UPDATE ON TRANSLATIONAL NEUROIMMUNOLOGY - RESEARCH OF ISNI 2018

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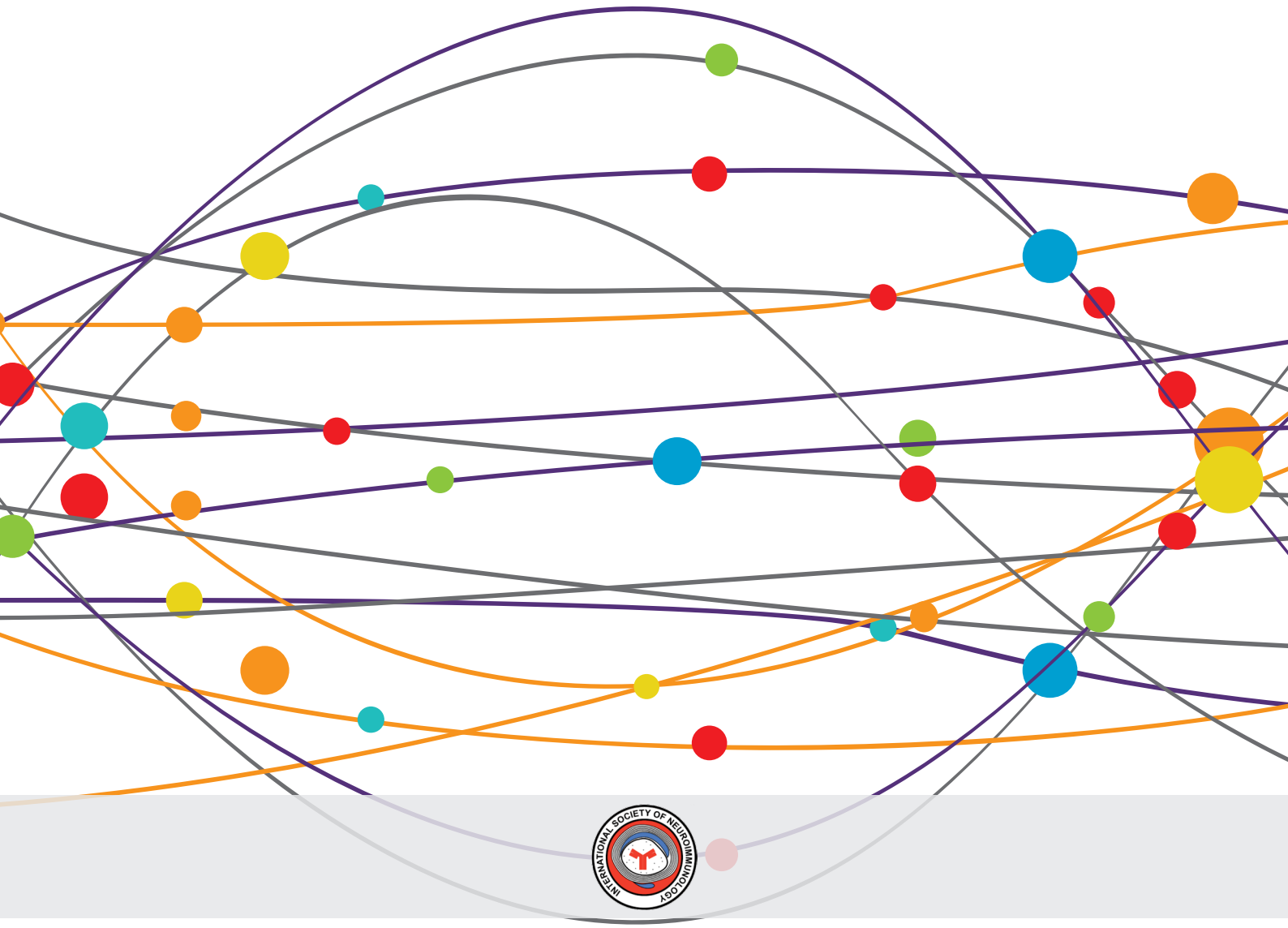

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UPDATE ON TRANSLATIONAL NEUROIMMUNOLOGY - RESEARCH OF ISNI 2018

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The 14th International Congress of Neuroimmunology, ISNI 2018, was held in August 2018 in Brisbane, Australia, and is a biennial event organized by the International Society of Neuroimmunology (ISNI). The theme of ISNI 2018 was "Travelling the Neuroimmunological Translational Highway", and the Congress highlighted many research discoveries that bridge the gap between basic and clinical sciences, and which impact our understanding of pathogenic immune-mediated mechanisms in diseases affecting the nervous system.

In this Research Topic, we aim to give a comprehensive overview of topics highlighted at the Congress, showcasing the current state of the field of neuroimmunology and where it is going in the near future.

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EDITORIAL
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Editorial: Update on Translational Neuroimmunology - Research of ISNI 2018

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Editorial on the Research Topic

Update on Translational Neuroimmunology - Research of ISNI 2018

With its roots firmly planted in the fields of CNS autoimmunity and infection, the rapidly growing field of neuroimmunology is branching out to include almost every area of neurophysiology and neurological disease to encompass brain development and function, psychiatric illnesses, inflammatory demyelinating diseases, cancer, infections, and neurodegeneration of the central and peripheral nervous systems. The nervous system is also functionally intimately interconnected, via immune mechanisms, to multiple other processes and organ systems including systemic inflammation, gut microbiome, and chronic pain, to name a few. The scope of neuroimmunology and its implications for the understanding of human health and disease, from diagnosis through therapeutics, was highlighted during the 14th Congress of the International Society for Neuroimmunology (ISNI) 2018, and the associated 2nd Global Schools of Neuroimmunology (GSNI), held together in August 2018 in Brisbane, Australia. The richness of topics covered is further illustrated through the series of related articles presented in this special issue of Frontiers in Neurology entitled "Update on Translational Neuroimmunology Research of ISNI 2018."

The Congress highlighted the importance taking the knowledge gained from translational research and clinical experience back into the research environment to gain further understanding of immune-mediated diseases affecting the human CNS and how to overcome them. During the meeting, dedicated symposia covered diverse topics of CNS and PNS autoimmunity, immune dysfunction, and immunotherapy, covering a wide range of neuroimmune interactions in health and disease. For example, disease topics ranged from immune-mediated conditions, such as multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD), myasthenia gravis, and autoimmune encephalopathies and neuropathies, to inflammation in conditions usually thought of as neurodegenerative, such as Alzheimer's disease (AD), Parkinson's disease (PD), motor neuron diseases, neuropsychiatric diseases, as well as traumatic injuries and infections by parasites and neurotropic RNA viruses including West Nile and Zika.

The involvement of immune mechanisms in psychiatric diseases was introduced at GSNI on the first day of the meeting and during the main meeting in talks by Lennox and Brown. In this issue, McCombe et al. review the emerging evidence that the peripheral immune system contributes to motor neurodegeneration in amytrophic lateral sclerosis (ALS), and implications for therapeutic targeting to manage the disease. This parallels current thinking in AD that peripheral inflammation influences inflammation in the brain. In a similar vein, Yates et al. review the current evidence suggesting that extracellular vesicles (EVs) shed from CNS cells, cross the blood brain

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barrier (BBB), and play a critical role in activating peripheral immune responses to traumatic CNS injury. Vilquin et al. describe the consequences of autoimmune attack against components of the neuromuscular junction in myasthenia gravis, focusing on the response of the ultimate target in this disease, the muscle. Fujii et al. turn their attention to investigating the link between allergic inflammation and neuropathic pain, describing a possible role for spinal cord glial inflammation and autoantibodies that target sensory neurons in the dorsal root ganglia.

The dramatic clinical success of B cell depleting immunotherapies in patients with MS, as well as CNS diseases that involve autoantibodies such as NMOSD, has focused research on understanding the critical roles of B cells, and their interactions with T cells, in the pathogenesis of MS and other neuroimmune diseases. B cells produce antibodies, present antigens to T cells, and produce pro-inflammatory and anti-inflammatory cytokines, and all these functions can contribute to CNS autoimmunity. At the Congress, The Immunology Lecture delivered by Vinuesa was dedicated to rare mutations that contribute to systemic autoimmunity. Focus was placed on genes that control mechanisms involved in the production, selection and elimination of memory B cells and antibody production, and specifically on the role of roquin, an E3 ubiquitin ligase, in the control of B cell-T cell interactions by T follicular helper cells (TFH) (1). In the Dale McFarlin Lecture delivered by Martin, we learnt that memory B cells increase the spontaneous autoproliferation of peripheral T helper (Th1) cells as well as non-classical Th1 cells (CCR6+ CXCR3+; Th17.1 cells) (2). Importantly, T cell autoproliferation is abnormally increased in MS patients during the remission phase of the disease and is likely to drive disease (2). B cells do this in a HLA-DR1-dependent manner, and depletion of B cells by anti-CD20 antibodies reduces T-cell proliferation, thereby providing one mechanistic basis for therapeutic B cell depletion in MS. The same group identified a new putative target autoantigen in MS, RASGRP2, expressed in both brain and B cells (2). Insights from pediatric-onset MS discussed by Bar-Or also explored novel antigenic targets and emphasized the importance of cellular interactions between memory B cells, T cells and myeloid cells (3). Autoantibody-mediated pathologies of the peripheral and central nervous systems were a main topic of the meeting discussed by Vincent, De Seze, Mathey, Kusunoki and Kiernan. In this issue, van Langelaar et al. further discuss the types of T cell-B cell interactions that might be important for MS pathogenesis, Greer et al. report that autoantibodies to the major myelin protein, proteolipid 181–230 peptides, are increased in a subgroup of MS patients and that their levels positively correlate with disease severity, while Young reviews the differential effects of antibodies to the GluN1 subunit of the glutamate NMDA receptor in pathogenesis of neurological diseases.

Neutrophils are amongst the first immune cells to reach the CNS in the MS model experimental autoimmune encephalomyelitis (EAE) and are present in the CNS during NMOSD and severe MS, but little is known of their role in disease pathogenesis. Vallieres showed that neutrophils adopt macrophage-like properties once entering the CNS in a B cell-dependent EAE model and to promote inflammation via the neutrophil-specific protease ASPRV1 (4). Moreover, Korn discussed mechanisms by which polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC), phenotypically related to neutrophils, control B cell accumulation and cytokine secretion during CNS autoimmunity (5). The involvement of other innate immune mechanisms in neurodegenerative disorders, such as heat shock proteins and the inflammasome, was also given attention by Amor, Issazadeh-Navikas, and Ting.

MS research is increasingly focused on myeloid cells and CNS-resident cells such as astrocytes, microglia, and oligodendrocytes, which contribute to disease pathology through additional mechanisms likely relevant for other neurologic diseases. One topic that remains hotly debated in the MS field is the identity of the antigen-presenting cell that is responsible for reactivating T cells in the CNS, and thereby licensing them to induce demyelinating lesions, since this is a potential target for more selective therapy. Various cell types, including dendritic cells, B cells, barrier-associated macrophages (BAM), and microglia are able to present antigen to T cells in the context of MHC class II, although which one is involved in the pathogenesis of EAE and MS still remains to be identified. Important targets are also the effector molecules communicating these interactions, as discussed by Becher for (GM-CSF) (6).

The functions of CNS-resident myeloid cell (microglia) in neuroinflammation continue to intrigue researchers. Unlike their peripheral counterparts (monocytes and macrophages), microglia perform essential functions in the CNS not only in pathologic states such as in repair of tissue damage, as discussed by Popovich, but also under normal physiologic conditions, such as shaping neuronal networks and clearing debris by phagocytosis. Loss of these functions during development has been implicated in schizophrenia and potentially in autism spectrum disorders, while loss of the same functions in the context of chronic inflammation may contribute to pathology in diseases such as MS and AD. For example, Waisman reported that selective depletion of A20 (an inhibitor of the transcription factor NF-κB) in microglia, was sufficient to induce spontaneous neuroinflammation and CNS infiltration by CD8+ T cells, pointing to a homeostatic function for microglia in the brain (7). The TAM family of receptor tyrosine kinases, Tyro3, Axl, and Mertk, has been implicated in demyelination, remyelination and MS susceptibility, and Kilpatrick reported that Mertk plays a role in beneficial microglia function in an experimental demyelination model, and that Tyro3 is important for developmental myelination of the CNS (8). In this issue, Benmamar-Badel et al. review data regarding a unique subset of microglia that is found in development, during normal aging and in diverse diseases and discuss the possible functional significance of these cells. It is clear that much more needs to be learnt regarding the differential functions of myeloid cell subtypes before these cells can be both effectively and safely targeted for therapy.

Evidence for inflammatory functions of CNS-resident oligodendrocyte precursor cells (OPC) was presented by Ransohoff wherein IL-17-induced Act1-NOTCH1 interactions in OPC promoted the inflammatory responses, cell proliferation

and inhibited OPC differentiation into mature oligodendrocytes thereby inhibiting remyelination. Genetic depletion of NOTCH1 in OPC in mice, or administration of a decoy peptide based on IL-17RA, were sufficient to inhibit Th17-induced EAE (9). Increasing complexity of astrocyte function in disease is also being appreciated. Liddelow reported that astrocytes can be converted by microglia into a neurotoxic phenotype in diseases such as AD and PD, and agents that inhibit the formation of neurotoxic astrocytes could be used to treat these so-far untreatable diseases (10). Quintana described a novel metabolic mechanism involving cytosolic phospholipase A2 interactions with mitochondrial antiviral signaling protein (MAVS), which leads to activation of NF-κB and drives pro-inflammatory activities of astrocytes in EAE and MS, while interfering with the metabolic support of neurons by astrocytes. These findings identified a candidate drug to be repurposed for therapeutic modulation of astrocyte pro-inflammatory activities, while they also provide a novel link between viral infections, metabolism, CNS inflammation and neurodegeneration (11).

Our current understanding of CNS immune privilege and CNS barriers, including the blood brain barrier (BBB), blood CSF barrier (B-CSF), and brain surface barrier, was reviewed by Engelhardt at GSNI and elaborated during the congress itself, where the perivascular space at the BBB was implicated as a main entry point for immune cell infiltration in MS (12). Brain barrier dysfunction also occurs in other neurodegenerative diseases. Aging of the choroid plexus was described by Schwartz in an experimental AD model and was associated with the immune dysfunction and cognitive defects that are characteristic of this disease (13). Targeting of the choroid plexus using blockers of immune-inhibitory checkpoints, such as PD-1, evoked IFN-γ dependent immune responses, which in turn improved the recruitment of monocyte-derived macrophages including so-called disease-associated macrophages (DAMs), into the CNS and reduced disease features (13). These findings provide another example where the immune system exerts protective functions in the CNS during disease. Drugs that protect barrier function represent promising new therapies for MS, and as described by Yong include approved drugs such as minocycline, a tetracycline antibiotic used to treat acne, and novel inhibitor analogs of extracellular matrix components called chondroitin sulfate proteoglycans (CSPGs) (14).

Well-deserved attention was given to the gut-CNS axis and, in particular, the role of gut microbiota in immune homeostasis of the brain and in triggering CNS autoimmunity. This field developed following a landmark discovery made in the field of neuroimmunology just one decade ago. Since then the functional relationships between the gut microbiome and its effects on CNS pathology driven by peripheral and resident cells have been subject to intense investigation, as introduced at GSNI by Barazini, and updated in the Congress by Wekerle, Miyake, Weiner, Quintana and Yamamura, and by recent reviews (15–18). This special issue includes reviews from Jogia and Ruitenberg on the significance of gut microbiota in traumatic spinal cord injury, and from Cady et al., on the importance of dietary phytoestrogens in protection in EAE and possibly MS.

Several talks highlighted the neuroimmunology of infectious diseases of both the peripheral and central nervous systems, including cerebral malaria by Idro and viral infections by Klein, Pender, Basu and Yamano.

The search for improved diagnostic biomarkers continues, and is a particular focus of articles published in this special issue. While MS is the most common inflammatory demyelinating disease of the CNS, other rare disorders include NMOSD and anti-MOG associated disease, and the need for accurate diagnosis is stressed since prognosis and treatment of these three diseases are different. In this issue, Prain et al. describe a clinically-based survey of NMOSD in Australia and New Zealand, and critically evaluate different assays for anti-AQP4 and anti-MOG antibodies used in the diagnosis of NMOSD and anti-MOG associated disease, respectively. In the case of anti-AQP4 antibody seronegative NMOSD, differential diagnosis from MS might be difficult. The current status of medical imaging research in MS and NMOSD, and its role in the diagnosis and management of these two diseases was discussed at the meeting by Stankoff and Paul, and is reviewed in this issue by Kuchling and Paul. Tea et al. together with the Australasian and New Zealand MOG Study Group, discuss data analysis tools for maximizing the diagnostic power of FACs cell-based assays that detect MOG autoantibodies. Gastaldi et al. critically report the results and main shortcomings of the 2018 Italian Neuroimmunology Association external quality assessment program (EQAP), which evaluated assays using a wide range of markers including oligoclonal bands, antibodies to intracellular and surface neuronal antigens, AQP4, MOG, and myelin-associated glycoprotein (MAG) antibodies, in different assay types used by 34 different laboratories. Jiang et al., describe improved novel biomarkers in the CSF of patients with autoimmune encephalitis for differential diagnosis between viral infections and autoimmune encephalitis. Masvekar et al. investigated whether apoptotic bodies/ apoptosomes, which are vesicles released from apoptotic cells, could represent a biomarker for MS, by measuring total and cell-specific apoptotic bodies in the CSF of MS patients by FACS using annexin V and antibodies to cell-specific markers.

Novel immunotherapeutic strategies for wide range of peripheral and central nervous system diseases, including neuropathies, myasthenia gravis, and pediatric CNS disorders were discussed during the meeting. The powerful therapeutic value of neural stem cells was the subject of The John Newsom David Lecture delivered by Martino (Ottoboni et al.) and The Rita Levi-Montalcini Neurobiology Lecture delivered by Bartlett (19), and is further highlighted in this special issue in a review by Ottoboni et al.. News of a phase I safety trial using allogenic human NSC in MS patients with progressive MS was shared by Martino, and strategies for further improvement of this approach by using autologous induced pluripotent stem cell derived NSC were considered. The homeostatic functions of NSC in brain inflammation and their contribution to remyelination and brain repair in experimental models was reported. Under conditions of inflammation, NSC are maintained in an immature state and secrete LIF, which in turn promotes remyelination. In a separate

report by Kilpatrick, oligodendrocyte-specific expression of the TAM receptor Tyro3 was necessary for remyelination in a demyelination model in mice (8). The possibility that these mechanisms interact to mediate CNS repair in demyelinating disease remains to be investigated. On the other hand, positive regulators of neurogenesis from NSC in the hippocampus are found to be stimulated by exercise and healthy lifestyle (19). The importance of microglial and astrocytic gap junction proteins in the modulation of CNS pathology was pointed out in talks by Suzumura and Kira. In this issue, Finardi et al., provide evidence that microRNAs miR106b-25 and miR17-92, which are upregulated in MS patient T regulatory cells, are involved in the development of experimental neuroinflammation and might represent novel therapeutic targets. In the context of glioma, in this issue Sarkar et al. provide evidence that the antibiotic demeclocycline reduces the growth of brain tumor initiating cells through direct and indirect effects via activation of myeloid cells.

