. H., Keller, M., Grubeck-Loebenstein, B., and Heinz, F. X. (2012). Age affects quantity but not quality of antibody responses after vaccination with an inactivated flavivirus vaccine against tick-borne encephalitis. *PLoS ONE* 7:e34145. doi: 10.1371/journal.pone.0034145

Stoll, G., Jander, S., Jung, S., Archelos, J., Tamatani, T., Miyasaka, M., et al. (1993). Macrophages and endothelial cells express intercellular adhesion molecule-1 in immune-mediated demyelination but not in wallerian degeneration of the rat peripheral nervous system. *Lab. Invest.* 68, 637–644.

Stoll, G., Schmidt, B., Jander, S., Toyka, K. V., and Hartung, H. P. (1991). Presence of the terminal complement complex (C5b-9) precedes myelin degradation in immune-mediated demyelination of the rat peripheral nervous system. *Ann. Neurol.* 30, 147–155. doi: 10.1002/ana.410300205

Stratton, J. A., Eaton, S., Rosin, N. L., Jawad, S., Holmes, A., Yoon, G., et al. (2020). Macrophages and associated ligands in the aged injured nerve: a defective dynamic that contributes to reduced axonal regrowth. *Front. Aging Neurosci.* 12:174. doi: 10.3389/fnagi.2020.00174

Susuki, K., Baba, H., Tohyama, K., Kanai, K., Kuwabara, S., Hirata, K., et al. (2007a). Gangliosides contribute to stability of paranodal junctions and ion channel clusters in myelinated nerve fibers. *Glia* 55, 746–757. doi: 10.1002/glia.20503

Susuki, K., Rasband, M. N., Tohyama, K., Koibuchi, K., Okamoto, S., Funakoshi, K., et al. (2007b). Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. *J. Neurosci.* 27, 3956–3967. doi: 10.1523/JNEUROSCI.4401-06

Suzuki, M. (2013). Peripheral neuropathy in the elderly. *Handb. Clin. Neurol.* 115, 803–813. doi: 10.1016/B978-0-444-52902-2.00046-1

Tanaka, T., Furutama, D., Sakai, R., Fujita, A., Kimura, F., Tagami, M., et al. (2007). Biological roles of anti-GM1 antibodies in patients with Guillain-Barré syndrome for nerve growth factor signaling. *Biochim. Biophys. Acta* 1772, 543–548. doi: 10.1016/j.bbadis.2007.01.011

Tang, H. Y., Chiu, D. T., Lin, J. F., Huang, C. Y., Chang, K. H., Lyu, R. K., et al. (2017b). Disturbance of plasma lipid metabolic profile in guillain-barre syndrome. *Sci. Rep.* 7:8140. doi: 10.1038/s41598-017-08338-7

Tang, H. Y., Ho, H. Y., Chiu, D. T., Huang, C. Y., Cheng, M. L., and Chen, C. M. (2017a). Alterations of plasma concentrations of lipophilic antioxidants are associated with Guillain-Barre syndrome. *Clin. Chim. Acta* 470, 75–80. doi: 10.1016/j.cca.2017.05.001

Terryberry, J. W., Thor, G., and Peter, J. B. (1998). Autoantibodies in neurodegenerative diseases: antigen-specific frequencies and intrathecal analysis. *Neurobiol. Aging* 19, 205–216. doi: 10.1016/s0197-4580(98)00049-9

Van den Berg, L. H., Mollee, I., Wokke, J. H., and Logtenberg, T. (1995). Increased frequencies of HPRT mutant T lymphocytes in patients with Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy: further evidence for a role of T cells in the etiopathogenesis of peripheral demyelinating diseases. *J. Neuroimmunol.* 58, 37–42. doi: 10.1016/0165-5728(94)00185-q

van Doorn, P. A., Ruts, L., and Jacobs, B. C. (2008). Clinical features, pathogenesis, and treatment of Guillain-Barré syndrome. *Lancet Neurol.* 7, 939–950. doi: 10.1016/S1474-4422(08)70215-1

Vasudev, A., Ying, C. T., Ayyadhury, S., Puan, K. J., Andiappan, A. K., Nyunt, M. S., et al. (2014). γ/δ T cell subsets in human aging using the classical α/β T cell model. *J. Leukoc. Biol.* 96, 647–655. doi: 10.1189/jlb.5A1213-650RR

Verdú, E., Ceballos, D., Vilches, J. J., and Navarro, X. (2000). Influence of aging on peripheral nerve function and regeneration. *J. Peripher. Nerv. Syst.* 5, 191–208. doi: 10.1046/j.1529-8027.2000.00026.x

Waksman, B. H., and Adams, R. D. (1955). Allergic neuritis: an experimental disease of rabbits induced by the injection of peripheral nervous tissue and adjuvants. *J. Exp. Med.* 102, 213–236. doi: 10.1084/jem.102.2.213

Wang, X., Zheng, X. Y., Ma, C., Wang, X. K., Wu, J., Adem, A., et al. (2014). Mitigated Tregs and augmented Th17 cells and cytokines are associated with severity of experimental autoimmune neuritis. *Scand. J. Immunol.* 80, 180–190. doi: 10.1111/sji.12201

Wanschitz, J., Maier, H., Lassmann, H., Budka, H., and Berger, T. (2003). Distinct time pattern of complement activation and cytotoxic T cell response in Guillain-Barré syndrome. *Brain 126,* 2034–2042. doi: 10.1093/brain/awg207

Webb, A. J., Brain, S. A., Wood, R., Rinaldi, S., and Turner, M. R. (2015). Seasonal variation in Guillain-Barré syndrome: a systematic review, meta-analysis and oxfordshire cohort study. *J. Neurol. Neurosurg. Psychiatr.* 86, 1196–1201. doi: 10.1136/jnnp-2014-309056

Weksler, M. E., and Szabo, P. (2000). The effect of age on the B-cell repertoire. *J. Clin. Immunol.* 20, 240–249. doi: 10.1023/a:1006659401385

Willison, H. J., and Yuki, N. (2002). Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125, 2591–2625. doi: 10.1093/brain/awf272

Winer, J., Hughes, S., Cooper, J., Ben-Smith, A., and Savage, C. (2002). gamma delta T cells infiltrating sensory nerve biopsies from patients with inflammatory neuropathy. *J. Neurol.* 249, 616–621. doi: 10.1007/s004150200072

Wu, M. X., Daley, J. F., Rasmussen, R. A., and Schlossman, S. F. (1995). Monocytes are required to prime peripheral blood T cells to undergo apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1525–1529. doi: 10.1073/pnas.92.5.1525

Wu, X., Wang, J., Liu, K., Zhu, J., and Zhang, H. L. (2016). Are Th17 cells and their cytokines a therapeutic target in guillain–barré syndrome? *Expert Opin. Ther. Targets* 20, 209–222. doi: 10.1517/14728222.2016.1086751

Xia, R. H., Yosef, N., and Ubogu, E. E. (2010). Selective expression and cellular localization of pro-inflammatory chemokine ligand/receptor pairs in the sciatic nerves of a severe murine experimental autoimmune neuritis model of Guillain–Barré syndrome. *Neuropathol. Appl. Neurobiol.* 36, 388–398. doi: 10.1111/j.1365-2990.2010.01092.x

Xin, J., Wainwright, D. A., Serpe, C. J., Sanders, V. M., and Jones, K. J. (2008). Phenotype of CD4+ T cell subsets that develop following mouse facial nerve axotomy. *Brain Behav. Immun.* 22, 528–537. doi: 10.1016/j.bbi.2007.10.006

Yang, Y. R., Liu, S. L., Qin, Z. Y., Liu, F. J., Qin, Y. J., Bai, S. M., et al. (2008). Comparative proteomics analysis of cerebrospinal fluid of patients with Guillain-Barré syndrome. *Cell. Mol. Neurobiol.* 28, 737–744. doi: 10.1007/s10571-007-9257-7

Yao, D., McGonigal, R., Barrie, J. A., Cappell, J., Cunningham, M. E., Meehan, G. R., et al. (2014). Neuronal expression of GalNAc transferase is sufficient to prevent the age-related neurodegenerative phenotype of complex ganglioside-deficient mice. *J. Neurosci.* 34, 880–891. doi: 10.1523/JNEUROSCI.3996-13

Yoshii, F., and Shinohara, Y. (1998). Natural killer cells in patients with Guillain-Barré syndrome. *J. Neurol. Sci.* 157, 175–178. doi: 10.1016/s0022-510x(98)00065-3

Yoshii, F., and Shinohara, Y. (2000). Impaired interleukin-2 response of mononuclear cells in Guillain-Barré syndrome. *Eur. J. Neurol.* 7, 303–307. doi: 10.1046/j.1468-1331.2000.00072.x

Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Odaka, M., Hirata, K., et al. (2004). Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barre syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11404–11409. doi: 10.1073/pnas.0402391101

Yuki, N., Yamada, M., Koga, M., Odaka, M., Susuki, K., Tagawa, Y., et al. (2001). Animal model of axonal Guillain-Barré syndrome induced by sensitization with GM1 ganglioside. *Ann. Neurol.* 49, 712–720. doi: 10.1002/ana.1012

Zettl, U. K., Gold, R., Hartung, H. P., and Toyka, K. V. (1994). Apoptotic cell death of T-lymphocytes in experimental autoimmune neuritis of the Lewis rat. *Neurosci. Lett.* 176, 75–79. doi: 10.1016/0304-3940(94)90875-3

Zhang, H. L., Azimullah, S., Zheng, X. Y., Wang, X. K., Amir, N., Mensah-Brown, E. P., et al. (2012). IFN-γ deficiency exacerbates experimental autoimmune neuritis in mice despite a mitigated systemic Th1 immune response. *J. Neuroimmunol.* 246, 18–26. doi: 10.1016/j.jneuroim.2012.02.011

Zhu, J., Bai, X. F., Mix, E., and Link, H. (1997). Cytokine dichotomy in peripheral nervous system influences the outcome of experimental allergic neuritis: dynamics of mRNA expression for IL-1 beta, IL-6, IL-10, IL-12, TNF-alpha, TNF-beta, and cytolysin. *Clin. Immunol. Immunopathol.* 84, 85–94. doi: 10.1006/clin.1997.4356

Zhu, J., Link, H., Weerth, S., Linington, C., Mix, E., and Qiao, J. (1994). The B cell repertoire in experimental allergic neuritis involves multiple myelin proteins and GM1. *J. Neurol. Sci.* 125, 132–137. doi: 10.1016/0022-510x(94)90025-6

Zou, L. P., Pelidou, S. H., Abbas, N., Deretzi, G., Mix, E., Schaltzbeerg, M., et al. (1999). Dynamics of production of MIP-1alpha, MCP-1 and MIP-2

and potential role of neutralization of these chemokines in the regulation of immune responses during experimental autoimmune neuritis in Lewis rats. *J. Neuroimmunol.* 98, 168–175. doi: 10.1016/s0165-5728(99)00100-9

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The Therapeutic Targets of Fingolimod (FTY720) Are Involved in Pathological Processes in the Frontal Cortex of Alzheimer's Disease Patients: A Network Pharmacology Study

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Background: The sphingosine-1-phosphate receptor (S1PR) modulator fingolimod (FTY720), which is commonly used as an immunomodulator in multiple sclerosis treatment, has recently been found to reduce pathological changes in the brain tissue of Alzheimer's disease (AD) animal models, but this has yet to be verified in human brain tissue. In this study, network pharmacology methods were applied to determine the potential pharmacological mechanisms of fingolimod in the frontal cortex of AD patients.

Methods: The pharmacological macromolecular targets of fingolimod and fingolimod phosphate were downloaded from SwissTarget and DrugBank. Systematic intersection analysis of the expression profiles of brain frontal cortex tissues (423 AD tissues and 266 control tissues) was performed to obtain AD-associated fingolimod targets (F-ADGs). Immune cell infiltration analysis and a primary mouse cortical culture RNA-seq drug screen database were used to identify immune-related F-ADGs and cortex-related F-ADGs. Then, the expression values of F-ADGs were correlated with the disease severity score (MMSE score) of AD patients to identify severity-related F-ADGs. We also analyzed miRNA expression microarray data in the frontal cortex of AD patients associated with disease severity to obtain severity-related F-ADG-miRNAs.

Results: A total of 188 F-ADGs were detected in the frontal cortices of AD patients and were enriched in biological processes such as synaptic signaling, inflammatory response, and response to oxygen-containing compounds. Eleven immune-related F-ADGs (like FPR1, BLNK.) and 17 cortex-related F-ADGs (like ALDH1L1, DUSP1.) were detected. Other F-ADGs, such as S1PR1 and GABBR2, although not classified into the above two categories, were still predicted by bioinformatics methods to play an important role in the development of AD. Two F-ADGs (GNAQ and MMP14) and 28 miRNAs (like miR- 323a-3p, miR-181a-5p.) were found to be associated with AD severity (MMSE 0-27 group). Fifteen F-ADGs (like ALDH1L1, FPR1, and IL6.) and 46 miRNAs (like miR-212-5p, miR-93-5p.) were found to be associated with mild or moderate dementia AD patients' severity (MMSE11-22 subgroup).

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Conclusions: Fingolimod may affect the brain frontal cortex function of AD patients in many different ways, such as affecting immune cell infiltration, nerve cell, or glial cell function, and synaptic function. miRNAs may also be involved. ALDH1L1, FPR1, S1PR1, and GABBR2 may be core drug targets.

Keywords: Alzheimer's disease, fingolimod, S1PR1, network pharmacology, GABA synapses, frontal cortex, ALDH1L1

INTRODUCTION

Fingolimod (FTY720), a sphingosine-1-phosphate receptor (S1PR) modulator, is the first oral drug approved by the Food and Drug Administration (FDA) for the treatment of relapsing-remitting multiple sclerosis (RRMS) (Brinkmann et al., 2010). Recently, fingolimod was reported to reduce the pathological changes in the brain tissue of Alzheimer's disease (AD) animal models (Angelopoulou and Piperi, 2019; Carreras et al., 2019; Jeśko et al., 2019), but this has yet to be verified in human brain tissues. The treatment effect of fingolimod in RRMS is commonly attributed to its ability to retain autoreactive lymphocytes in the lymph nodes and prevent them from entering the circulation and central nervous system (CNS) by downregulating the expression of S1PR1 on T lymphocytes (Hunter et al., 2016; Huwiler and Zangemeister-Wittke, 2017). However, as fingolimod is a lipophilic molecule, it can cross the blood-brain barrier (BBB) (Asle-Rousta et al., 2014; Hunter et al., 2016) and has been proven to exert direct effects in the CNS of RRMS patients and in animal models of degenerative diseases of the CNS, including inhibiting microglial activation, reducing astrocyte proliferation, reducing the loss of dendritic spines, and preventing excitotoxic neuronal death (Hunter et al., 2016). Since these pathophysiological processes have also been observed in AD patients, fingolimod is speculated to also have therapeutic effects on the pathological processes of AD patients.

To date, most of the studies that have explored the mechanism of action of fingolimod in AD animal models have focused on the effect of fingolimod on β-amyloid precursor protein (APP) metabolism and β-amyloid (Aβ) protein aggregation (Tonelli et al., 2010; Takasugi et al., 2011, 2013). However, due to the complexity of the pathologic processes of AD, current animal models cannot fully recapitulate AD, particularly late-onset disease. Therefore, in AD-related research, direct research in human brain tissue is necessary. However, there is currently no report directly demonstrating fingolimod function in the brain tissue of AD patients. Network pharmacology methods provide new approaches to more comprehensively elucidate the mechanisms underlying the pharmacological effects of drugs in specific diseases (Hopkins, 2008) and a possible technique to directly study human brain tissue. In this research, we used network pharmacology methods to determine the potential pharmacological mechanism underlying the treatment effect of fingolimod in AD patients and to identify potential targets of fingolimod in the brain tissues of AD patients.

MATERIALS AND METHODS

Study Design and Rationale

In this study, the pharmacological macromolecular targets of fingolimod and fingolimod phosphate (i.e., fingolimod-associated genes, FGs) were downloaded from SwissTarget and DrugBank. Systematic intersection analysis of the expression profiles of brain frontal cortex tissues (423 AD tissues and 266 control tissues) was performed to obtain AD-associated genes (ADGs) and fingolimod AD-associated targets (F-ADGs). Immune cell infiltration analysis and a primary mouse cortical culture RNA-seq drug screen database were used to identify immune-related F-ADGs and cortex-related F-ADGs. Then, the expression values of F-ADGs were correlated with the disease severity score (MMSE score) of AD patients to identify severity-related F-ADGs. We also analyzed miRNA expression microarray data in the frontal cortex of AD patients associated with disease severity to obtain severity-related F-ADG-miRNAs. A flow chart of the experimental method is shown in **Figure 1**. In this study, we used the same method to analyze the data of the frontal cortex, temporal cortex and entorhinal cortex of AD patients. The main body of this article mainly used the frontal cortex data. The temporal cortex and entorhinal cortex data were shown in the **Supplementary Materials**.

FGs

The chemical structures of fingolimod and its metabolic product fingolimod phosphate were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). SwissTargetPrediction (Gfeller et al., 2013; Daina et al., 2019) (http://www.swisstargetprediction.ch/) was used to predict the pharmacological targets of fingolimod and fingolimod phosphate based on their chemical structures, and DrugBank (https://go.drugbank.com/) was used to collect experimentally confirmed fingolimod drug targets. In SwissTargetPrediction, pharmacological targets with a feasibility >0.1 were identified. UniProt (https://www.UniProt.org/) was used to standardize the naming of the drug targets. STRING (version 11.0) was used to retrieve protein-protein interaction (PPI) information with the retrieval condition "Species" set to "*Homo sapiens,*" the "confidence" set to "high (>0.7)," and the maximum number of interactions for the "1st shell" and "2nd shell" set to "no more than 20 interactors." The target genes of fingolimod and fingolimod phosphate and the proteins that were predicted to interact with these target genes are collectively referred to as FGs.

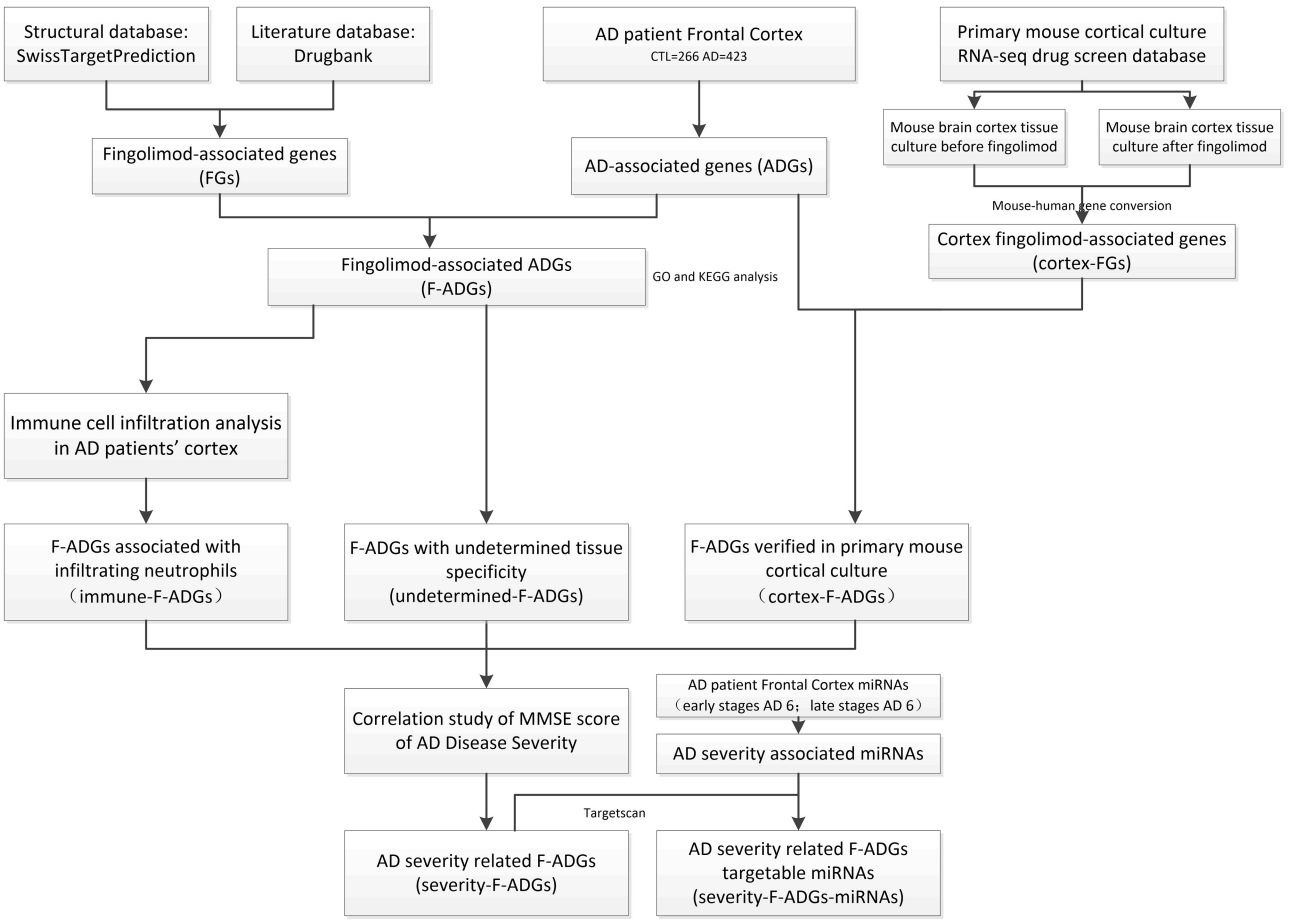

FIGURE 1 | Flowchart of the analysis of the effect of fingolimod against AD. AD, Alzheimer's disease; CTL, control; FGs, fingolimod-associated genes; ADGs, AD-associated genes; F-ADGs, fingolimod-associated AD-associated genes.

ADGs

Original microarray data associated with AD patients were retrieved from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) database by searching for the keyword "Alzheimer." Exhaustive and non-redundant data for the frontal cortex were retrieved from eight datasets (GSE12685, GSE36980, GSE48350, GSE5281, GSE53890, GSE66333, GSE33000, and GSE118553) from 423 AD patients and 266 normal controls. All processed expression data were merged and then normalized with the "sva" package in R. Only genes (probes) for which <50% of the gene expression data were missing were retained. Then, the data from normal controls and AD patients were compared to identify ADGs. The "limma" package in R was used for differential expression analysis, and the Benjamini-Hochberg's method was used to correct for multiple comparisons. A |fold change| >1.2 and $p < 0.05$ were considered to indicate significantly differentially expressed genes.

F-ADGs, PPI Analysis, and Gene Function Analysis

The FGs and ADGs were compared to identify AD-associated fingolimod targets (F-ADGs). STRING (version 11.0) was used for PPI analysis. After repeats were eliminated, a "confidence score" >0.7 was set to design the PPI network. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of F-ADGs were performed using WebGestalt (http://www.webgestalt.org/) (Liao et al., 2019). The following parameters were used in the enrichment analysis: minimum number of IDs in the category: 5; maximum number of IDs in the category: 2000; FDR method: BH; significance level: Top 20 and $p < 0.01$.

Analysis of Immune Cell Infiltration in the Frontal Cortices of AD Patients

CIBERSORTx (https://cibersortx.stanford.edu/) (Newman et al., 2019) was used to estimate the percentages of various cell populations in the cortex of each AD patient. A signature matrix of each cell type was generated with the default settings. Bulk RNA-seq data were deconvoluted using the signature matrix with S-batch correction to remove variances between different sequencing platforms. Two-tailed unpaired Student's *t*-test was used to analyse differences in the abundances of various cell types between AD patients and normal controls. The *p*-values were corrected for multiple testing using the

Benjamini-Hochberg method. $P < 0.05$ was considered to indicate statistical significance.

Cortex-Related FGs and Cortex-Related F-ADGs

To better identify the direct effects of fingolimod on the nervous system and eliminate interference by immune infiltration, we used the "Primary mouse cortical culture RNA-seq drug screen database" (http://bigbear.med.uottawa.ca:1000/) (Hadwen et al., 2018). We compared the gene expression profiles of mouse brain cortex tissue cultures before and after treatment with fingolimod and obtained mouse cortex-related fingolimod-associated genes (cortex-related FGs). Then, we used Mouse Genome Informatics (http://www.informatics.jax.org/) to transform the mouse gene sets into the corresponding human gene sets and obtained the cortex-related fingolimod-associated genes (cortex-related FGs). Then, we used the intersection of cortex-related FGs and ADGs to obtain cortex-related F-ADGs.

Analysis of the Undetermined F-ADGs

The F-ADGs that were not classified as immune-related F-ADGs or cortex-related F-ADGs were defined as undetermined F-ADGs. The Minimal Common Oncology Data Elements (MCODE) method was used for network analysis to select core molecular modules from the PPI network. The screening criterion for the core modules was twice the average degree of the "MCODE score." The degree cut-off was set to 2, the node score cut-off was set to 0.2, the K-core was set to 2 and the maximum depth was set to 100. Undetermined F-ADGs with MCODE scores > 16 were considered key genes and were subjected to GO functional analysis.

Correlation Analysis of F-ADG Expression and the Clinical Severity of AD

The frontal cortex expression profiles of 14 AD patients were correlated with their Mini-Mental State Exam (MMSE) scores, which ranged from 0 to 27. At the same time, we conducted a stratified analysis of subgroups of patients with mild and moderate dementia (MMSE score 10–23). In this study, 9 patients with MMSE scores from 11–22 were collected in this subgroup. Pearson's correlation analysis was used to assess the correlation between the expression values of the F-ADGs and the MMSE scores of the AD patients. $R > 0.7$ indicated a very strong linear correlation, $0.5 < R \leq 0.7$ indicated a significant linear correlation, $0.3 < R \leq 0.5$ indicated a low linear correlation and $R \leq 0.3$ indicated no linear correlation. $P < 0.05$ was considered statistically significant.

Analysis of the miRNAs Targeted by AD Severity-Related F-ADGs

First, we downloaded the GSE48552 dataset from the GEO database, which contains miRNA expression data of the frontal cortices of 6 early-stage AD patients and 6 late-stage AD patients. Then, we compared the frontal cortex miRNA expression data of early- and late-stage AD patients to obtain miRNAs related to the severity of AD. TargetScan (http://www.targetscan.org/vert_71/) (Lewis et al., 2005) is widely used to predict possible targets of miRNAs by pairing conserved 8-mer, 7-mer, and 6-mer sites within the seed region of miRNAs. In this study, the severity-related F-ADGs were applied to TargetScan to predict possible targeted miRNAs. Then, we intersected these possible targeted miRNAs with the AD severity-associated miRNAs to obtain the severity-related F-ADG-miRNAs.

RESULTS

FGs

A total of 36 pharmacological targets of fingolimod or fingolimod phosphate (4 confirmed and 32 predicted based on chemical structures) from the TargetScan database and 11 targets from the DrugBank database were identified. Using the STRING database, 1,054 proteins that interact with these targets were identified and designated FGs (**Supplementary Material 1**).

ADGs

A total of 2,149 genes that were differentially expressed in the frontal cortices of AD patients compared to healthy controls were identified as ADGs; 1,046 of these genes were upregulated and 1,103 were downregulated in AD patients compared to normal controls (**Supplementary Material 2**). At the same time, we also analyzed the data of the temporal and entorhinal cortexes of AD patients in the same way (**Supplementary Material 2, Supplementary Figure 1**).

F-ADGs

Of the 2,156 ADGs identified in the frontal cortex, 164 genes (100 upregulated and 64 downregulated) were FGs, suggesting that they are targets of fingolimod and were designated F-ADGs. A total of 575 interacting edges were formed consisting of 110 F-ADGs in the PPI network (**Figure 2**, **Supplementary Material 3**).

Gene Function and KEGG Pathway Enrichment Analysis of F-ADGs

In the frontal cortex, the 188 identified F-ADGs were enriched in biological processes such as synaptic signaling, inflammatory response, and response to oxygen-containing compounds, and KEGG pathways such as neurotrophin signaling pathways, dopaminergic synapses, and glutamatergic synapses (**Figure 3**, **Supplementary Material 4**).

Immune-Related F-ADGs

Immune infiltration analysis showed that compared with normal controls, AD patients had higher neutrophil infiltration ($p < 0.05$) and lower plasma cell infiltration ($p < 0.05$) in the frontal lobe brain tissue. By comparing the signal intensity of infiltrating cells with the expression signal intensity of F-ADGs, we found that no F-ADGs showed a correlation with plasma cells, while 11 F-ADGs showed a correlation with neutrophils. Among the 11 F-ADGs related to neutrophils, ANGPTL4, FPR1, CDKN1A, and SHC1 were positively correlated with neutrophil infiltration, while MCM4, CLEC7A, FEN1, BLNK, MBTPS2, SST, and TM7SF2 were negatively correlated with neutrophil infiltration. We believe that the four genes that are positively related to the signal intensity associated with neutrophil infiltration may have a

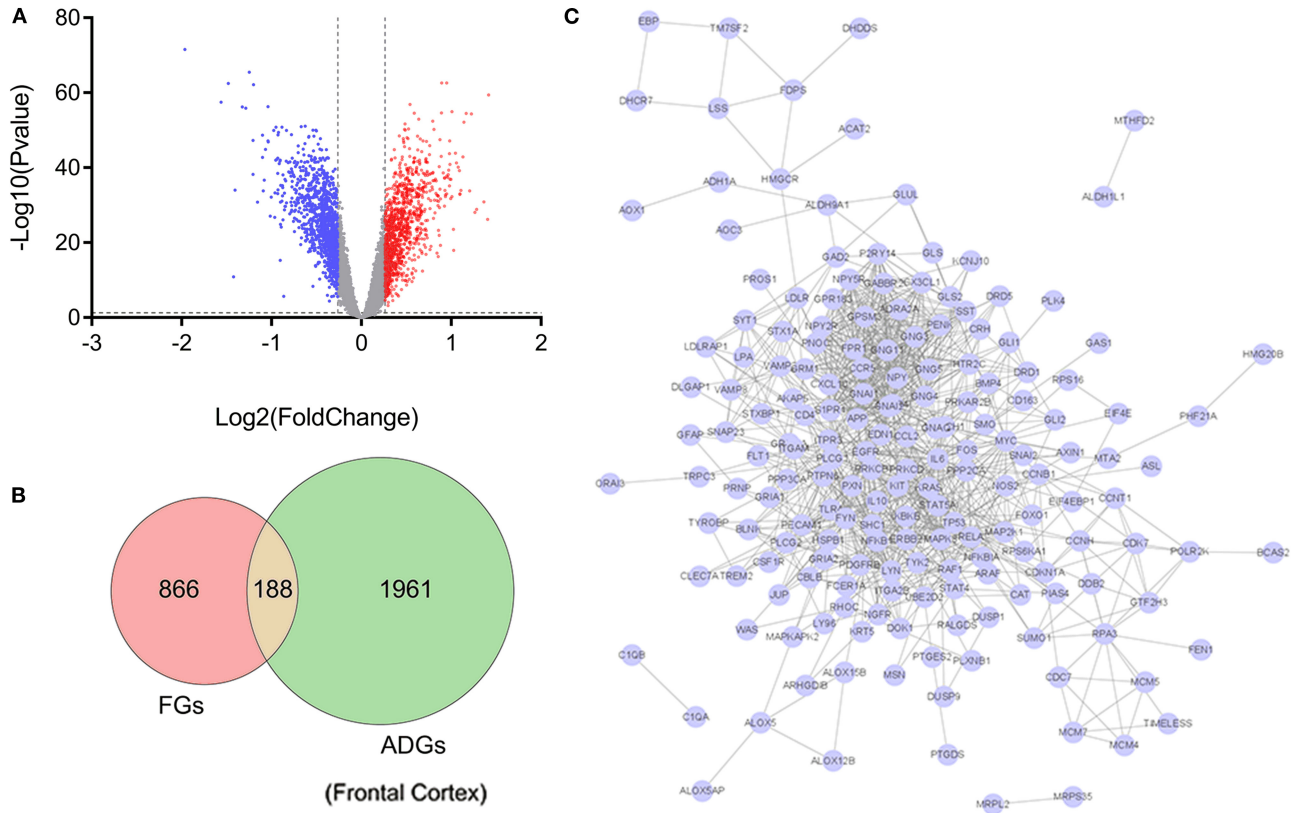

FIGURE 2 | Pharmacological targets of fingolimod and FGs. **(A)** Volcano map showing the differentially expressed genes in the frontal cortices of AD patients compared to normal controls. Significantly upregulated genes are shown in red, significantly downregulated genes are shown in blue, and unchanged genes are shown in gray. **(B)** Venn diagram showing FGs, ADGs, and F-ADGs. **(C)** PPI networks of F-ADGs. Each node represents an F-ADG.

more direct correlation with neutrophil infiltration and are more likely to be targets of fingolimod that regulate the infiltration of neutrophils in the frontal lobe of AD patients (**Figure 4**, **Supplementary Material 5**).

Cortex-Related F-ADGs

In the primary mouse cortical culture RNA-seq drug screen database, we compared the mouse brain cortex expression data before fingolimod and after fingolimod treatment, conducted mouse-human gene name conversion, and obtained 74 cortex-related FGs. Then, we compared the cortex-related FGs with ADGs and obtained 17 cortex-related F-ADGs. The PPI analysis of cortex-related F-ADGs showed that VIM, MMP14, and FLNA were key genes in the network, suggesting that these genes may be targets of fingolimod that act directly on the nerve tissue of AD patients. GO analysis showed that the cortex-related F-ADGs were enriched in functions such as response to oxidative stress, cell adhesion, and negative regulation of apoptotic process (**Figure 5**, **Supplementary Material 6**).

Undetermined F-ADGs

A total of 175 F-ADGs were classified as undetermined F-ADGs. The MCODE method divided undetermined F-ADGs into 11 parts, and the genes in the first part showed the strongest functional correlation. It is worth noting that S1PR1, the classic target of fingolimod, was also included in the first part, which suggested that these functionally related genes may be regulated by fingolimod at the same time. Functional enrichment analysis showed that the undetermined F-ADGs in the first part were enriched in KEGG pathways such as the chemokine signaling pathway, GABAergic synapses, and morphine addiction (**Figure 6**, **Supplementary Material 7**).

Severity-Related F-ADGs

The correlation between the MMSE score of 14 AD patients, which ranged from 0 to 27, was collected. Two F-ADGs (GNAQ and MMP14) were found to be correlated with MMSE scores and were designated severity-related F-ADGs.

At the same time, we conducted a stratified analysis of subgroups of patients with mild and moderate dementia (9 patients with MMSE scores from 11 to 22). The correlation between the MMSE score of 9 AD patients, which ranged from 11 to 22, was collected. Fifteen F-ADGs were found to be strongly correlated with MMSE scores and were designated severity-related F-ADGs. Among the cortex-related F-ADGs, ALDH1L1 was positively correlated with the severity of AD (negatively correlated with the MMSE score). Among the immune-related

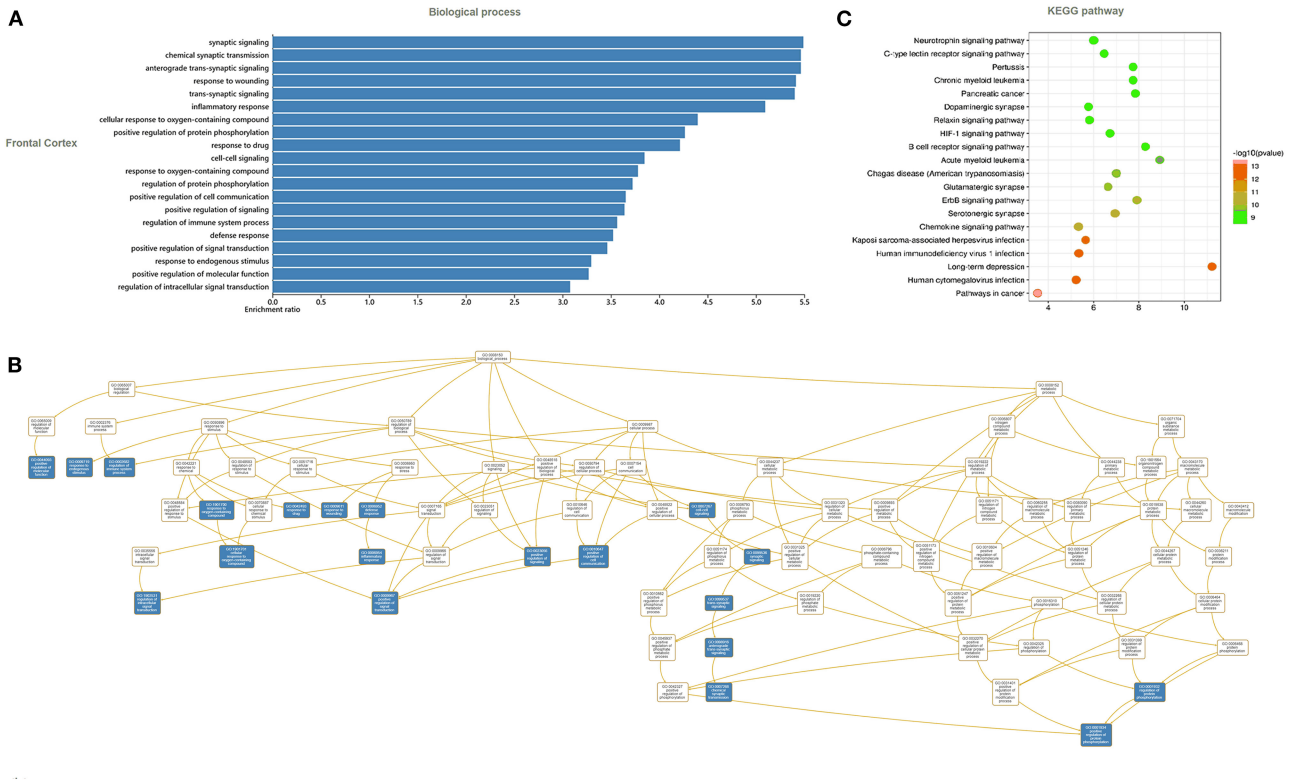

FIGURE 3 | GO and KEGG analyses of F-ADGs. **(A)** Histogram showing GO enrichment analysis of F-ADGs. **(B)** Directed acyclic graph showing GO enrichment analysis of F-ADGs. **(C)** Bubble chart showing KEGG pathway enrichment analysis of F-ADGs.

F-ADGs, SHC1, CDKN1A, and FPR1 were positively correlated with the severity of AD, while BLNK was negatively correlated with the severity of AD. Among the undetermined F-ADGs, CCNT1, DUSP9, HSPB1, IL6, LDLR, CCL2, and FLT1 were positively correlated with the severity of AD, while KCNJ10, JUP and LDLRAP1 were negatively correlated with the severity of AD (**Figure 7**, **Supplementary Material 8**).

Severity-Related F-ADG-miRNAs

A total of 270 miRNAs were found to be differentially expressed in the frontal cortex in the early and late stages of AD. Among them, 169 had lower expression in the early stage of AD, and 101 had higher expression. These miRNAs were recorded as AD severity-associated miRNAs. In MMSE score 0–27 group, the 2 severity-related F-ADGs (GNAQ and MMP14) were found to be targeted by 28 miRNAs (like miR- 323a-3p and miR-181a-5p.); In mild and moderate dementia AD subgroup (MMSE scores from 11 to 22), among the 15 severity-related F-ADGs obtained above, 11 were found to be targeted by 46 AD severity-associated miRNAs. These AD severity-associated miRNAs were recorded as severity-related F-ADG-miRNAs (**Figure 8**, **Supplementary Material 9**).

DISCUSSION

AD is the most common cause of dementia in the world, and there are no effective therapies that can halt disease progression (Rossor, 1993; Brookmeyer et al., 2018; Weller and Budson, 2018). The S1PR modulator fingolimod (FTY720), which is an immunomodulator used in the treatment of multiple sclerosis, was recently reported to reduce the pathological changes in the brain tissue of AD animal models, but this has yet to be verified in human brain tissue. In this study, we used network pharmacology to identify possible fingolimod drug targets in the frontal cortex of AD patients. We found that fingolimod may affect the pathological processes in the CNS of AD patients by affecting the number and function of neurons and astrocytes, the infiltration of neutrophils in the frontal cortices, cell apoptosis, GABA synaptic function, and miRNA interactions, among others (**Figure 9**).

ALDH1L1 is one of the key drug targets discovered in this study. ALDH1L1 was predicted to be targeted by fingolimod in both the human drug target database and mouse cortex drug target database. Since the correlation between this gene and fingolimod has been reported in studies of these two different species, we believe that the discovery has a high degree of credibility. Since ALDH1L1 is commonly viewed as a pan-astrocytic constitutive marker (Yoon et al., 2017), it is possible that fingolimod may increase the number of astrocytes in the CNS. However, it is worth noting that the increase in the number of astrocytes is a double-edged sword for AD patients. On the one hand, maintenance of the number of healthy astrocytes is necessary for stable brain function, including regulation of synaptic activity, neuronal metabolism, and

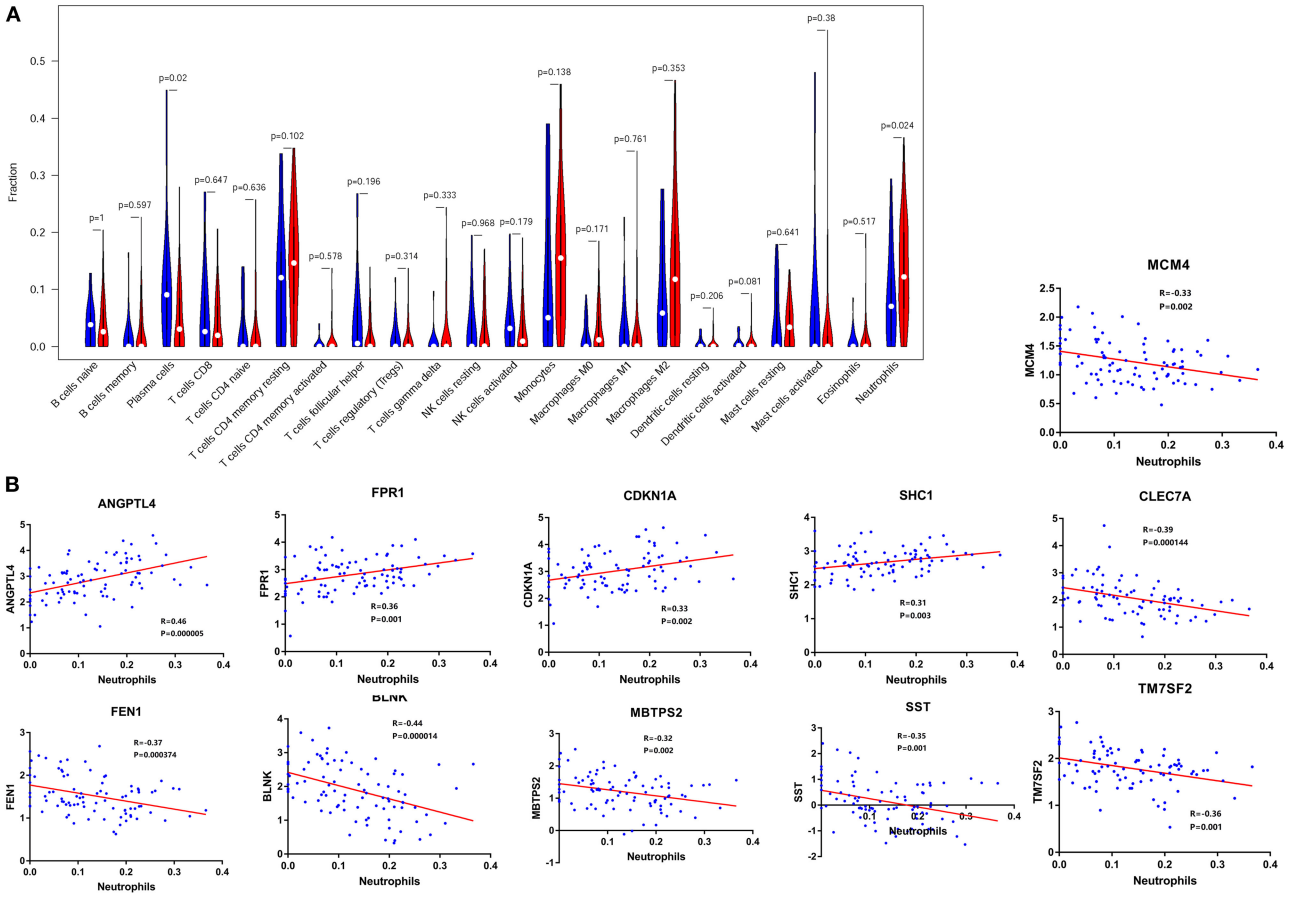

FIGURE 4 | Neutrophil-related F-ADGs. **(A)** Violin plot of frontal cortex immune cell infiltration in AD patients. **(B)** Correlation analysis of the expression levels of neutrophil-related F-ADGs and neutrophil signals. The blue dots indicate the expression levels of the genes in each sample.

regional blood supply. On the other hand, overproliferation of astrocytes may aggravate a series of pathological damages in AD patients, including glutamate excitotoxicity, impaired synaptic plasticity, reduced carbon delivery to neurons for oxidative phosphorylation, and dysregulated linkages between neuronal energy demand and regional blood supply. Therefore, we cannot conclude whether this potential function of fingolimod in mediating astrocyte proliferation mentioned above is beneficial or harmful to AD patients (Habib et al., 2020). Similarly, as the results of the severity-related F-ADGs of this study also suggested that the expression level of ALDH1L1 was positively correlated with the severity of AD, it is unclear whether this would be a beneficial compensation mechanism or a cause of aggravation. In addition, it is worth noting that in this study, we found that miR-212-5p, which has a mutual targeting relationship with ALDH1L1, has a lower content in brain tissue in late AD than in early AD, which is consistent with the trend that ALDH1L1 expression increases with the severity of the disease. However, because a lower miR-212 level has been proven to protect elderly patients from dementia (Hadar et al., 2018), we believe that the elevated ALDH1L1 in AD patients may reduce the level of miR-212 in the brain tissue by acting as a miRNA sponge to play a neuroprotective function, and fingolimod may increase this beneficial function by increasing ALDH1L1 levels in brain tissue.

Another key target proposed in this study is DUSP1. This gene was also predicted to be regulated by fingolimod in both the human database and the mouse data cortical culture database. Data from the mouse database showed that the expression of DUSP1 in the cerebral cortex of mice increased after fingolimod treatment. At the same time, the expression of DUSP1 in the temporal cortices and entorhinal cortices of AD patients was also found to be higher than that of normal controls (**Supplementary Figure 2**). In the human cortex, DUSP1 protein expression correlates with tau phosphorylation (Arango-Lievano et al., 2016), synaptic defects and cognitive decline in subjects diagnosed with AD. Therefore, if fingolimod can truly increase the expression level of DUSP1 in the brain tissue of AD patients, it is reasonable to believe that fingolimod can affect the pathogenesis of AD by regulating tau phosphorylation.

Among immune-related F-ADGs were 11 gene targets related to neutrophil infiltration. Among them, the four genes ANGPTL4, FPR1, CDKN1A, and SHC1 were positively correlated with neutrophil signaling, and we believe that these genes are more likely to be directly related to neutrophil

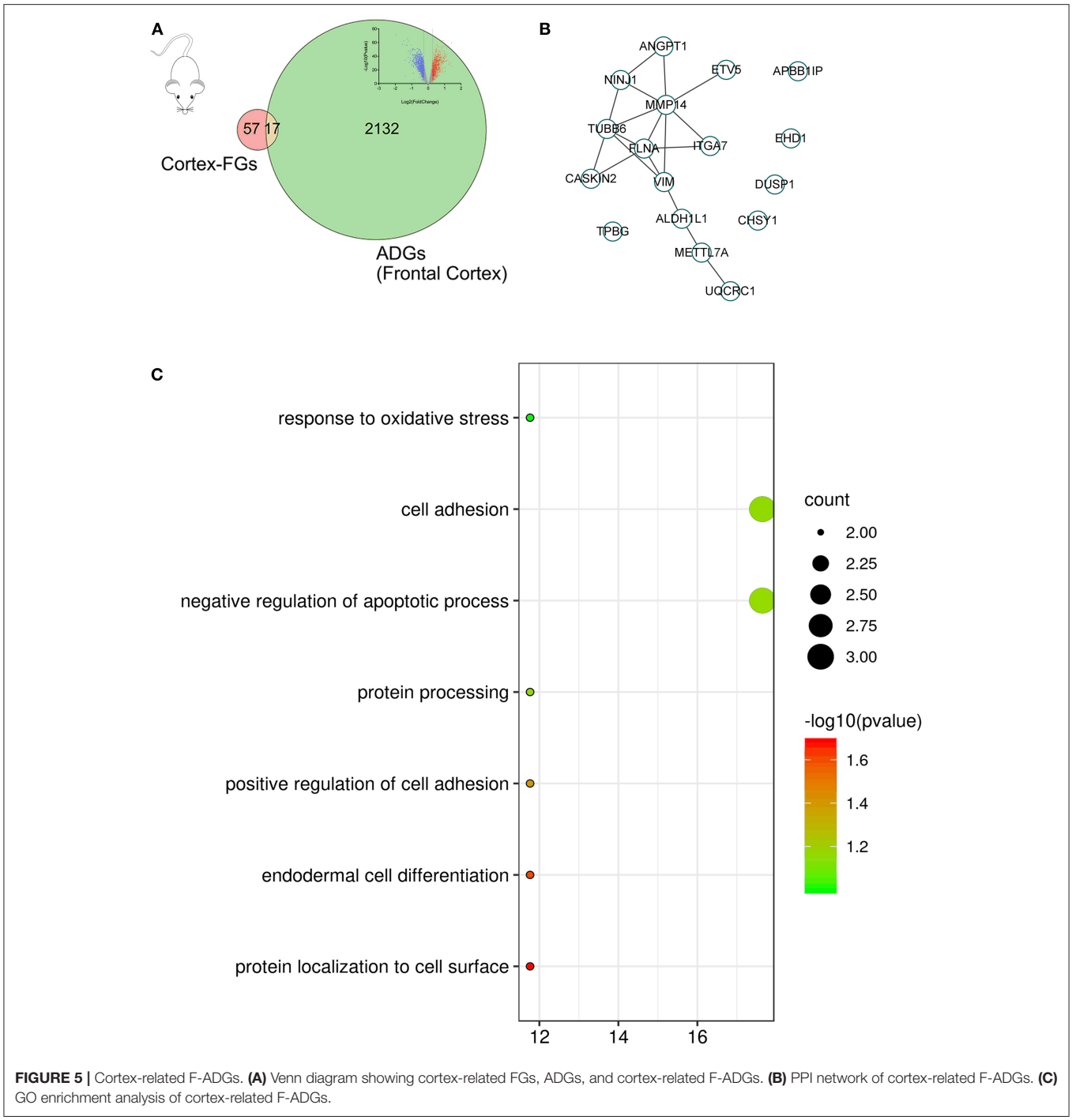

FIGURE 5 | Cortex-related F-ADGs. **(A)** Venn diagram showing cortex-related FGs, ADGs, and cortex-related F-ADGs. **(B)** PPI network of cortex-related F-ADGs. **(C)** GO enrichment analysis of cortex-related F-ADGs.

infiltration. Angiopoietin-like 4 (ANGPTL4) is a hypoxia-induced factor that is upregulated and secreted by human cortical astrocytes under hypoxic conditions (Chakraborty et al., 2018). Therefore, the increased expression of ANGPTL4 in AD patients is consistent with the increase in the expression of the abovementioned astrocyte-related genes in the brain tissue of AD patients compared to normal controls. ANGPTL4 can increase vascular permeability (Gomez Perdiguero et al., 2016), so fingolimod may regulate inflammatory cell infiltration in the CNS by targeting ANGPTL4. Formyl peptide receptor (FPR) is a G protein-coupled chemokine receptor that is mainly expressed in phagocytic leukocytes (Krepel and Wang, 2019). In related studies on colorectal tumors (Li et al., 2017), FPR1 mRNA expression has been confirmed to be related to tumor serous membrane infiltration, and FPR1 protein is expressed in colorectal epithelium and tumor-infiltrating

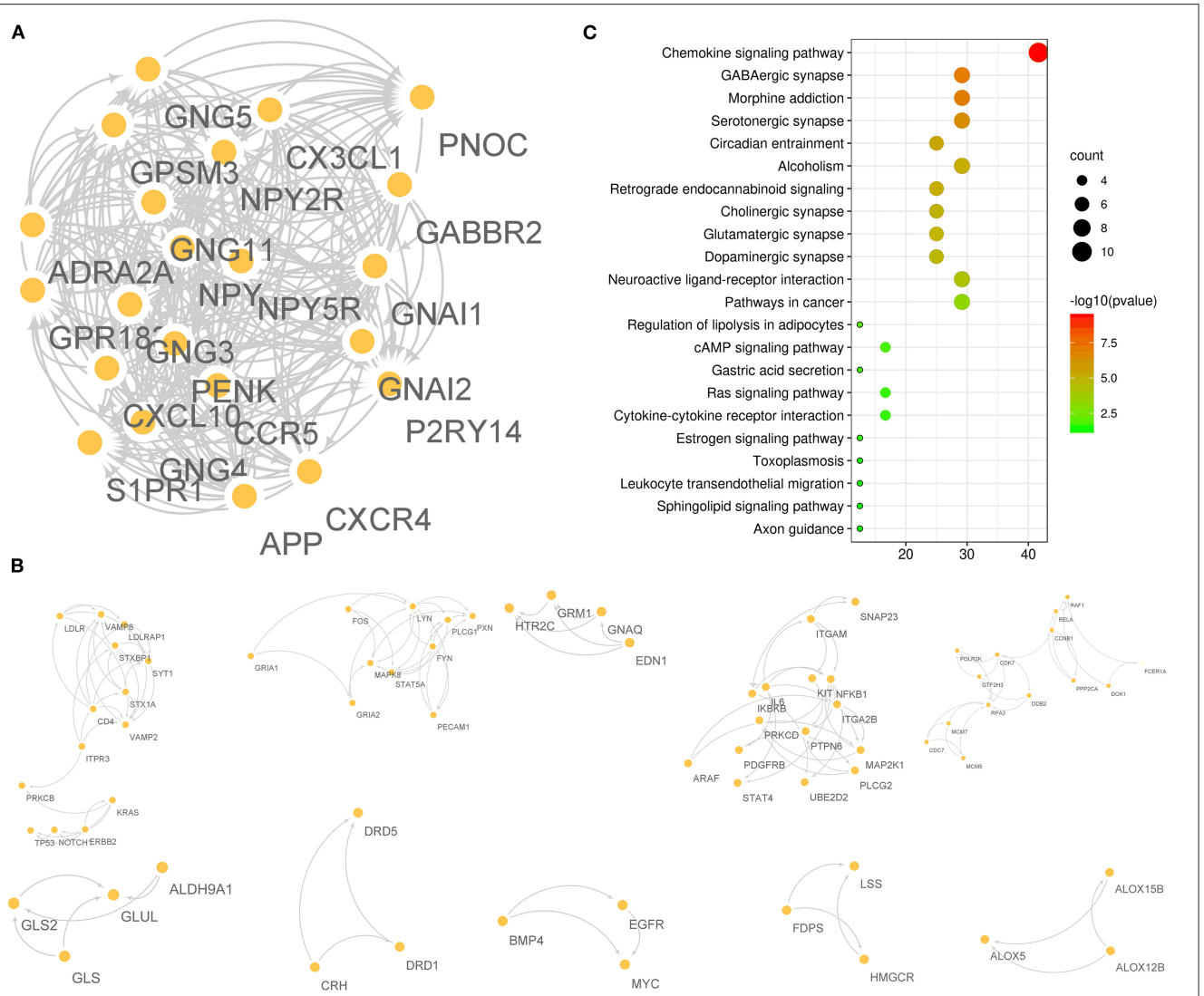

FIGURE 6 | Undetermined F-ADGs. **(A)** PPI network of the 1st gene group of undetermined F-ADGs. **(B)** PPI network of the 2nd–11th gene groups of undetermined F-ADGs. **(C)** KEGG enrichment analysis of undetermined F-ADGs.

neutrophils/macrophages (Chen et al., 2017). In this study, it was found that the FPR1 signal in AD brain tissue increased, which was related to neutrophil infiltration and was positively correlated with the severity of AD. Therefore, if fingolimod can reduce the expression of FPR1 as predicted in this study, it may improve the occurrence and development of AD by reducing the adverse effects of immune infiltration.