Further advancing the spirit of translational research in the neuroimmunology field, the importance of improved animal models of autoimmunity and infection was highlighted in the talks by Liblau, Baker, De Seze, Klein and O'Connor. Several new animal models have been developed to recapitulate findings from the clinic and to aid deeper mechanistic studies into disease pathogenesis. A humanized mouse model for Rasmussen's encephalitis was generated by transplanting patient PBMC's into immunodeficient NSG mice, as reported by Prat (20), as were B cell models for MS and NMOSD, including one in which sequences encoding human AQP 4 antibody isolated from an NMOSD patient were knocked into the mouse heavy chain locus as reported by Kuchroo (21), and another EAE model induced by immunization with AQP4 201-220 peptide in AQP4 KO mice as reported by Zamvil (22). In this issue, Giannoccaro et al. review results and the limitations of current animal models of autoantibody-mediated neurological diseases, and discuss the increasing evidence that maternal antibodies to neuronal surface antigens in the maternal circulation can reach the fetal brain during gestation causing neurodevelopmental disorders.

In summary, this collection represents the broad range of NeuroImmunology research presented at the 14th Congress of the International Society for Neuroimmunology (ISNI) 2018, and the associated 2nd Global Schools of Neuroimmunology (GSNI), highlighting new developments in this rapidly moving field, as well as unanswered research questions and unmet clinical needs. This growing body of research sets up the stage for the upcoming ISNI/GSNI 2021 meeting in Nice, France.

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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AQP4 Antibody Assay Sensitivity Comparison in the Era of the 2015 Diagnostic Criteria for NMOSD

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We have compared five different assays for antibodies to aquaporin-4 in 181 cases of suspected Neuromyelitis optica spectrum disorders (NMOSD) and 253 controls to assess their relative utility. As part of a clinically-based survey of NMOSD in Australia and New Zealand, cases of suspected NMOSD were referred from 23 centers. Clinical details and magnetic imaging were reviewed and used to apply the 2015 IPND diagnostic criteria. In addition, 101 age- and sex-matched patients with multiple sclerosis were referred. Other inflammatory disease ($n = 49$) and healthy controls ($n = 103$) were also recruited. Samples from all participants were tested using tissue-based indirect immunofluorescence assays and a subset were tested using four additional ELISA and cell-based assays. Antibodies to myelin oligodendrocyte glycoprotein (MOG) were also assayed. All aquaporin-4 antibody assays proved to be highly specific. Sensitivities ranged from 60 to 94%, with cell-based assays having the highest sensitivity. Antibodies to MOG were detected in 8/79 (10%) of the residual suspected cases of NMOSD. Under the 2015 IPND diagnostic criteria for NMOSD, cell-based assays for aquaporin-4 are sensitive and highly specific, performing better than tissue-based and ELISA assays. A fixed cell-based assay showed near-identical results to a live-cell based assay. Antibodies to MOG account for only a small number of suspected cases.

Keywords: neuromyelitis optica, autoantibody, aquaporin, myelin oligodendrocyte glycoprotein, astrocytopathy, demyelination

INTRODUCTION

Neuromyelitis optica spectrum disorders (NMOSD) (1) encapsulate a variety of defined neurological clinical presentations associated with autoantibodies to aquaporin-4 (AQP4) (2). Detection of antibodies to AQP4 is of immense value in the accurate diagnosis and management of NMOSD, which represent about 1% of central nervous system (CNS) inflammatory disease (3).

The current diagnostic criteria for NMOSD permit the inclusion of AQP4 antibody negative cases, but this requires additional radiological criteria (1).

Myelin oligodendrocyte glycoprotein (MOG) antibody-related demyelinating disease is emerging as another antibody mediated inflammatory disorder of the CNS which shares some overlapping features with NMOSD (4). In particular, a predilection for lesions of the optic nerve and spinal cord is seen in both conditions (5, 6). However, there are some very clear clinical distinctions between the two disorders. MOG antibody-related demyelinating disease accounts for up to one third of cases of pediatric demyelinating disease, often presenting with acute disseminated encephalomyelitis, a clinical picture that is rare in NMOSD (7). In addition, the distribution of the spinal cord lesions is subtly different with lesions of the high cervical spine (C1/2) being seen in NMOSD and lesions extending all the way to the conus being seen in MOG antibody-related demyelinating disease (8).

We recently performed a nationwide prevalence survey of NMOSD across Australia and New Zealand (9). We have compared the relative utility of a variety of AQP4 antibody assays and studied the prevalence of positivity for MOG antibodies in this population, with the aim of guiding best laboratory practice and interpretation of results for clinicians.

METHODS

Case Ascertainment

Possible cases of NMOSD were identified through a network of 23 neurology clinics specializing in demyelinating diseases of the CNS (ICD-10 G35-G37) across Australia and New Zealand. These centers match the population distribution of both countries. Participating centers referred cases to the coordinating center in Queensland if they had features suggestive of NMOSD as previously described (9). Cases were excluded if no serum sample was supplied and results of prior AQP4 antibody testing were not available, insufficient clinical data to make a diagnosis were supplied or if an alternate diagnosis became apparent. All subjects provided written informed consent. Institutional human research ethics committee approval was obtained for all participating sites. The period of data collection was from 1 January 2011 to 31 December 2013. The 2015 International Panel for NMO Diagnosis (IPND) diagnostic criteria for NMOSD (ICD-10 G36) were applied retrospectively.

Referring neurologists were also requested to recruit age- and sex-matched patients with multiple sclerosis, who did not have any of the features suggestive of NMOSD. Additional controls consisted of patients with other inflammatory diseases (infectious and rheumatological) and healthy blood donors. The other inflammatory diseases included infections (varicella, systemic CMV, infectious mononucleosis), Sjögren's syndrome and systemic lupus erythematosus. All participants gave written, informed consent to participation in this study and the study protocol was approved by the Human Research Ethics Committee at all participating sites.

Demographic details (age and gender) together with clinical details sufficient to confirm a diagnosis of NMOSD or MS were collected, including relapse history and MR imaging as previously described (9). Cases were then defined as "NMOSD" (meeting seropositive or seronegative 2015 IPND criteria) (1), "suspected NMOSD" (cases having features suggestive of NMOSD but not meeting 2015 IPND criteria), or "MS" (meeting 2010 McDonald criteria with no features suggestive of NMOSD) (10). The remaining control groups were other inflammatory disease and healthy blood donors.

Antibody Assays

Any prior AQP4 antibody testing results were collected using a standard questionnaire in all cases. Serum samples were obtained and tested for AQP4 antibodies using indirect immunofluorescence staining techniques on mouse, rat, or monkey brain tissue and rat or mouse kidney sections at one of four testing sites (see **Supplementary Table 1** for details). A subset of samples was also tested using an ELISA kit (RSRTM, UK), as well as two fixed cell-based slide kits from Euroimmun® and a live cell based assay (11). The two slides each consisted of two chips of HEK cells transfected with M1 and M23 isoforms of AQP4 in one and M23 AQP4 and MOG in the other (Euroimmun®, Germany). The tissue-based indirect immunofluorescence testing was undertaken in 4 centers across Australia. The ELISA, and fixed-cell based assays were performed by the Autoimmunity section of the Division of Immunology, Pathology Queensland Central Laboratory, Brisbane, Australia, as per the manufacturer's instructions. The live cell-based assay was performed in the Nuffield Department of Clinical Neurosciences, Oxford, UK, as previously described (12). All assays were performed by researchers blinded to the final diagnostic status of the cases and results from Brisbane and Oxford were collated by a blinded third party based in Cambridge, UK, who then distributed the final combined results to all parties. The typical outputs of the tissue-based and live cell-based assays are shown in **Figure 1**. MOG antibodies were detected using three different assays: a commercial fixed cell-based assay (Euroimmun®, Germany), a live cell-based assay, and a live cell-based fluorescence activated cell sorting (FACS) assay. The fixed cell-based assay was performed as per the manufacturer's instructions. The live cell-based assay was performed in the Nuffield Department of Clinical Neurosciences, Oxford, UK, as previously described (13) and the FACS assay was performed at the Westmead Immunology Laboratory, Sydney, Australia as previously described (14). Seropositivity for AQP4 or MOG antibodies was defined as either a positive result on any of the tissue-based indirect immunofluorescent assays or a positive result on at least 2 cell-based assays (including repeated FACS assay for MOG antibodies).

Statistics

Results are presented as n/N (%) of positive or negative antibody assays in cases and controls. Non-parametric statistics were used to assess differences in the demographic distribution of cases and controls. The optimal cut-off for the ELISA antibody level was assessed using receiver operator characteristic (ROC) curve analysis. Sensitivity and specificity with 95% confidence intervals (CI) were used to assess utility of the assays. Degree of

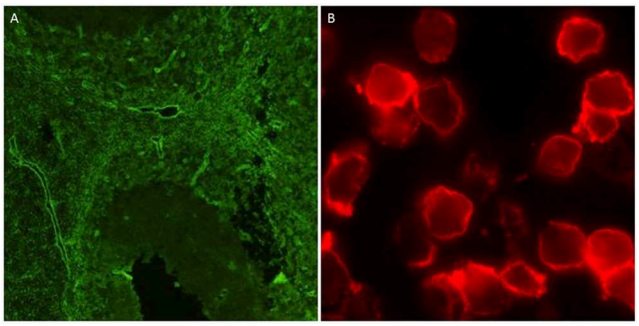

FIGURE 1 | Positive outputs for AQP4 antibodies using tissue-based indirect immunofluorescence on mouse cerebellum **(A)**, and live cell-based assay **(B)**.

TABLE 1 | Demographic details of cases and controls.

Group tested	*N*	Gender, female n/N (%)	Age, years median (range)
CASES			
NMOSD	80	71/80 (89)	47 (19-85)
Suspected NMOSD	101	68/101 (67)*	40 (15 – 72)*
CONTROLS			
Multiple sclerosis	101	86/101 (85)	46 (16 – 73)
Inflammatory disease	49	30/49 (61)*	59 (21 – 97)*
Blood donors	103	N/A	N/A
Overall	253	116/150 (77)*	49.5 (16 – 97)

**Statistically significantly different from NMOSD cases ($p < 0.05$). NMOSD, neuromyelitis optica spectrum disorders.*

agreement between the assays was assessed using Cohen's kappa coefficient. All statistical analysis was performed using SPSS® v24 (IBM®, US).

RESULTS

In total, 189 cases of suspected NMOSD were referred. Of these 8/189 (4%) were excluded due to lack of an available serum sample. Of 181 suspected NMOSD cases, 80 met the 2015 IPND diagnostic criteria for NMOSD. Of these, 73/80 (91%) were seropositive for AQP4 antibodies and 7/80 (9%) were seronegative, leaving 101 suspected NMOSD cases. Not all of the seronegative NMOSD cases were tested with all assays. There were 108 cases of MS referred of which 7/108 (6%) had no serum available, leaving 101 included MS controls. Serum was available for 49 inflammatory disease and 103 blood donor controls. The inflammatory disease controls included the following: systemic lupus erythematosus (15), Sjögren's syndrome (8), cytomegalovirus infection (9), Epstein-Barr virus infection (7), and varicella zoster infection (6). The demographic details for cases and controls are given in **Table 1**. There were no statistically significant differences in gender ($X^2 = 0.503$, $p = 0.478$) or age distribution (Mann-Whitney U $p = 0.145$) between NMOSD cases and MS controls, indicating that our age- and sex-matching strategy had been effective. No data were available for the blood donor controls as these samples were provided anonymously as required by Australian Red Cross. The inflammatory disease controls were older, but when combined with the MS controls were not significantly different to NMOSD cases. The proportion of females in inflammatory disease controls (61%) compared with NMOSD cases (89%) was significantly lower ($X^2 = 13.548$, $p < 0.001$). When MS and inflammatory disease controls were combined the proportion of females increased (77%), but remained significantly different ($X^2 = 4.474$, $p = 0.034$).

ROC curve analysis (see **Figure 2**) of the ELISA test kit results showed an optimal cut-off of equal to or >10 (arbitrary units), which had a sensitivity of 60% (95% CI 45–98%) and specificity of 97% (95% CI 93–98%). This level was used to determine positivity on the ELISA assay.

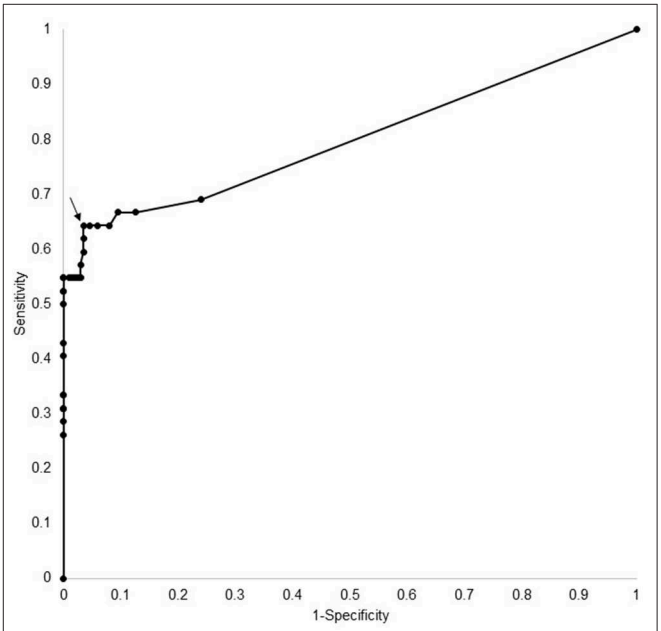

FIGURE 2 | ROC curve analysis for most appropriate cut off (arrow) for ELISA AQP4 assay.

Tissue-based indirect immunofluorescence testing for AQP4 antibodies was performed in 424/434 (98%) of cases and controls. A cell-based AQP4 assay was performed in 307/434 (71%) of cases and controls. The sensitivity for various assays in NMOSD and suspected NMOSD together with their specificity in the various control groups and overall controls is given in **Table 2**. The results of the Euroimmun® M1 and M23 biochips on a shared slide proved to be identical and so these results have been considered together. The most sensitive assays were the fixed and live cell-based assays, which gave very similar results (see **Table 2** and **Supplementary Table 2**). The overall sensitivity of the live cell-based assay was 92% (95% CI 78–97%) and specificity was

TABLE 2 | Sensitivity and specificity of autoantibody assays.

Group tested	*N*	T-IIF	ELISA	EI-M1/M23	EI-CBA	Ox-CBA	MOG
CASE SENSITIVITY—n +ve/*N* (%)							
NMOSD [95% CI for sensitivity]	80	62/78 (78) [69–87]	25/42 (60) [45–73]	38/42 (90) [78–96]	34/36 (94) [82–99]	33/36 (92) [78–97]	0/48 (0) [0–7]
Suspected NMOSD	101						8/79 (10)
CONTROL SPECIFICITY—n –ve/*N* (%)							
Suspected NMOSD	101	99/99 (100)	62/64 (97)	61/64 (95)	42/43 (98)	49/49 (100)	
Multiple sclerosis	101	98/98 (100)	48/48 (100)	48/48 (100)	20/20 (100)	21/21 (100)	52/52 (100)
Inflammatory disease	49	49/49 (100)	43/49 (88)	49/49 (100)	49/49 (100)	49/49 (100)	48/49 (98)
Blood donors	103	99/100 (99)	102/103 (99)	103/103 (100)	103/103 (100)	82/82 (100)	89/90 (99)
Overall [95% CI for specificity]	354	346/346 (99.7) [98–100]	255/264 (97) [94–98]	242/245 (99) [97–100]	214/215 (99.5) [97–100]	201/201 (100) [98–100]	189/191 (99) [96–100]

T-IIF, tissue-based indirect immunofluorescence; ELISA, enzyme linked immunosorbent assay; EI M1/M23, Euroummun® M1/M23 biochip slide; EI-CBA, Euroimmun® AQP4 fixed cell-based assay; Ox-CBA, Oxford AQP4 live cell-based assay; MOG, myelin oligodendrocyte glycoprotein antibody assay; NMOSD, neuromyelitis optica spectrum disorders.

100% (95% CI 98–100%). Whilst less sensitive (78% [95% CI 69–87%]), the tissue-based indirect immunofluorescence assay also proved to be very specific (99.6% [95% CI 98–100%]). The ELISA test was positive in 6 inflammatory disease controls, but none of the blood donor or MS controls. The ELISA assay proved to be the least sensitive (60% [95%CI 45–98%]) and least specific (97% [95% CI 93–98%]).

The degree of concordance between assays was generally high, and particularly so for the cell-based assays, as shown in **Table 3**. In the suspected NMOSD cases, there were 5 cases who were positive on the Euroimmun® M1/M23 assay or the ELISA assay alone. As these cases were negative on all other cell-based assays they were not included in the NMOSD cases and remained as suspected NMOSD. Inclusion of the suspected NMOSD cases as controls for the calculation of specificity did not significantly change the results.

Amongst suspected NMOSD cases, 8 were positive for MOG antibodies. One of these was also positive for both the AQP4 and MOG biochips on the same fixed cell-based assay. This case was negative for all other cell-based assays for AQP4 antibodies and was confirmed as positive for MOG antibodies by FACS assay and so was not considered to be a case of NMOSD, but rather as a case of MOG antibody-related demyelinating disease. Thus, we did not identify any AQP4 and MOG antibody double positive cases. One MOG antibody positive case met the clinical/MRI 2015 IPND criteria for a diagnosis of NMOSD, but was considered as a MOG antibody-related demyelinating disease case. When the sensitivity and specificity analysis was restricted to cases with testing available for all assays (AQP4 and MOG) results were not significantly different (**Supplementary Tables 2, 3**). We observed a clear correlation between the number of positive tests (tissue and cell-based assays) and the ELISA antibody level (**Figure 3**). However, antibody levels >100 were seen in a few samples with only one positive result on the other assays.

TABLE 3 | Concordance and agreement for AQP4 antibody assays.

Assay	T-IIF	ELISA	EI M1/M23	EI AQP4
ELISA	121/141 (86)			
	0.556	n/a		
	<0.001			
EI M1/M23	131/141 (93)	121/141 (86)		
	0.790	**0.605**	n/a	
	<0.001	*<0.001*		
EI AQP4	132/141 (94)	122/141 (87)	136/141 (96)	
	0.808	**0.620**	**0.904**	n/a
	<0.001	*<0.001*	*<0.001*	
Ox AQP4	134/141 (95)	122/141 (87)	136/141 (96)	139/141 (99)
	0.847	**0.612**	**0.902**	**0.960**
	<0.001	*<0.001*	*<0.001*	*<0.001*

All data presented as: Concordance n/N (%); bold values represent the Cohen's kappa coefficient; italic value represent the P-value; n/a, not applicable.
T-IIF, tissue-based indirect immunofluorescence; ELISA, enzyme linked immunosorbent assay; EI M1/M23, Euroummun® M1/M23 biochip slide; EI-CBA, Euroimmun® AQP4 fixed cell-based assay; Ox-CBA, Oxford AQP4 live cell-based assay.