Dysfunction of the GABAergic system may cause cognitive impairment in humans (Solas et al., 2015). In severe AD patients, GABA levels have been shown to be significantly reduced, which may be the cause of AD behavior and psychological symptoms (Calvo-Flores Guzmán et al., 2018). Increasing evidence supports the remodeling of GABAergic synapses in the AD brain, which may begin in the early stages of disease pathogenesis last through the entire course of AD (Mandal et al., 2017). Therefore, GABAergic synapses may become effective molecular targets for AD drug development and pharmacological treatment. In this study, the functional enrichment of F-ADGs resulted in the enrichment of GABAergic synapses, which suggests that fingolimod may exert pharmacological effects on GABA synapses that undergo pathological changes during the pathogenesis of AD. It is worth noting that in the undetermined F-ADG group analysis, S1PR1, the currently recognized main target of fingolimod, was found to be closely related to GABA synaptic function. In addition, the expression of S1PR1 was found to be increased in the frontal cortices, temporal cortices, and entorhinal cortices of AD patients, which suggests that S1PR1 is widely involved in the pathological changes of the AD cerebral cortex (**Supplementary Material 2, 3**, **Supplementary Figure 2**). Therefore, in the CNS, fingolimod phosphate may act by directly targeting S1PR1 on the surface of the CNS and may act by influencing GABAergic synaptic function. Notably, oral

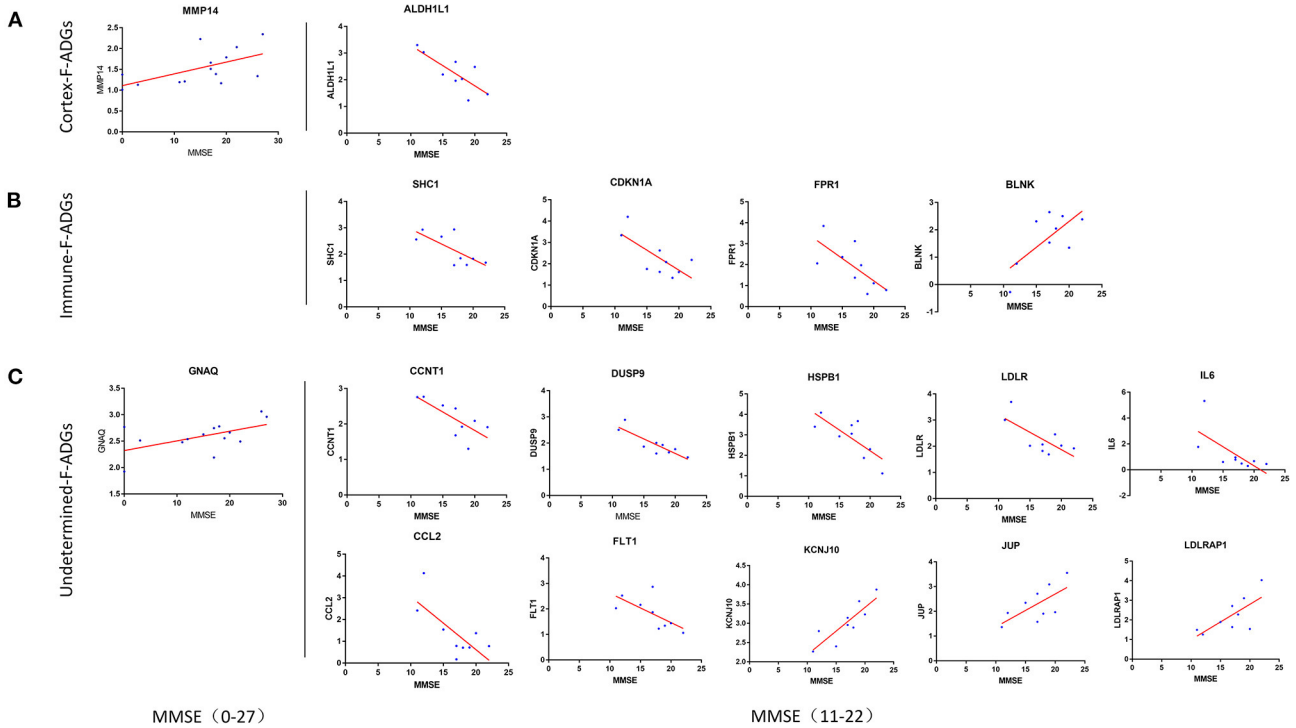

FIGURE 7 | Severity-related F-ADGs **(A)** Cortex-related F-ADGs, **(B)** immune-related F-ADGs, and **(C)** undetermined F-ADGs. Correlation analysis of the expression levels of F-ADGs and MMSE scores as a measure of AD clinical severity. Each group includes F-ADGs from MMSE (0–27) group and F-ADGs from MMSE (11–22) subgroup. MMSE scores are plotted on the x-axis. Gene expression values are plotted on the y-axis. The blue dots indicate the expression levels of the core genes in each sample.

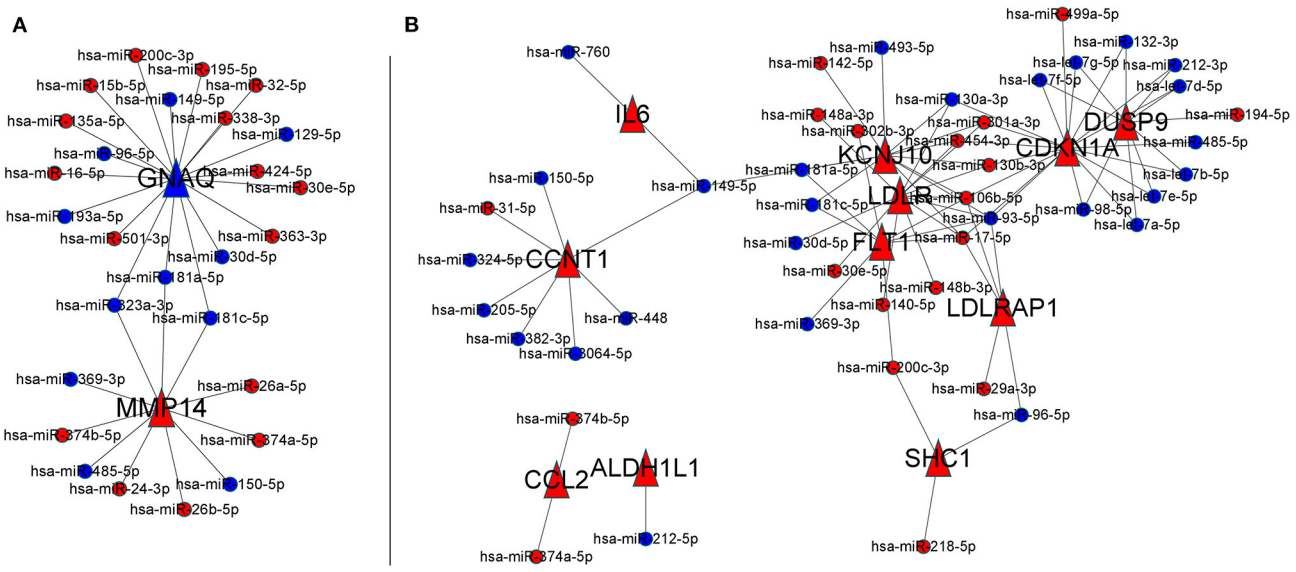

FIGURE 8 | Severity-related F-ADG-miRNAs **(A)** AD patients with MMSE score 0–27 group, **(B)** mild or moderate dementia AD patients subgroup (MMSE score 11–22). The red dots indicate miRNAs that are upregulated in late AD; the blue dots indicate miRNAs that are downregulated in late AD; and the red triangle indicates the mRNA that is upregulated with the worsening of AD.

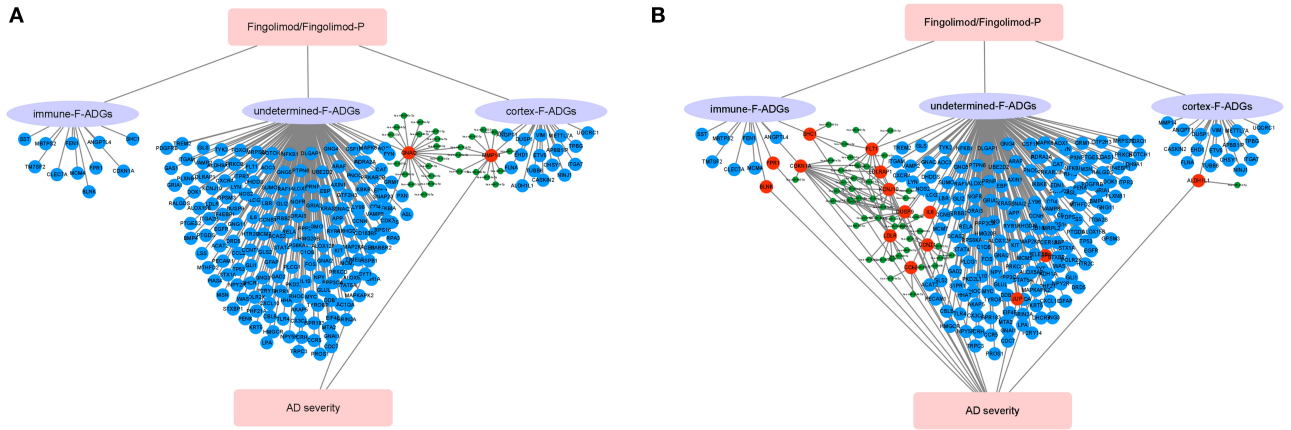

FIGURE 9 | Schematic diagram of the synaptic action of fingolimod in AD patients. **(A)** AD patients with MMSE score 0-27 group, **(B)** mild or moderate dementia AD patients subgroup (MMSE score 11-22). The red circles represent F-ADGs that are related to the severity of AD; the blue circles represent F-ADGs that are not related to the severity of AD; and the green circles represent miRNAs.

administration of fingolimod has been reported to alter synaptic function in the brain tissues of experimental autoimmune encephalomyelitis (EAE) mice (Gillingwater, 2012; Rossi et al., 2012).

In addition, fingolimod has been shown to reduce TNF-induced expression of B-cell activating factor (BAFF) and chemokine interferon-γ inducible protein 10 (CXCL10), which have immunoregulatory effects, in astrocytes (Hoffmann et al., 2015). This study revealed that the expression of CXCL10 was higher in the frontal cortices of AD patients than normal controls, suggesting that the abnormal increase in CXCL10 expression in AD is involved in the development and progression of the disease and that fingolimod could play a role in immune regulation in AD-affected brains.

In addition, this study found that one of the ways that fingolimod affects the brain tissue of AD patients could be through miRNAs. DUSP9, CCNT1, KSNJ10, LDLR, and FLT1 all have a large number of potential miRNA targets. This also suggests the importance of non-coding RNAs in the study of the pharmacological mechanism of fingolimod. More research on different types of non-coding RNAs is necessary. Moreover, neurons may contain an array of non-germline variants, including single-nucleotide variants (SNVs), copy number variants (CNVs), DNA breaks, inversions, and translocations. The methodology does not take into account the effects of these variables in a highly complex pathological context. This is one of the limitations of this study, and further research needs to be conducted from more perspectives to explore the function of fingolimod in the brain tissue of AD patients.

In this study, each of the two parts of Severity-related F-ADG-miRNAs and Severity-related F-ADGs was divided into two parts: the all sample group (MMSE0-27) and the mild or moderate dementia subgroup (MMSE 11-22). We found that fewer severity-related F-ADGs were found in the all sample group, GNAQ and MMP14, while more severity-related genes appeared in the mild or moderate dementia subgroup. We believe that two reasons may have contributed to this phenomenon. On the one hand, patients with excessive dementia may have interference from other complications, such as infections and metabolic diseases, which may have more effects on nerve cell gene expression levels beyond the pathological process of AD. On the other hand, the correlation between the expression level of F-ADGs and AD severity may only occur in some specific disease periods, rather than the entire course of AD. For example, some genes may only be positively correlated with disease severity in the early stage of AD, but not correlated or even negative correlated with the disease severity in the middle or late stages. Therefore, it is necessary to have the stratified study of AD patients at different stages of the disease on the basis of a comprehensive study of all patients with MMSE scores. A variety of MMSE scoring standards are used to grade the severity of dementia patients. An accepted way is: Normal cognition (24 and higher), Mild dementia (19–23), Moderate dementia (10–18), and Severe dementia (9 and lower) (Crum et al., 1993). Based on this classification method, our previous analysis of patients with 11–22 points can be regarded as a stratified analysis after the overall analysis, that is, a stratified analysis for mild dementia and moderate dementia. At the same time, we also tried to conduct separate stratified analysis on the "Severe dementia group" and the "no dementia group." But in "Severe dementia" there are only two patients with 0 points and one patient with 3 points, and "no dementia" has only two samples of 26 points and 27 points. Neither can perform an ideal correlation analysis. Therefore, in this study, only the all sample group (MMSE 0-27) and the mild or moderate dementia subgroup (MMSE 11-22) were included.

Limitations

This study has a number of limitations. First, this study is a bioinformatics analysis, and the predicted fingolimod drug targets are only potential drug targets. However, the *in vivo* effects of fingolimod on these predicted targets and the true impact on the pathogenesis of AD after treatment still need to be verified in further animal and clinical trials. Since the samples included in this study come from multiple databases, some clinical

data, and MMSE scores may have been generated by different standards, which may cause bias and affect the results of the study. Moreover, neurons may contain an array of non-germline variants, including SNVs, CNVs, DNA breaks, inversions, and translocations. Therefore, in the future, additional large-scale and clinical studies of AD patients receiving fingolimod treatment are necessary.

CONCLUSION

Fingolimod may affect the brain frontal cortex function of AD patients in many different ways, such as affecting immune cell infiltration, nerve cell or astrocyte proliferation, and GABAergic synapse function. miRNAs may also be involved. ALDH1L1, DUSP1, S1PR1, and GABBR2 may be core drug targets.

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/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

The study was conceived and designed by PY, YX, TW, DZ and GL. Data collection was carried out by PY. Processing and analysis of the imaging data was performed by PY and YX. PY led the statistical analysis. All authors contributed to interpretation of the data, preparation of the manuscript, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.609679/full#supplementary-material

REFERENCES

Angelopoulou, E., and Piperi, C. (2019). Beneficial effects of fingolimod in Alzheimer's disease: molecular mechanisms and therapeutic potential. *Neuromol. Med*. 21, 227–238. doi: 10.1007/s12017-019-08558-2

Arango-Lievano, M., Peguet, C., Catteau, M., et al., (2016). Deletion of neurotrophin signaling through the glucocorticoid receptor pathway causes tau neuropathology. *Sci. Rep*. 6:37231. doi: 10.1038/srep37231

Asle-Rousta, M., Kolahdooz, Z., Dargahi, L., Ahmadiani, A., and Nasoohi, S. (2014). Prominence of central sphingosine-1-phosphate receptor-1 in attenuating abeta-induced injury by Fingolimod. *J. Mol. Neurosci*. 54, 698–703. doi: 10.1007/s12031-014-0423-3

Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., et al. (2010). Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat. Rev. Drug Discov.* 9, 883–897. doi: 10.1038/nrd3248

Brookmeyer, R., Abdalla, N., Kawas, C. H., and Corrada, M. M. (2018). Forecasting the prevalence of preclinical and clinical Alzheimer's disease in the United States. *Alzheimers Dement.* 14, 121–129. doi: 10.1016/j.jalz.2017.10.009

Calvo-Flores Guzmán, B., Vinnakota, C., Govindpani, K., Waldvogel, H. J., Faull, R. L. M., and Kwakowsky, A. (2018). The GABAergic system as a therapeutic target for Alzheimer's disease. *J. Neurochem*. 146, 649–669. doi: 10.1111/jnc.14345

Carreras, I., Aytan, N., Choi, J. K., Tognoni, C. M., Kowall, N. W., Jenkins, B. G., et al. (2019). Dual dose-dependent effects of Fingolimod in a mouse model of Alzheimer's disease. *Sci. Rep*. 9:10972. doi: 10.1038/s41598-019-47287-1

Chakraborty, A., Kamermans, A., van Het Hof, B., Castricum, K., Aanhane, E., van Horssen, J., et al. (2018). Angiopoietin like-4 as a novel vascular mediator in capillary cerebral amyloid angiopathy. *Brain* 141, 3377–3388. doi: 10.1093/brain/awy274

Chen, K., Bao, Z., Gong, W., Tang, P., Yoshimura, T., and Wang, J. M. (2017). Regulation of inflammation by members of the formyl-peptide receptor family. *J. Autoimmun* 85, 64–77. doi: 10.1016/j.jaut.2017.06.012

Crum, R. M., Anthony, J. C., Bassett, S. S., and Folstein, M. F. (1993). Population-based norms for the Mini-Mental Status Examination by age and educational level. *JAMA* 269, 2386–2391. doi: 10.1001/jama.1993.03500180078038

Daina, A., Michielin, O., and Zoete, V. (2019). SwissTargetPrediction: updated data and new features for efficient prediction of protein targets of small molecules. *Nucl. Acids Res*. 47, W357–W364. doi: 10.1093/nar/gkz382

Gfeller, D., Michielin, O., and Zoete, V. (2013). Shaping the interaction landscape of bioactive molecules. *Bioinformatics* 29, 3073–3079. doi: 10.1093/bioinformatics/btt540

Gillingwater, T. H. (2012). Targeting synaptic pathology in multiple sclerosis: Fingolimod to the rescue?. *Br. J. Pharmacol*. 165, 858–860. doi: 10.1111/j.1476-5381.2011.01612.x

Gomez Perdiguero, E., Liabotis-Fontugne, A., Durand, M., Faye, C., Ricard-Blum, S., Simonutti, M., et al. (2016). ANGPTL4-αvβ3 interaction counteracts hypoxia-induced vascular permeability by modulating Src signalling downstream of vascular endothelial growth factor receptor 2. *J. Pathol.* 240, 461–471. doi: 10.1002/path.4805

Habib, N., McCabe, C., Medina, S., Varshavsky, M., Kitsberg, D., Dvir-Szternfeld, R., et al. (2020). Disease-associated astrocytes in Alzheimer's disease and aging. *Nat. Neurosci.* 23, 701–706. doi: 10.1038/s41593-020-0624-8

Hadar, A., Milanesi, E., Walczak, M., Puzianowska-Kuznicka, M., Kuznicki, J., Squassina, A., et al. (2018). SIRT1, miR-132 and miR-212 link human longevity to Alzheimer's disease. *Sci. Rep.* 8:8465. doi: 10.1038/s41598-018-26547-6

Hadwen, J., Schock, S., Mears, A., Yang, R., Charron, P., Zhang, L., et al., (2018). Transcriptomic RNAseq drug screen in cerebrocortical cultures: toward novel neurogenetic disease therapies. *Hum. Mol. Genet.* 27, 3206–3217. doi: 10.1093/hmg/ddy221

Hoffmann, F. S., Hofereiter, J., Rubsamen, H., Melms, J., Schwarz, S., Faber, H., et al. (2015). Fingolimod induces neuroprotective factors in human astrocytes. *J. Neuroinflam.* 12:184. doi: 10.1186/s12974-015-0393-6

Hopkins, A. L. (2008). Network pharmacology: the next paradigm in drug discovery. *Nat. Chem. Biol.* 4, 682–690. doi: 10.1038/nchembio.118

Hunter, S. F., Bowen, J. D., and Reder, A. T. (2016). The direct effects of Fingolimod in the central nervous system: implications for relapsing multiple Sclerosis. *CNS Drugs* 30, 135–147. doi: 10.1007/s40263-015-0297-0

Huwiler, A., and Zangemeister-Wittke, U. (2017). The sphingosine 1-phosphate receptor modulator Fingolimod as a therapeutic agent: recent findings and new perspectives. *Pharmacol. Therap.* 185, 34–49. doi: 10.1016/j.pharmthera.2017.11.001

Jeśko, H., Wencel, P. L., Lukiw, W. J., and Strosznajder, R. P. (2019). Modulatory effects of fingolimod (FTY720) on the expression of sphingolipid metabolism-related genes in an animal model of Alzheimer's Disease. *Mol. Neurobiol.* 56, 174–185. doi: 10.1007/s12035-018-1040-x

Krepel, S. A., and Wang, J. M. (2019). Chemotactic ligands that activate G-Protein-coupled formylpeptide receptors. *Int. J. Mol. Sci.* 20:3426. doi: 10.3390/ijms20143426

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20. doi: 10.1016/j.cell.2004.12.035

Li, S. Q., Su, N., Gong, P., Zhang, H-B., Liu, J., Wang, D., et al. (2017). The expression of formyl peptide receptor 1 is correlated with tumor invasion of human colorectal cancer. *Sci. Rep.* 7:5918. doi: 10.1038/s41598-017-06368-9

Liao, Y., Wang, J., Eric, J., Jaehnig, E. J., Shi, Z., and Zhang, B. (2019). WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Res.* 47, W199–W205. doi: 10.1093/nar/gkz401

Mandal, P. K., Kansara, K., and Dabas, A. (2017). The GABA-working memory relationship in Alzheimer's disease. *J. Alzheimers Dis. Rep.* 1, 43–45. doi: 10.3233/ADR-170003

Newman, A. M., Steen, C. B., Liu, C. L., Gentles, A. J., Chaudhuri, A. A., Scherer, F., et al. (2019). Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat. Biotechnol.* 37, 773–782. doi: 10.1038/s41587-019-0114-2

Rossi, S., Lo Giudice, T., De Chiara, V., Musella, A., Studer, V., Motta, C., et al. (2012). Oral Fingolimod rescues the functional deficits of synapses in experimental autoimmune encephalomyelitis. *Br. J. Pharmacol.* 165, 861–869. doi: 10.1111/j.1476-5381.2011.01579.x

Rossor, M. (1993). Alzheimer's disease. *BMJ.* 307, 779–782. doi: 10.1136/bmj.307.6907.779

Solas, M., Puerta, E., and Ramirez, M. J. (2015). Treatment options in Alzheimer's disease: The GABA story. *Curr. Pharm. Des.* 21, 4960–4971. doi: 10.2174/1381612821666150914121149

Takasugi, N., Sasaki, T., Ebinuma, I., Osawa, S., Isshiki, H., Takeo, K., et al. (2013). FTY720/Fingolimod, a sphingosine analogue, reduces amyloid-beta production in neurons. *PLoS ONE* 8:e64050. doi: 10.1371/journal.pone.0064050

Takasugi, N., Sasaki, T., Suzuki, K., Osawa, S., Isshiki, H., Hori, Y., et al. (2011). BACE1 activity is modulated by cell-associated sphingosine-1-phosphate. *J. Neurosci.* 31, 6850–6857. doi: 10.1523/JNEUROSCI.6467-10.2011

Tonelli, F., Lim, K. G., Loveridge, C., Long, J., Pitson, S. M., Tigyi, G., et al. (2010). FTY720 and (S)-FTY720 vinylphosphonate inhibit sphingosine kinase 1 and promote its proteasomal degradation in human pulmonary artery smooth muscle, breast cancer and androgen-independent prostate cancer cells. *Cell Signal.* 22, 1536–1542. doi: 10.1016/j.cellsig.2010.05.022

Weller, J., and Budson, A. (2018). Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Res* 7:F1000. doi: 10.12688/f1000research.14506.1

Yoon, H., Walters, G., Paulsen, A. R., and Scarisbrick, I. A. (2017). Astrocyte heterogeneity across the brain and spinal cord occurs developmentally, in adulthood and in response to demyelination. *PLoS ONE* 12:e0180697. doi: 10.1371/journal.pone.0180697

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ORIGINAL RESEARCH
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Immune Profiling of Parkinson's Disease Revealed Its Association With a Subset of Infiltrating Cells and Signature Genes

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Parkinson's disease (PD) is an age-related and second most common neurodegenerative disorder. In recent years, increasing evidence revealed that peripheral immune cells might be able to infiltrate into brain tissues, which could arouse neuroinflammation and aggravate neurodegeneration. This study aimed to illuminate the landscape of peripheral immune cells and signature genes associated with immune infiltration in PD. Several transcriptomic datasets of substantia nigra (SN) from the Gene Expression Omnibus (GEO) database were separately collected as training cohort, testing cohort, and external validation cohort. The immunoscore of each sample calculated by single-sample gene set enrichment analysis was used to reflect the peripheral immune cell infiltration and to identify the differential immune cell types between PD and healthy participants. According to receiver operating characteristic (ROC) curve analysis, the immunoscore achieved an overall accuracy of the area under the curve (AUC) = 0.883 in the testing cohort, respectively. The immunoscore displayed good performance in the external validation cohort with an AUC of 0.745. The correlation analysis and logistic regression analysis were used to analyze the correlation between immune cells and PD, and mast cell was identified most associated with the occurrence of PD. Additionally, increased mast cells were also observed in our *in vivo* PD model. Weighted gene co-expression network analysis (WGCNA) was used to selected module genes related to a mast cell. The least absolute shrinkage and selection operator (LASSO) analysis and random-forest analysis were used to analyze module genes, and two hub genes RBM3 and AGTR1 were identified as associated with mast cells in the training cohort. The expression levels of RBM3 and AGTR1 in these cohorts and PD models revealed that these hub genes were significantly downregulated in PD. Moreover, the expression trend of the aforementioned two genes differed in mast cells and dopaminergic (DA) neurons. In conclusion, this study not only exhibited a landscape of immune infiltrating patterns in PD but also identified mast cells and two hub genes associated with the occurrence of PD, which provided potential therapeutic targets for PD patients (PDs).