DISCUSSION

We have conducted a rater-blinded comparison of 5 different assays for antibodies to AQP4 in a population of cases with suspected NMOSD and a variety of controls. Consistent with previous studies (11, 12, 16, 17) we have found that the sensitivity of cell-based assays, both fixed and live cell-based assays, was higher (90–94%) than for either an ELISA assay (60%) or tissue indirect immunofluorescence (78%). The sensitivity of cell-based assays was at the higher end of previously reported data for studies using the 2006 Wingerchuk or earlier diagnostic criteria for NMOSD in adult, Caucasian populations (64–98%)

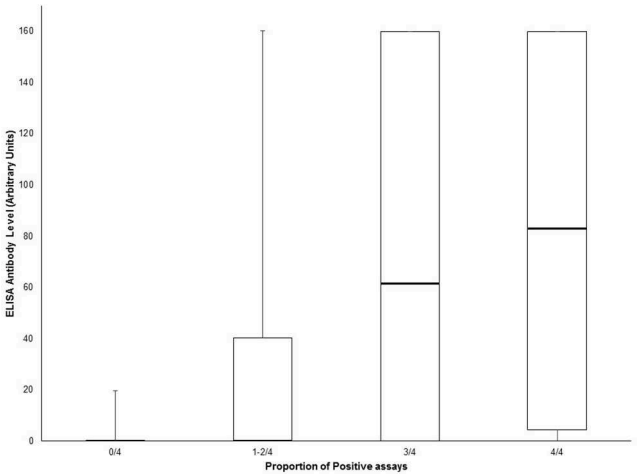

FIGURE 3 | Box and whisker plot of ELISA antibody levels according to the proportion of positive AQP4 assays (tissue-based indirect immunofluorescence, Euroimmun® M1/M23 biochip slide, Euroimmun® AQP4/MOG biochip slide and Oxford live cell-based assay). Central bar shows the median, boxes represent interquartile range, and whiskers indicate range.

(11, 12, 15, 18–20). This likely reflects the stricter radiological requirements of the 2015 IPND criteria. In addition to having typical presenting attacks, cases must also fulfill additional MRI criteria for the commoner presenting lesions (e.g., longitudinally extensive spinal cord lesion, long optic nerve lesion or area postrema lesion on imaging). All assays proved to be highly specific (≥97%) with the Euroimmun® fixed cell-based AQP4 and live cell based assays showing 100% specificity. False positives were more common amongst cases with MS and other inflammatory diseases with the ELISA assay having the highest false positive rate. This finding has also been noted previously (17). The concordance between assays, particularly for the cell-based assays was high.

The status of cases with positive results for one cell-based assay in the remaining suspected NMOSD cases remains uncertain. This may reflect greater sensitivity in "true positives." However, they may also represent false positives. Currently, there is no means to determine the true status of these cases although repeat testing over time may prove useful. The fact that the number of positive tests correlates well with the ELISA antibody level suggests that false negative results may occur when antibody levels are low, reflecting a sensitivity issue. However, the possibility of this being due to lower specificity of these assays cannot be discounted.

Amongst suspected NMOSD cases who were seronegative for AQP4 antibodies and did not meet the 2015 IPND diagnostic criteria for NMOSD 8/79 (10% [95% CI 5–19%]) were positive for MOG antibodies. This is again consistent with previous studies that have shown positivity rates for MOG antibodies in this population of 8–32% (17, 19, 21, 22). Specificity for MOG antibodies was 190/191 (99% [95% CI 96–100%]). No cases were positive for both AQP4 (on more than one assay) and MOG antibodies.

One advantage of the present study was that all cases were identified clinically by clinicians experienced in diagnosing inflammatory disease of the CNS and not based upon the results of laboratory testing, which introduces an inherent bias and the potential for low pre-test probability. The fact that not all cases were assessed using all assays is a weakness in this study, but when the analysis was restricted to only cases tested for all AQP4 antibody assays the results were not significantly different. The finding of identical results for the M1 and M23 AQP4 antibody assays is contrary to prior studies which have indicated a higher sensitivity for the M23 isoform (18). However, another recent study found the same result (23). The lack of clinical inclusion criteria for rarer presentations which had not been defined at the time of this study (e.g., area postrema lesion) is a further weakness of this study. Cases with these features were included and the numbers of missed cases is likely to have been small. However, depending on the relative frequency of positive AQP4 antibodies in these cases this could have had an impact on the reported sensitivity. There is no data to suggest that the rate of seropositivity in these cases would be different.

We have confirmed the high sensitivity and specificity for a wide range of AQP4 antibody assays in identifying NMOSD. The high sensitivity is to be expected, because of the inclusion of positive AQP4 antibodies as a part of the diagnostic criteria in the presence of a single characteristic presentation (1). The higher sensitivity of cell-based assays makes these preferable over other AQP4 assays in the identification of NMOSD. The fact that more than half of all suspected NMOSD cases are negative for both AQP4 and MOG antibodies remains a diagnostic dilemma. The issue of whether these cases are false negatives on the available assays or represent phenocopies of NMOSD remains unresolved. It is possible that yet more antibodies remain to be identified in this patient population or that a T-cell mediated process more akin to that hypothesized for MS pathology may be responsible for these cases (24). The high specificity of both AQP4 and MOG antibody assays means that in clinical practice, where there is a characteristic clinical presentation, a positive antibody result can be taken as being indicative of NMOSD or MOG antibody-related demyelinating disease respectively. Caution should be applied in the setting of concurrent inflammatory diseases, due to potential false positive results. The recent 2015 IPND criteria identify a closely defined group of NMOSD cases suitable for research purposes, but leaves a wider group of cases with a similar phenotype unclassified.

DATA AVAILABILITY STATEMENT

De-identified, individual level data for cases where all assays were performed is provided as **Supplementary Table 2**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Griffith University HREC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DA, MHB, SBh, SBl, MB, KB, BB, SAB, WB, CB, HB, WC, CC, LC, AC, RD, CD, KD, DG, SHa, RH, AH, SHe, SHo, AGK, TK, JK, CK, CL, RM, MM, DM, PM, CO'G, JDEP, MP, JP, JDP, KP, SWR, SS, JL-S, CS, RS, MS, JSp, JSt, IS, BT, AV, SV, MWa, PW, EW, RJW, and RCW conceived and designed the study. DA, MHB, SBh, SBl, MB, KB, BB, FB, SAB, WJB, WB, CB, HB, WC, CC, LC, AC, RD, CD, DF, DG, SH, RH, AH, SHe, SHo, AK, TK, JK, CK, AJK, M-WL, CL, RM, MM, DM, PM, CO'G, JDEP, MF-P, MP, JP, JDP, KP, SR, SWR, SS, JL-S, CS, RS, MS, JSp, JSt, IS, BT, SV, MWa, PW, EW, RJW, RCW, MWo, and EY played a major role in collecting data. FB, SAB, WB, KD, KP, SR, JSt, and PW conducted the analyses. KP, WB, and SAB prepared the initial draft. MHB, BB, FB, SAB, CB, HB, WC, LC, RD, KD, DG, SHo, AGK, TK, JK, RM, MM, DFM, PM, CO'G, JDEP, MF-P, MP, JP, JDP, SR, SWR, JL-S, CS, RS, MS, JSp, JSt, IS, BT, AV, SV, PW, EW, and MWo contributed to revisions. All authors approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01028/full#supplementary-material

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Systemic Immune Response to Traumatic CNS Injuries—Are Extracellular Vesicles the Missing Link?

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Inflammation following traumatic injury to the central nervous system (CNS) persists long after the primary insult and is known to exacerbate cell death and worsen functional outcomes. Therapeutic interventions targeting this inflammation have been unsuccessful, which has been attributed to poor bioavailability owing to the presence of blood-CNS barrier. Recent studies have shown that the magnitude of the CNS inflammatory response is dependent on systemic inflammatory events. The acute phase response (APR) to CNS injury presents an alternative strategy to modulating the secondary phase of injury. However, the communication pathways between the CNS and the periphery remain poorly understood. Extracellular vesicles (EVs) are membrane bound nanoparticles that are regulators of intercellular communication. They are shed from cells of the CNS including microglia, astrocytes, neurons and endothelial cells, and are able to cross the blood-CNS barrier, thus providing an attractive candidate for initiating the APR after acute CNS injury. The purpose of this review is to summarize the current evidence that EVs play a critical role in the APR following CNS injuries.

Keywords: extracellular vesicles, traumatic brain injury, spinal cord injury, inflammation, acute phase response

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INTRODUCTION

Acute CNS injuries, including traumatic brain and spinal cord injury (TBI; SCI), as well as stroke, are a major global burden (1, 2). These neurological disorders have a collective global incidence rate of 500–700 per 100,000 people (3), and have extremely high morbidity, requiring lifelong subsequent care at a substantial financial and emotional cost (4, 5). Whilst the primary causes of TBI and SCI, and even to some extent stroke, are largely unavoidable, the ensuing secondary injury and ongoing inflammatory response can significantly worsen outcome and could be amenable to therapeutic intervention (6–9). The mechanisms that promote the inflammatory response to injury, and the communication pathways that convey messages about CNS health status to the systemic immune system, are the subject of intense investigation, but it is becoming clear that extracellular vesicles (EVs) play a pivotal role.

Acute CNS Injury—Primary vs. Secondary Injury

Damage to the CNS following a neurotraumatic event occurs in two distinct phases (7, 10, 11). The primary phase is largely mechanical, whereby the physical insult causes direct structural damage to

neuronal tissue and the vasculature, resulting in immediate cell death, and hemorrhage, ischemia and/or oedema.

The primary phase occurs within a short window of time, whereas the secondary phase has been shown to persist for days, weeks, even months after the injury (12, 13). Although not damaged directly during the initial insult, CNS tissue surrounding the injury is highly vulnerable to secondary damage (10, 14). Hypoxia, excitotoxicity, free radical formation, breakdown of blood-CNS barriers and release of proteases, all contribute to further cell death (10, 15). Moreover, activated microglia and astrocytes, as well as infiltrating leukocytes from the periphery, release cytokines and chemokines that create a pro-inflammatory microenvironment (6, 7, 13). Collectively, this results in the progressive destruction of CNS tissue, known as "bystander tissue damage", which considerably impairs functional recovery (16).

Previous studies utilizing rodent models have shown that the secondary phase of traumatic CNS injury is dependent on the acute phase response (APR), a systemic inflammatory response occurring predominantly in the liver (17). In response to CNS damage, hepatic expression of pro-inflammatory mediators significantly increases as early as 2 h post-insult (17–21). In turn, these mediators trigger the mobilization and priming of leukocytes from the bone marrow, which then translocate to the site of injury, as well as seemingly uninvolved peripheral organs. The spleen releases its reservoir of pro-inflammatory monocytes and increases expression of IFN-γ, TNF, and IL-6 amongst others (22–24). Systemic inflammatory response syndrome (SIRS) which can lead to multi-organ dysfunction syndrome (MODS) is also not uncommon in patients (25–29). Concurrent immunosuppression of the adaptive immune components is often observed (30, 31), leaving patients also highly susceptible to infections. Peripheral immune responses thus significantly increase patient mortality and morbidity.

Interestingly, suppression of the peripheral inflammatory response has been shown to ameliorate CNS inflammation (20, 32–35). Modulation of the APR by targeting the production of acute phase proteins, or Kupffer cell depletion, both reduce neutrophil recruitment to the CNS in models of TBI and SCI (20, 33). Therefore, suppression of the APR may offer an alternative strategy of minimizing tissue loss and functional deficits after traumatic CNS injuries. However, it must be acknowledged that modulating systemic inflammation is complex; paradoxically, exacerbating periphery inflammation has similarly been shown to reduce lesion size and leukocyte infiltration of the CNS post-injury (36, 37). As such, it has been suggested that the systemic response can also serve as an immune "distraction", redistributing leukocyte populations from the injured CNS to other sites, although it remains unclear to where the leukocytes redistribute (17). It is likely that timing of the inflammatory insult is key, and improving our understanding of it will ease therapeutic targeting.

The initiation signal for the activation of the peripheral response is unclear. Both humoral and neuronal methods have been investigated, yet vagotomized animals still exhibit an APR (38, 39), and thus far no consistent molecular candidates have been identified that can fully explain this response (40). There is growing evidence that extracellular vesicles, novel mediators of communication between distant organs, provide the missing link.

Extracellular Vesicles

Extracellular vesicles (EVs) is a general term that defines all cell-derived particles encapsulated in a lipid bilayer, which are enriched for proteins, lipids, and nucleic acids (41–44). They are typically classified according to their biogenesis (**Figure 1**); apoptotic bodies (1,000–5,000 nm) are released from the plasma membrane as part of programmed cell death, microvesicles (150–1,000 nm) are blebbed from the cell membrane, whilst exosomes (40–150 nm) are generated via the endolysosomal pathway and stored in multivesicular bodies (MVB) prior to release by exocytosis.

Whilst EVs have been investigated as a phenomenon for more than 30 years, the significant role EVs play in intercellular communication is only just being recognized. Indeed, a plethora of studies have identified EVs as important mediators of not only normal physiology, but also of pathology. They have been shown to be released from almost all cell types, including neurons (45, 46), microglia (47, 48), astrocytes (35), and CNS endothelial cells (49). EVs have also been isolated from almost all bodily fluids, including cerebrospinal fluid (CSF) (50, 51) and plasma (52). They have shown a unique capacity to disseminate information around the body, including across the blood-CNS barrier (35), to exert their effects both locally and systemically to distant organs, making them attractive candidate mediators of CNS-to-immune communication following injury.

EV-mediated cell communication has been associated in a number of neurological diseases, where they have been shown to be vectors of pathogenic proteins, propagating both Alzheimer's and Parkinson's disease (53–56). In brain cancers, EVs derived from tumor cells have been shown to act locally in facilitating proliferation, growth and angiogenesis (57–60), as well as distally in other organs aiding metastasis (61). In turn, distal cancers are able to metastasize to the brain via EVs as well (61–63). In the periphery, circulating EVs isolated from LPS-treated animals have been shown to induce gliosis and expression of pro-inflammatory molecules in the brains of naïve mice (64). Moreover, EVs released from stimulated brain endothelial cells have been shown to induce hepatic TNF and CXCL1 expression in naïve rats, in turn inducing a sickness behavior phenotype (49). Together, these studies suggest the presence of a CNS-periphery communicatory axis that is mediated by EVs. As such, investigating EVs in the context of traumatic CNS injuries is of great interest. Here, we will evaluate the current evidence that EVs mediate the communicatory pathways between the CNS and the periphery following traumatic CNS injury.

TRAUMATIC BRAIN INJURY (TBI)

TBI is a devastating disorder, affecting over 55 million people globally (2). The current lack of available treatments is commonly attributed to gaps in our knowledge of the secondary phase

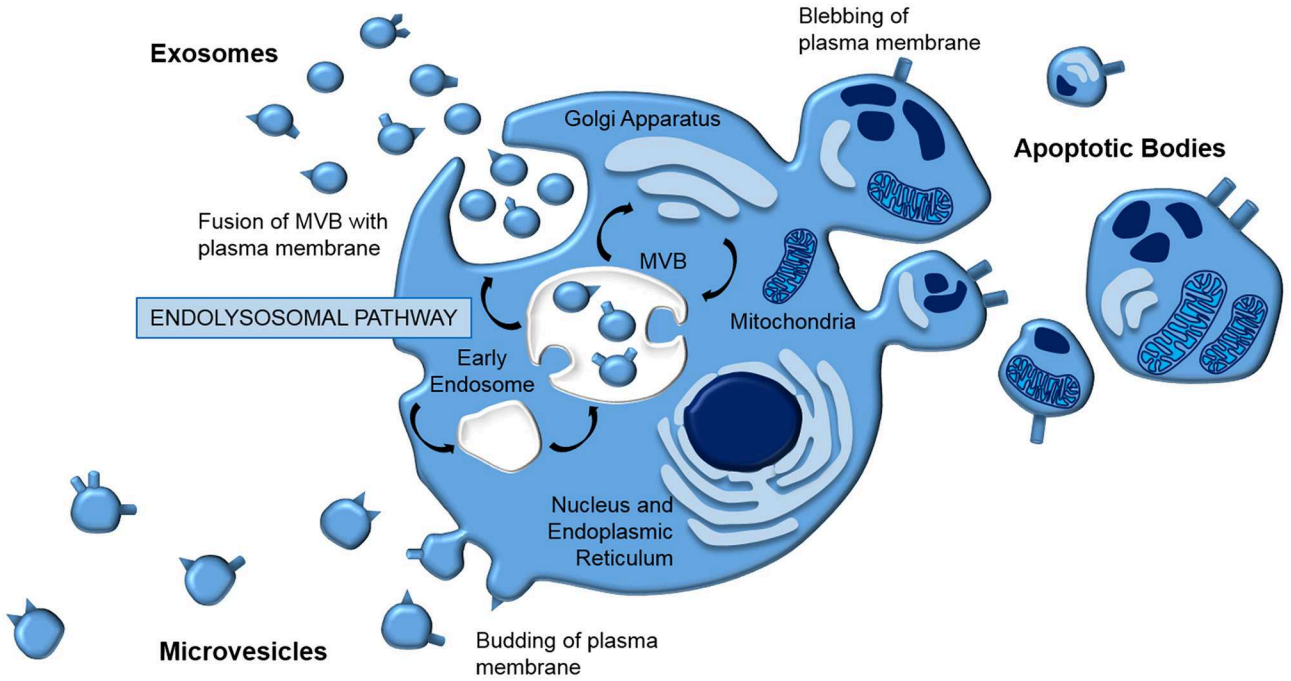

FIGURE 1 | EV biogenesis. EVs are typically classed according to their biogenesis. Apoptotic bodies and microvesicles are released from the plasma membrane in blebbing and budding mechanisms, respectively. In contrast, exosomes are generated by the endolysomal pathway; internal budding of an endosome results in a multivesicular body which fuses with the plasma membrane, releasing exosomes by exocytosis.

of injury (14). Human clinical data has confirmed that TBI induces a robust inflammatory response in the periphery which is predictive of poor outcome (65). In conjunction, numerous studies have consistently demonstrated that circulating EVs are significantly elevated in TBI patients during the acute phase of injury (66, 67). For example, Nekludov et al. (66) showed a transcranial gradient in EV concentration; more EVs were detected in the cerebrovenous compared to arterial blood, indicating that the increase in circulating EVs originated from the brain. Increases in EVs in the circulation of patients with TBI have been reflected in rodent studies (35, 48, 49, 52, 68, 69). Hazelton et al. (52) showed an increase in plasma EVs during the first 24 h after TBI, whilst Couch et al. (49) and Dickens et al. (35) both showed increases in an IL-1β model of inflammatory focal brain lesions. Critically, inhibition of EV release from the CNS has been shown to attenuate the systemic response to brain inflammation, and subsequently inhibit leukocyte infiltration (35). Nekludov et al. further demonstrated that whilst leukocyte- and platelet-derived EVs were increased after injury, the circulating EVs were predominantly of endothelial origin, the concentration of which was 7-fold greater than in healthy controls. Dickens et al. (35) showed however that a proportion of plasma EVs released after striatal IL-1β injection are derived from astrocytes, and that these translocate to the liver, spleen, and lung, further linking EV-mediated signaling with the APR following CNS injury. Microglia and astrocytes both release EVs in response to DAMP-mediated activation with ATP (47). In turn, microglia-derived EVs enriched for IL-1β have been reported in the plasma of TBI patients (48). From these studies, it is easy to assume that EV population changes are due to increased release from cells of the CNS. However, EVs derived from hematopoietic cells can also signal to the brain, and their uptake here was exacerbated by peripheral inflammation (70). Delineating the origin of EVs could identify the critical players in CNS-periphery communication, and may identify a specific cellular target for EV-based therapeutics.