Keywords: Parkinson's disease, RBM3, AGTR1, mast cell, immune cell infiltration

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INTRODUCTION

Parkinson's disease (PD) is an age-related and the second most common neurodegenerative disorder characterized clinically by classic motor symptoms, including resting tremor, bradykinesia, rigidity, and non-motor symptoms, including insomnia, constipation, and pathologically by the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta and the occurrence of Lewy bodies containing α-synuclein (Cao et al., 2011; Fasano et al., 2015; Kalia and Lang, 2015). The etiology of PD has not been completely determined, involving a complex interaction between various genetic and environmental factors (Kalia and Lang, 2015). Furthermore, the pathogenesis of PD has not yet been fully understood.

Mounting evidence indicates that neuroinflammation is one of the vital features in both PD patients (PDs) and animal models of PD, the hallmarks of which are the presence of activated microglia and astrocyte that occur together with the loss of DA neurons in the midbrain (Cao et al., 2011; Calabrese et al., 2018; Stephenson et al., 2018). Microglia and astrocyte could be activated by multiple factors related to PD, such as the pivotal PD-associated genes [α-synuclein (SNCA), Parkin, deglycase (DJ-1), leucine-rich repeat kinase 2 (LRRK2)] and neurotoxins (rotenone and methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Wang et al., 2015). Besides, neuroinflammation may play significant roles in pathogenetic mechanisms of PD, including the deposition of protein aggregates, injury of oxidative stress, impairment of mitochondrial function, disruption of calcium homeostasis, and abnormal iron deposition (He et al., 2020; Picca et al., 2020).

Deep researches accumulating the changes of peripheral immunity in PD patients have captured increasing attention and is gradually (Cao et al., 2011; Kannarkat et al., 2013; Lee et al., 2017). These changes are not only reflected in the types and numbers of peripheral immune cells, but also in the impact on PD-related organs, such as the intestinal tract and central nervous system (CNS; Travagli et al., 2020). Under physiological conditions, the peripheral immune cells are hardly detectable in CNS due to the existence of a blood-brain barrier (BBB). However, the pathological processes of PD could break down BBB, and BBB breakdown leads to increased infiltration of peripheral immune cells into CNS, which has been identified as one of the major contributing factors for PD (Pan and Nicolazzo, 2018; Sweeney et al., 2018). The increased infiltration of peripheral immune cells may induce excessive microglial inflammation, oxidative stress, and cytotoxicity that exacerbate neurodegeneration in PD (Wang et al., 2015; Stephenson et al., 2018; Kustrimovic et al., 2019). In a word, the current evidence indicates that there is a close relationship between the peripheral immune system and the progression of PD.

Nevertheless, the infiltration of peripheral immune cells during PD remains largely unknown, which highlights the significance to illuminate the crosstalk between the peripheral immune system and neuroinflammation in PD. Single-sample gene set enrichment analysis (ssGSEA) was developed to analyze the statistical enrichment score of each sample according to the expression of these sets of metagenes, i.e., non-overlapping sets of genes that are representative for specific immune cell subpopulations (Charoentong et al., 2017). In the present study, we used ssGSEA to calculate the immunescores of peripheral immune cells in the samples of PD patients (PDs) and healthy controls (HCs) based on their gene expression profiles available from public databases, which provided a new angle to uncover the cell types and the extent of peripheral immune cell infiltration in PD. We also identified two immune-based signature genes, RNA-Binding Motif Protein 3 (RBM3) and Angiotensin II Receptor Type 1 (AGTR1), to provide promising targets for improving the prognosis of PD patients.

MATERIALS AND METHODS

Gene Expression Data and Processing

As shown in **Figure 1**, we searched the Gene Expression Omnibus (GEO[1]) for the following keywords: "(Parkinson's disease) and (Substantia nigra)" and collected several datasets in March 2020 (Barrett et al., 2013). According to the description of these datasets, three array datasets with the same platform GPL570 were analyzed as the internal cohort in our study, including GSE7621, GSE20141, and GSE49036. The raw "CEL" files of these microarray data were downloaded, normalized, and log2 transform with "affy" packages (Gautier et al., 2004). After processing, the batch effect of three datasets was removed with "sva" packages (Leek et al., 2012), with 41 PD patients and 25 HCs subsequently included. For external validation, two array datasets with the same platform GPL96 were selected as the external cohort in our study, including GSE20163 and GSE20164. The same preprocessing procedures were performed on the two datasets as mentioned above, with 14 PD patients and 14 HCs subsequently involved; and two datasets profiled by next-generation sequencing, GSE114517, and GSE133101, were also used to verify our signature genes. The information of these datasets is listed in **Table 1**.

Immune Cell Infiltration Score

The ssGSEA was introduced to quantify the relative infiltration of immune cell types in substantia nigra of each PD patients and HCs according to the expression of metagenes that are representative of specific immune cells (Jia et al., 2018); and we selected the metagene set of 28 peripheral immune cell types that were deeply researched and widely accepted, and the metagene set was shown in **Supplementary Table 1** (Charoentong et al., 2017). The relative abundance of each immune cell type was represented by an enrichment score in ssGSEA analysis that was performed using "GSEA" package (Subramanian et al., 2005). Then two samples unequal variance two-tailed *t*-test was used to analyze the immunoscores of PDs and HCs to determine differential immune cell types (p-value < 0.05) between the two groups. We analyzed the correlations among these immune cell types and disease states and employed a Logistic regression model to evaluate the associations between immune cell types and disease occurrence using the "glmnet" package (Friedman et al., 2010). The receiver

[1]http://www.ncbi.nlm.nih.gov/geo

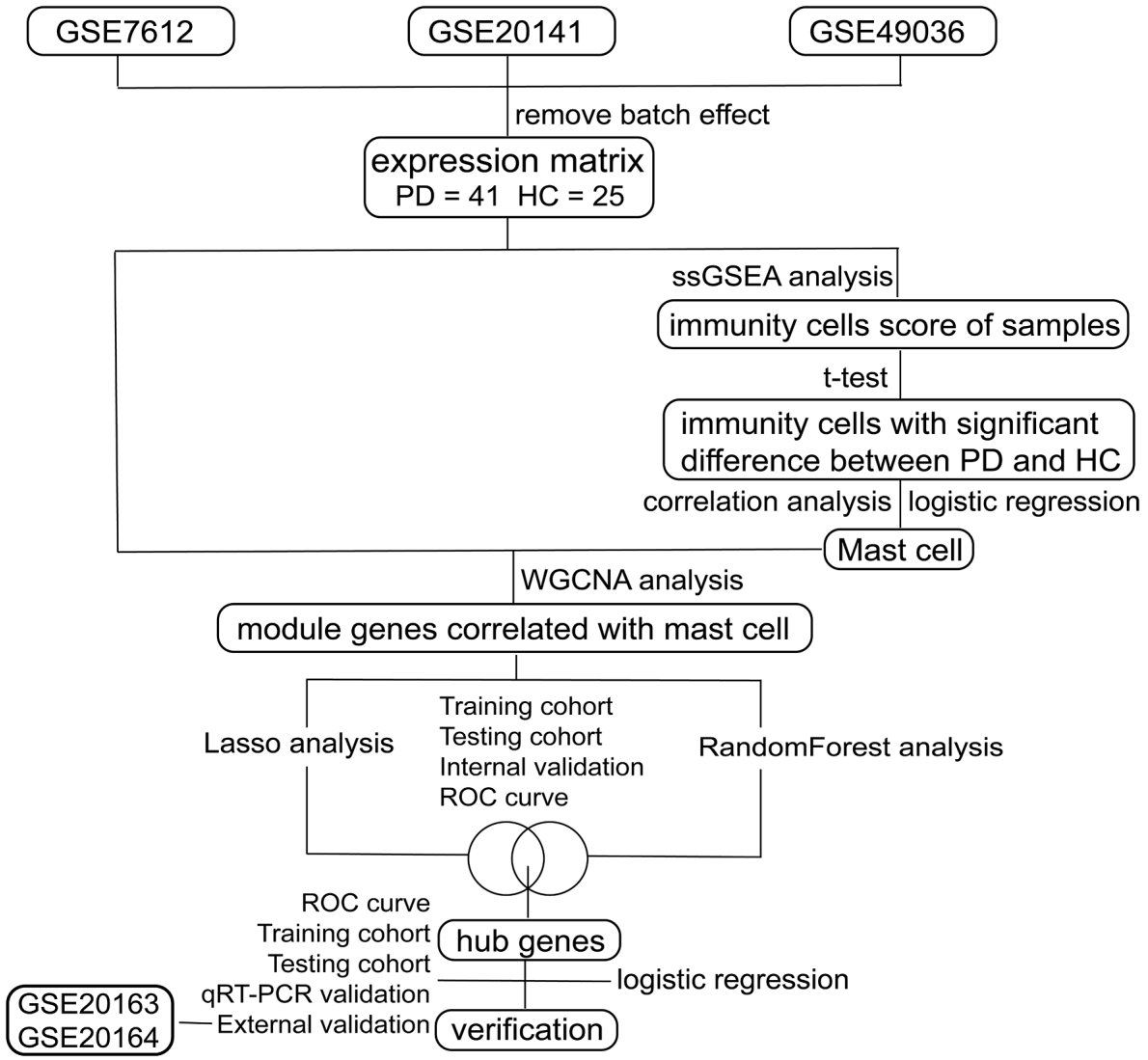

FIGURE 1 | Flow chart of this study.

operating characteristic (ROC) curve was used to explore the sensitivity and specificity of the aforementioned model using the "ROCR" package (Sing et al., 2005).

Weighted Gene Co-expression Network Analysis

All genes of the datasets were used for weighted gene co-expression network analysis (WGCNA) using the "WGCNA" package (Langfelder and Horvath, 2008). WGCNA was used to explore the relationships between expression modules and clinical features. The immunoscores and disease state were regarded as clinical features. According to the algorithm, we tested the independence and the average connectivity degree of different modules with different power values (the power value ranging from 1 to 20). The appropriate power value was determined when the degree of independence was 0.85 and the module construction proceeded with the appropriate power value. The minimum number of genes was set as 40 for the high reliability of the results. Module-trait associations were estimated using the correlation between module eigengenes and clinical features, which facilitates the identification of expression modules highly correlated to clinical features. Then, we selected two expression modules that had significant positive and negative correlations with clinical features and extracted the corresponding genes' information of these modules to perform subsequent analysis.

Identification and Validation of Signature Genes

To remove confounding genes and screen key genes without a relationship between each other, the least absolute shrinkage and selection operator (LASSO) analysis was performed to find

TABLE 1 | The information of Gene Expression Omnibus (GEO) datasets.

GEO datasets	Platform	Method	Tissue	PD samples	HC samples
GSE7621	GPL570	Microarray	Substantia nigra	16	9
GSE20141	GPL570	Microarray	Substantia nigra	10	8
GSE49036	GPL570	Microarray	Substantia nigra	15	8
GSE20164	GPL96	Microarray	Substantia nigra	6	5
GSE20163	GPL96	Microarray	Substantia nigra	8	9
GSE114517	GPL18573	NGS	Substantia nigra	17	12
GSE133101	GPL18573	NGS	Substantia nigra	15	10

Abbreviations: PD, Parkinson's disease; HC, healthy control; NGS, next-generation sequencing.

the optimal gene list. Moreover, to select out more convincing key genes, we also chose random forest (Liaw and Wiener, 2002) for feature selection, which has been widely used and can precisely calculate the importance of each feature in the dataset. RF and LASSO analysis were performed to analyze genes' information of two modules selected from WGCNA analysis using "randomForest" and "glmnet" packages (Liaw and Wiener, 2002; Friedman et al., 2010). Internal datasets consisting of GSE7621, GSE20141, and GSE49036 were randomly divided into the training cohort and the testing cohort by the ratio of 7-3. The intersection of two gene lists analyzed by RF and LASSO analysis in training cohort were used to construct a logistic regression model to explore the correlation of disease occurrence and these genes and were analyzed by ROC curve and confusion matrix to verify the sensitivity and specificity of the model in the testing cohort, in the internal cohort and external one.

In vivo Mouse Model Experiments and Behavioral Tests

Male C57BL/6 mice (weighing 20–30 g) were purchased from Shanghai SLAC Laboratory Animal, housed, and maintained at constant temperature and humidity with a 12 h light/dark cycle in Tongji University. Eight-week-old mice (six per group) were injected a daily i.p. injection of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sigma–Adrich, St. Louis, MO, USA; 30 mg/kg) or saline treatment for 5 days. Motor impairments were tested with rotarod tests and pole tests. In the rotarod tests, mice were trained for 2 min at a speed of 4 r.p.m. and then performed three trials for a maximum of 4 min with increasing speed starting from 4 r.p.m. to 40 r.p.m. The pole tests were performed with a wooden pole (50 cm high, 0.5 cm in diameter, wrapped with gauze to prevent slipping) with a wooden ball at the top. After training and acclimatization, mice were tested with the pole three times for the total time it took for the mouse to get from the top to the bottom.

Tissue Preparation

After treatment and behavioral test, mice (three per group) intended for immunofluorescence (IF) staining analysis were euthanized and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were postfixed for 24 h in 4% PFA at 4°C and transferred to a solution of 30% sucrose in PBS for 24 h at 4°C. The coronal section of SN and STR was sectioned as 10 μm sections on a cryostat (Leica CM3050) and kept on polylysine-coated slides at −80°C. The mouse brains intended for cell lysis (three per group) were transcardially perfused with ice-cold PBS and later performed western blotting.

Cell Co-culture and Drug Treatment

Human neuroblastoma SH-SY5Y cells and human mast cells HMC-1 (560) were kindly provided by Dr. Jingxing Zhang (Tongji Hospital, Tongji University School of Medicine, Shanghai, China) and Prof. Furong Gao (Tongji University School of Medicine, Shanghai, China), and they were cultured in Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) mixed with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C in a humidified incubator (Thermo Fisher Scientific, Wilmington, MA, USA) supplied with 5% CO_2. To contribute to the two-cell co-culture system, 1 ml of 1.5×10^5 cells/ml of SH-SY5Y cells and 1 ml of 1.5×10^5 cells/ml of HMC-1 cells were co-cultured for 24 h directly or by using Transwell 12-well plates with 0.4 μm pore polyester membrane insert (Corning, NY, USA).

To contribute PD cell culture model *in vitro*, 1.5 ml of 1×10^5 cells/ml of SH-SY5Y cells were cultured in 12-well plates for approximately 24 h and were then respectively treated with 0.1% dimethyl sulfoxide (Sigma–Adrich, St. Louis, MO, USA) containing 1 μmol/L (μM) rotenone (Sigma–Adrich, St. Louis, MO, USA) for 24 h, 1 mmol/L (mM) 1-methyl-4-phenylpyridinium (MPP^+; Sigma–Adrich, St. Louis, MO, USA) for 24 h or 100 μmol/L (μM) 6-hydroxydopamine (6-OHDA; Sigma–Adrich, St. Louis, MO, USA) for 24 h (Feng et al., 2015; Zhang et al., 2017; Kim et al., 2018) After being digested and washed with phosphate buffer saline, SH-SY5Y cells were subjected to further treatment and analysis. Each experiment was repeated at least three times.

RNA Extraction and Quantitative Real Time-PCR

For the quantitation of AGTR1 and RBM3 gene expression, the total SH-SY5Y cell RNA was extracted using RNAiso Plus (9109, TaKaRa, Dalian, China) following the manufacturer's instructions. Quantitative real-time PCR was carried out using the AceQ Universal SYBR qPCR Master Mix (Q411, Vazyme, Biotech, Nanjing, China). Primer sequences are listed in **Supplementary Table 2**. Relative expression levels of genes were calculated by ΔΔCt method normalized to β-Actin compared with control samples.

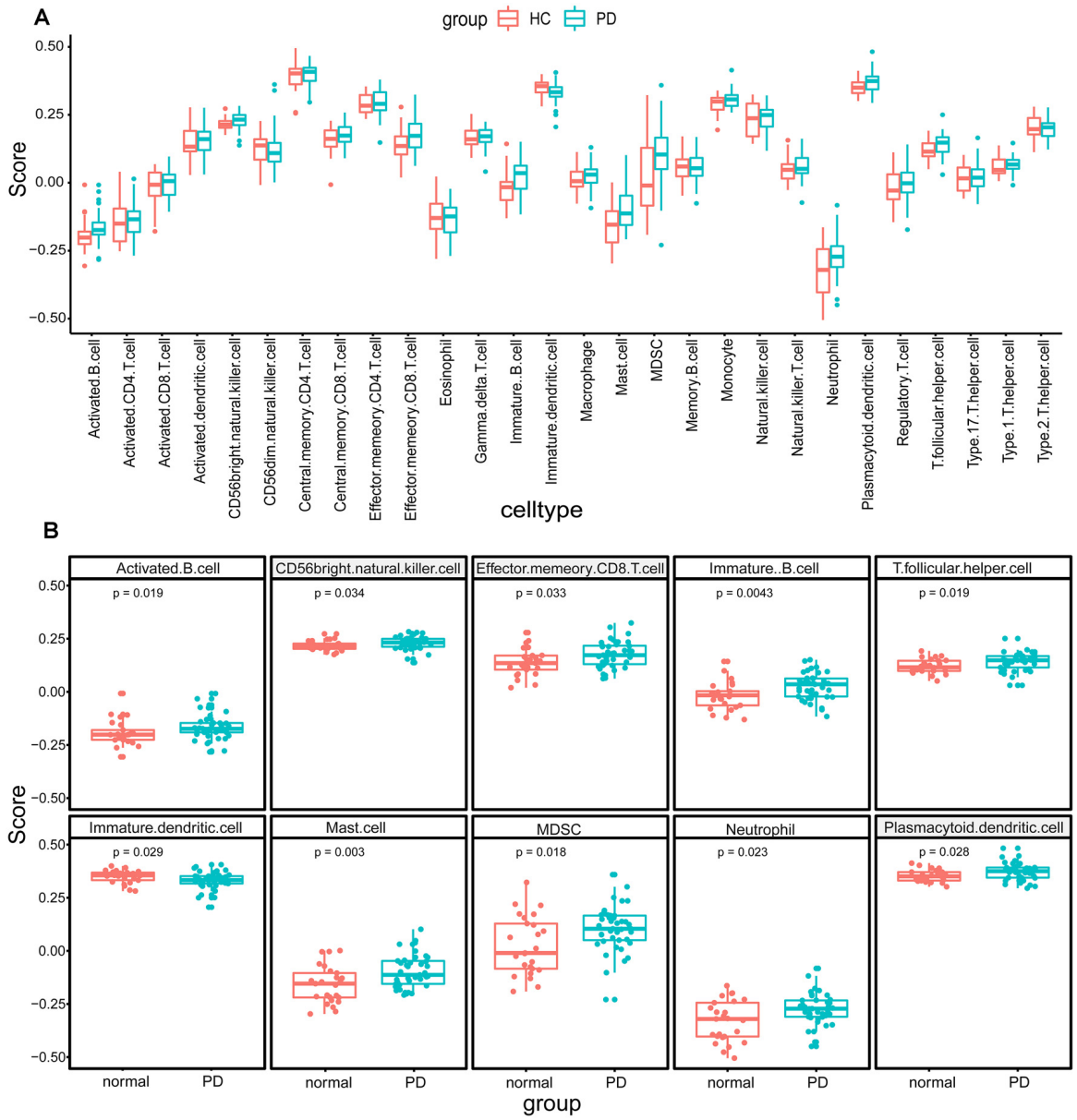

FIGURE 2 | The immune cell infiltration analysis of substantia nigra between Parkinson's disease patients (PDs) and healthy controls (HCs). **(A)** The landscape of immune cell infiltration based on expression data from the Gene Expression Omnibus (GEO) database. **(B)** The immune cell types with significant differences between PDs and HCs.

Western Blot Analysis

Cultured cells and brain tissues were lysed with RIPA lysis buffer (Beyotime, Wuhan, China) supplemented with protease inhibitor cocktail (Roche, Switzerland) and phosphatase inhibitor cocktail (Roche, Switzerland). BCA assay kits (Beyotime, Shanghai, China) were used to measure total protein concentrations. Then, 50–750 μg protein for each sample was separated on 12.5% SDS polyacrylamide gels with SDS running buffer [25 mM Tris (pH 8.3), 250 mM glycocoll, and 0.1% SDS]. The proteins were transferred onto 0.2 PVDF membranes (Merck Millipore, Darmstadt, Alemanha) with transfer buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycocoll, and 20% methyl alcohol]. After being blocked in 5% skim milk for an hour, the membrane bars were incubated with specific primary antibodies overnight at 4°C, and then the membrane bars were washed in 0.1% TBST three times and incubated with secondary antibodies for an hour at room temperature. Antibodies are listed in **Supplementary Table 3**. After being washed in 0.1% TBST three times, the protein signals were detected with an ECL Western Blotting Substrate kit (Thermo Fisher Scientific, Wilmington, MA, USA) on the ImageQuant LAS 4000mini system. ImageJ software was applied for quantitative analysis of band density.

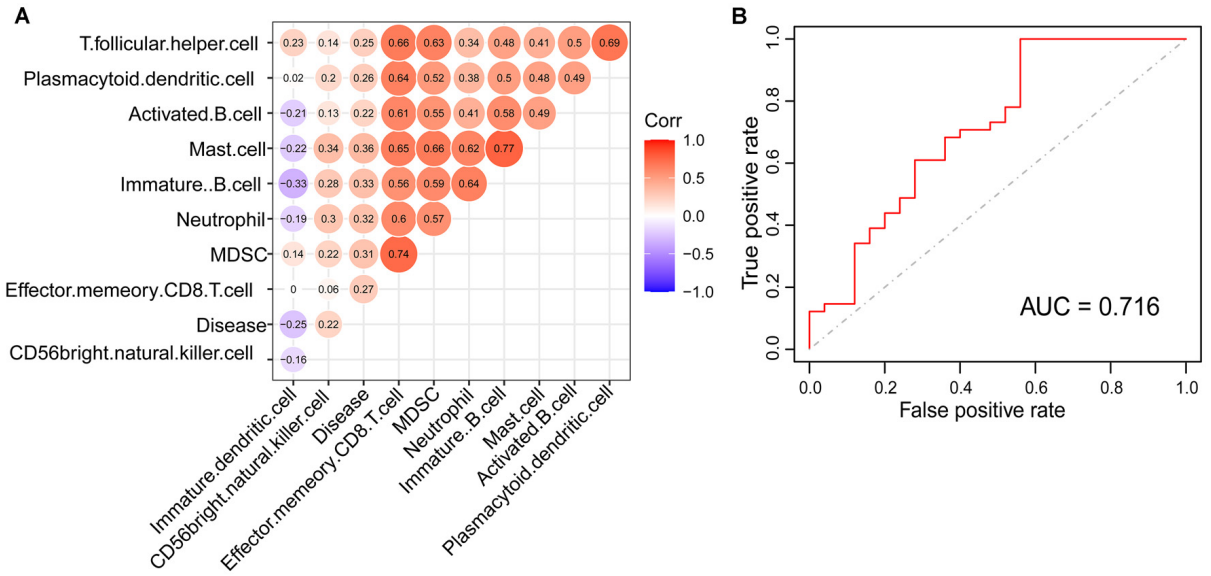

FIGURE 3 | Mast cell was identified to be tightly correlated with PD. **(A)** The correlation of disease state and immune cell types with significant differences between PDs and HCs. **(B)** The receiver operating characteristic (ROC) curve of logistic model for verifying the association between mast cell and the occurrence of PD.

Immunofluorescence Staining

Cultured cells on coverslips and brain slices were fixed with 4% paraformaldehyde in PBS for 15 min, washed with PBS three times, and then permeabilized in 0.1–0.3% Triton X-100 for 30–60 min. After blocking in 3% donkey serum for an hour, cells and tissues were incubated with primary antibodies overnight at 4°C. Then, cells and tissues were incubated with corresponding secondary antibodies for an hour at room temperature. Antibodies are listed in **Supplementary Table 3**. The nuclei were stained by DAPI (1:1,000; Roche, Switzerland) for 15 min at room temperature. Images were captured by fluorescence microscopy (OLYMPUS BX53).

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Each experiment was replicated at least three times. Data visualization and analysis were performed with GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed using either student's *t*-test or one-way ANOVA. Significant difference among groups was assessed as ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Immune Cell Infiltration Landscape of Substantia Nigra Tissue in PDs and HCs

The research flowchart is shown in **Figure 1**. Based on the profiling data of 25 HCs and 41 PDs, the immune cell infiltration landscape of substantia nigra was constructed through ssGSEA with 28 immune cell types identified (**Figure 2A**). Notably, the infiltrating levels of 10 kinds of immune cells were significantly different between PDs and HCs, containing activated B cell, CD56 bright natural killer cell, effector memory CD8 T cell, immature B cell, T follicular helper cell, immature dendritic cell, mast cell, myeloid-derived suppressor cell (MDSC), neutrophil and plasmacytoid dendritic cell (**Figure 2B**). Together, these results suggest that the immune infiltration patterns in SN tissue of PDs might be altered during the progression of the disease.

Mast Cell Could Be the Key Immunocyte Associated With the Occurrence of PD

Based on the immunoscore, we obtained immune cell types that differed between PDs and HCs. According to the correlation analysis of these immune cells and disease states, mast cells were most associated with the occurrence of PD (**Figure 3A**). The Logistic regression analysis based on these immune cell types revealed that mast cells were significantly correlated to the occurrence of PD, and the ROC curve of mast cells was drawn to assess the predictive accuracy with the area under the curve (AUC) = 0.716 (**Figure 3B**); and it was already reported that the number and activation of mast cells in PD brain slices had a higher level compared to non-PD control brain slices (Kempuraj et al., 2019). Then based on the WGCNA that was used to explore the relationships between gene expression modules and clinical features, we found that the modules significantly correlated to mast cells and disease states were the same (**Figure 4**). The purple module was positively correlated to a disease state (r = 0.26, p = 0.04) and mast cell (r = 0.59, p = 2e-07), and the turquoise module was observably negatively correlated to a disease state (r = 0.29, p = 0.02) and mast cells (r = 0.41, p = 7e-04). In aggregate, these results indicate that mast cells could be the key immunocyte and were most associated with the occurrence of PD.

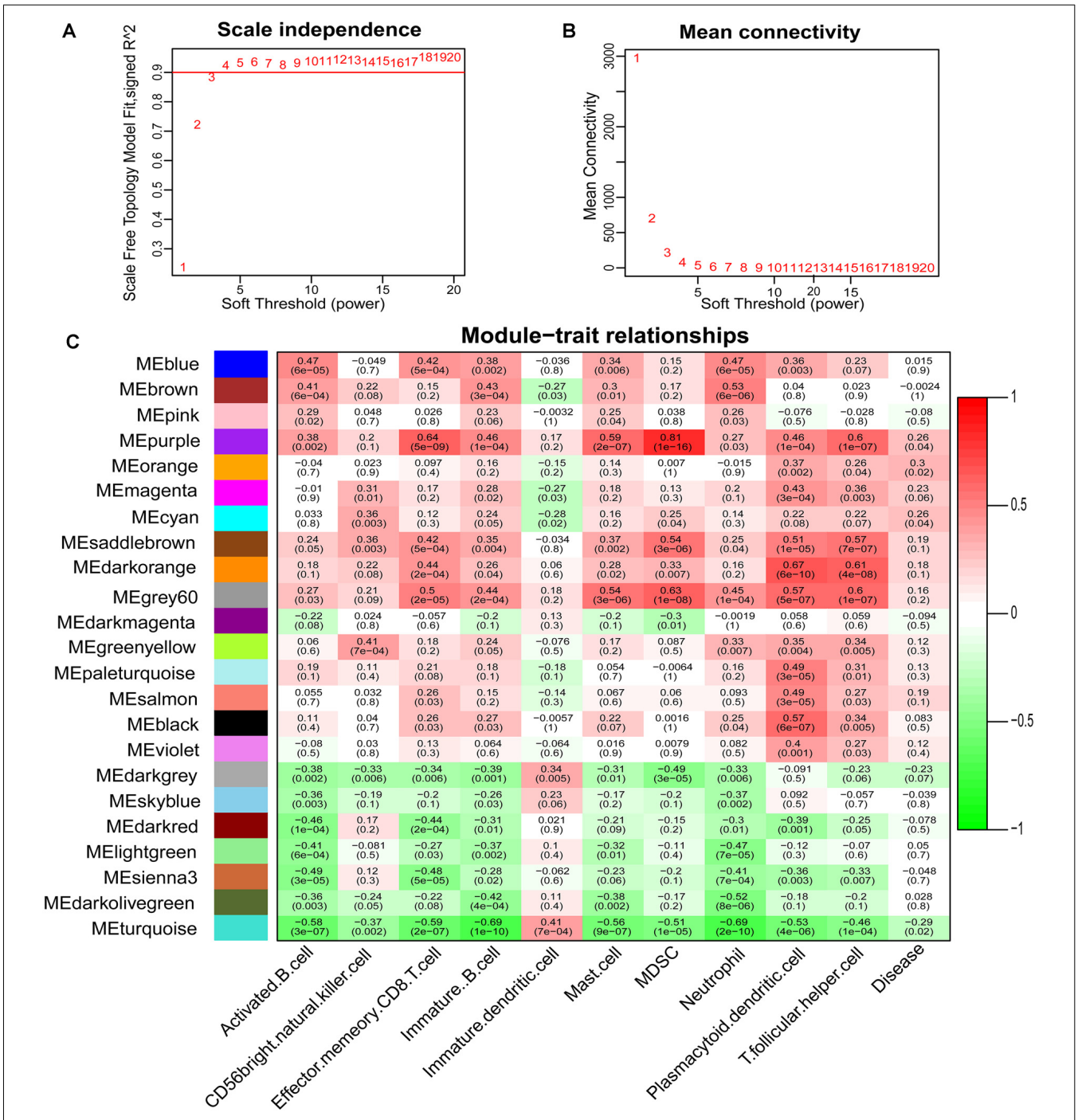

FIGURE 4 | Determination of soft-thresholding power in the weighted gene co-expression network analysis (WGCNA) and identification of modules. **(A)** Analysis of the scale-free fit index for various soft-thresholding powers. **(B)** Analysis of the mean connectivity for various soft-thresholding powers. **(C)** Heatmap of the correlation between module eigengenes and clinical traits of PD.

Multiple Algorithms Collectively Revealed That RBM3 and AGTR1 Were Associated With Mast Cell and PD

To explore signature genes associated with the immune infiltration and occurrence of PD, the genes' information of the above two modules were used to explore key genes through LASSO and RF analyses. Eight genes were identified as key genes based on LASSO analysis (**Table 2**, **Figure 5A**), and PDs and HCs could be distinguished according to the model constructed by these genes in the testing cohort (**Figures 5B,C**). We selected the

TABLE 2 | The key gene list of LASSO and RF analysis.

Analysis	Genes
LASSO	**RBM3**, **AGTR1**, PSPH, PAK6, MTMR9, KANK4, CALN1, TCERG1L
RF	**AGTR1**, CNTN6, PCSK1, KIAA1191, **RBM3**, RIT2, UNC13C, RIIAD1, KCNJ6, SLC35D3, CHPF2, NUPR1, SGSH, MAN1C1, RBM17, DCC, DNM1L, TPBG, CCDC117, LOC101928307, DLL1, HSPA6, UBE2N, ABCA5, ROBO2, KLHL1, SEMA3G, SRA1, PLCXD2, DLK1

Abbreviations: LASSO, least absolute shrinkage and selection operator analysis; RF, random forest analysis. These genes in LASSO analysis are equally important. These genes in RF analysis are sorted by the parameter of increase in node purity. The two genes highlighted in bold are the overlap of the results of RF and Lasso analysis.

top 30 candidate genes from the result of RF analysis (**Table 2**, **Figure 6A**), and the RF model based on these genes could also distinguish PDs from HCs in the testing cohort (**Figures 6B,C**). The intersection genes of the two results gained from LASSO and RF analyses, RBM3 and AGTR1, were regarded as key genes that were associated with mast cells and PD. Then the correlation analysis also indicated that RBM3 and AGTR1 were related to disease states and mast cells (**Figure 6D**). Collectively, these data revealed that RBM3 and AGTR1 were associated with mast cells and disease states.

Logistic Regression Model Identified That RBM3, AGTR1 Could be Signature Genes Related to the Occurrence of PD

To identify signature genes related to the occurrence of PD, a Logistic regression model was constructed based on the expression level of RBM3 and AGTR1 in the training cohort. A ROC curve was used to evaluate the efficacy of the model in the testing cohort, the internal cohort, and the external one. The AUC of the testing cohort was 0.883 (95% CI: 0.722–1.000; **Figure 7A**). Moreover, the AUC of the internal cohort was 0.897 (95% CI: 0.823–0.970; **Figure 7B**), and the AUC of the external cohort was 0.745 (95% CI: 0.553–0.937; **Figure 7C**). A confusion matrix was also used to evaluate the model in the internal cohort (**Figure 7D**, **Table 3**) and the external cohort (**Figure 7E**, **Table 3**). Together, these results indicate that RBM3 and AGTR1 could be signature genes of PD.

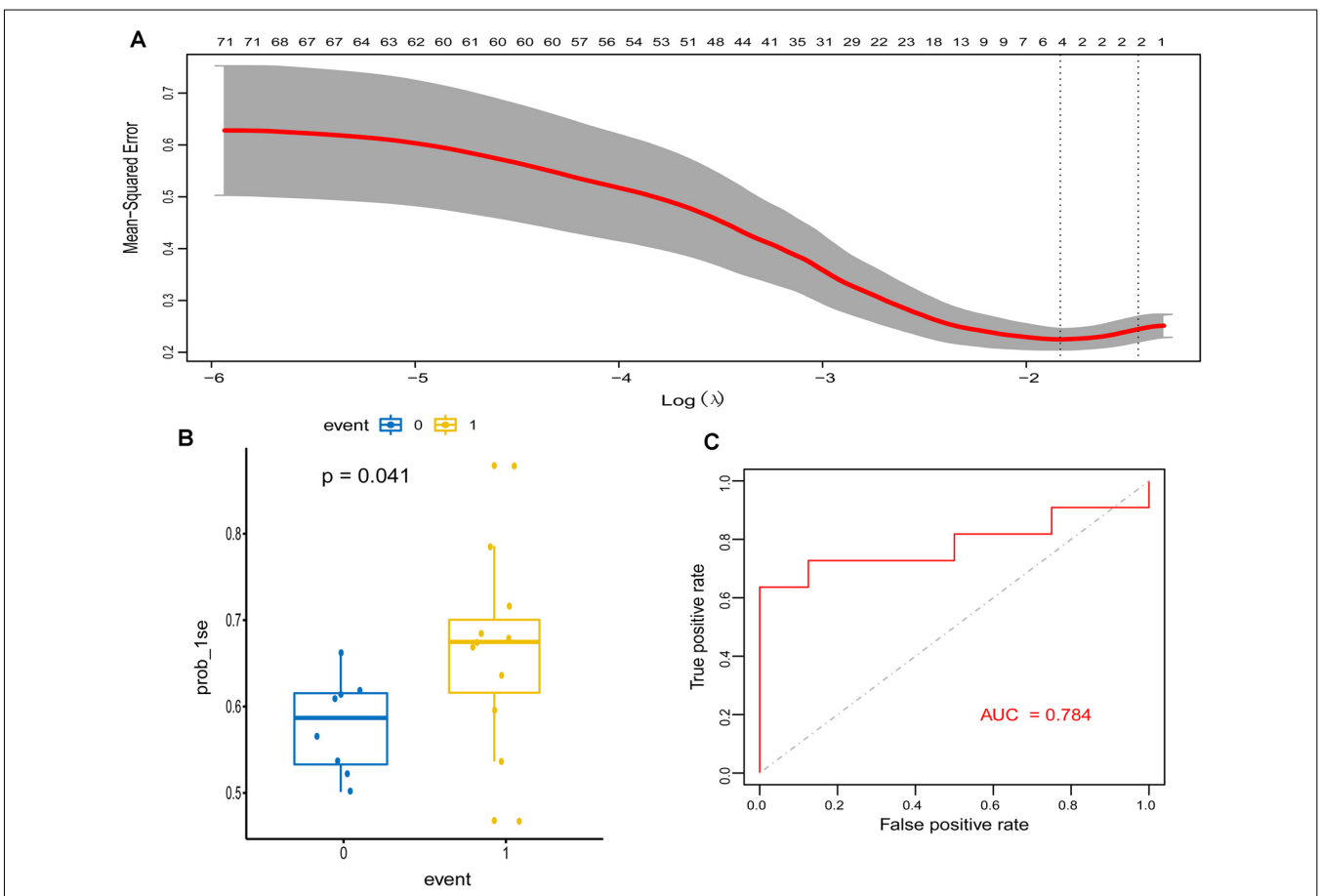

FIGURE 5 | Construction and validation of the least absolute shrinkage and selection operator (LASSO) model in PD patients. **(A)** The relationship between cross-validated mean square error and model size. Partial likelihood deviance is plotted against log (λ), where λ is the tuning parameter. Dotted vertical lines were drawn at the optimal values by minimum criteria and 1-s.e. criteria, and we selected 1-s.e. criteria to construct the model. **(B)** The distribution of the LASSO model in the testing cohort. **(C)** ROC curve of LASSO model for differentiating PDs from HCs of the testing cohort.

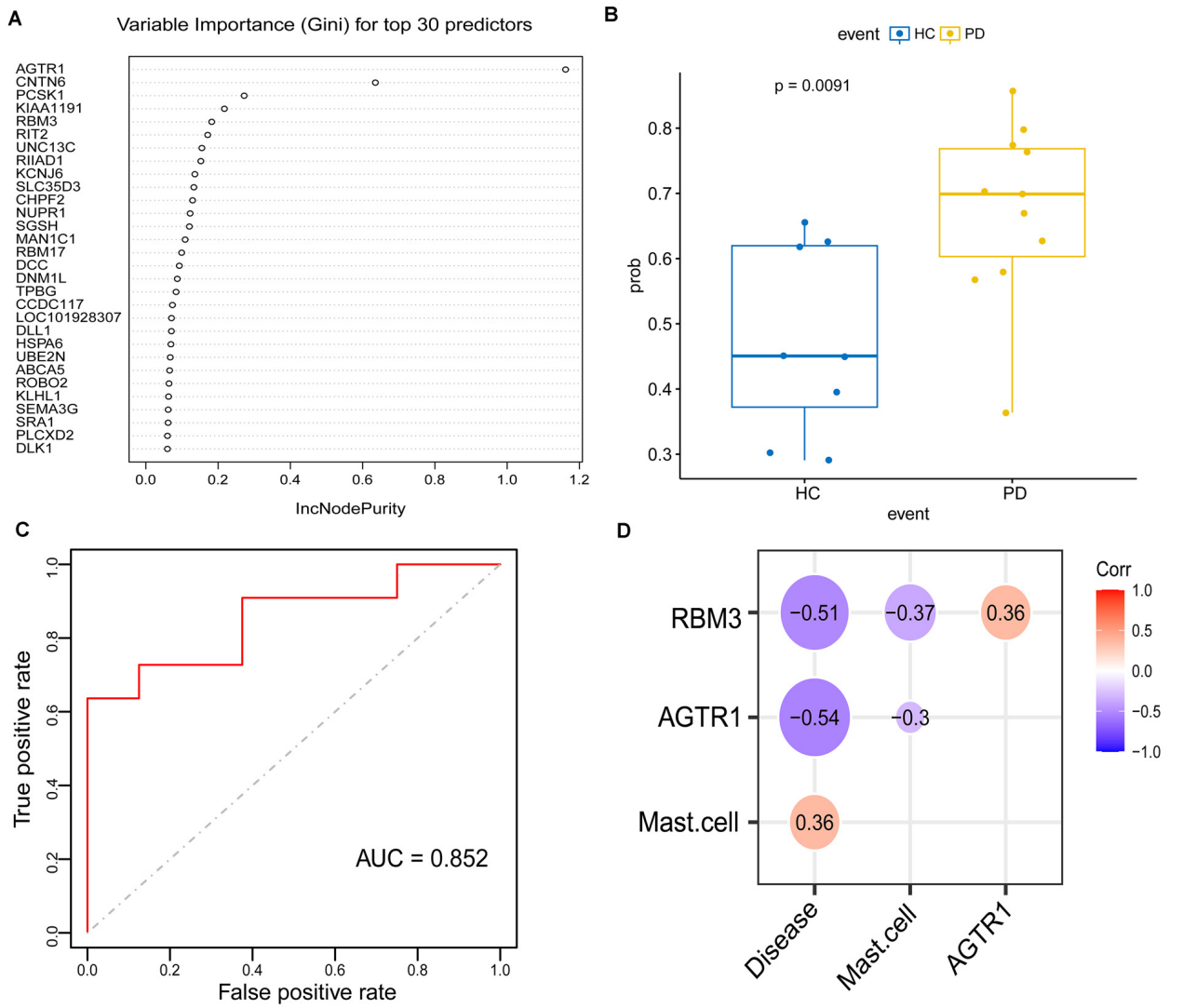

FIGURE 6 | Construction and validation of the random forest (RF) model in PDs. **(A)** Top 30 genes based on the parameter of increase in node purity in RF analysis. **(B)** The distribution of the RF model is based on these 30 genes in the testing cohort. **(C)** ROC curve of RF model for differentiating PDs from HCs of the testing cohort. **(D)** The correlation between the intersection gene of lasso and RF analysis (RBM3, AGTR1), mast cell, and disease state.

RBM3 and AGTR1 Were Differentially Expressed Genes Between PDs and HCs

To better verify the aforementioned results, we compared the expression levels of RBM3 and AGTR1 in all the array datasets, including 55 PDs and 39 HCs, and the expression levels of RBM3 and AGTR1 in PDs were significantly downregulated compared with HCs (**Figures 8A,B**). The relative expression levels of AGTR1 in next-generation sequencing data (**Figures 8C,E**) also had a significant difference between PDs and HCs, but the relative expression levels of RBM3 in PDs only shown the lower level without significant differences (**Figures 8D,F**). In a word, low expression of AGTR1 and RBM3 in PD patients was not only found in the microarray profiling cohort, but also in the next-generation sequencing cohort.

RBM3 and AGTR1 Were Also Differentially Expressed Genes in PD Models

Moreover, we constructed *in vitro* and *in vivo* PD models to verify the result. The expression levels of RBM3 and AGTR1 in PD cell models based on SH-SY5Y cell line and two PD-related neurotoxins, Rotenone and MPP $^{+}$, also showed a similar trend,

TABLE 3 | The model index of confusion matrix based on the internal cohort and the external cohort.

Index	Cohorts	
	The internal cohort	The external cohort
Accuracy	0.8182	0.7143
Precision	0.9143	0.7500
Sensitivity	0.7805	0.6429
Specificity	0.8800	0.7857

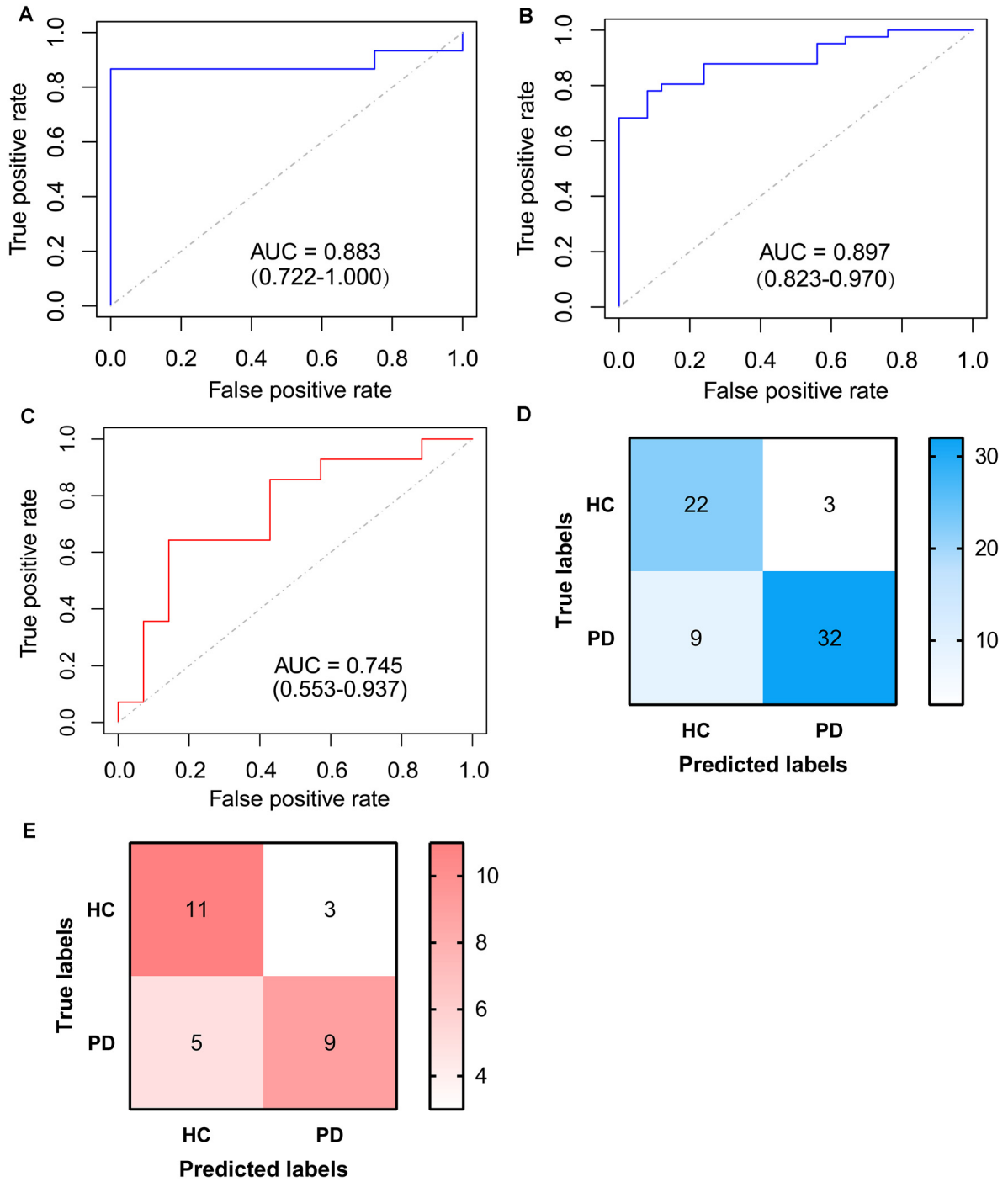

FIGURE 7 | ROC curve of logistic model based on RBM3 and AGTR1. **(A)** ROC curve of logistic model for differentiating PDs from HCs of the testing cohort. **(B)** ROC curve of logistic model for differentiating PDs from HCs of the internal cohort. **(C)** ROC curve of logistic model for differentiating PDs from HCs of the external cohort. **(D)** Confusion matrix based on the internal cohort. **(E)** Confusion matrix based on the external cohort.

but the trend of RBM3 and AGTR1 was unavailable in SH-SY5Y cell treated with 6-OHDA (**Figures 8G–I**, **9A–D**). Interestingly, compared with the two-cell co-culture system by Transwell, in the co-culture system that was based directly on the co-culture of SH-SY5Y and HMC-1, the expression levels of these two genes in SH-SY5Y and HMC-1 respectively showed more significant differences comparing with the control group (**Figures 8J–M**, **9A–F**). After successfully constructing and evaluating MPTP subacute models (**Figures 10A–E**), we analyzed the expression levels of AGTR1 and RBM3 in SN of MPTP mice, which were

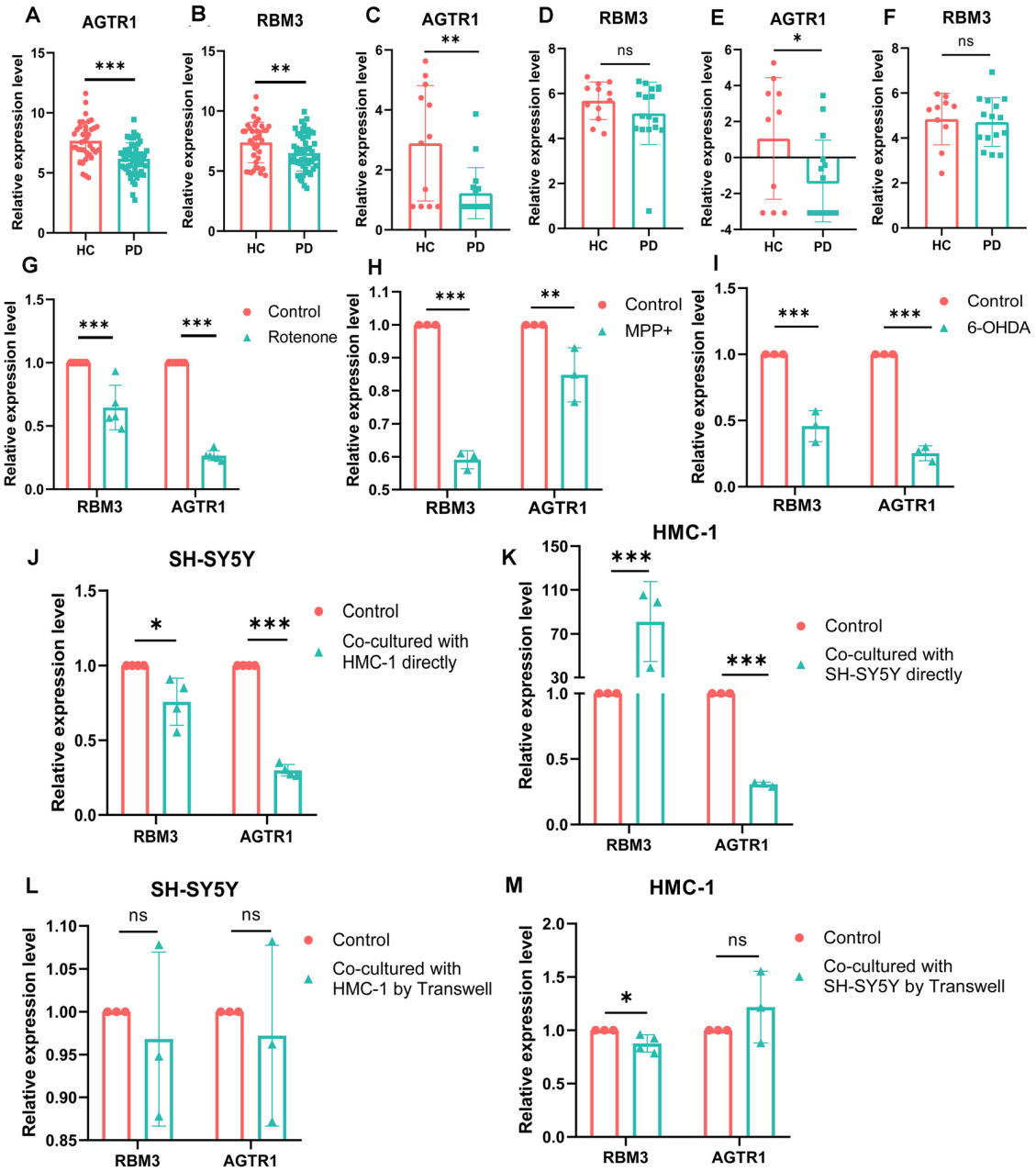

FIGURE 8 | The mRNA relative expression levels of RBM3 and AGTR1 in GEO datasets and PD cell culture model. **(A)** The expression levels of AGTR1 of PDs and HCs in a total of five array datasets. **(B)** The expression levels of RBM3 of PDs and HCs in a total of five array datasets. **(C)** The expression levels of AGTR1 of PDs and HCs in GSE114517. **(D)** The expression levels of RBM3 of PDs and HCs in GSE114517. **(E)** The expression levels of AGTR1 of PDs and HCs in GSE133101. **(F)** The expression levels of RBM3 of PDs and HCs in GSE133101. **(G)** The expression levels of RBM3 and AGTR1 in cell culture model *in vitro* constructed by SH-SY5Y cell using rotenone. **(H)** The expression levels of RBM3 and AGTR1 in cell culture model *in vitro* constructed by SH-SY5Y cell using MPP^+. **(I)** The expression levels of RBM3 and AGTR1 in cell culture model *in vitro* constructed by SH-SY5Y cell using 6-OHDA. **(J)** The expression levels of RBM3 and AGTR1 in SH-SY5Y cell co-cultured with HMC-1 cell directly. **(K)** The expression levels of RBM3 and AGTR1 in HMC-1 cell co-cultured with SH-SY5Y cell directly. **(L)** The expression levels of RBM3 and AGTR1 in SH-SY5Y cell co-cultured with HMC-1 cell by Transwell. **(M)** The expression levels of RBM3 and AGTR1 in HMC-1 cell co-cultured with SH-SY5Y cell by Transwell (ns $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control group).

significantly downregulated compared with the control group (**Figures 10F–H**, **11A,D**). According to co-localization staining, the expression levels of AGTR1 and RBM3 in dopaminergic neurons labeled by tyrosine hydroxylase (TH) of MPTP mice also showed the downregulated trend (**Figures 10I,J**), but the trend was unavailable in mast cells labeled by CD117, MAR-1, Tryptase, and Chymase simultaneously of MPTP mice (**Figures 11B,C,F**, **12A–H**). Furthermore, CD117/MAR-1,