Functional analysis of plasma EVs from models of brain injury determined that plasma EVs were pro-inflammatory and able to induce a systemic inflammatory response in naïve rats, in the absence of CNS injury (49). It has been established that EVs are capable of interacting with granulocytes and lymphocytes; they have been shown to carry MHC class I and II, and contribute to antigen presentation (71–75). Therefore, they may directly activate the peripheral immune system through receptor-ligand mechanisms. Moreover, microvesicles and apoptotic bodies are enriched for phosphatidylserine (PS) on the outer leaflet, which not only assists in promoting budding, but also encourages uptake by macrophages and dendritic cells (76, 77). This is highly relevant considering the ongoing apoptosis of CNS cells post-injury. Kumar et al. (48) showed that EVs depleted of their content with the surfactant PEG-TB had lost their ability to activate microglia *in vitro*, making it clear that the composition of EVs is vital for them to exert their effect.

As well as surface chemistry, EV cargo appears to be key to the function of the EVs after TBI. Plasma EVs isolated from TBI patients were found to have distinct and unique protein profiles in comparison to those isolated from healthy controls (68, 78). When exogenous pro-inflammatory EVs were administered intravenously to a model of TBI, the EVs were found to exacerbate both the APR, and the subsequent neuroinflammation and pathology (52). Importantly, this response was dependent on the cellular origin of the EVs. Particles derived from macrophages had the greatest effect on hepatic expression of pro-inflammatory molecules, as well as infiltrating neutrophils in the brain, compared to those from endothelial cells and plasma samples. Cargo analysis revealed differential miRNA content in the different EVs, suggesting the particles exert their effect through transfer of specific genetic transcripts. Indeed, Dickens et al. (35) identified that miRNA in astrocyte-derived EVs target the PPAR-α pathway, leading to increased NFκB activity and cytokine production in the liver. However, EVs have been found to be enriched for pro-inflammatory molecules themselves, including cytokines, chemokines, and inflammasome proteins. Inflammatory EVs have been reported to transport IL-1β (47, 48, 79, 80), IL-6 and CCL2 (81), as well as chemokine receptors, such as CCR5 (82). Collectively, these studies suggest a more direct mechanism of initiating and propagating inflammation.

In addition to the activation of a systemic inflammatory response, TBI-associated coagulopathy (TBI-AC) has been associated with EV signaling (83). Following injury, TBI patients often develop a hypercoagulable state, leading to an increased risk of thrombosis (84–86). This has been associated with increased mortality (84), and platelet dysfunction has been reported to play a causal role (83). It is thought that the circulating EV population is predominantly shed from platelets (87), and these platelet-derived particles have greater procoagulant activity than platelets themselves (88). TBI induces the release of EVs from platelets (66, 67, 69), and circulating microparticles following TBI were shown to have procoagulant properties *ex vivo* (89). Moreover, Tian et al. (69) were able to reproduce systemic coagulopathy in uninjured mice through adoptive transfer of TBI plasma EVs. Together, these data indicate that platelet-derived EVs may be responsible for TBI-AC, which could be attributed to the exposure of PS on the outer EV leaflet. It is also likely that brain-derived particles interact with platelets directly to promote systemic coagulation and thrombosis. Astrocyte- and neuronal-derived EVs have been isolated from the blood of TBI animals, and were found to be procoagulant in phenotype (69). Thus, EV-mediated changes in systemic function are not limited to alterations in inflammatory status after injury.

SPINAL CORD INJURY (SCI)

In comparison to brain pathologies, the role of EVs following SCI has been somewhat overlooked. Whilst systemic inflammation has been well-documented in SCI patients (26, 90–93), studies have focused on its contribution to functional outcome rather than the manner in which it is communicated. To our knowledge, there is currently no data that describes changes in the circulating EV population and their influence on pathophysiology of SCI. That being said, EVs have been isolated from the CSF of deceased SCI patients (94). These EVs were found to be enriched for the inflammasome-associated proteins NLRP1, caspase-1, and ASC, suggesting a pro-inflammatory phenotype. The authors speculated that these EVs may be able to trigger an innate immune response *in vivo,* which would correspond with TBI associated data, however, EV-mediated effects on systemic inflammation and immune activation were not investigated. These authors additionally demonstrated that neuronal exosomes loaded with siRNA could localize to the lesion epicenter following SCI when injected systemically, further supporting the hypothesis of an EV-mediated CNS-periphery communicatory axis.

Preliminary, unpublished data from our group suggest that SCI induces a significant increase in plasma-derived EVs during the acute phase of injury, which is consistent with human and animal models with brain injuries. However, it is necessary to determine the specific role of these SCI-induced changes in the circulating EV population in propagating peripheral inflammation and the subsequent effect on lesion development. Whilst TBI data may provide some insight, it must be acknowledged that the overall impact on the APR and lesion progression is likely to be different (17). Anatomically, the distribution of gray and white matter, as well as the distribution and phenotype of microglia are quite different in the spinal cord compared to the brain. Moreover, they both respond differently to traumatic injury in that the blood-spinal cord barrier (BSCB) shows greater breakdown after trauma compared to the blood-brain barrier (BBB), and also that there is increased local CXC chemokine expression and recruitment of neutrophils to the parenchyma of the spinal cord compared to the brain. Regarding the systemic response, peripheral administration of the PPARα agonist fenofibrate blocked the APR and neutrophil recruitment to the brain after an intrastriatal microinjection of IL-1β injection (35), however it was found to be an ineffective treatment in experimental SCI (95). These differences must be taken into consideration when assessing the impact of EV signaling following injury, as manipulation of the cascade after SCI may have differential effects on lesion progression and patient recovery compared to TBI.

EVS AS THERAPY

It is clear that interrupting EV signaling may be useful to treat inflammation, but some groups have also used the EVs themselves as a therapeutic agent, specifically EVs derived from stem cells. This strategy is certainly attractive, circumventing the ethical issues with embryonic and fetal stem cells, as well as being less invasive with low or no tumorigenicity. Moreover, the ability to use autografted stem cells will eliminate the risk of rejection. Most studies to date have almost exclusively utilized EVs released by mesenchymal stem cells (MSCs), and these have consistently been shown to improve functional recovery and behavior deficits in models of TBI (96, 97) and SCI (98–100). EVs derived from progenitor cells, such as endothelial colony-forming cells (101) and neural stem cells (102), appear to have similar neuroprotective effects in

animal models. Kobayashi et al. (103) demonstrated that EVs derived from induced pluripotent stem cells (iPSCs) were able to both increase angiogenesis and the rate of wound closure in a model of skin wound healing. Whether iPSC-EVs have therapeutic potential in the context of TBI/SCI remains to be investigated.

The mechanisms underlying the neuroprotective actions of stem cell-derived EVs are currently under investigation. To date, they have been shown to be internalized by endothelial cells (101), neurons (104), astrocytes (104), oligodendrocytes (105), and microglia (106) in the CNS, suggesting they may exert their effect directly. However, improvements after injury are not necessarily due to prevention of cell death, as no change in lesion volume has often been reported (97, 107). Rather, EVs may exert their effect by stimulating endogenous restorative mechanisms that promote recovery. Zhang et al. (97) have shown MSC-EVs enhanced vascular density and neurogenesis, with a concurrent reduction in brain inflammation in a TBI model. Increased angiogenesis has also been shown in a model of SCI (108), following treatment with MSC-EVs. One potential mechanism that has been proposed is the transfer of miRNAs (11). Xin et al. (104) demonstrated that EV-associated miR-133b transferred to astrocytes and neurons was responsible for stimulating neurite outgrowth in their stroke model, and that inhibition of miRNA machinery proteins attenuated this effect (109). Exosomal miR-17-92 (109, 110), miR-134 (105), and miR-124-3p (111) have additionally been implicated in neuroprotection. Bioengineering MSCs to produce EVs overexpressing these transcripts are currently under investigation (110, 112–114). In the majority of these studies, EVs are administered intravenously to the periphery which is important as MSC-EVs have been shown to additionally modulate the systemic immune response following traumatic CNS injuries. In a model of SCI, improvements in locomotor function have been attributed to suppression of the systemic immune response by stimulated MSC-EVs, as circulating neutrophils were reduced and monocytes were retained in the spleen (100). MSC-EVs have been shown to localize to this organ (106), and splenectomies improve neurological outcomes in models of SCI (22); it would be of interest to investigate the effect of MSC-EVs in injury models with splenectomy to determine if their beneficial effect remains.

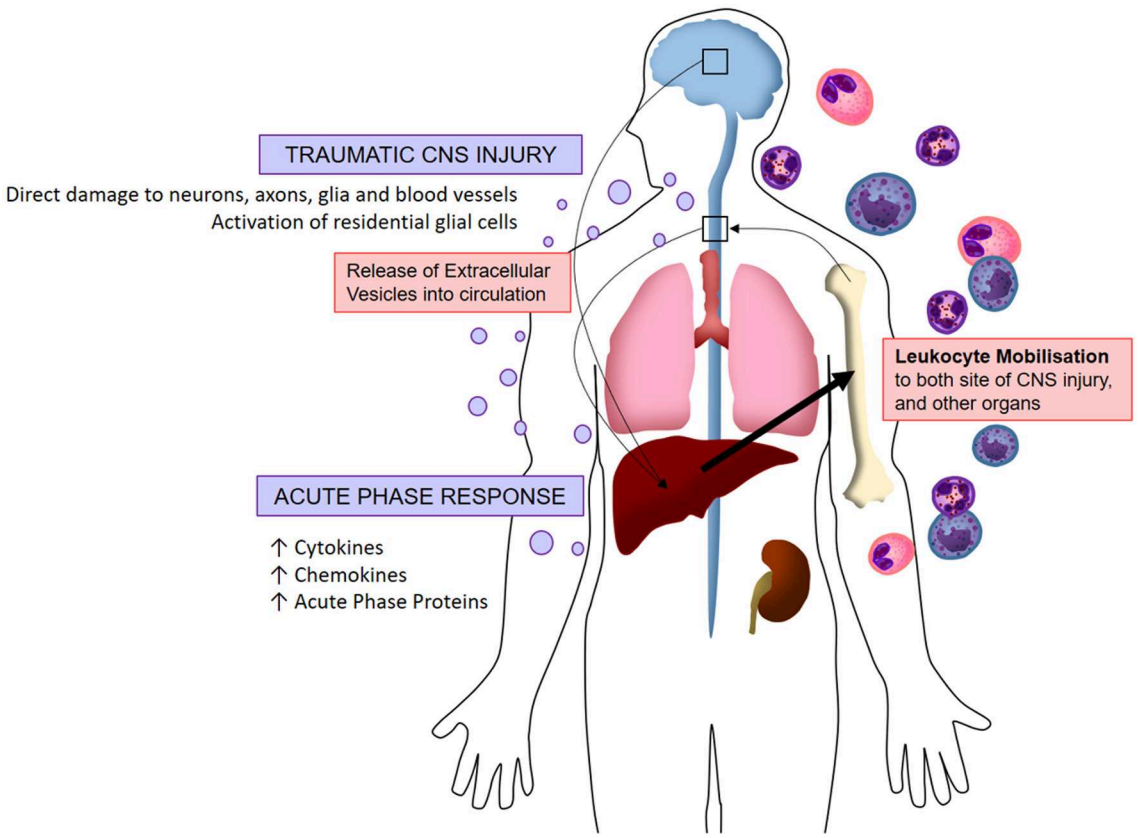

FIGURE 2 | Visualized hypothesis of EV-mediated systemic inflammation response to traumatic CNS injury. Acute traumatic injuries to the brain and spinal cord induce the release of extracellular vesicles into circulation. These EVs localize to peripheral organs whereby they induce the production of pro-inflammatory molecules (chemokines, cytokines, acute phase proteins), in turn stimulating the mobilization of leukocytes which infiltrate both the CNS and peripheral organs. This systemic immune response is referred to as the acute phase response.

CONCLUSIONS

In the last decade, interest in EVs has increased exponentially for both biomarker and therapeutic purposes, as more studies identify EV signaling as a key component of normal physiology and pathology. However, whilst fields such as gynecology have led the way, the investigation of the role that EVs play in the context of acquired neurological diseases is relatively new. Here, we have discussed how it has been consistently shown that the circulating EV population is altered by trauma to the CNS (**Figure 2**). The collected evidence presented here suggests that EVs mediate the systemic response following CNS injury, and that manipulation of this pathway can protect the CNS from secondary damage. However, our understanding of the underlying mechanisms and the consequences of manipulation of the EV population, is limited, and fundamental questions remain. For instance, it is unclear whether EV biogenesis after injury is different from the mechanisms that govern basal EV production. It also remains unclear whether the absolute number of EVs in the circulation is the most important factor, or whether the enrichment of circulating EVs from CNS-derived populations, that is barely detectable in the periphery without specific markers, is more important. Moving forward, it is clear that the role of EVs in the pathogenesis of systemic inflammation following CNS injury warrants further investigation to underpin development of successful therapeutic strategies and improve functional outcomes.

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Quantifications of CSF Apoptotic Bodies Do Not Provide Clinical Value in Multiple Sclerosis

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Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that leads to the death of neurons and oligodendrocytes, which cannot be measured in living subjects. Physiological cellular death, otherwise known as apoptosis, progresses through a series of stages which culminates in the discharge of cellular contents into vesicles known as apoptotic bodies (ABs) or apoptosomes. These ABs can be detected in bodily fluids as Annexin-V-positive vesicles of 0.5–4.0 μm in size. In addition, the origin of these ABs might be detected by staining for cell-specific surface markers. Thus, we investigated whether quantifications of the total and CNS cell-specific ABs in the cerebrospinal fluid (CSF) of patients provided any clinical value in MS. Extracellular vesicles, from CSF of 64 prospectively-acquired subjects, were collected in a blinded fashion using ultra-centrifugation. ABs were detected by flow cytometry using bead-enabled size-gating and Annexin-V-staining. The origin of these ABs was further classified by staining the vesicles for cell-specific surface markers. Upon unblinding, we evaluated the differences between diagnostic categories and correlations with clinical measures. There were no statistically significant differences in the numbers of total or any cell-specific ABs across different disease diagnostic subgroups and no significant correlations with any of the tested clinical measures of CNS tissue destruction, disability, MS activity, and severity (i.e., rates of disability accumulation). Overlap of cell surface markers suggests inability to reliably determine origin of ABs using antibody-based flow cytometry. These negative data suggest that CNS cells in MS either die by non-apoptotic mechanisms or die in frequencies indistinguishable by current assays from apoptosis of other cells, such as immune cells performing immunosurveillance in healthy conditions.

Keywords: multiple sclerosis, cerebrospinal fluid, apoptotic bodies, clinical outcomes, flow cytometry, cell surface markers

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INTRODUCTION

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS), leading to the demyelination of axons and neurodegeneration. Alongside traumatic brain injury, it is the most frequent cause of neurological disability in young adults (1). Findings from prior studies led to the hypothesis that MS can be largely divided into two stages, starting with the inflammatory phase in the periphery and later entering into the neurodegenerative phase (2). Although great progress has been made in understanding the inflammatory components of the disease, the neurodegenerative components are still obscure.

Currently, there are two main ways to measure neurodegenerative process in living human subjects: structural imaging and measurement of neurofilament light chain protein (NFL) (3, 4). Structural (MRI) imaging identifies CNS tissue destruction as brain/spinal cord atrophy. However, MRI imaging fails to provide cellular or molecular information and therefore cannot reliably measure the loss of crucial cell types such as neurons and oligodendrocytes, especially when loss of these CNS cells may be masked by infiltration of CNS tissue by immune cells or by compensatory astrogliosis. With emergence of ultra-sensitive Single Molecule Analysis (Simoa) NFL assay (5, 6) blood (serum or plasma) NFL levels can be measured both in healthy subjects and MS patients. In MS NFL levels are increased during MS activity and they can weakly predict subsequent MS progression on a group, but not individual levels (4, 7–11). Thus, there is still a need to develop biomarkers of neuronal and oligodendroglial injury/loss that can be applied on a patient level.

Pathology studies have shown the presence of apoptotic cells, mainly oligodendrocytes and neurons, at the site of MS lesions (2, 12, 13), suggesting that CNS cell apoptosis plays an important role in the irreversible neurological disability during the progressive stage of MS. This hypothesis was further supported by animal modeling with antiapoptotic protein B-cell lymphoma-2 (Bcl-2)-overexpressing transgenic mice; in comparison with wild type (WT) these transgenic mice showed reduced experimental autoimmune encephalomyelitis (EAE)-severity, despite similar inflammatory response (14).

Apoptotic cells progress through a series of stages including chromatin condensation, DNA fragmentation, membrane blebbing, and cell shrinkage, which all culminate in the discharge of cellular contents into extracellular vesicles, known as apoptotic bodies (ABs) or apoptosomes (15, 16). Previous studies have tried to isolate and identify ABs from subject's body fluid and use them as markers of respective disease-related degenerative processes (17–20).

Thus, the goal of the current study was to identify the presence of apoptotic cells in the CNS of living subjects by measuring the total and cell-specific ABs in cerebrospinal fluid (CSF) of patients. Additionally, we asked whether densities of total or cell-specific ABs differentiate MS from healthy donors (HDs), and within MS patient cohorts correlate with clinical measures of CNS tissue destruction, disability, MS activity and severity (i.e., rates of disability accumulation).

MATERIALS AND METHODS

Cell Cultures and Treatments

Human neuroblastoma cells (SK-N-SH; ATCC# HTB-11, Manassas, VA) were cultured on poly-L-lysine (PLL; Sigma-Aldrich, St. Louis, MO) -coated plates (Costar, Corning, NY), in Dulbecco's modified Eagles medium (DMEM; Gibco, Gaithersburg, MD) supplemented with fetal bovine serum (FBS; Gemini Bio-Products, Sacramento, CA), and sodium pyruvate (Lonza, Walkersville, MD). Cells were either left untreated (Control) or treated with Staurosporine (0.5 μM; R&D Systems Inc., Minneapolis, MN). Twenty four hours after treatment, culture supernatants were collected and stored on ice until further use. Cells were washed with phosphate-buffered saline (PBS; Gibco, Gaithersburg, MD) and detached from plate using

TABLE 1 | Subjects' demographics data based on their disease diagnosis.

	Diagnosis	HD	NIND	OIND	CIS	RR-MS	P-MS
N	Female/male	4/6	1/4	0/12	2/0	11/6	11/7
Age	Average	43.8	42.2	56.8	53.3	46.2	60.6
	SD	12.1	13.1	13.3	6.1	10.3	6.0
	Range	24.3–60.5	26.4–60.3	24.5–70.0	48.9–57.6	24.2–66.5	49.9–70.0
Clinical disease activity	Active/non-active	NA	NA	NA	NA	4/13	2/16
COMRIS-CTD	Average	NA	NA	NA	NA	11.7	15.9
	SD	NA	NA	NA	NA	7.4	6.2
	Range	NA	NA	NA	NA	2.2–24.2	1.5–25.1
EDSS	Average	NA	NA	NA	NA	2.4	5.3
	SD	NA	NA	NA	NA	1.6	1.8
	Range	NA	NA	NA	NA	1.0–6.5	2.5–7.5
CombiWISE	Average	NA	NA	NA	NA	20.5	43.7
	SD	NA	NA	NA	NA	12.3	16.4
	Range	NA	NA	NA	NA	6.9–51.2	20.5–70.0
MS-DSS	Average	NA	NA	NA	NA	1.3	2.2
	SD	NA	NA	NA	NA	0.8	1.0
	Range	NA	NA	NA	NA	0.5–3.4	0.5–4.0
CombiWISE slope	Average	NA	NA	NA	NA	1.7	1.9
	SD	NA	NA	NA	NA	1.5	1.5
	Range	NA	NA	NA	NA	−1.2–4.6	−1.1–4.3

trypsin-EDTA solution (Sigma-Aldrich); detached cells were pelleted and stored on ice until further use.

CSF Collection and Processing

All subjects were recruited under IRB-approved protocols (Comprehensive Multimodal Analysis of Neuroimmunological Diseases of the Central Nervous System, ClinicalTrials.gov Identifier: NCT00794352; and Evaluation and Follow-up of Patients with Cryptococcosis, ClinicalTrials.gov Identifier: NCT00001352) and all patients provided written informed consent. CSF from subjects were collected per standardized operating procedures (21). CSF aliquots were prospectively labeled using alphanumeric code, stored on ice until further use and analyzed in a blinded fashion.

Isolation of Apoptotic Bodies

ABs were isolated from culture supernatants and CSF as previously described (19, 22–24). Briefly, cells were isolated and removed by pelleting at 335 g for 10 min. To remove cell-debris, cell-free supernatants were centrifuged at 1,000 g for 10 min; followed by another centrifugation at 2,000 g for 30 min to pellet ABs. Pelleted ABs were resuspended and washed with PBS.

Flow Cytometry

ABs were stained with Annexin V-FITC, and cells were stained with Annexin V-FITC and Propidium Iodide (TACS® Annexin V Kit; Trevigen Inc., Gaithersburg, MD) as per manufacturer's instructions. CSF ABs were also stained for CNS cell-specific surface markers to identify their origin: We used cell-surface markers previously employed in isolation of human CNS cells from brain specimens using immune-panning, and validated by cell-specific RNA profiles (25, 26): CD90 (Neuronal surface marker; Human CD90/Thy1 APC-conjugated Antibody; R&D Systems, Minneapolis, MN; Clone # Thy-1A1), HepaCAM (Astroglial surface marker; Human HepaCAM Antibody; R&D Systems; Clone # 419305; tagged with DyLight 405; Novus Biologicals, Centennial, CO), GalC (Oligodendroglial surface

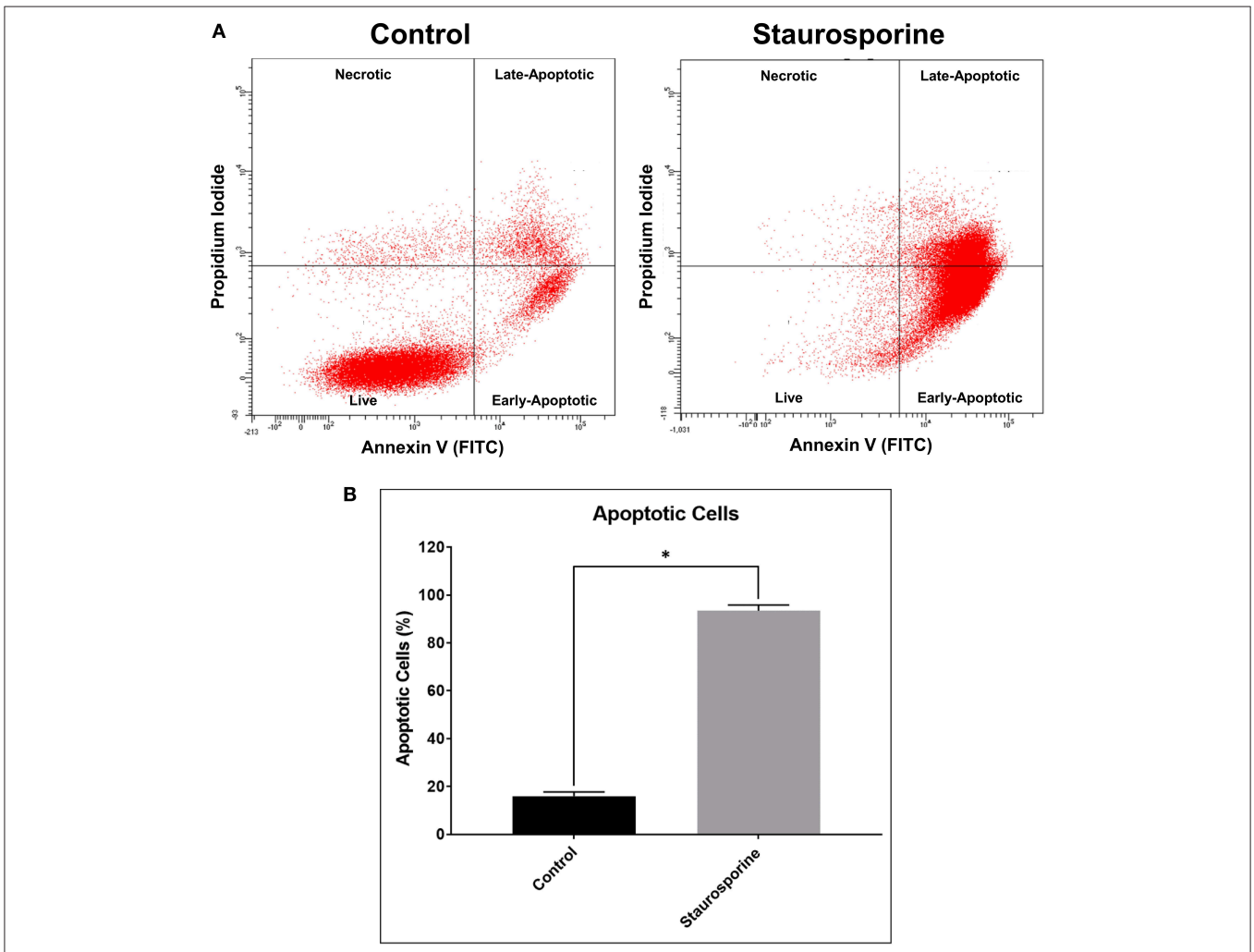

FIGURE 1 | (A) Representative flow cytometry images of cells stained with Annexin V-FITC and propidium iodide after control or Staurosporine (0.5 μM) treatment for 24 h. **(B)** Plot of apoptotic cells (%). The error bars represent standard deviation ($n = 6$); data were analyzed using Wilcoxon test, $P = 0.031$. *$p < 0.05$.

marker; Anti-Galactocerebroside Antibody; EMD Millipore, Burlington, MA; Clone # mGalC; tagged with PerCP-Cy5.5; Novus Biologicals), CD31 (Endothelial cell surface marker (27); Human CD31/PECAM-1 PE-conjugated Antibody; R&D Systems; Clone # 9G11), and CD14 (Myeloid lineage cell surface marker (28); Alexa Fluor® 700 anti-human CD14 Antibody;

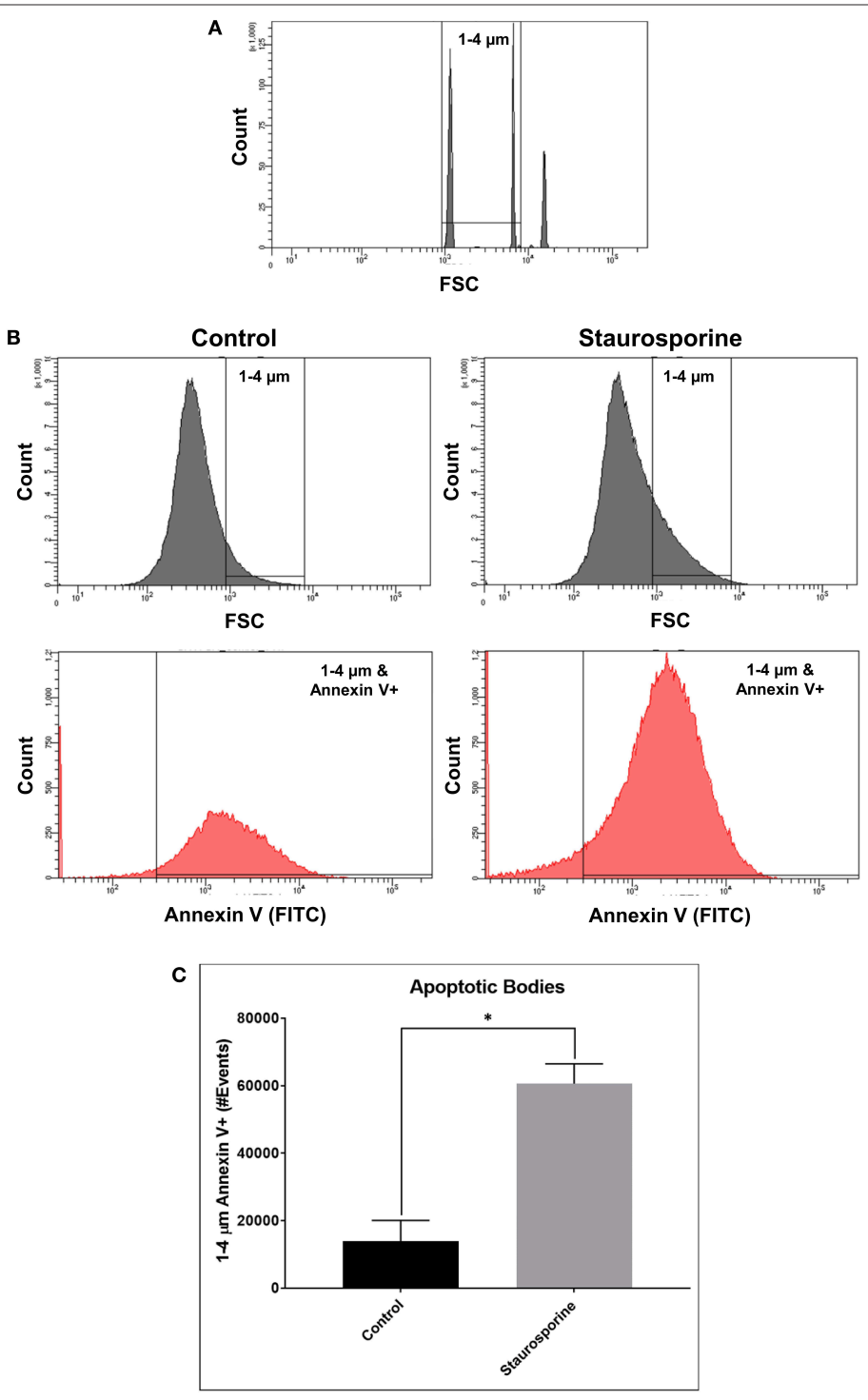

FIGURE 2 | (A) Flow cytometry images of size calibration beads (1, 4, and 6 µm beads). **(B)** Representative flow cytometry images indicating process of ABs identification using size gate (1–4 µm) and Annexin V-FITC staining, from cell culture supernatants after control or Staurosporine (0.5 µM) treatment. **(C)** Plot of ABs (1–4 µm and Annexin V-positive events). The error bars represent standard deviation ($n = 6$); data were analyzed using Wilcoxon test, $P = 0.031$. $^*p < 0.05$.

BioLegend, San Diego, CA; Clone # HCD14). Briefly, after wash with PBS, pelleted ABs were resuspended in Annexin V-FITC and fluorescence-tagged antibodies against cell-specific surface markers in Annexin V-binding buffer (provided with TACS® Annexin V Kit) and incubated in dark for 15 min at room temperature. Stained ABs were washed with binding buffer and then analyzed using fluorescence-activated flow cytometer (BD LSR II Flow Cytometer, BD Biosciences, San Jose, CA). Gating on ABs included size gate [1–4 μm (29)], using Flow Cytometry Size Calibration Kit (ThermoFisher Scientific, Grand Island, NY). The vesicles in 1–4 μm size gate were further analyzed for Annexin V and cell-specific surface markers' staining.

Subjects' Demographics Data

A total of 64 CSF samples were analyzed. After unblinding diagnostic codes, this cohort consisted of healthy donors (HD, $n = 10$), non-inflammatory neurological disorders (NIND, $n = 5$), other inflammatory neurological disorders (OIND, $n = 12$; mainly, comprised of Cryptococcal Meningitis patients), clinically isolated syndrome that did not yet fulfill MS diagnostic criteria (CIS, $n = 2$), relapsing-remitting MS (RR-MS, $n = 17$), and progressive MS [P-MS, comprised of both secondary- (SP-MS) and primary-progressive MS (PP-MS), $n = 18$] (**Table 1**). MS diagnostic subgroups (CIS, RR-MS, SP-MS, and PP-MS) were classified using McDonald's criteria, 2010 revisions (30). MS cohort (both RR- and P-MS) was further separated based on disease activity (active vs. non-active MS) using clinical relapses and new contrast-enhancing or new MRI lesions.

Statistical Analyses

ABs data for subjects' CSF samples were acquired with the operator blinded to subjects' clinical diagnoses. After data acquisition for all subjects, ABs per ml of CSF were compared across disease diagnostic subgroups (HD, NIND, OIND, RR-MS, and P-MS) using one-way ANOVA; as we have acquired CSF samples from only two CIS subjects, they were not included in analyses. Also, within MS subjects, ABs per ml of CSF were compared across disease activity (active vs. non-active

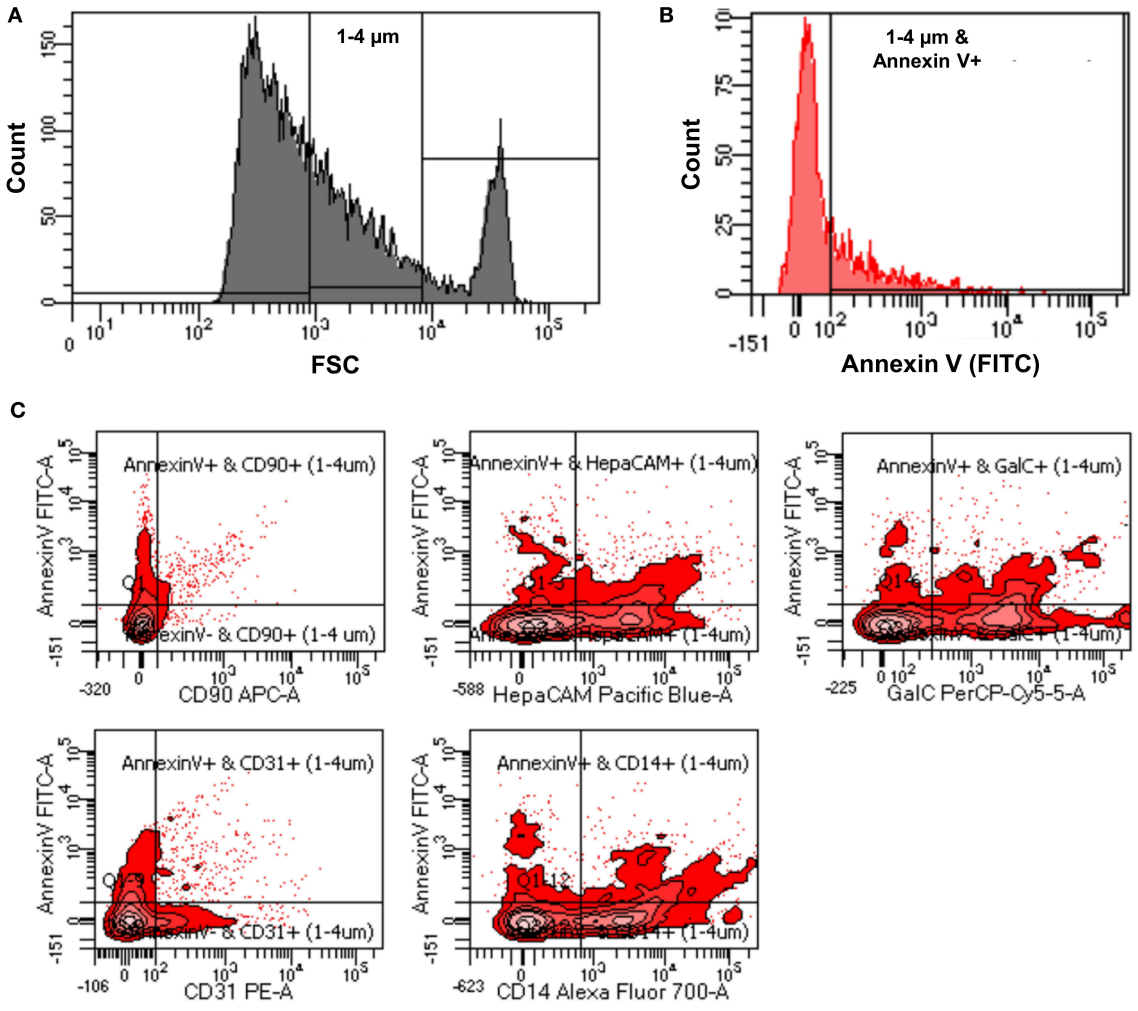

FIGURE 3 | (A) Representative flow cytometry image indicating how size gate was used to select 1-4 μm vesicles from patients' CSF samples. **(B)** Representative flow cytometry image showing process of identification of ABs from 1 to 4 μm sized vesicles using Annexin V-FITC staining (1–4 μm and Annexin V-positive events). **(C)** Representative flow cytometry images showing how origin of ABs was identified using CNS cell-specific surface markers staining.

MS) using non-parametric (Mann–Whitney) test. Within MS subjects, ABs per ml of CSF were correlated with machine-learning-optimized clinical and imaging measures of CNS tissue destruction [Composite MRI scale of CNS tissue destruction, COMRIS-CTD (31)], disability [Expanded Disability Status Scale, EDSS (32) and Combinatorial Weight-Adjusted Disability Scale, CombiWISE (33)], severity [Multiple Sclerosis Disease Severity Scale, MS-DSS (34)], and disability progression slopes (CombiWISE Slope) derived from linear regression models from CombiWISE measurements during longitudinal follow-up after LP collection using Spearman correlation analysis (GraphPad Prism 7; GraphPad Software Inc., La Jolla, CA).