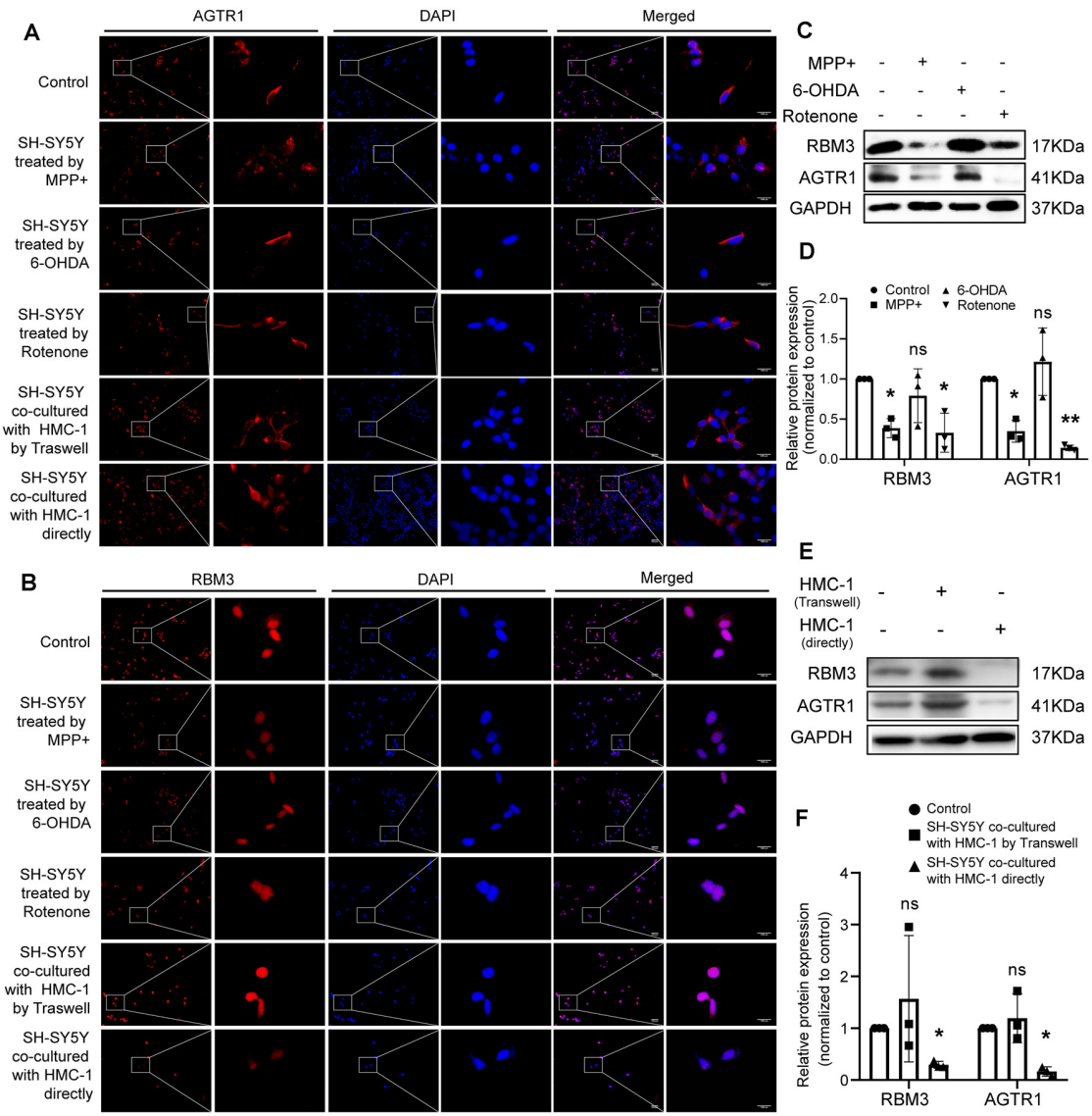

FIGURE 9 | The protein relative expression levels of RBM3 and AGTR1 in PD cell culture model. **(A)** AGTR1 expression level in each treated group was detected by fluorescence microscope after immunofluorescence staining. Scale bars: 100 μm; 1,000 μm. **(B)** RBM3 expression level in each treated group was detected by fluorescence microscope after immunofluorescence staining. **(C,D)** Western blot analyses of specific genes' expression in SH-SY5Y cells treated by MPP+, 6-OHDA, and Rotenone. GAPDH was used as an endogenous control. **(E,F)** Western blot analyses of specific genes' expression in SH-SY5Y cells co-cultured with HMC-1 by Transwell or directly. GAPDH was used as an endogenous control (ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, vs. Control group).

Tryptase, and Chymase staining also verified the increased infiltration of mast cells in MPTP mice (**Figures 11B,C,E, 12A–F**). Taken together, these results further confirmed that the levels of RBM3 and AGTR1 in neurocytes could be significantly downregulated in both *in vivo* and *in vitro* PD models, and the changes could be shown in neuronal cells directly contacted Should it be "connected" with mast cells.

DISCUSSION

The arrival of peripheral immune cells at the CNS may have a role in modulating these microglial functions such that subsequent stimuli produce exaggerated responses, and thus affect the outcome in CNS injury and disease (Prinz and Priller, 2017; Greenhalgh et al., 2020; Urban et al., 2020). All lines of evidence provided, it might be speculated that a key trigger to PD pathogenesis is the peripheral immune system that could affect the neuroinflammation of CNS to induce and promote the process of PD (Sim et al., 2020). In this study, using high throughput data and bioinformatic techniques, we provided more robust evidence for the potential roles of infiltrating immunocytes in PD as well as their key molecules, which might be helpful to further illustrate the correlations between the peripheral immune system and PD.

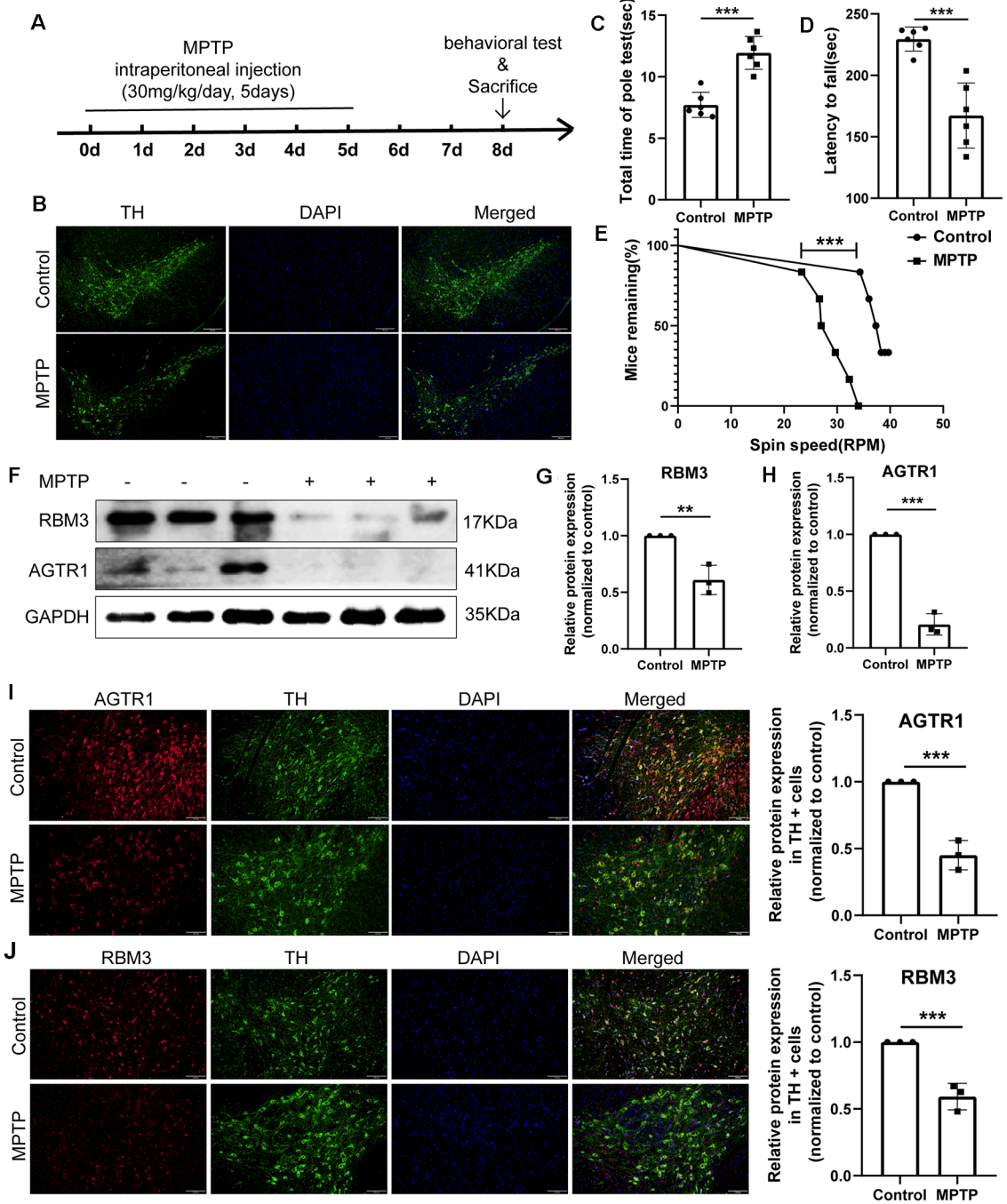

FIGURE 10 | The relative protein expression levels of RBM3 and AGTR1 in the PD animal model. **(A)** The flowchart of the construction of the MPTP subacute model, behavioral tests, and sacrifice. **(B)** Tyrosine hydroxylase (TH) staining of the substantia nigra (SN) of the above mice. Scale bars: 200 μm. **(C)** Pole tests were conducted by a blinded observer after MPTP treatment. **(D,E)** Rotarod tests were conducted by a blinded observer after MPTP treatment. **(F–H)** Western blot analyses of RBM3 and AGTR1 in SN of the above mice. **(I)** The co-localization of AGTR1 and TH in SN of the control group and MPTP group was detected by fluorescence microscope after immunofluorescence staining. Scale bars: 100 μm. **(J)** The co-localization of RBM3 and TH in SN of two groups was detected by fluorescence microscope (**$p < 0.01$, ***$p < 0.001$ vs. Control group).

It is generally accepted that T-cell infiltration participated in nigrostriatal dopaminergic neurodegeneration in models of PD (González and Pacheco, 2014). Based on our analysis of the immune infiltration, 10 kinds of immune cells in PDs were identified to be significantly different from those in HCs, which provided new insights into the infiltrating patterns of immunocytes in PD. Mast cell was the most eye-catching immunocyte that had wide correlations with other immune

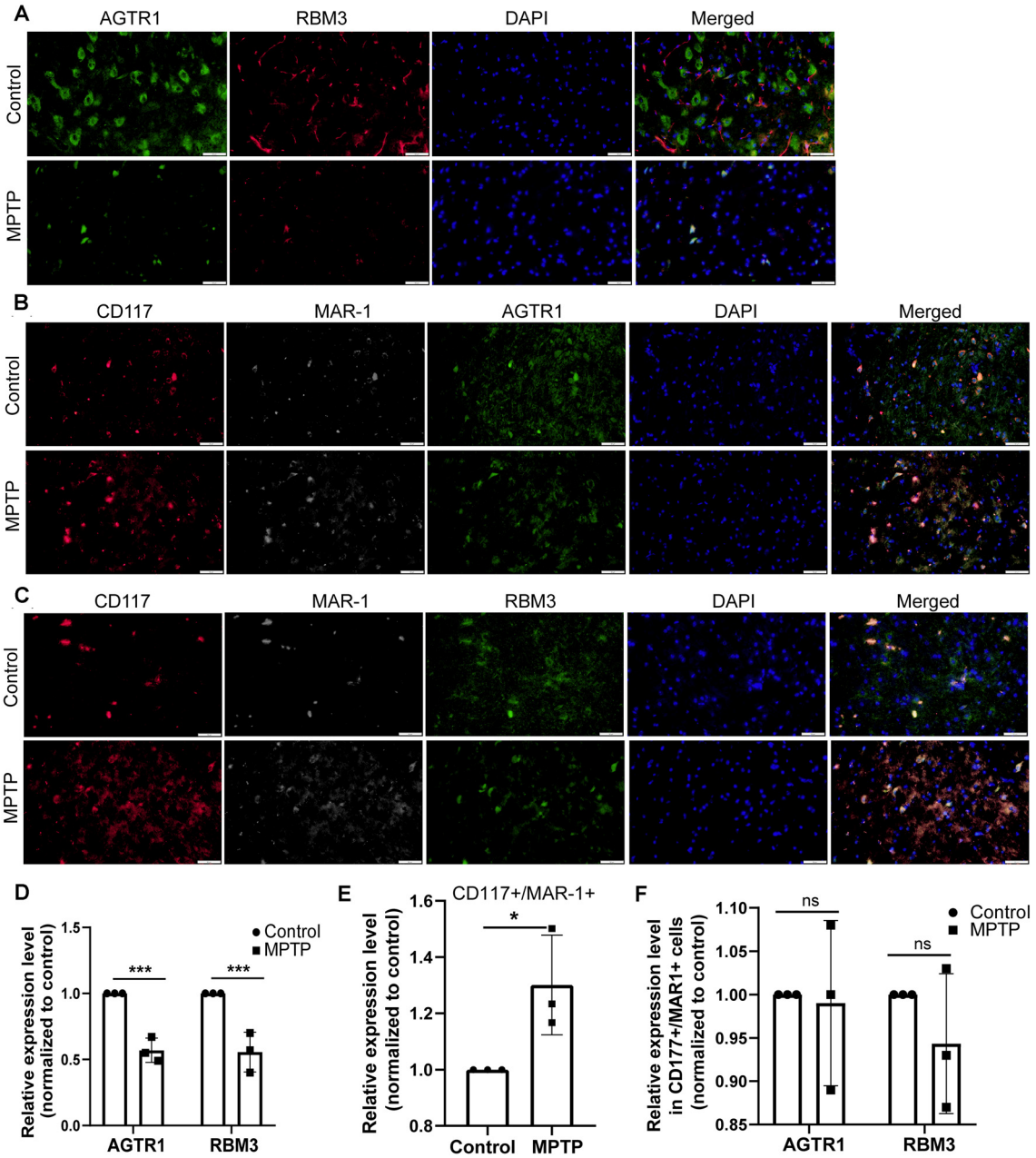

FIGURE 11 | The relative protein expression levels of RBM3 and AGTR1 in the PD animal model. **(A)** The co-localization of AGTR1 and RBM3 in SN of two groups was detected by fluorescence microscope. Scale bars: 50 μm. **(B)** The co-localization of AGTR1, MAR-1, and CD117 in SN of two groups was detected by fluorescence microscope. **(C)** The co-localization of RBM3, MAR-1, and CD117 in SN of two groups was detected by fluorescence microscope. **(D)** The relative expression levels of AGTR1 and RBM3 in SN of the two groups are based on the co-localization of AGTR1 and RBM3. **(E)** The relative co-expression level of CD117/MAR-1 in SN of two groups based on the co-localization of CD117 and MAR-1. **(F)** The relative expression levels of AGTR1 and RBM3 in SN of two groups separately based on the co-localization of AGTR1/CD117/MAR-1 and RBM3/CD117/ MAR-1 (ns $p > 0.05$, *$p < 0.05$, ***$p < 0.001$ vs. Control group).

cells and disease states. PD brains showed increased number and activation of mast cells compared with normal brains of non-PD control in both human tissues and animal models (Kempuraj et al., 2019). Our results proved the increased number and activation of mast cells in PD models through the staining of CD117, MAR-1, Chymase, and Tryptase. These proteases from mast cells could activate Protease-activated receptor-2 (PAR-2) that is expressed in neuronal and glial cells and is involved in the development and progression of PD and upregulate neuroinflammation (Liu et al., 2014; Kempuraj et al., 2019; Widera et al., 2019). Additionally, tryptase induces the recruitment and accumulation of mast cells at the site of inflammation through the activation of PAR-2 (Liu et al., 2016). The changes of mast cells also occur together with the

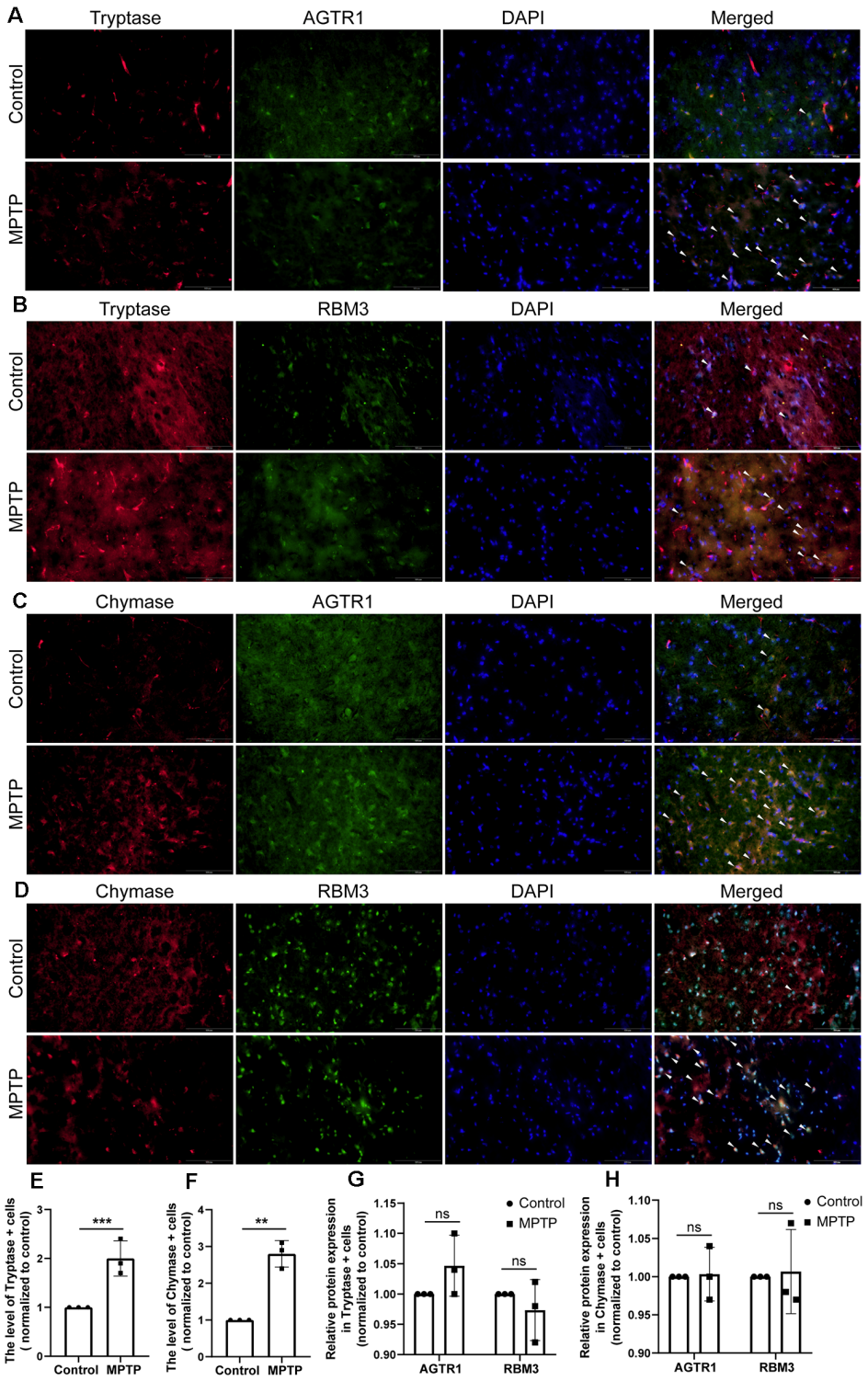

FIGURE 12 | The relative protein expression levels of RBM3 and AGTR1 in the PD animal model. **(A)** The co-localization of AGTR1 and Tryptase in SN of two groups was detected by fluorescence microscope. Scale bars: 100 μm. **(B)** The co-localization of RBM3 and Tryptase in SN of two groups was detected by fluorescence microscope. **(C)** The co-localization of AGTR1 and Chymase in SN of two groups was detected by fluorescence microscope. **(D)** The co-localization of RBM3 and Chymase in SN of two groups was detected by fluorescence microscope. **(E)** The relative expression levels of Tryptase in SN of two groups. **(F)** The relative expression levels of Chymase in SN of two groups. **(G)** The relative expression levels of AGTR1 and RBM3 in SN of two groups separately based on the co-localization of AGTR1/Tryptase and RBM3/ Tryptase. **(H)** The relative expression levels of AGTR1 and RBM3 in SN of two groups separately based on the co-localization of AGTR1/Chymase and RBM3/ Chymase (ns $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control group).

activation of microglial and astrocyte. Mast cells can selectively release proinflammatory cytokines/chemokines and neuroactive mediators in pathophysiological conditions (Skaper et al., 2014; Hendriksen et al., 2017; Kempuraj et al., 2018). These cytokines not only could active microglial and astrocytes through matched receptors, but also break down BBB and attract peripheral immune cells to increase inflammatory infiltration (Hendriksen et al., 2017). The glial cells can also interact with mast cells and accelerate neuroinflammation, and the crosstalk may act as a novel therapeutic target for PD.

RBM3 is a neuroprotective cold-shock protein and can improve neurological function. According to previous researches, knockdown of RBM3 can aggravate apoptosis inducted by rotenone and 1-methyl-4-phenylpyridinium (MPP+), while overexpression of RBM3 not only reduces this apoptosis, but also restores structural synaptic plasticity, and mediates neuroprotection against rotenone by inhibiting the MAPK signaling of p38, JNK, and ERK (Peretti et al., 2015; Yang et al., 2018, 2019). In a word, the relationship between RBM3 and PD is worthy of further investigation.

The angiotensin II type 1 receptor (AT1R) is coded by AGTR1 and is termed as the component of the renin-angiotensin system. Compared with the matched controls, radiolabeled AT1R recognition site levels were significantly decreased by approximately 70%, 70%, and 90% in the caudate nucleus, putamen, and SN (Ge and Barnes, 1996); and total cellular AT1R expression in SN DA neurons is reduced with disease progression (Zawada et al., 2015). However, AT1R upregulation can lead to the release of pro-inflammatory cytokines and subsequent inflammation to induce dopaminergic cell death and dysfunction, and its antagonist could reduce dopaminergic neuron degeneration introduced by a neurotoxin in SN (Grammatopoulos et al., 2007; Sathiya et al., 2013). The results of model-based studies seem to be contrary to the clinical phenomenon, but it also indicates that the role of AGTR1 in PD is complex and needs further exploration.

Our study had several limitations that should be acknowledged. First, the method combined with metagenes and ssGSEA could not accurately identify immune cell subtypes from bulk RNA-Seq data, since some of these metagenes are controversial to represent specific immune cell subpopulations. Although, we analyzed the expression of RBM3 and AGTR1 in dopaminergic neurons and mast cells in PD models using co-localization staining, the relationship between other cell types and these two genes remains unclear. It is necessary to use single-cell RNA-Seq or other methods for verifying our results and illustrating the roles of RBM3 and AGTR1 in different cell types of PD. Second, we did not illustrate the correlations between immune infiltration and clinical symptoms due to the lack of complete clinical information in the included datasets. Then, although we collected multiple datasets in the public database, the number of samples in the present study was not large enough, which required further real-world studies to verify the current results. Besides these, the current results also should be validated in human PD brain specimens. Although mast cell plays key roles in PD, the source of mast cell is unknown and need to be studied. Finally, although our findings were verified in *in vitro* and *in vivo* PD models and other published researches, functional experiments based on models that could better imitate the real state of PD, like induced pluripotent stem cells from PD patients and transgenic animal models are still necessary to reveal the roles of RBM3 and AGTR1 played in PD.

In conclusion, based on the gene expression profiling, the immune landscape of PDs was preliminarily constructed. This study revealed core immunocytes and molecules significantly associated with peripheral immune cell infiltration in SN of PD patients. Our findings will help enhance our cognition of immune cell infiltration heterogeneity and complexity, as well as provide promising targets for immunotherapy in patients with PD.

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 authors.

AUTHOR CONTRIBUTIONS

XZ, ZS, YL, LJ, and SL designed the study. XZ, SX, QL, and CL performed the data analysis. XZ, ZS, and SX wrote the manuscript. YL, LJ, and SL reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.605970/full#supplementary-material.