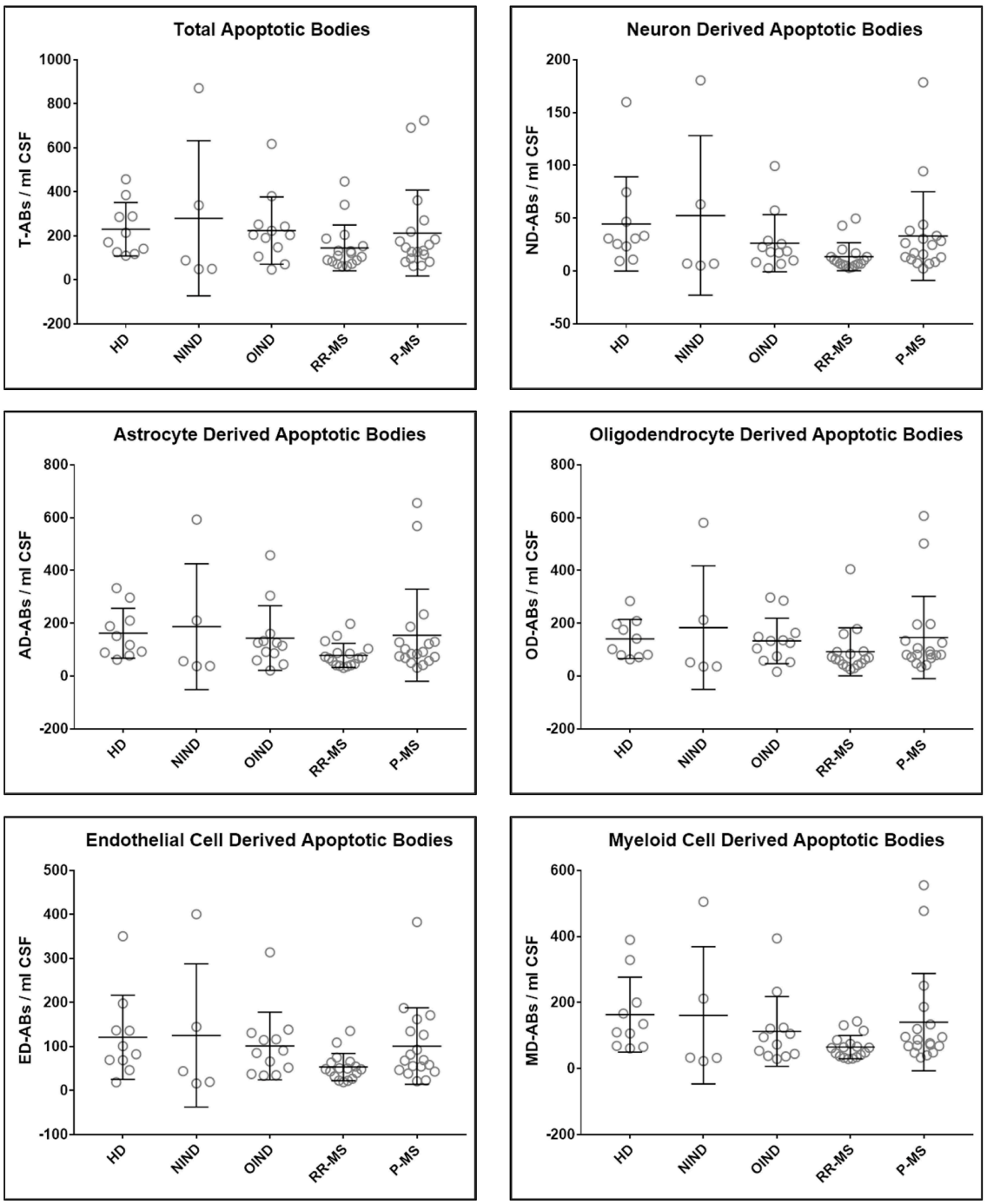

FIGURE 4 | Plots of total and CNS cell-specific ABs adjusted for CSF volume (ABs/ml CSF). Each point represents individual subject; and error bars represent standard deviation (*n*: HD = 10, NIND = 5, OIND = 12, RR-MS = 17, and P-MS = 18); data were analyzed using one-way ANOVA.

RESULTS

In vitro Model Validation

We validated our "identification and assessment of Abs" model using human neuronal cell line (SK-N-SH) cultures. As a positive control for induction of apoptosis we used Staurosporine treatment (0.5 μM, 24 h). Apoptotic cells were identified by staining with Annexin V and PI and were analyzed using flow cytometry. According to manufacturer's (TACS® Annexin V Kit) instructions both Annexin V and PI-negative cells are live, only Annexin V-positive cells are early-apoptotic, both Annexin V- and PI-positive cells are late-apoptotic and only PI-positive cells are necrotic (**Figure 1A**). After Staurosporine treatment, the % of apoptotic cells was significantly elevated (**Figure 1B**).

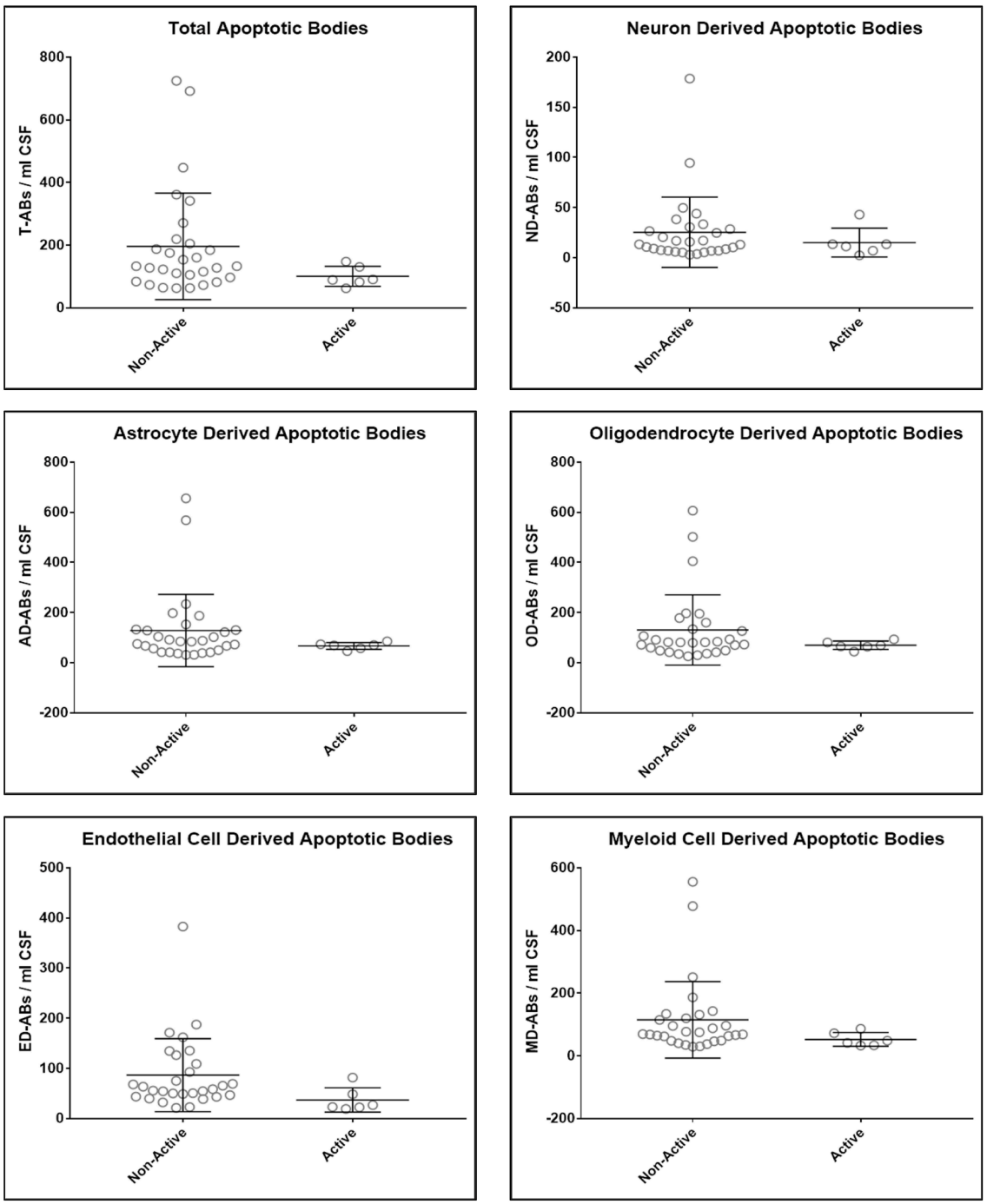

FIGURE 5 | Plots of total and CNS cell-specific ABs adjusted for CSF volume (ABs/ml CSF). Each point represents individual MS subject; and error bars represent standard deviation (*n*: non-active MS = 29 and Active MS = 6); data were analyzed using nonparametric (Mann–Whitney) test.

Quantifying the induction of apoptosis by Staurosporine in our culture conditions, we next sought to quantify ABs in cell culture supernatants in order to demonstrate that our assay could differentiate between the release of ABs from control and Staurosporine-treated cultures. To this end, size gates [1–4 μm, an average size of ABs (29)] were applied in combination with Annexin V staining. First, 1–4 μm size gates were set using 1, 4, and 6 μm beads (**Figure 2A**; Flow Cytometry Size Calibration Kit, ThermoFisher Scientific). Within 1–4 μm vesicles ABs were identified as Annexin V positive (**Figure 2B**). Total ABs were quantified (1–4 μm and Annexin V-positive events); after Staurosporine treatment the total number of ABs in cell culture supernatants were significantly elevated (**Figure 2C**).

Analyses of CSF Apoptotic Bodies

Verifying the flow-cytometry-based ABs detection in cell culture supernatants, we next applied the same assay to prospectively-acquired CSF samples. As described in the methods, extracellular vesicles were collected using the differential centrifugation approach. 1–4 μm vesicles were selected using size gate set by known sized beads (**Figure 3A**). From these vesicles total ABs were detected using Annexin V staining (1–4 μm and Annexin V-positive events; **Figure 3B**).

While total number of ABs in the CSF may be clinically useful, we considered the possibility that apoptosis occurs in the intrathecal compartment also under physiological conditions: e.g., any activated immune cell may cross the blood brain barrier as a part of active immunosurveillance mechanism. Such immune cell (e.g., T lymphocyte), if not re-activated in the CNS compartment, may either re-cycle back to blood via lymphatic system, or undergo apoptosis as part of physiological termination of the immune response (35, 36). Thus, we envisioned that the assay that could identify the cellular origin of ABs may have significantly higher clinical utility. To this end, we employed fluorescently-tagged antibodies specific for surface markers of different CNS cells. Selected surface markers/antibodies were previously validated as CNS cell-types-specific, because they were used to isolate specific CNS cells (i.e., neurons, oligodendrocytes, astrocytes, microglia, and endothelial cells) from human brain samples via immunopanning. Subsequent sequencing of thus-isolated CNS cell types validated that expected cell-specific transcripts were only expressed in appropriate CNS cell-type (25, 26).

The utilized cell-specific markers were: CD90+ ABs (neurons derived ABs), HepaCAM+ ABs (astrocytes derived ABs), GalC+ ABs (oligodendrocytes derived ABs), CD31+ ABs (endothelial cells derived ABs), and CD14+ ABs (myeloid cells derived ABs) (**Figure 3C**). Total number of ABs and cell-specific ABs were adjusted for CSF volume to obtain the number of ABs per ml of CSF (ABs/ml).

Upon unblinding the diagnostic categories, we observed no statistically significant differences in number of total as well as CNS cell-specific ABs across disease diagnostic subgroups or MS activity (**Figures 4**, **5**). However, while using non-overlapping cell-surface markers (i.e., each selected cell surface marker is specific for one CNS cell type and should not be expressed on any other CNS cells), we observed substantial co-expression of these markers on individual ABs. This overlap could be quantified by how much the sum of cell-specific ABs exceeds total ABs (**Figure 4** and **Supplementary Data File 1**). Because the sum of cell-specific ABs always exceeded total number of ABs, we conclude that ABs most likely exhibited non-specific binding of antibodies. High non-specific antibody binding is a well-known problem affecting apoptotic cells, as apoptosis-induced changes in plasma cell membrane upregulate "eat me" signals recognized by phagocytes, including enhanced, non-specific binding of antibodies (37–39).

Consequently, we observed no correlations between the numbers/concentrations of total or any cell-specific ABs with accurate clinical and imaging measures CNS tissue destruction, disability, MS severity, and disability progression (**Table 2**).

DISCUSSION

MS has been studied extensively regarding the inflammatory component of disease (40). However, neurodegenerative component of MS, or immune-mediated destruction of specific CNS cells cannot be measured in living subjects. In this study, we attempted to analyze apoptosis in living subjects by assessing ABs in CSF. While there have been previous attempts to

TABLE 2 | Correlation analysis (Spearman *r* and *P*-values) between adjusted total (T-ABs) and CNS cell-specific ABs (ND-ABs, neurons; AD-ABs, astrocytes; OD-ABs, oligodendrocytes; ED-ABs, endothelial cells; and MD-Abs, myeloid cells) in subjects' CSF (ABs/ml CSF) and their clinical measures of CNS tissue destruction, disability, and severity.

		T-ABs	ND-ABs	AD-ABs	OD-ABs	ED-ABs	MD-ABs
COMRIS-CTD	Spearman *r*	−0.20	−0.02	−0.10	−0.13	−0.19	−0.08
	P-value	0.25	0.89	0.57	0.46	0.26	0.66
EDSS	Spearman *r*	−0.07	0.23	0.07	0.01	0.07	0.15
	P-value	0.69	0.19	0.70	0.97	0.68	0.38
CombiWISE	Spearman *r*	−0.11	0.25	0.07	0.02	0.00	0.13
	P-value	0.53	0.14	0.68	0.92	0.99	0.45
MS-DSS	Spearman *r*	−0.18	0.04	−0.08	−0.09	−0.13	−0.04
	P-value	0.29	0.84	0.64	0.63	0.44	0.82
CombiWISE slope	Spearman *r*	−0.29	0.05	−0.05	−0.08	−0.30	−0.08
	P-value	0.09	0.80	0.76	0.64	0.08	0.65

analyze blood/serum extracellular vesicles as markers of CNS disorders (41–43), there is no evidence for the presence of CNS ABs in blood/serum; this is likely due to their large size (0.5–4 μm) (16, 24, 29, 44) which prevents ABs from crossing the blood brain barrier (BBB) or their rapid elimination from the blood by the splenic or hepatic reticulo-endothelial system. Moreover, blood/serum naturally has a basal level of ABs from immune system cells which arise during regular immune responses (45, 46).

Our *in-vitro* studies validated that selected flow-cytometry assay measures ABs released to culture supernatants. Additionally, using CNS cell-specific surface markers previously validated in human immunopanning isolation of specific CNS cell-types provided high expectation that enumeration of CNS cell-specific ABs may be of clinical value. Unfortunately, after breaking the diagnostic codes we observed no differences between diagnostic categories and no correlations with any clinical or imaging outcomes of disability, CNS tissue destruction or MS severity. While some may argue that our study was under-powered to detect differences between diagnostic categories, we had good representation of subjects from all four diagnostic categories and found no biologically plausible trends. We concluded that expanding our dataset using the same assay would be futile, as such test could never be applied on a patient-level and therefore cannot outperform current tests such as NFL.

There are several possible interpretations of our negative results: as cell-surface proteins are often shed during apoptosis (47–49) and changes in cell membrane structure induced by apoptosis increase non-specific binding of antibodies (37–39), accurate determination of the origin of apoptotic bodies using flow cytometry may not be possible. The interference from non-specific binding is supported by the observed overlap of multiple CNS cell-type specific surface markers on the individual ABs. If non-specific antibody binding, rather than shedding of cell-surface markers from apoptotic cells was the main cause of our negative results, then attempting to use alternative reagents for detection of cell-surface molecules, such as DNA-aptamers (50) may be of use. Unfortunately, such alternative reagents are not commercially available for validated CNS cell-surface markers. Flow cytometry may also not be an ideal method for analyzing ABs, as older flow cytometers have low resolution for subcellular particles. Our employment of enhanced gating guided by size beads and validation of our assay in cell-culture supernatants mitigated this impediment.

The fate of ABs after their release from the CNS cells is unknown; while some may be secreted to the CSF via extracellular fluid, others, perhaps most, are likely phagocytosed closer to their origin (51–53). Especially, in the context of pro-inflammatory environment rich in myeloid cells such as activated microglia and infiltrating macrophages, this local capture of ABs may be much more efficient in MS and OIND controls than in healthy subject, mitigating expected differences between diagnostic categories. Thus, CSF concentrations of ABs may not reliably reflect their CNS origin.

We conclude that measuring ABs in the CSF using flow cytometry does not provide desired clinical value. We present our negative report in an effort to prevent other investigators from pursuing this path without incorporating substantial technical advancement that may mitigate problems identified in our study. Thus, a need to develop CNS cell-specific biomarkers reflective of neurodegenerative mechanisms associated with CNS diseases remains.

DATA AVAILABILITY STATEMENT

The flow cytometry data of patients' CSF ABs and their demographic and clinical information are provided in **Supplementary Data File 1**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NIH-CNS-IRB. Written informed consent to participate in the study was provided either by participant or by his/her legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BB and RM designed the study. RM, JM, and JP performed the experiments. RM, PW, and BB analyzed the data. RM wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, 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/fneur.2019.01241/full#supplementary-material

Supplementary Data File 1 | Flow cytometry data for all subjects' CSF samples including their MRI and clinical measures (COMRIS-CTD, EDSS, CombiWISE, MS-DSS, and CombiWISE Slope), clinical diagnosis and demographics (age and gender).

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Novel Neuropathic Pain Mechanisms Associated With Allergic Inflammation

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Allergic diseases are associated with central and peripheral nervous system diseases such as autism spectrum disorders and eosinophilic granulomatosis with polyangiitis, which frequently causes mononeuritis multiplex. Thus, it is possible that patients with an atopic constitution might develop multifocal inflammation in central and peripheral nervous system tissues. In a previous study in Japan, we reported a rare form of myelitis with persistent neuropathic pain (NeP) in patients with allergic disorders. However, the underlying mechanism of allergic inflammation-related NeP remains to be elucidated. First, we analyzed the effect of allergic inflammation on the nociceptive system in the spinal cord. Mice with atopy showed microglial and astroglial activation in the spinal cord and tactile allodynia. In a microarray analysis of isolated microglia from the spinal cord, endothelin receptor type B (EDNRB) was the most upregulated cell surface receptor in mice with atopy. Immunohistochemical analysis demonstrated EDNRB expression was upregulated in microglia and astroglia. The EDNRB antagonist BQ788 abolished glial activation and allodynia. These findings indicated that allergic inflammation induced widespread glial activation through the EDNRB pathway and NeP. Second, we investigated whether autoantibody-mediated pathogenesis underlies allergic inflammation-related NeP. We detected specific autoantibodies to small dorsal root ganglion (DRG) neurons and their nerve terminals in the dorsal horns of NeP patients with allergic disorders. An analysis of IgG subclasses revealed a predominance of IgG2. These autoantibodies were mostly colocalized with isolectin B4- and P2X3-positive unmyelinated C-fiber type small DRG neurons. By contrast, immunostaining for S100β, a myelinated DRG neuron marker, showed no colocalization with patient IgG. Immunoprecipitation and liquid chromatography-tandem mass spectrometry identified plexin D1 as a target autoantigen. Patients with anti-plexin D1 antibodies often present with burning pain and thermal hyperalgesia. Immunotherapies, including plasma exchange, are effective for NeP management. Therefore, anti-plexin D1 antibodies may be pathogenic for immune-mediated NeP, especially under allergic inflammation conditions. Thus, allergic inflammation may induce NeP through glial inflammation in the spinal cord and the anti-plexin D1 antibody-mediated impairment of small DRG neurons.