REFERENCES

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., et al. (2013). NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Res.* 41, D991–D995. doi: 10.1093/nar/gks1193

Calabrese, V., Santoro, A., Monti, D., Crupi, R., Di Paola, R., Latteri, S., et al. (2018). Aging and Parkinson's disease: inflammaging, neuroinflammation and biological remodeling as key factors in pathogenesis. *Free Radic. Biol. Med.* 115, 80–91. doi: 10.1016/j.freeradbiomed.2017.10.379

Cao, J.-J., Li, K.-S., and Shen, Y.-Q. (2011). Activated immune cells in Parkinson's disease. *J. Neuroimmune Pharmacol.* 6, 323–329. doi: 10.1007/s11481-011-9280-9

Charoentong, P., Finotello, F., Angelova, M., Mayer, C., Efremova, M., Rieder, D., et al. (2017). Pan-cancer immunogenomic analyses reveal genotype-immunophenotype relationships and predictors of response to checkpoint blockade. *Cell Rep.* 18, 248–262. doi: 10.1016/j.celrep.2016.12.019

Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E., and Pfeiffer, R. F. (2015). Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* 14, 625–639. doi: 10.1016/S1474-4422(15)00007-1

Feng, Y., Liu, T., Dong, S.-Y., Guo, Y.-J., Jankovic, J., Xu, H., et al. (2015). Rotenone affects p53 transcriptional activity and apoptosis *via* targeting SIRT1 and H3K9 acetylation in SH-SY5Y cells. *J. Neurochem.* 134, 668–676. doi: 10.1111/jnc.13172

Friedman, J., Hastie, T., and Tibshirani, R. (2010). Regularization paths for generalized linear models *via* coordinate descent. *J. Stat. Softw.* 33, 1–22. doi: 10.18637/jss.v033.i01

Gautier, L., Cope, L., Bolstad, B. M., and Irizarry, R. A. (2004). Affy—analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20, 307–315. doi: 10.1093/bioinformatics/btg405

Ge, J., and Barnes, N. M. (1996). Alterations in angiotensin AT1 and AT2 receptor subtype levels in brain regions from patients with neurodegenerative disorders. *Eur. J. Pharmacol.* 297, 299–306. doi: 10.1016/0014-2999(95)00762-8

González, H., and Pacheco, R. (2014). T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases. *J. Neuroinflammation* 11:201. doi: 10.1186/s12974-014-0201-8

Grammatopoulos, T. N., Jones, S. M., Ahmadi, F. A., Hoover, B. R., Snell, L. D., Skoch, J., et al. (2007). Angiotensin type 1 receptor antagonist losartan, reduces MPTP-induced degeneration of dopaminergic neurons in substantia nigra. *Mol. Neurodegener.* 2:1. doi: 10.1186/1750-1326-2-1

Greenhalgh, A. D., David, S., and Bennett, F. C. (2020). Immune cell regulation of glia during CNS injury and disease. *Nat. Rev. Neurosci.* 21, 139–152. doi: 10.1038/s41583-020-0263-9

He, J., Zhu, G., Wang, G., and Zhang, F. (2020). Oxidative stress and neuroinflammation potentiate each other to promote progression of dopamine neurodegeneration. *Oxid. Med. Cell. Longev.* 2020:6137521. doi: 10.1155/2020/6137521

Hendriksen, E., van Bergeijk, D., Oosting, R. S., and Redegeld, F. A. (2017). Mast cells in neuroinflammation and brain disorders. *Neurosci. Biobehav. Rev.* 79, 119–133. doi: 10.1016/j.neubiorev.2017.05.001

Jia, Q., Wu, W., Wang, Y., Alexander, P. B., Sun, C., Gong, Z., et al. (2018). Local mutational diversity drives intratumoral immune heterogeneity in non-small cell lung cancer. *Nat. Commun.* 9:5361. doi: 10.1038/s41467-018-07767-w

Kalia, L. V., and Lang, A. E. (2015). Parkinson's disease. *Lancet* 386, 896–912. doi: 10.1016/S0140-6736(14)61393-3

Kannarkat, G. T., Boss, J. M., and Tansey, M. G. (2013). The role of innate and adaptive immunity in Parkinson's disease. *J. Parkinsons Dis.* 3, 493–514. doi: 10.3233/JPD-130250

Kempuraj, D., Selvakumar, G. P., Zaheer, S., Thangavel, R., Ahmed, M. E., Raikwar, S., et al. (2018). Cross-talk between glia, neurons and mast cells in neuroinflammation associated with Parkinson's disease. *J. Neuroimmune Pharmacol.* 13, 100–112. doi: 10.1007/s11481-017-9766-1

Kempuraj, D., Thangavel, R., Selvakumar, G. P., Ahmed, M. E., Zaheer, S., Raikwar, S. P., et al. (2019). Mast cell proteases activate astrocytes and glia-neurons and release interleukin-33 by activating p38 and ERK1/2 MAPKs and NF-κB. *Mol. Neurobiol.* 56, 1681–1693. doi: 10.1007/s12035-018-1177-7

Kim, H.-Y., Jeon, H., Kim, H., Koo, S., and Kim, S. (2018). *Sophora flavescens* aiton decreases MPP^+-induced mitochondrial dysfunction in SH-SY5Y cells. *Front. Aging Neurosci.* 10:119. doi: 10.3389/fnagi.2018.00119

Kustrimovic, N., Marino, F., and Cosentino, M. (2019). Peripheral immunity, immunoaging and neuroinflammation in Parkinson's disease. *Curr. Med. Chem.* 26, 3719–3753. doi: 10.2174/0929867325666181009161048

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 9:559. doi: 10.1186/1471-2105-9-559

Lee, H., James, W. S., and Cowley, S. A. (2017). LRRK2 in peripheral and central nervous system innate immunity: its link to Parkinson's disease. *Biochem. Soc. Trans.* 45, 131–139. doi: 10.1042/BST20160262

Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E., and Storey, J. D. (2012). The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 28, 882–883. doi: 10.1093/bioinformatics/bts034

Liaw, A., and Wiener, M. (2002). Classification and regression by randomForest. *R News* 2, 18–22. Available online at: https://journal.r-project.org/archive/r-news.html

Liu, P., Sun, L., Zhao, X.-L., Zhang, P., Zhao, X.-M., and Zhang, J. (2014). PAR2-mediated epigenetic upregulation of α-synuclein contributes to the pathogenesis of Parkinson's disease. *Brain Res.* 1565, 82–89. doi: 10.1016/j.brainres.2014.04.014

Liu, X., Wang, J., Zhang, H., Zhan, M., Chen, H., Fang, Z., et al. (2016). Induction of mast cell accumulation by tryptase *via* a protease activated receptor-2 and ICAM-1 dependent mechanism. *Mediators Inflamm.* 2016:6431574. doi: 10.1155/2016/6431574

Pan, Y., and Nicolazzo, J. A. (2018). Impact of aging, Alzheimer's disease and Parkinson's disease on the blood-brain barrier transport of therapeutics. *Adv. Drug Deliv. Rev.* 135, 62–74. doi: 10.1016/j.addr.2018.04.009

Peretti, D., Bastide, A., Radford, H., Verity, N., Molloy, C., Martin, M. G., et al. (2015). RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. *Nature* 518, 236–239. doi: 10.1038/nature14142

Picca, A., Calvani, R., Coelho-Junior, H. J., Landi, F., Bernabei, R., and Marzetti, E. (2020). Mitochondrial dysfunction, oxidative stress, and neuroinflammation: intertwined roads to neurodegeneration. *Antioxidants* 9:647. doi: 10.3390/antiox9080647

Prinz, M., and Priller, J. (2017). The role of peripheral immune cells in the CNS in steady state and disease. *Nat. Neurosci.* 20, 136–144. doi: 10.1038/nn.4475

Sathiya, S., Ranju, V., Kalaivani, P., Priya, R. J., Sumathy, H., Sunil, A. G., et al. (2013). Telmisartan attenuates MPTP induced dopaminergic degeneration and motor dysfunction through regulation of α-synuclein and neurotrophic factors (BDNF and GDNF) expression in C57BL/6J mice. *Neuropharmacology* 73, 98–110. doi: 10.1016/j.neuropharm.2013.05.025

Sim, K.-Y., Im, K. C., and Park, S.-G. (2020). The functional roles and applications of immunoglobulins in neurodegenerative disease. *Int. J. Mol. Sci.* 21:5295. doi: 10.3390/ijms21155295

Sing, T., Sander, O., Beerenwinkel, N., and Lengauer, T. (2005). ROCR: visualizing classifier performance in R. *Bioinformatics* 21, 3940–3941. doi: 10.1093/bioinformatics/bti623

Skaper, S. D., Facci, L., and Giusti, P. (2014). Mast cells, glia and neuroinflammation: partners in crime? *Immunology* 141, 314–327. doi: 10.1111/imm.12170

Stephenson, J., Nutma, E., van der Valk, P., and Amor, S. (2018). Inflammation in CNS neurodegenerative diseases. *Immunology* 154, 204–219. doi: 10.1111/imm.12922

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U S A* 102, 15545–15550. doi: 10.1073/pnas.0506580102

Sweeney, M. D., Sagare, A. P., and Zlokovic, B. V. (2018). Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nat. Rev. Neurol.* 14, 133–150. doi: 10.1038/nrneurol.2017.188

Travagli, R. A., Browning, K. N., and Camilleri, M. (2020). Parkinson disease and the gut: new insights into pathogenesis and clinical relevance. *Nat. Rev. Gastroenterol. Hepatol.* 17, 673–685. doi: 10.1038/s41575-020-0339-z

Urban, S. L., Jensen, I. J., Shan, Q., Pewe, L. L., Xue, H.-H., Badovinac, V. P., et al. (2020). Peripherally induced brain tissue-resident memory $CD8^+$ T cells mediate protection against CNS infection. *Nat. Immunol.* 21, 938–949. doi: 10.1038/s41590-020-0711-8

Wang, Q., Liu, Y., and Zhou, J. (2015). Neuroinflammation in Parkinson's disease and its potential as therapeutic target. *Transl. Neurodegener.* 4:19. doi: 10.1186/s40035-015-0042-0

Widera, D., Martínez Aguilar, R., and Cottrell, G. S. (2019). Toll-like receptor 4 and protease-activated receptor 2 in physiology and pathophysiology of the nervous system: more than just receptor cooperation? *Neural Regen. Res.* 14, 1196–1201. doi: 10.4103/1673-5374.251290

Yang, H.-J., Shi, X., Ju, F., Hao, B.-N., Ma, S.-P., Wang, L., et al. (2018). Cold shock induced protein RBM3 but not mild hypothermia protects human SH-SY5Y neuroblastoma cells from MPP^+-induced neurotoxicity. *Front. Neurosci.* 12:298. doi: 10.3389/fnins.2018.00298

Yang, H.-J., Zhuang, R.-J., Li, Y.-B., Li, T., Yuan, X., Lei, B.-B., et al. (2019). Cold-inducible protein RBM3 mediates hypothermic neuroprotection against neurotoxin rotenone *via* inhibition on MAPK signalling. *J. Cell. Mol. Med.* 23, 7010–7020. doi: 10.1111/jcmm.14588

Zawada, W. M., Mrak, R. E., Biedermann, J., Palmer, Q. D., Gentleman, S. M., Aboud, O., et al. (2015). Loss of angiotensin II receptor expression in dopamine neurons in Parkinson's disease correlates with pathological progression and is accompanied by increases in Nox4- and 8-OH guanosine-related nucleic acid oxidation and caspase-3 activation. *Acta Neuropathol. Commun.* 3:9. doi: 10.1186/s40478-015-0189-z

Zhang, J., Cai, Q., Jiang, M., Liu, Y., Gu, H., Guo, J., et al. (2017). Mesencephalic astrocyte-derived neurotrophic factor alleviated 6-OHDA-induced cell damage *via* ROS-AMPK/mTOR mediated autophagic inhibition. *Exp. Gerontol.* 89, 45–56. doi: 10.1016/j.exger.2017.01.010

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Evidence for Peripheral Immune Activation in Parkinson's Disease

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Background: Accumulating evidence has revealed that peripheral immunity is involved in Parkinson's disease (PD). However, the results regarding the percentage of T-cell subsets are inconsistent, and the changes of immunoglobins levels have been seldom studied in PD patients.

Methods: Serum levels of the percentage of T-cell subsets and immunoglobulins were measured in 761 PD patients and 761 age- and gender-matched healthy controls. The correlations between the variables of peripheral immune activation (PIA) and the clinical characteristics of PD were analyzed using correlation analysis.

Results: The pooled results showed that PD patients had higher proportional levels of CD3+ T and CD4+ T lymphocytes than healthy controls. CD8+ T cell percentages were similar in PD patients and controls, and the CD4/CD8 ratio was significantly higher in the PD population. No significant differences in IgG, IgA, or IgM levels between these two groups were found. CD4+ T cell percentage was inversely correlated with the H&Y stage, and IgG level was positively correlated with disease duration and UPDRS part III. Subgroup analyses showed that these associations existed in female patients, but not in male patients.

Conclusion: The enhanced immune activation in the peripheral system is indicated in PD, and dynamic alterations in CD4+ T cell percentage and IgG level suggest an active role for peripheral immunity in the disease progression, especially in female PD patients.

Keywords: humoral immune, cellular immune, Parkinson's disease, disease process, peripheral immunity

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INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder, and the role of adaptive immune system and humoral immunity needs to be clarified in PD; profiling of peripheral T cells and immunoglobulin may be able to provide insight into biologic markers of disease. Adaptive immunity has become implicated as a pathogenic factor in the onset and progression of PD. The infiltration of immune cells in the central nervous system (CNS), particularly the substantia nigra (SN), was identified in both postmortem PD patients and animal models (Reynolds et al., 2007; Tufekci et al., 2011). These brain-invading immune cells consisted of a heterogeneous population of both CD8+ and CD4+ T cells. Studies had found a significant increase in the density of CD8+ and CD4+ T cells in the SN of PD patients compared with age-matched control

subjects (Brochard et al., 2009). Active T cells are detected not only in the CNS, but also in the peripheral blood (Rodrigues et al., 2014). Both clinical study and experimental model exhibit blood-brain barrier (BBB) dysfunction and T lymphocytes' recruitment to the CNS in PD. Besides direct function at the inflammatory site, the peripheral immune cells may get involved in the pathogenesis of PD systemically. Interestingly, CNS is reported to be actively interacts with the peripheral immune system (Aloisi, 2001; Wirenfeldt et al., 2011). Previous studies reported that PD patients with stage from I to III, or stage from I to V evaluated using the Hoehn and Yahr (H&Y) scale, displayed an altered population of peripheral T-lymphocyte than age-matched control subjects (Hisanaga et al., 2001; Baba et al., 2005). Studies have shown the involvement of peripheral adaptive immunity in PD (Kustrimovic et al., 2018; De Francesco et al., 2021), and the decrease of overall numbers of lymphocytes in PD patients (Bas et al., 2001; Calopa et al., 2010). Moreover, the peripheral immune change in PD patients was evidenced by decreased percentage of CD3+ T lymphocytes and CD4+ T lymphocytes in peripheral blood (Cen et al., 2017; Rocha et al., 2018; Sun et al., 2019). The reduced circulating CD4+ T lymphocytes in PD patients were shown to be caused by the reduction in Th2, Th17, and Treg (Kustrimovic et al., 2018), and the reduction of CD4+ T cells was worsened with increasing clinical severity (Stevens et al., 2012). A meta-analysis included 943 PD patients and found that the numbers of CD3+ and CD4+ T lymphocyte subsets were decreased in PD patients (Jiang et al., 2017). PD patients were also found to have a lower CD4+:CD8+ ratio than age-matched controls (Bas et al., 2001; Baba et al., 2005). However, another study did not find any difference in the percentage of CD4+ or CD8+ T lymphocytes and the CD4+:CD8+ ratio between PD patients and controls (Rocha et al., 2018). Furthermore, some studies found that PD patients showed higher levels of CD4+ or CD8+ T lymphocytes in peripheral blood (Garfias et al., 2019).Therefore, the results of the composition of T cells in the blood of PD patients are not consistent (Jiang et al., 2017); probably due to the relatively small sample size and ethnic variations. In addition, a Meta-analysis included 21 case-control studies, and the sample size of these studies varied from 20 to 82. Among these studies 16 were from the Asian population, and five were from the Caucasian population; a significant heterogeneity was observed in the analysis of T lymphocytes, and one reason of heterogeneity may be ascribed to ethnic variations (Jiang et al., 2017). The present study will focus on the changes in the percentage of T-cell subsets in PD patients relative to age/gender-matched healthy controls, trying to shed some light on whether adaptive immune responses are altered in PD.

Given the chronic inflammatory nature of PD, humoral immunity could play another role in disease progression. Lewy bodies (LB) shows strong immunolabelling for immunoglobulin G (IgG) and about one-third of DA neurons in SN show surface immunoreactivity for IgG (Sanchez-Guajardo et al., 2013), and the surface coating of SN DA neurons with IgG may target them for early degradation in the disease process (Orr et al., 2005). In the mouse model, the AAV-induced intra-nigral expression of human wild-type α-synuclein leads to significant IgG deposition, indicating that humoral immunity is involved in the degenerative process (Theodore et al., 2008). Humoral immunity and the secreted immunoglobulins can affect at sites far from the actual location of secretion, antibodies against α-synuclein have been found in sera and cerebrospinal fluid (CSF) of PD patients (Papachroni et al., 2007). Another study showed that sera from PD patients contained disease-specific auto-antibodies, because characteristic alterations of 10 auto-antibodies could distinguish PD from normal aging, Alzheimer's disease (AD), multiple sclerosis, and breast cancer with accuracies over 85%, and these 10 auto-antitbodies included PTCD2, HSH2D, Myotilin, Elongation factor 1-alpha 1, ICAM4, FRMD8, CTLA-4/Fc, PABPC3, Fibronectin 1, and TRIM21 (Han et al., 2012).

Yet another study found that the frequency of anti-synuclein antibodies in the peripheral blood serum of sporadic PD patients is not different from those in healthy controls (Papachroni et al., 2007). This is the first study that focusing on the change of serum immunoglobulins relative to age/gender-matched healthy controls, trying to provide more insight into the role of immunoglobulins in PD pathogenesis.

Since functional profiling of peripheral immune activities could provide important disease biomarkers, we conducted this study to investigate peripheral immune abnormalities in PD. The current study was designed: (1) to measure the percentage of T cell subset, and the levels of serum immunoglobulins IgG, IgM, IgA in PD patients and normal controls and (2) to test whether the peripheral immune activation (PIA) state of PD patients would correlate with disease characteristics.

MATERIALS AND METHODS

Patients and Healthy Individual

This study was performed within the Department of Neurology, West China Hospital of Sichuan University; a large, regional referral movement disorders clinic in Southwest China. A total of 761 PD patients who met the current consensus criteria for PD (Postuma et al., 2015) were recruited and followed from May 2006 to November 2018. A total of 761 Age- and gender-matched healthy controls were recruited from the medical examination center of West China Hospital; the control subjects did not have any neurological conditions or systemic diseases, such as hypertension and diabetes. All patients and control subjects gave their written informed consent, and the Ethical Committee of West China Hospital of Sichuan University approved this study.

Exclusion Criteria

Patients who showed evidence of systemic inflammation in clinical examination or biochemical blood tests (increased number of white blood cells, high concentration of C-reactive protein, or high erythrocyte sedimentation rate) were excluded. Patients with manifestations of monoclonal gammopathy, non-malignant endocrine abnormalities, neoplastic disorders, auto-antibodies (high-titer GM1 ganglioside antibody), and infection (HIV-1, hepatitis B and C, varicella-zoster, syphilis, borreliosis) were also

excluded. None of the patients had a history of using anti-inflammatory drugs, steroids, acetylsalicylic acid or statins during the past 2 months before enrollment. Any patient with a family history of PD or common chronic diseases was also excluded.

Collection and Processing of Blood Specimens

In accordance with study protocols, peripheral blood samples (∼9 ml each set) were obtained by venipuncture performed between 9:00 and 11: 00 am after fasting from midnight from each patient and healthy controls. Blood was collected into a sterile 7.5 ml tube with a clot activator and double gel for transport (BD Vacutainer, SST, REF 367987). All the experiments in this study were conducted in the Division of Clinical Immunology, West China Hospital, affiliated with Sichuan University. Flow cytometric assay for analysis of cell viability was performed. The lymphocyte subsets were identified and determined using the BD FACSCanto II Flow Cytometer [Becton Dickinson (BD) Biosciences, Heidelberg, Germany]. For each sample, at least 10,000 cells were analyzed and the percentage of the cells expressing CD3+, CD4+ and CD8+ markers were evaluated. The levels of immunoglobulins IgG, IgM, and IgA were quantified by means of nephelometry on an Image 800 nephelometer (Beckman Coulter, Fullerton, CA, United States). A portion of each collected sample was analyzed to detect infectious diseases. According to the manufacturer's instructions, all the tests were performed by board-certified laboratory technicians who were blinded to clinical data.

Clinical Evaluation

We collected clinical data regarding sex, age at onset, disease duration, diagnostic delay, personal history, chronic disease history, treatment regimen and motor complications through a standardized personal interview. The diagnostic delay is defined as the duration from the first appearance of motor symptoms to clinical diagnosis of PD. We made a detailed record of chronic disease history, such as hypertension, diabetes, and hyperlipemia. The use of antiparkinson drug was recorded at the time of enrollment and L-DOPA equivalent daily doses (LEDD) were evaluated according to the guidelines (Tomlinson et al., 2010). Patients who reported one or more of the following symptoms, including wearing-off, delayed-ON, no-ON, random ON-OFF, early morning/nocturnal akinesia, peak-dose or diphasic dyskinesias, were judged to have motor complications. The Unified PD Rating Scale (UPDRS) part III and H&Y stage were used to evaluate the motor severity (Hoehn and Yahr, 1967; Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease, 2003). The severity of non-motor symptoms (NMSs) was assessed with the Non-Motor Symptom Scale (NMSS) (Chaudhuri et al., 2007). The Chinese version of the NMSS includes nine domains and 30 items, and it is a valuable measure to assess the frequency and severity of NMS in Chinese PD patients (Wang et al., 2009). The quality of life (QoL) of PD patients was evaluated with the Chinese version of the PD Questionnaire 39 (PDQ-39) (Jenkinson et al., 1997).

Statistical Analysis

The distribution of the detected variables of PIA, including percentage of T lymphocyte subset and levels of IgG, IgM, and IgA were tested for normality using skewness and kurtosis, Shapiro–Wilk and Shapiro–Francia tests. The Student's *T*-test was applied to analyze the difference in the detected variables of PIA for comparisons between PD patients and controls. Chi-square tests compared sex ratios between PD and control groups. Spearman's rho correlation was used to calculate the correlations between the detected PIA variables and the H&Y stage, while Pearson's correlation was applied to evaluate the correlations with UPDRS part III, NMSS, and PDQ-39. Multivariate general linear models (GLMs) were used to investigate the associations with clinical variables, with CD4+ T lymphocyte percentage or IgG level as an independent variable. Clinical variables included in the GLMs were as follows: age at assessment, sex, disease duration, UPDRS part III, and LEDD. All data was presented in the form of mean ± standard deviation, and they were analyzed using SPSS 17.0. A p-value of < 0.05 was considered statistically significant.

RESULTS

The demographic features of included PD patients and controls were listed in **Table 1**. In the 761 PD patients, the sex ratio (male/female) was 395/366; the mean age at examination was 61.77 ± 11.19 years, which were not significantly different from controls. The results of the detected variables of PIA and the demographic data in PD patients were summarized in **Table 1**. Analysis of the T cell subset showed that PD patients had significantly higher proportional levels of CD3+ T and CD4+ T lymphocytes than healthy controls. CD8+ T cell percentages were similar in PD patients and controls. Consequently, the CD4/CD8 ratio was significantly higher in the PD population (**Table 1**). The T cell subset was evaluated separately according to gender. In both PD patients and controls, increased percentages

TABLE 1 | Comparison of peripheral immune variables in PD patients and matched controls.

	PD patients n = 761	Controls n = 761	*p-value*
Age-of-onset	61.77 ± 11.19	61.67 ± 11.16	0.8670
Male/female	395/366	395/366	1
UPDRS part III	33.29 ± 15.12		
H&Y stage	2.44 ± 0.75		
NMSS	46.22 ± 36.00		
LEDD	376.52 ± 315.53		
PDQ-39	40.00 ± 27.80		
CD3%	63.36 ± 10.90	59.75 ± 12.53	**< 0.0001**
CD4%	36.50 ± 8.95	32.17 ± 8.83	**< 0.0001**
CD8%	22.57± 8.41	22.77± 8.93	0.6562
CD4/CD8	1.95± 1.75	1.64± 0.82	**< 0.0001**
IgG	12.25± 3.09	12.51± 3.60	0.0962
IgM	1227± 1305	1173± 745.9	0.2713
IgA	2212± 1065	2309± 1316	0.0798

Bold values mean that the difference is statistically significant.

of CD3+ T and CD4+ T lymphocyte populations were found in females (**Table 2**). The differences in age at assessment, disease duration, UPDRS part III, H&Y stage, NMSS, PDQ-39, and LEDD between female PD patients and male PD patients were evaluated, and the results showed that the differences in these variables were not significant (**Table 2**). We further examined the correlations between T cell subset and clinical data, including disease duration, UPDRS part III, H&Y stage, NMSS, LEDD,

TABLE 2 | Comparison of peripheral immune variables according to gender of PD patients and matched controls.

Variables	PD patients			Controls		
	Male	Female	*p-value*	Male	Female	*p-value*
CD3%	62.28 ± 11.16	64.68 ± 10.36	**0.0022**	58.02 ± 12.96	61.63 ± 11.79	**<0.0001**
CD4%	35.42 ± 9.18	37.71 ± 8.49	**0.0004**	30.89 ± 9.27	33.56 ± 8.10	**<0.0001**
CD8%	22.50 ± 8.57	22.67 ± 8.64	0.7903	22.63 ± 9.26	22.91 ± 8.56	0.6681
CD4/CD8	1.96 ± 2.26	1.93 ± 0.95	0.8364	1.61 ± 0.86	1.68 ± 0.77	0.2316
IgG	12.21 ± 3.42	12.31 ± 2.61	0.6411	12.29 ± 3.93	12.80 ± 3.10	**0.0299**
IgM	1155 ± 1638	1317 ± 685.4	0.0587	1079 ± 746.5	1292 ± 728.6	**<0.0001**
IgA	2218 ± 1163	2205 ± 928.1	0.8494	2167 ± 2355	2220 ± 2518	0.2111

Bold values mean that the difference is statistically significant.

TABLE 3 | Clinical characteristics of PD patients and the associations with T cell subsets and immunoglobulins levels.

Variables	CD3%		CD4%		CD8%		CD4/CD8		IgG		IgM		IgA	
	r	*p*-value	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Disease duration	−0.073	0.175	−0.033	0.542	−0.029	0.586	0.050	0.360	**0.036**	**0.011**	−0.068	0.206	−0.001	0.990
UPDRS part III	−0.063	0.240	0.097	0.074	0.047	0.385	−0.019	0.719	**0.117**	**0.035**	−0.085	0.116	−0.035	0.514
H&Y stage	−0.099	0.067	**−0.121**	**0.024**	0.033	0.539	0.068	0.207	0.065	0.226	−0.079	0.146	−0.018	0.743
NMSS	−0.021	0.703	−0.037	0.496	0.074	0.170	−0.059	0.273	0.078	0.147	−0.063	0.215	0.063	0.242
LEDD	−0.064	0.236	−0.060	0.762	−0.016	0.762	0.020	0.715	−0.004	0.941	−0.099	0.067	−0.046	0.392
PDQ-39 total	−0.046	0.406	−0.037	0.509	0.019	0.738	−0.014	0.806	0.057	0.308	−0.92	0.095	−0.090	0.095

Bold values mean that the difference is statistically significant.

TABLE 4 | Clinical characteristics of female PD patients and the associations with T cell subsets and immunoglobulins levels.

Variables	CD3%		CD4%		CD8%		CD4/CD8		IgG		IgM		IgA	
	r	*p*-value	r	*p*-value	r	*p*-value	*r*	*p*-value	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Disease duration	−0.108	0.178	−0.104	0.192	0.010	0.896	0.020	0.801	**0.173**	**0.029**	−0.082	0.303	−0.028	0.726
UPDRS part III	−0.087	0.278	**−0.165**	**0.038**	0.073	0.361	−0.067	0.406	0.113	0.158	0.054	0.500	−0.016	0.843
H&Y stage	−0.139	0.082	**−0.201**	**0.011**	0.039	0.627	−0.106	0.183	0.039	0.630	−0.123	0.124	0.037	0.644
NMSS	−0.064	0.426	−0.093	0.246	0.043	−0.074	0.354	0.273	0.019	0.808	−0.143	0.072	0.072	0.371
LEDD	−0.144	0.072	**−0.172**	**0.031**	0.019	0.809	−0.075	0.347	0.017	0.827	−0.085	0.287	−0.084	0.294
PDQ-39 total	0.075	0.351	**−0.194**	**0.017**	−0.046	0.575	−0.022	0.789	0.086	0.293	−0.153	0.061	−0.067	0.402

Bold values mean that the difference is statistically significant.

TABLE 5 | Clinical characteristics of male PD patients and the associations with T cell subsets and immunoglobulins levels.

Variables	CD3%		CD4%		CD8%		CD4/CD8		IgG		IgM		IgA	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Disease duration	0.052	0.482	0.019	0.797	−0.069	0.349	0.077	0.299	0.105	0.153	−0.066	0.369	0.026	0.726
UPDRS part III	−0.030	0.680	−0.038	0.604	0.031	0.675	0.017	0.819	0.117	0.113	−0.045	0.093	−0.045	0.540
H&Y stage	−0.063	0.394	0.049	0.507	0.023	0.753	−0.029	0.692	0.092	0.210	−0.043	0.562	−0.065	0.381
NMSS	0.006	0.940	−0.001	0.992	0.100	0.177	−0.047	0.524	0.137	0.061	−0.006	0.936	0.046	0.529
LEDD	0.003	0.969	0.024	0.744	−0.046	0.530	0.098	0.185	−0.023	0.752	−0.111	0.131	−0.005	0.946
PDQ-39 total	0.056	0.459	0.066	0.378	0.069	0.359	−0.009	0.904	0.037	0.626	−0.065	0.392	0.089	0.239

and PDQ-39. The results showed that CD4+ T cell percentage in PD patients was inversely correlated with the H&Y stage (p = 0.024) (**Table 3**). PD patients in a more advanced stage had a lower CD4+ T cell percentage. However, we did not find a significant association between CD4+ T cell percentage and sex, disease duration, UPDRS part III, and LEDD in GLMs. Still, a relationship between CD4+ T cell percentage and age at assessment was identified (p = 0.002). In the subgroup analyses of patients with different gender, CD4+ T cell percentage was found to be inversely correlated with UPDRS part III, H&Y stage, LEDD, and PDQ-39 in female patients, but not in male patients (**Tables 4**, **5**). Female patients with a lower CD4+ T cell percentage were more likely to experience more severe motor symptoms and worse quality of life. In addition, the subgroup analyses using GLMs also showed that CD4+ T cell percentage was correlated to age at assessment in female patients (p = 0.003).