Keywords: neuropathic pain, allergic inflammation, glia, endothelin, plexin D1

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INTRODUCTION

Allergic diseases are associated with central and peripheral nervous system diseases such as autism spectrum disorders (1–3) and eosinophilic granulomatosis with polyangiitis, which frequently causes mononeuritis multiplex (4, 5). These observations indicate that patients with an atopic constitution develop multifocal inflammation in central nervous system (CNS) and peripheral nervous system (PNS) tissues (6).

We previously reported a rare form of myelitis with persistent neuropathic pain (NeP) in Japanese patients with allergic diseases (7, 8). Nationwide surveys have found that this form of myelitis is widely distributed in Japan (6, 9). Similar cases have also been reported in Western countries (10, 11). Patients with this form of myelitis as well as atopy often showed cervical cord involvement, mainly in the posterior lesion, and exhibited sensory impairment including NeP in all four limbs (6, 9). We found a loss of myelin and axon and eosinophil infiltration in biopsied spinal cord lesions from these patients (12, 13). Thus, we designated this form of myelitis "atopic myelitis (AM)" and established diagnostic criteria (14). Definite AM is defined as: (1) patients meeting the absolute criteria [myelitis with unknown etiology; positivity for allergen-specific IgE; and absence of brain MRI lesions fulfilling the Barkhof criteria for MS (15)] plus the pathological criteria (spinal cord biopsy samples showing existence of perivascular lymphocyte cuffings with various degrees of eosinophil infiltration, sometimes accompanied by granuloma); or (2) patients meeting the absolute criteria plus at least two of the three supporting positive criteria [present or past history of atopic disease; serum hyperIgEemia; increased level of interleukin (IL)-9 or eotaxin in cerebrospinal fluid (CSF)] plus one supporting negative criterion (no oligoclonal bands in CSF). Probable cases of AM are defined as: (1) patients meeting the absolute criteria plus one of the supporting positive criteria plus the one supporting negative criterion; or (2) patients meeting the absolute criteria plus at least two of the supporting positive criteria. In patients with AM, there were significant positive correlations between disease duration and Kurtzke Expanded Disability Status Scale score (16) and Sensory Functional scale score (17). However, the underlying mechanism of allergic inflammation-related NeP remains to be elucidated.

Recent studies have established a crucial role of immune system activation in modulation of NeP (18, 19). Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon gamma (IFNγ), IL-1β, IL-6, and IL-17, were shown to be elevated in sera and CSF of patients with NeP (20, 21). Because receptors for these cytokines are expressed on sensory neurons, pro-inflammatory cytokines may exert direct effects on nociceptive sensory neurons and induce NeP. Moreover, treatment with anti-inflammatory cytokines, such as IL-4 and IL-10, was reported to alleviate NeP in animal models (22, 23). Moreover, passive transfer of Th1 cells to athymic nude rats lacking mature T cells enhanced pain hypersensitivity in the recipient mice (24). In contrast, passive transfer of polarized Th2 cells attenuated pain hypersensitivity in the recipient mice. These findings suggest that Th2-dominant allergic inflammation may be protective for NeP. However, in clinical practice, we often encounter patients with both allergic disease and severe NeP (6), suggesting that other NeP mechanisms are operative. Accumulating evidence indicates that activation of spinal microglia, resident macrophages in the CNS, is crucial for NeP generation and modulation (25, 26). Peripheral nerve damage induces microglial activation in the dorsal horn of the spinal cord. Activated microglial mediators in the spinal dorsal horn, such as TNF-α, IL-1β, and brain-derived neurotrophic factor (BDNF), increase excitatory synaptic transmission and cause NeP via neuron-glial interactions (27). We further focused on B cell hyperactivation, which induces NeP through production of autoantibodies against antigens in the somatosensory pathway in response to the allergic condition (19, 28). Indeed, autoantibodies against sensory neurons were detected in autoimmune diseases associated with pain, such as Guillain–Barré syndrome (29) and complex regional pain syndrome (CRPS) (30), and depletion of B cells reduced NeP in CRPS model mice (31).

In this Mini Review, we will discuss the possible NeP mechanisms associated with allergic inflammation, on the basis of findings from animal models of allergic disease and autoantibodies against sensory neurons of patients with allergic diseases.

ALLERGIC INFLAMMATION INDUCES NEUROPATHIC PAIN THROUGH THE ACTIVATION OF GLIAL CELLS

First, we analyzed the effect of allergic inflammation on the nociceptive system of the spinal cord in an animal model of allergic disease (32). We induced atopic diathesis, bronchial asthma, or atopic dermatitis in C57BL/6 mice by intraperitoneal sensitization with ovalbumin (OVA) (50 μg) and aluminum hydroxide hydrate (2 mg) on days 0, 7, and 14 (atopic diathesis model), followed by nasal aspiration of OVA solution (2.5 mg/ml) for 4 consecutive days (days 15–18) (bronchial asthma model) or direct OVA application (100 μg) on tape-stripped skin (atopic dermatitis model). Mice with atopy showed microglial and astroglial activation in the dorsal horn of the spinal cord. A higher expression of FBJ murine osteosarcoma viral oncogene homolog B (FosB), a neuronal activation marker, was also seen in the dorsal horn of mice with atopy compared with mice without atopy. Additionally, we found activated endothelial cells and extravasation of serum albumin in atopic mice, suggesting blood–brain barrier (BBB) impairment. There was neither demyelination nor axonal degeneration in the spinal cord of mice with atopy. We used von Frey filaments to evaluate tactile allodynia in mice with atopy (33) and found that atopy model mice had severe tactile allodynia.

In a microarray analysis of isolated microglia from the spinal cord of mice with atopy, microglia showed an augmented pro-inflammatory signature, including IL-1β, CD38, and prostaglandin-endoperoxide synthase 2, which are known to be upregulated in activated microglia (34, 35). Endothelin receptor type B (EDNRB) was the most upregulated cell surface microglial receptor in mice with atopy. Immunohistochemical analysis confirmed that EDNRB expression was upregulated in microglia

and astroglia, and that spinal cord neurons did not express EDNRB. Meanwhile, endothelin receptor type A (EDNRA), another main receptor for endothelin, was not detected in microglia, astroglia, and neurons of the spinal cord of atopic mice. We further found increased levels of endothelin-1 (ET-1), an EDNRB ligand, in serum by ELISA, and observed marked up-regulation of ET-1 in alveolar epithelial cells and epidermis of atopic mice by immunohistochemistry. We then analyzed whether a selective EDNRB antagonist, BQ-788, would affect glial activation and tactile allodynia in atopic mice. BQ-788 treatment abolished microglial, astroglial, and neuronal activation and allodynia. Because the neuronal expression of EDNRB was not detected in atopic mice, the EDNRB antagonist primarily acted on microglia and astroglia rather than neurons. Thus, microglia and astroglia are important for the emergence of allergic inflammation-related NeP via the ET-1/EDNRB pathway.

We also conducted a neuropathological examination of autopsied spinal cord lesions from a patient with AM. We found microglial and astroglial activation in the dorsal horn of the spinal cord and the loss of myelin and axons, as seen in previously biopsied AM cases (12, 13). EDNRB expression was upregulated in microglia and astroglia, similar to in our atopy model mice. Moreover, we found elevated serum ET-1 levels in AM patients compared with healthy controls without atopy. Together, these findings indicate that allergic inflammation induces widespread glial activation, which persistently activates the nociceptive system in the spinal cord via the ET-1/EDNRB pathway.

ANTI-PLEXIN D1 ANTIBODY-RELATED NEUROPATHIC PAIN IN PATIENTS WITH ALLERGIC DISEASES

Allergic inflammation can enhance autoantibody production (28) and plasma exchange has been reported to improve NeP in patients with AM (6, 36). Therefore, we investigated whether an autoantibody-mediated mechanism underlies allergic inflammation-related NeP.

We screened novel autoantibodies against dorsal root ganglion (DRG) neurons and the dorsal horn, which are involved in generating NeP, in patients with various neurologic diseases including AM, using a tissue-based indirect immunofluorescence assay (IFA) (37). We found specific autoantibodies against small DRG neurons and their nerve terminals in the dorsal horn of the spinal cord (37), and these autoantibodies were more frequently detected in patients with NeP than subjects without NeP (10% vs. 0%; $p < 0.05$). IgG subclass analysis revealed a predominance of IgG2, which weakly activates complement. These autoantibodies mostly colocalized with isolectin B4 (IB4)- and P2X3-positive unmyelinated C-fiber type small DRG neurons. By contrast, immunostaining for S100β, a myelinated DRG neuron marker, showed no colocalization with patient IgG. These findings showed that NeP patients' IgG binding was restricted to unmyelinated DRG neurons. In the dorsal horn of the spinal cord, patient IgG axonal staining colocalized with a lamina I marker calcitonin gene-related peptide (CGRP) and lamina II marker IB4. Therefore, IgG binding in patients with anti-small DRG neuron antibodies was restricted to the superficial dorsal horn (laminae I and II). These autoantibodies also bound to vasoactive intestinal peptide (VIP)-positive postganglionic parasympathetic nerve fibers in the skin. In western blotting (WB) using mouse DRG, these autoantibodies recognized a common 220 kDa band. Liquid chromatography-tandem mass spectrometry with immunoprecipitates revealed plexin D1 was the autoantigen.

Plexin D1 is a receptor for semaphorin 3E, an axon guidance factor and immune regulator (38) expressed in the nervous system, B cells, macrophages, endothelial cells, and skin (38). Given that the presence of plexin D1 in DRG sensory neurons has not been investigated, we assessed the expression of plexin D1 in human DRG sensory neurons (37). Immunohistochemical analysis of human DRG and spinal cord tissues with an anti-human plexin D1 antibody revealed that plexin D1 was expressed in small DRG neurons and the superficial dorsal horn. The immunostaining of small DRG neurons and spinal dorsal horn by IgG from all anti-small DRG neuron antibody-positive patients was removed by pre-incubation with recombinant human plexin D1 extracellular domain in a concentration-dependent manner (37). Therefore, we confirmed plexin D1 is a relevant autoantigen. Additionally, plexin D1 extracellular domain contains antigenic epitopes for autoantibody recognition. Then, we performed a propidium iodide (PI) assay to assess plasma membrane permeability using dissociated mouse DRG neurons and heat-inactivated sera from NeP patients with anti-plexin D1 antibodies. Heat-inactivated sera from NeP patients with anti-plexin D1 antibodies showed a significant increase in the percentage of PI-positive cells compared with those without anti-plexin D1 antibodies (37). These findings suggest that anti-plexin D1 IgG2 antibodies may invade the DRG where the BBB and blood–nerve barrier are absent, bind to plexin D1 on the surface of unmyelinated C-fiber type DRG neurons, and impair the plasma membranes of small pain-conveying neurons, resulting in their dysfunction.

In **Table 1**, we have summarized the clinical features of patients with anti-plexin D1 antibodies based on our previous study (37). The patients with anti-plexin D1 antibodies were predominantly female, although the difference in anti-plexin D1 antibody positivity rates between female and male patients with NeP was not significant (12.3 vs. 5.4%; $p = 0.33$). The age at onset was relatively young. The clinical courses were relapsing or fluctuating. The underlying neurological diseases of 11 patients with anti-plexin D1 antibodies included atopic myelitis, neuromyelitis optica spectrum disorders, multiple sclerosis, neurosarcoidosis, and erythromelalgia. The common comorbidities in patients with anti-plexin D1 antibodies were allergic diseases and collagen diseases. The patients commonly developed burning pain, thermal hyperalgesia, and peripheral vascular dysfunction symptoms. The current perception threshold test showed abnormalities of C-fibers. Plasma exchange and intravenous methylprednisolone pulse therapy were effective for NeP management. These findings suggest that anti-plexin D1 antibodies may be pathogenic in immune-mediated NeP, especially under allergic inflammation conditions.

TABLE 1 | Clinical findings for 11 patients with anti-plexin D1 antibodies.

Characteristic	Summary
Female sex, number (%)	9 (81.8)
Age at onset, mean ± SD (range), years	26.3 ± 13.3 (12–53)
Underlying diseases, number (%)	AM 6 (54.5), NMOSD 2 (18.2), RRMS 1 (9.1), neurosarcoidosis 1 (9.1), erythromelalgia 1 (9.1)
Coexisting disorders, number (%)	Allergic diseases 10 (90.9), collagen-vascular diseases 4 (36.4), malignant neoplasms 1 (9.1)
Clinical course, number (%)	Relapsing 9 (81.8), fluctuating 2 (18.2)
Neurological manifestations, number (%)	NeP 11 (100), sensory impairment 11 (100), motor weakness 10 (90.9), hyperreflexia 10 (90.9), peripheral vascular autonomic dysfunction symptoms 5 (45.5), hand muscle atrophy 2 (18.2), visual impairment 2 (18.2)
Quality of NeP, number (%)	Burning 6 (54.5), tingling 6 (54.5), thermal hyperalgesia 5 (45.5), allodynia 2 (18.2), pinprick hyperalgesia 2 (18.2), squeezing 2 (18.2)
Electrophysiological findings, number (%)[a]	MEP abnormality of CNS 8 (72.7), CPT abnormality of C-fiber 6 (100), SEP abnormality of CNS 4 (36.4), SEP abnormality of PNS 3 (27.3), NCS abnormality 3 (33.3), QSART abnormality 1 (100)
Immunotherapy response for NeP, number (%)[b]	Improved 7 [mPSL pulse 4 and mPSL pulse plus PE 3] (100)

[a]*Percentage among tested patients who underwent each electrophysiological examination.* [b]*Percentage among patients treated with various immunotherapies. CNS, central nervous system; CPT, current perception threshold; MEP, motor-evoked potentials; mPSL, methylprednisolone; NCS, nerve conduction study; NeP, neuropathic pain; NMOSD, neuromyelitis optica spectrum disorders; PE, plasma exchange; PNS, peripheral nervous system; QSART, quantitative sudomotor axon reflex test; RRMS, relapsing-remitting multiple sclerosis; SEP, somatosensory-evoked potentials.*

HYPOTHETICAL MECHANISMS UNDERLYING ALLERGIC INFLAMMATION-RELATED NEUROPATHIC PAIN

Glial Activation in Allergic Inflammation

Allergic diseases are associated with a risk for autism spectrum disorders (ASD) and attention-deficit and hyperactivity disorder (ADHD) (1, 2, 39). Moreover, microglia and autoantibodies against brain proteins are also associated with the pathogenesis of ASD (40–42). A recent transcriptome study using cortical tissue samples from patients with ASD showed microglial activation in cortical tissues of ASD patients (43). In an animal model of ASD, microglia from the offspring of mothers with allergic asthma exhibited epigenomic alterations in dysregulated genes (44). Therefore, allergic inflammation may contribute to the pathogenesis of ASD through microglial activation. However, it is unknown how allergic inflammation causes microglial activation. ASD children had significantly higher serum levels of anti-myelin basic protein (MBP) and anti-myelin-associated glycoprotein (MAG) antibodies than healthy children and the levels of autoantibodies against MBP and MAG were significantly correlated with the presence of allergic symptoms (45). Therefore, allergic inflammation might induce the production of autoantibodies against neurons and glial cells, which leads to CNS damage. However, no specific autoantibodies produced by allergic inflammation have been identified.

In our previous study (32), expression of EDNRB was upregulated in spinal microglia and astroglia from atopic mice and an autopsied AM case. By contrast, expression of EDNRA was not detected in microglia and astroglia of atopic mice. In the normal condition, expression of EDNRA in the spinal cord is observed in vascular smooth muscle cells and the superficial dorsal horn (primary afferent nerve fibers), while expression of EDNRB in the spinal cord is observed in radial glia, a small population of astroglia, ependymal cells, and vascular endothelial cells (46) (**Supplementary Table 1**). Therefore, allergic inflammation can induce overexpression of EDNRB in microglia and astroglia in the spinal cord.

We also found an overproduction of ET-1 in sera, alveolar epithelial cells, and skin tissues from atopic mice and elevated serum ET-1 in patients with AM. Previous studies reported increased ET-1 expression in the epidermis of atopic dermatitis patients (47) and the bronchial epithelium of asthma patients (48). Additionally, several studies reported that ET-1 attenuated BBB permeability (49). Therefore, the overproduction of ET-1 in inflamed tissues may induce BBB hyperpermeability and activate microglia and astroglia via the ET-1/EDNRB pathway in allergic inflammation. Then, glial activation might activate second-order sensory neurons in the dorsal horn of the spinal cord, causing NeP (**Figure 1**).

A previous study showed that the ET-1/EDNRB pathway has dual effects on the nociceptive system in response to pathological conditions (50). The ET-1/EDNRB pathway exhibited pro-nociceptive effects in inflammatory pain models (51, 52). Furthermore, because ET-1 enhances capsaicin-induced release of substance P and CGRP, as nociceptive mediators, from isolated sensory neurons without EDNRB expression, ET-1 induced pro-nociceptive effects independently of EDNRB (53). In contrast, the ET-1/EDNRB pathway exerted anti-nociceptive effects in a subcutaneous hindpaw ET-1 injection model (54) and a bone cancer model (55). In our atopic mice, the ET-1/EDNRB pathway exhibited pro-nociceptive effects. Although EDNRA is normally expressed in small DRG neurons while EDNRB is expressed in satellite glial cells and myelinating Schwann cells surrounding axons (56) (**Supplementary Table 1**), we have not investigated the PNS expression of EDNRA and EDNRB in our atopic mice. Further studies are required to achieve a deeper understanding of the nociceptive effects of ET-1 in allergic inflammation.