The levels of immunoglobulins IgG, IgM, and IgA were determined by routine laboratory testing. The results showed no significant differences in IgG, IgM, and IgA level between PD patients and controls (**Table 1**). The subgroup analysis by gender revealed that the levels of immunoglobulins IgG, IgM, and IgA were not significantly different between male and female PD patients (**Table 2**). Furthermore, IgG level was positively correlated with disease duration and UPDRS part III (p = 0.011 and 0.031, respectively) (**Table 3**). Higher IgG level was associated with longer disease duration and more severe motor symptoms. Furthermore, the results of GLMs showed that IgG level was significant correlated with disease duration (p = 0.011). In subgroup analyses, a positive relationship between IgG level and disease duration was found in female PD patients (**Tables 4**, **5**), and GLMs also found a positive association between IgG level and disease duration in female PD patients (p = 0.049).

DISCUSSION

Recent studies have indicated that the activity of the immune system in the CNS of PD patients, however; few studies to date have explored the status of peripheral immune response. To our knowledge, the current study included the largest sample in determining whether peripheral immune abnormalities are involved in PD by evaluating the T cell subsets and determining the humoral immunity in PD patients and healthy subjects.

In this study, we found that compared to healthy controls, PD patients had an increase in the percentage of CD3+ and CD4+ T lymphocyte, no change in CD8+ T cell subpopulation, and consequently an increase in the CD4+/CD8+ ratio. Considering the T lymphocyte subsets, CD3+ T cells represent the total lymphocytes, CD4+ T cells represent the helper T cells and are associated with anti-inflammation, but CD8+ T cells represent the cytotoxic T cells and are related with pro-inflammation, and a declining of CD4+/CD8+ ratio indicates an impaired immune system (Luz Correa et al., 2014). Our findings suggested the enhanced immune activation in the peripheral system. Furthermore, persistently activated peripheral immune system were also observed in amyotrophic lateral sclerosis (ALS), multiple system atrophy (MSA), and AD (Shalit et al., 1995; Chen et al., 2014; Chen et al., 2016; Kempuraj et al., 2016; Cao et al., 2020). However, previous studies reported a reduction in the number of CD4+ cells in PD patients (Bas et al., 2001; Baba et al., 2005; Niwa et al., 2012), and a meta-analysis also showed the decreased numbers of CD3+ and CD4+ lymphocyte subsets in PD patients (Jiang et al., 2017). The present study was focusing the percentage of the T cell subset, which could not represent the numbers of T cells. A study identified a significant decrease in the number of CD4+ lymphocytes in PD patients, but did not find any significant difference in the percentage of CD4+ cells between PD patients and healthy controls (Kustrimovic et al., 2016). Previous studies found an ongoing loss of CD4+ T cells as the disease progresses in PD patients (Stevens et al., 2012), and PD patients with cognitive impairment had a higher number of circulating lymphocytes than patients with normal cognitive function (Magistrelli et al., 2020), and the heterogeneity of the PD patients may account for the discrepancies. In the present study, we found an increase in the percentage of CD4+ T lymphocyte in PD patients. Similarly, previous studies have found a slightly increased proportion of CD4+ T cells in AD patients (Shalit et al., 1995; Richartz-Salzburger et al., 2007). Systemic CD4+ T cells must be recruited to the CNS to modify potentially destructive neuro-inflammation, and the increase of CD4+ T cells proportion may represent the compensatory mechanism. Furthermore, dopaminergic antiparkinson drugs may have had a role in regulating the populations of lymphocytes in PD. An *in vivo* study found that levodopa treatment could inhibit the number of peripheral IFN-γ-producing T cells (Carr et al., 2003). In addition, a recent study found that the percentage of CD3+ T cells was higher, but the percentage of CD4+ T cells was lower in PD patients treated with antiparkinson drugs than drug naïve (Kustrimovic et al., 2016). However, another study reported that PD patients with or without levodopa treatment had similarly decreased lymphocyte counts (Bas et al., 2001). In the present study, we only found an association between the percentage of CD4+ T cells and LEDD; a higher dose of antiparkinson drugs was related to lower percentage of CD4+ T cells. Therefore, the influence of antiparkinsonian treatment on peripheral immunity should be assessed in future study.

According to the theory of Braak's staging in PD, the pathogenesis is indicated to develop from the peripheral system to the CNS (Braak et al., 1996), and peripheral immune activation has been suggested to play a role in the etiology of PD. The infiltration of T cells in the brain has been reported in both postmortem PD patients and animal models, and these brain-invading lymphocytes are composed of a heterogeneous population of both CD4+ and CD8+ cells (Farkas et al., 2000; Brochard et al., 2009; Tufekci et al., 2011). Furthermore, the brain parenchyma migration of peripheral leukocytes is found to be tightly regulated at the level of the BBB (Alvarez et al., 2011), and breakdown of the BBB was indicated in multiple pathways of neurodegeneration, including AD, PD, and MSA (Sweeney et al., 2018). In the present study, CD4+ T cell percentage was found to be inversely correlated with the H&Y stage. Previous study have found that the numbers of CD4+ T cells were negatively correlated with UPDRS and H&Y stage (Stevens et al., 2012). As the disease progressed to an advanced stage, the CD4+ T cell

subpopulation tends to be decreased; this reduction may reflect the attempt of the CNS to recruit CD4+ T lymphocytes from the periphery to modulate the brain immunological reaction. The infiltration of CD4+ T lymphocytes was indicated to be related to a decrease in the phagocytic activity of microglia (Sommer et al., 2016), and the specific involvement of the CD4+ T lymphocytes was demonstrated by the dopaminergic neuroprotection observed when: deleting CD4 cells in the MPTP mouse (Brochard et al., 2009) and the MHC-II global knockout mouse (Harms et al., 2013). However, we had to notice that the association between CD4+ T cell percentage and H&Y stage was relatively weak, and the results of GLMs did not support the findings. In addition, the inverse association between CD4+ T cell percentage and UPDRS part III, H&Y stage, and PDQ-39 was only identified in female PD patients. Increasing evidence points to gender differences as an important determinant of the susceptibility to develop PD and the clinical and therapeutic management of PD (Picillo et al., 2017; Cerri et al., 2019). Sex hormones, specifical estrogen, were shown to influence PD pathogenesis and play a vital role in PD differences between men and women (Jurado-Coronel et al., 2018). Estrogen can CD4+ T lymphocytes development and function, particularly their ability to produce selected cytokine profiles (Pernis, 2007).

In the present study, we only analyzed the percentage of T-cell subsets, but we had to notice that the transcription factor (TF) gene expression profile and the phenotypes of CD4+ T cells also represented the peripheral immune abnormalities in PD. For the TF gene expression, studies have shown that PD patients had higher levels of STAT6, GATA3, and FOXP3, and lower levels of TBX21, STAT3, STAT4, RORC, and NR4A2 than control subjects. In addition, patients with inidiopathic REM sleep behavior disorder (iRBD) were also had a similar TF gene expression with PD patients (De Francesco et al., 2021). Since iRBD was considered to be the strongest risk factor for prodromal PD, the altered TF gene expression in iRBD suggested early involvement of peripheral immunity in PD. Furthermore, PD patients with or without motor complications had different TF gene expression in CD4+ T cells; indicating the associations with disease progression (Contaldi et al., 2020). There are two phenotypes of CD4+ T cells, the proinflammatory phenotypes (Th1 and Th17), and the anti-inflammatory phenotypes (Th2 and the Treg). PD patients had a functional Th1-biased immune response in the peripheral immune system (Kustrimovic et al., 2018), and PD patients who had higher Th1 and a dysregulation of Treg tend to be more vulnerable to develop cognitive dementia (Magistrelli et al., 2020). Study also showed the associations of dopaminergic Receptors on Naïve CD4+ T cells and memory lymphocytes to motor impairment in PD patients (Kustrimovic et al., 2016). Combined with our findings, these studies' results indicate that peripheral adaptive immunity is involved in PD.

In the present study, our study indicated that the serum levels of IgG, IgA, and IgM in PD patients did not differ from those in control subjects. Similarly, our previous study also found that IgG and IgM concentrations were not significantly different between ALS patients and healthy controls (Chen et al., 2014). We conducted a pilot study that showed no differences in serum immunoglobulin levels between ALS and PD patients (data not shown). Therefore, PD seems to share similar features with other neurodegenerative diseases with regard to peripheral immunity activation. However, a role for humoral immunity has also been proposed in PD. Immunoglobulins are found to colocalize with pigmented DA neurons, and LB in PD brains shows strong immunolabelling for IgG (Orr et al., 2005). A previous study injected immunoglobulins from PD patients into the SN of rats, and found that these immunoglobulins induced perivascular inflammation, microgliosis, and loss of TH+ neurons in the SN (Chen et al., 1998). Furthermore, DA neurons' surface coating with IgG could target them for degradation early in PD progress (Orr et al., 2005). Researchers used AAV to induce intraneural expression of human wild type α-synuclein, and found a significant IgG deposition, indicating that humoral immunity and the BBB breakdown are involved in the pathogenesis of PD (Cao et al., 2010). Supporting this, we also found that IgG level was positively correlated with disease duration and UPDRS part III; as the disease progressed the increase of IgG may occur spontaneously with the deposition of aggregated α-synuclein and the DA neurons loss. Studies have shown that all humans have a great number of serum IgG auto-antibodies, but this number can be influenced by three factors: age, gender, and disease (Nagele et al., 2013). In the present study, we found a positive relationship between IgG level and disease duration in female patients, but further research is required to understand the influence of gender in systemic humoral immune responses in PD.

The principal role of neuroinflammation is indicated in the progression of PD, and the presence of activated microglia and the elevated cytokine levels in CNS are associated with neuroinflammation. Also, overstimulated immune system due to the gut dysbiosis and the enhanced intestinal permeability can persuade systemic inflammation in PD (Dogra et al., 2021). Therefore, the bidirectional communication between neuroinflammation and the peripheral immunity is involved in the pathobiology of PD (Kustrimovic et al., 2019).

Several limitations of this study need to be addressed. First, CD4+ T lymphocytes may be proinflammatory (Th 1 and Th17), or anti-inflammatory (Th2 and Treg). However, in the present study, we did not evaluate the imbalance of CD4+ T cell subsets. Second, a peculiar TF gene expression profile can reflect the imbalance of CD4+ T cells in PD. Therefore, it would be more convincing to examine the expression of peripheral TFs, including TBX21, STAT1, STAT3, STAT4, STAT6, RORC, GATA3, FOXP3, NR4A2, MEF2s, Nurr1, Pitx3, and En1/2. Third, we did not evaluate antiparkinsonian treatment's effect on the periphery immunity in the present study.

CONCLUSION

This study validated the enhanced immune activation in the peripheral system of PD. In addition, dynamic alterations in CD4+ T cell percentage and IgG level indicated an active role for peripheral immunity in the disease progression and neuronal health. A better understanding of the peripheral immune response is likely to have therapeutic implications; further prospective studies are required to confirm our findings.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XC contributed the major role in the acquisition of data, analyzed the data, and drafted the manuscript for intellectual content. WF, RO, JL, JY, JF, BC, YC, and QW contributed to data collection. HS interpreted the data and revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

REFERENCES

Aloisi, F. (2001). Immune function of microglia. *Glia* 36, 165–179. doi: 10.1002/glia.1106

Alvarez, J. I., Dodelet-Devillers, A., Kebir, H., Ifergan, I., Fabre, P. J., Terouz, S., et al. (2011). The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science* 334, 1727–1731. doi: 10.1126/science.1206936

Baba, Y., Kuroiwa, A., Uitti, R. J., Wszolek, Z. K., and Yamada, T. (2005). Alterations of T-lymphocyte populations in Parkinson disease. *Park. Relat. Disord.* 11, 493–498. doi: 10.1016/j.parkreldis.2005.07.005

Bas, J., Calopa, M., Mestre, M., Molleví, D. G., Cutillas, B., Ambrosio, S., et al. (2001). Lymphocyte populations in Parkinson's disease and in rat models of parkinsonism. *J. Neuroimmunol.* 113, 146–152. doi: 10.1016/s0165-5728(00)00422-7

Braak, H., Braak, E., Yilmazer, D., de Vos, R. A., Jansen, E. N., and Bohl, J. (1996). Pattern of brain destruction in Parkinson's and Alzheimer's diseases. *J. Neural. Transm.* 103, 455–490.

Brochard, V., Combadière, B., Prigent, A., Laouar, Y., Perrin, A., Beray-Berthat, V., et al. (2009). Infiltration of CD4+ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease. *J. Clin. Invest.* 119, 182–192.

Calopa, M., Bas, J., Callen, A., and Mestre, M. (2010). Apoptosis of peripheral blood lymphocytes in Parkinson patients. *Neurobiol. Dis.* 38, 1–7. doi: 10.1016/j.nbd.2009.12.017

Cao, B., Chen, X., Zhang, L., Wei, Q., Liu, H., Feng, W., et al. (2020). Elevated Percentage of CD3(+) T-Cells and CD4(+)/CD8(+) Ratios in Multiple System Atrophy Patients. *Front. Neurol.* 11:658. doi: 10.3389/fneur.2020.00658

Cao, S., Theodore, S., and Standaert, D. G. (2010). Fcgamma receptors are required for NF-kappaB signaling, microglial activation and dopaminergic neurodegeneration in an AAV-synuclein mouse model of Parkinson's disease. *Mol. Neurodegener.* 5:42. doi: 10.1186/1750-1326-5-42

Carr, L., Tucker, A., and Fernandez-Botran, R. (2003). In vivo administration of L-dopa or dopamine decreases the number of splenic IFN gamma-producing cells. *J. Neuroimmunol.* 137, 87–93. doi: 10.1016/s0165-5728(03)00047-x

Cen, L., Yang, C., Huang, S., Zhou, M., Tang, X., Li, K., et al. (2017). Peripheral Lymphocyte Subsets as a Marker of Parkinson's Disease in a Chinese Population. *Neurosci. Bull.* 33, 493–500. doi: 10.1007/s12264-017-0163-9

Cerri, S., Mus, L., and Blandini, F. (2019). Parkinson's Disease in Women and Men: What's the Difference? *J. Park. Dis.* 9, 501–515.

Chaudhuri, K. R., Martinez-Martin, P., Brown, R. G., Sethi, K., Stocchi, F., Odin, P., et al. (2007). The metric properties of a novel non-motor symptoms scale for Parkinson's disease: results from an international pilot study. *Mov. Disord.* 22, 1901–1911.

Chen, S., Le, W. D., Xie, W. J., Alexianu, M. E., Engelhardt, J. I., Siklós, L., et al. (1998). Experimental destruction of substantia nigra initiated by Parkinson disease immunoglobulins. *Arch. Neurol.* 55, 1075–1080. doi: 10.1001/archneur.55.8.1075

Chen, W. W., Zhang, X., and Huang, W. J. (2016). Role of neuroinflammation in neurodegenerative diseases (Review). *Mol. Med. Rep.* 13, 3391–3396. doi: 10.3892/mmr.2016.4948

Chen, X., Feng, W., Huang, R., Guo, X., Chen, Y., Zheng, Z., et al. (2014). Evidence for peripheral immune activation in amyotrophic lateral sclerosis. *J. Neurol. Sci.* 347, 90–95. doi: 10.1080/17482960600989343

Contaldi, E., Magistrelli, L., Milner, A. V., Cosentino, M., Marino, F., and Comi, C. (2020). Expression of Transcription Factors in CD4+T Cells as Potential Biomarkers of Motor Complications in Parkinson's Disease. *J. Park. Dis.* doi: 10.3389/fnins.2018.00612 [Epub online ahead of print].

De Francesco, E., Terzaghi, M., Storelli, E., Magistrelli, L., Comi, C., Legnaro, M., et al. (2021). CD4+ T-cell Transcription Factors in Idiopathic REM Sleep Behavior Disorder and Parkinson's Disease. *Mov. Disord.* 36, 225–229. doi: 10.1002/mds.28137

Dogra, N., Mani, R. J., and Katare, D. P. (2021). The Gut-Brain Axis: Two Ways Signaling in Parkinson's Disease. *Cell. Mol. Neurobiol.* 2021, 1066–1067.

Farkas, E., De Jong, G. I., de Vos, R. A., Jansen Steur, E. N., and Luiten, P. G. (2000). Pathological features of cerebral cortical capillaries are doubled in Alzheimer's disease and Parkinson's disease. *Acta Neuropathol.* 100, 395–402. doi: 10.1007/s004010000195

Garfias, S., Tamaya Domínguez, B., Toledo Rojas, A., Arroyo, M., Rodríguez, U., Boll, C., et al. (2019). Peripheral blood lymphocyte phenotypes in Alzheimer and Parkinson's diseases. *Neurologia* S0213-4853, 30013–30011.

Han, M., Nagele, E., DeMarshall, C., Acharya, N., and Nagele, R. (2012). Diagnosis of Parkinson's disease based on disease-specific autoantibody profiles in human sera. *PLoS One* 7:e32383. doi: 10.1371/journal.pone.0032383

Harms, A. S., Cao, S., Rowse, A. L., Thome, A. D., Li, X., Mangieri, L. R., et al. (2013). MHCII is required for alpha-synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration. *J. Neurosci.* 33, 9592–9600. doi: 10.1523/jneurosci.5610-12.2013

Hisanaga, K., Asagi, M., Itoyama, Y., and Iwasaki, Y. (2001). Increase in peripheral CD4 bright+ CD8 dull+ T cells in Parkinson disease. *Arch. Neurol.* 58, 1580–1583. doi: 10.1001/archneur.58.10.1580

Hoehn, M. M., and Yahr, M. D. (1967). Parkinsonism: onset, progression and mortality. *Neurology* 17, 427–442. doi: 10.1212/wnl.17.5.427

Jenkinson, C., Fitzpatrick, R., Peto, V., Greenhall, R., and Hyman, N. (1997). The Parkinson's Disease Questionnaire (PDQ-39): development and validation of a Parkinson's disease summary index score. *Age Ageing* 26, 353–357. doi: 10.1093/ageing/26.5.353

Jiang, S., Gao, H., Luo, Q., Wang, P., and Yang, X. (2017). The correlation of lymphocyte subsets, natural killer cell, and Parkinson's disease: a meta-analysis. *Neurol. Sci.* 38, 1373–1380. doi: 10.1007/s10072-017-2988-4

Jurado-Coronel, J. C., Cabezas, R., Ávila Rodríguez, M. F., Echeverria, V., García-Segura, L. M., and Barreto, G. E. (2018). Sex differences in Parkinson's disease: features on clinical symptoms, treatment outcome, sexual hormones and genetics. *Front. Neuroendocrinol.* 50, 18–30. doi: 10.1016/j.yfrne.2017.09.002 doi: 10.1016/j.yfrne.2017.09.002

Kempuraj, D., Thangavel, R., Natteru, P. A., Selvakumar, G. P., Saeed, D., Zaheer, S., et al. (2016). Neuroinflammation Induces Neurodegeneration. *J. Neurol. Neurosurg. Spine.* 1:1003.

Kustrimovic, N., Comi, C., Magistrelli, L., Rasini, E., Legnaro, M., Bombelli, R., et al. (2018). Parkinson's disease patients have a complex phenotypic and functional Th1 bias: cross-sectional studies of CD4+ Th1/Th2/T17 and Treg in drug-naïve and drug-treated patients. *J. Neuroinflammation* 15:205.

Kustrimovic, N., Marino, F., and Cosentino, M. (2019). Peripheral Immunity, Immunoaging and Neuroinflammation in Parkinson's Disease. *Curr. Med. Chem.* 26, 3719–3753. doi: 10.2174/0929867325666181009161048

Kustrimovic, N., Rasini, E., Legnaro, M., Bombelli, R., Aleksic, I., Blandini, F., et al. (2016). Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease. *Sci. Rep.* 6:33738.

Luz Correa, B., Ornaghi, A. P., Cerutti Muller, G., Engroff, P., Pestana Lopes, R., da, S. F. I., et al. (2014). The inverted CD4:CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults. *Neuroimmunomodulation* 21, 206–212. doi: 10.1159/000356827

Magistrelli, L., Storelli, E., Rasini, E., Contaldi, E., Comi, C., Cosentino, M., et al. (2020). Relationship between circulating CD4+ T lymphocytes and cognitive impairment in patients with Parkinson's disease. *Brain Behav. Immun.* 89, 668–674. doi: 10.1016/j.bbi.2020.07.005

Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease (2003). The Unified Parkinson's Disease Rating Scale (UPDRS): status and recommendations. *Mov. Disord.* 18, 738–750. doi: 10.1002/mds.10473

Nagele, E. P., Han, M., Acharya, N. K., DeMarshall, C., Kosciuk, M. C., Nagele, R. G., et al. (2013). Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One* 8:e60726. doi: 10.1371/journal.pone.0060726

Niwa, F., Kuriyama, N., Nakagawa, M., and Imanishi, J. (2012). Effects of peripheral lymphocyte subpopulations and the clinical correlation with Parkinson's disease. *Geriatr Gerontol. Int.* 12, 102–107. doi: 10.1111/j.1447-0594.2011.00740.x

Orr, C. F., Rowe, D. B., Mizuno, Y., Mori, H., and Halliday, G. M. (2005). A possible role for humoral immunity in the pathogenesis of Parkinson's disease. *Brain* 128, 2665–2674. doi: 10.1093/brain/awh625

Papachroni, K. K., Ninkina, N., Papapanagiotou, A., Hadjigeorgiou, G. M., Xiromerisiou, G., Papadimitriou, A., et al. (2007). Autoantibodies to alpha-synuclein in inherited Parkinson's disease. *J. Neurochem.* 101, 749–756. doi: 10.1111/j.1471-4159.2006.04365.x

Pernis, A. B. (2007). Estrogen and CD4+ T cells. *Curr. Opin. Rheumatol.* 19, 414–420. doi: 10.1097/bor.0b013e328277ef2a

Picillo, M., Nicoletti, A., Fetoni, V., Garavaglia, B., Barone, P., and Pellecchia, M. T. (2017). The relevance of gender in Parkinson's disease: a review. *J. Neurol.* 264, 1583–1607.

Postuma, R. B., Berg, D., Stern, M., Poewe, W., Olanow, C. W., Oertel, W., et al. (2015). MDS clinical diagnostic criteria for Parkinson's disease. *Mov. Disord.* 30, 1591–1601. doi: 10.1002/mds.26424

Reynolds, A. D., Banerjee, R., Liu, J., Gendelman, H. E., and Mosley, R. L. (2007). Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease. *J. Leukoc. Biol.* 82, 1083–1094. doi: 10.1189/jlb.0507296

Richartz-Salzburger, E., Batra, A., Stransky, E., Laske, C., Köhler, N., Bartels, M., et al. (2007). Altered lymphocyte distribution in Alzheimer's disease. *J. Psychiatr. Res.* 41, 174–178. doi: 10.1016/j.jpsychires.2006.01.010

Rocha, N. P., Assis, F., Scalzo, P. L., Vieira, ÉL. M., Barbosa, I. G., de Souza, M. S., et al. (2018). Reduced Activated T Lymphocytes (CD4+CD25+) and Plasma Levels of Cytokines in Parkinson's Disease. *Mol. Neurobiol.* 55, 1488–1497. doi: 10.1007/s12035-017-0404-y

Rodrigues, M. C., Sanberg, P. R., Cruz, L. E., and Garbuzova-Davis, S. (2014). The innate and adaptive immunological aspects in neurodegenerative diseases. *J. Neuroimmunol.* 269, 1–8. doi: 10.1016/j.jneuroim.2013.09.020

Sanchez-Guajardo, V., Barnum, C. J., Tansey, M. G., and Romero-Ramos, M. (2013). Neuroimmunological processes in Parkinson's disease and their relation to alpha-synuclein: microglia as the referee between neuronal processes and peripheral immunity. *ASN Neuro.* 5, 113–139.

Shalit, F., Sredni, B., Brodie, C., Kott, E., and Huberman, M. (1995). T lymphocyte subpopulations and activation markers correlate with severity of Alzheimer's disease. *Clin. Immunol. Immunopathol.* 75, 246–250. doi: 10.1006/clin.1995.1078

Sommer, A., Fadler, T., Dorfmeister, E., Hoffmann, A. C., Xiang, W., Winner, B., et al. (2016). Infiltrating T lymphocytes reduce myeloid phagocytosis activity in synucleinopathy model. *J. Neuroinflammation* 13:174.

Stevens, C. H., Rowe, D., Morel-Kopp, M. C., Orr, C., Russell, T., Ranola, M., et al. (2012). Reduced T helper and B lymphocytes in Parkinson's disease. *J. Neuroimmunol.* 252, 95–99.

Sun, C., Zhao, Z., Yu, W., Mo, M., Song, C., Si, Y., et al. (2019). Abnormal subpopulations of peripheral blood lymphocytes are involved in Parkinson's disease. *Ann. Transl. Med.* 7:637. doi: 10.21037/atm.2019.10.105

Sweeney, M. D., Sagare, A. P., and Zlokovic, B. V. (2018). Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nat. Rev. Neurol.* 14, 133–150. doi: 10.1038/nrneurol.2017.188

Theodore, S., Cao, S., McLean, P. J., and Standaert, D. G. (2008). Targeted overexpression of human alpha-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease. *J. Neuropathol. Exp. Neurol.* 67, 1149–1158. doi: 10.1097/nen.0b013e31818e5e99

Tomlinson, C. L., Stowe, R., Patel, S., Rick, C., Gray, R., and Clarke, C. E. (2010). Systematic review of levodopa dose equivalency reporting in Parkinson's disease. *Mov. Disord.* 25, 2649–2653. doi: 10.1002/mds.23429

Tufekci, K. U., Genc, S., and Genc, K. (2011). The endotoxin-induced neuroinflammation model of Parkinson's disease. *Park. Dis.* 2011:487450.

Wang, G., Hong, Z., Cheng, Q., Xiao, Q., Wang, Y., Zhang, J., et al. (2009). Validation of the Chinese non-motor symptoms scale for Parkinson's disease: results from a Chinese pilot study. *Clin. Neurol. Neurosurg.* 111, 523–526. doi: 10.1016/j.clineuro.2009.02.005

Wirenfeldt, M., Babcock, A. A., and Vinters, H. V. (2011). Microglia - insights into immune system structure, function, and reactivity in the central nervous system. *Histol. Histopathol.* 26, 519–530.

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