Mechanism of Anti-plexin D1 Antibody Production in Allergic Inflammation

Although NeP patients with anti-plexin D1 antibodies have various underlying neurological diseases, they have common coexisting comorbidities, mainly allergic diseases (37), that enhance the production of autoantibodies (28). Therefore, the production of anti-plexin D1 antibodies is considered to be associated with allergic inflammation. Interestingly, the anti-plexin D1 IgG main subclass was IgG2, which predominantly recognizes carbohydrate epitopes (57). Plexin D1 is heavily

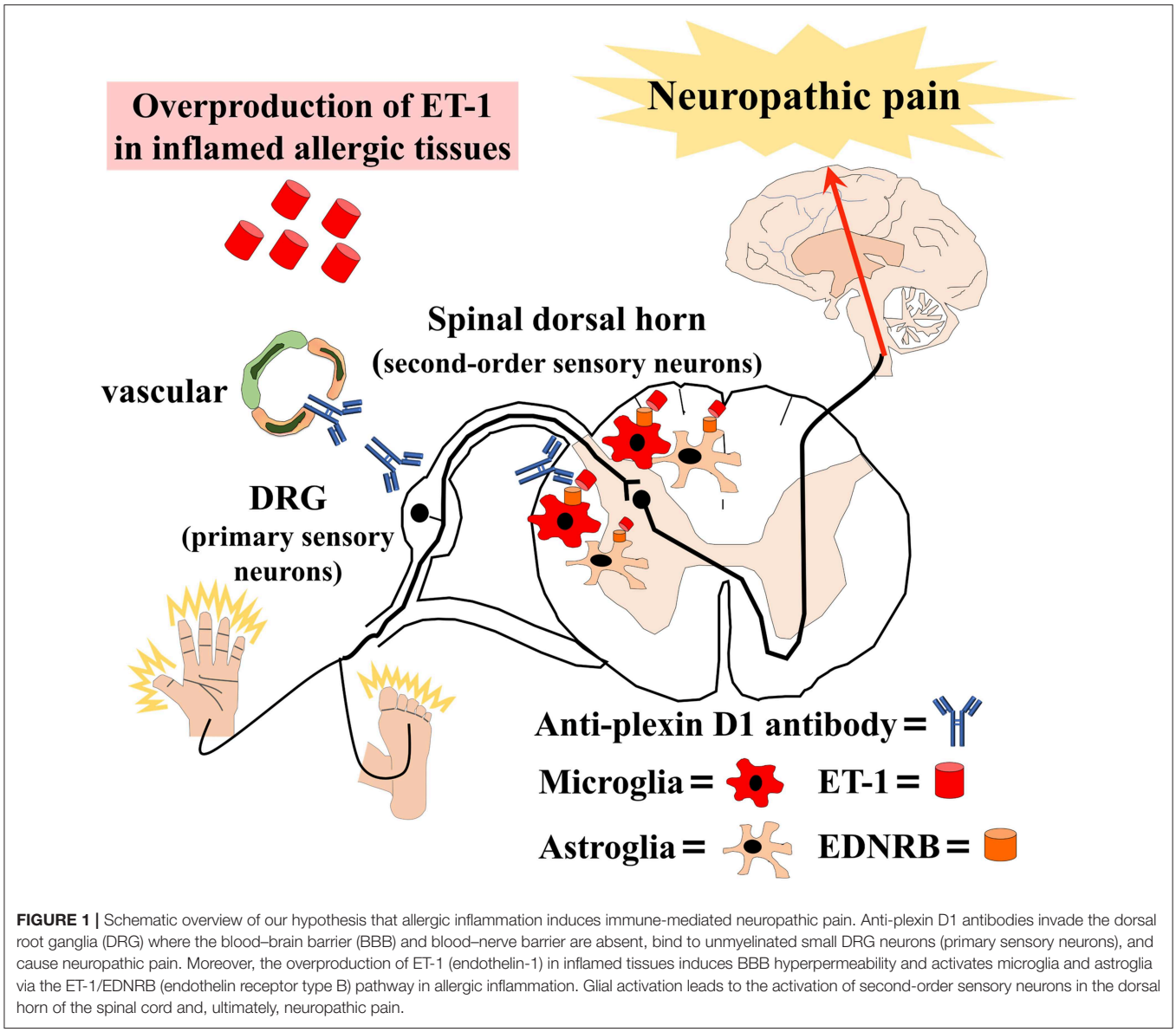

FIGURE 1 | Schematic overview of our hypothesis that allergic inflammation induces immune-mediated neuropathic pain. Anti-plexin D1 antibodies invade the dorsal root ganglia (DRG) where the blood–brain barrier (BBB) and blood–nerve barrier are absent, bind to unmyelinated small DRG neurons (primary sensory neurons), and cause neuropathic pain. Moreover, the overproduction of ET-1 (endothelin-1) in inflamed tissues induces BBB hyperpermeability and activates microglia and astroglia via the ET-1/EDNRB (endothelin receptor type B) pathway in allergic inflammation. Glial activation leads to the activation of second-order sensory neurons in the dorsal horn of the spinal cord and, ultimately, neuropathic pain.

glycosylated, especially at the extracellular IPT/TIG3 domain, which is the same region that immunoprecipitates as identified by mass spectrometry (37). IgG2 is preferentially produced against polysaccharides of environmental microorganisms. AM patients frequently have high levels of IgE antibodies to mite antigens, such as *Dermatophagoides pteronyssinus* (*Dpt*) and *Dermatophagoides farinae*, which are also heavily glycosylated (6, 9). Of note, IgG2 antibodies were reported to comprise up to 50% of antibodies against *Dpt* in atopic patients with high levels of anti-*Dpt* IgE antibodies (58). Thus, allergic inflammation may facilitate anti-plexin D1 antibodies through the molecular mimicry of carbohydrates such as plexin D1 and environmental allergens, including *Dpt*. IgG2 is a low inducer of complement activation and antibody-dependent cell-mediated cytotoxicity compared with IgG1 (57, 59), which might explain the observation that anti-plexin D1 antibody-positive NeP patients, especially AM patients, experience only minor disabilities other than NeP (6).

Action of Anti-plexin D1 Antibodies

Neurological manifestations of NeP patients with anti-plexin D1 antibodies commonly include burning pain and thermal hyperalgesia (37). These symptoms reflect C-fiber type DRG neuron impairment (60). Because anti-plexin D1 antibodies specifically bind to C-fiber DRG neurons, anti-plexin D1 antibodies might be the cause of C-fiber type DRG neuron impairment and NeP. Indeed, in our *in vitro* study, anti-plexin D1 antibodies induced membrane hyperpermeability and cellular swelling of DRG neurons independent of complement activation. Because plexin D1 regulates cytoskeleton stability through actin polymerization (61), anti-plexin D1 antibodies may induce

complement-independent cytotoxicity to DRG neurons through the dysregulation of cytoskeleton stability.

CONCLUSION

On the basis of the above-mentioned findings, we propose that increased humoral immunity in allergic individuals may cause anti-plexin D1 antibody production through molecular mimicry with environmental allergens (**Figure 1**). Anti-plexin D1 antibodies can invade the DRG where the blood–nerve barrier is absent and damage primary pain-conducting neurons, triggering NeP. In addition, allergy may induce the activation of spinal microglia and astroglia via the ET1/EDNRB pathway, which might activate second-order sensory neurons and predispose allergic individuals to NeP. Although there is no evidence of a direct interaction between the ET-1/EDNRB and semaphorin/plexin D1 pathways, activation of the ET-1/EDNRB pathway may allow anti-plexin D1 antibodies to invade the CNS via the hyperpermeable BBB. Plasma exchange can remove circulating serum ET-1 and anti-plexin D1 antibodies, and ameliorate NeP associated with allergic inflammation.

Given that the prevalence of allergic diseases has been increasing over recent decades (62), we predict that allergic inflammation-related neurological diseases will also increase. Therefore, a better understanding of the neuro-immune interactions in allergic diseases might lead to novel therapeutic approaches to treat allergy-related neurological diseases.

AUTHOR CONTRIBUTIONS

TF, RY, and JK: study concept and design, manuscript development, writing, and funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01337/full#supplementary-material

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MINI REVIEW
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The Muscle Is Not a Passive Target in Myasthenia Gravis

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Myasthenia gravis (MG) is a rare autoimmune disease mediated by pathogenic antibodies (Ab) directed against components of the neuromuscular junction (NMJ), mainly the acetylcholine receptor (AChR). The etiological mechanisms are not totally elucidated, but they include a combination of genetic predisposition, triggering event(s), and hormonal components. MG disease is associated with defective immune regulation, chronic cell activation, inflammation, and the thymus is frequently abnormal. MG is characterized by muscle fatigability that is very invalidating and can be life-threatening when respiratory muscles are affected. MG is not cured, and symptomatic treatments with acetylcholinesterase inhibitors and immunosuppressors are life-long medications associated with severe side effects (especially glucocorticoids). While the muscle is the ultimate target of the autoimmune attack, its place and role are not thoroughly described, and this mini-review will focus on the cascade of pathophysiologic mechanisms taking place at the NMJ and its consequences on the muscle biology, function, and regeneration in myasthenic patients, at the histological, cellular, and molecular levels. The fine structure of the synaptic cleft is damaged by the Ab binding that is coupled to focal complement-dependent lysis in the case of MG with anti-AChR antibodies. Cellular and molecular reactions taking place in the muscle involve several cell types as well as soluble factors. Finally, the regenerative capacities of the MG muscle tissue may be altered. Altogether, the studies reported in this review demonstrate that the muscle is not a passive target in MG, but interacts dynamically with its environment in several ways, activating mechanisms of compensation that limit the pathogenic mechanisms of the autoantibodies.

Keywords: myasthenia, muscle, neuromuscular junction, acetylcholine receptor, autoimmunity, cytokines, transcriptome

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INTRODUCTION

Autoimmune Myasthenia Gravis (MG) is characterized by muscular weakness aggravated by exercise and improved by rest. The symptoms fluctuate, which makes the clinical diagnosis difficult. MG is mediated by antibodies (Ab) to components of the neuromuscular junction (NMJ), the muscle is thus the target of the autoimmune attack. About 85% of MG patients present Ab against the acetylcholine receptor (AChR) (1). In about 5% of MG patients, the autoreactive Ab target the muscle-specific kinase (MuSK) protein (2), which is involved in the clustering of AChRs (3). More recently, the agrin receptor LRP_4 (low-density lipoprotein receptor-related protein 4), which forms a complex with MuSK, has been recognized as a novel autoantigen in a small proportion of MG patients without anti-AChR or -MuSK Ab (4). Antibodies to cortactin and agrin (5, 6) have been described, but their presence is most often concomitant to one of the other types of Ab.

MG is a complex disease to which genetic predispositions and defects of the immune system contribute (7–9). Thymic abnormalities are frequently found in the subgroup of MG with anti-AChR Ab but not in that with anti-MuSK Ab (10), and thymectomy has clinically favorable effects in AChR-MG (11), but not in MuSK-MG (12). MG patients with anti-AChR Ab can be classified in several subgroups according to the age of onset, the gender, thymic pathology, and anti-AChR antibodies [Reviewed in (13)]. While the defects of the immune system are richly described (7, 14, 15), reviews on the mechanisms taking place at the level of the muscle tissue are more sporadic (16–18), therefore we will focus on this aspect.

ULTRASTRUCTURAL AND PHYSIOLOGICAL CHANGES OF THE NMJ IN MG

The development and maintenance of the NMJ are primarily dependent on the agrin-MuSK-LRP_4 signaling system (19, 20). LRP_4 and MuSK are anchored in the post-synaptic membrane. Agrin, secreted by the nerve terminal, binds to LRP_4, which then binds to the extracellular domain of MuSK, resulting in phosphorylation and activation of MuSK (19). Phosphorylated MuSK recruits then Dok-7, an adaptor protein that becomes phosphorylated and recruits additional signaling molecules essential for synapse formation and AChR clustering (21).

Detailed structure and mechanism of the NMJ have been described in several reviews (22–25). Briefly, the post-synaptic membrane is characterized by deep junctional folds, the top of which are rich in AChRs, while voltage-gated Na+ channel (VGSCs) are concentrated in the depths [Review in (22, 24)]. There are ∼10,000 AChR per square micrometer on the muscle surface in the motor plate, whereas the concentration is negligible outside the synaptic area. At the presynaptic side, 150,000–300,000 vesicles contain a quantum of acetylcholine (ACh) each (∼10 000 molecules). Upon local depolarization, one quantal content (about 20 vesicles) is released in the synaptic cleft. The binding of ACh to AChRs induces an entry of Na+ into the muscle fibers, causing the local depolarization of the membrane and forming the endplate potential (EPP). The EPP stimulates the opening of the VGSCs, and upon reaching the firing threshold, a further influx of Na+ ions ensues, and the action potential spreads along the muscle fiber. It reaches and opens the stocks of intracellular calcium that finally trigger the muscle contraction (**Figure 1A**). In the healthy NMJ, the amplitude of EPP exceeds the threshold necessary to produce an action potential in the muscle. The ratio between the actual EPP and the threshold required to generate an action potential represents the safety factor of neuromuscular transmission, which is especially important during intense activation of the NMJ (26). In humans, the safety factor is about two, whereas it is higher in rodents or feline (27).

In AChR-MG disease, morphometric analysis reveals degenerative changes of the postsynaptic regions with widening and simplification of synaptic clefts and accumulation of debris in the synaptic zone (28, 29) (**Figure 1B**). In addition, nerve terminals are often smaller than normal size, and their sprouting may be observed (28). The degradation of the post-synaptic membrane results in a decrease in the expression of the AChR and the VGSCs channels, both contributing to the significant reduction of the safety factor: (1) EPP is lowered by the partial loss of functional AChRs and (2) the firing threshold is raised due to the reduction in the density of the sodium channels (30). During prolonged synaptic activity, as the quantal content of ACh normally runs down, the summation of EPP falls below the threshold, and they can no longer trigger the action potential of the muscle fibers (**Figure 1B**, numbers 1, 2, 3, 4). Then, several NMJ will present perithreshold EPP and intermittent transmission failures concomitantly, and the summation of several progressive blocks of NMJ transmission will lead to the MG symptoms (31).

Interestingly, the extraocular muscles (EOM) have physiologically less developed post-synaptic folding, hence a lower baseline safety factor, which could explain their high predisposition to dysfunction in MG (32). Furthermore, in ocular MG, these muscles are susceptible to complement-mediated attack due to a deficiency in complement-inhibitory proteins of the EOM and orbital tissue (33).

MECHANISMS OF ACTION OF THE Ab

Anti-AChR Ab

The pathogenicity of anti-AChR Ab has been shown by their ability to transfer the disease to control animals (34) and to reduce the number of α-bungarotoxin binding sites in myotube cultures (35). There is no correlation between the clinical severity of the disease and the Ab titer, but there is a correlation between the Ab titer and the ability of the sera to degrade AChR *in vitro* (36). However, in patients with immunosuppressive treatment, the changes in the level of anti-AChR antibodies is correlated with the clinical score (37).

Anti-AChR Ab can reduce the expression of muscle AChR by several mechanisms (**Figure 1B**): (1) removal of AChRs due to cross-linking and subsequent internalization (number 2); (2) functional AChR block (number 3), and (3) activation of complement with formation of membrane-attack complexes (MAC) that cause focal lysis (number 4) [Review in (38)]. Anti-AChR Ab are mainly IgG1 and IgG3 isotypes that bind the complement. This mechanism is likely the most pathogenic one: (a) there is an inverse relationship between the integrity of junctional folds and the abundance of C9, one molecule of the MAC (39); (b) mice mutated for complement factors (C3, C4, C5, C6) develop a lower incidence of MG upon active immunization, and their NMJ does not harbor the MAC [Review in (38)]; (c) Some patients with refractory MG have significant, often rapid, improvement in symptoms when treated with eculizumab, that inhibits the cleavage of C5 (40); (d) NMJ degradation decreases the safety factor and the efficacy of the transmission (41).

Anti-MuSK Ab

As a receptor tyrosine kinase, MuSK interacts with a plethora of proteins and downstream pathways, some of which involved in nuclear anchoring, gene transcription, Wnt interactions,

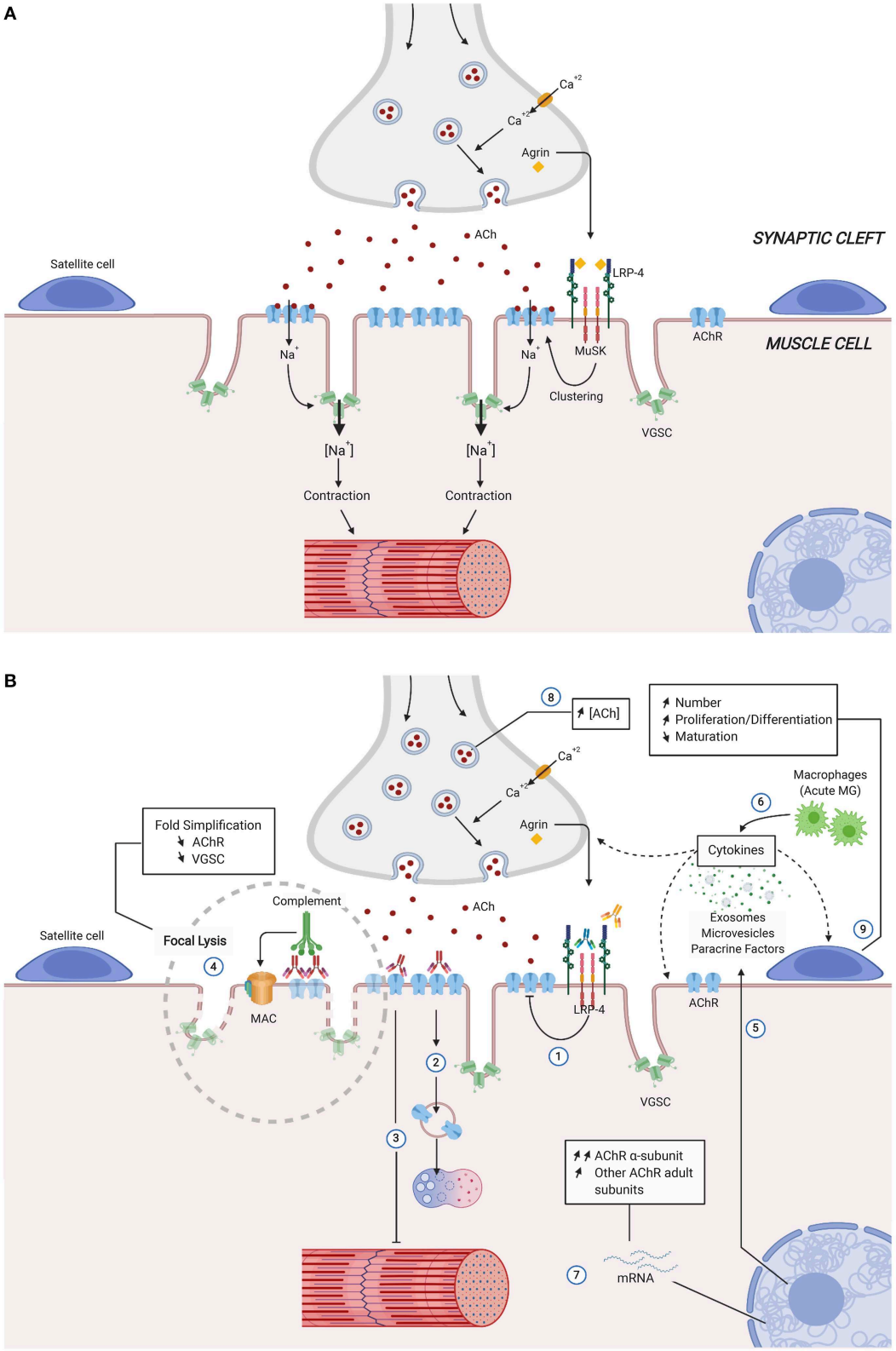

FIGURE 1 | Simplified structure and function of the neuromuscular junction **(A)** and physiological Changes in autoimmune Myasthenia Gravis **(B)**. Direct, indirect consequences of the various autoantibodies and compensatory mechanisms, are identified by numbers. Anti-MuSK and anti-LRP$_4$ autoantibodies act mainly by

(Continued)

FIGURE 1 | inhibiting AChR clustering (1). Anti-AChR antibodies reduce the expression of muscle AChR by removal of AChRs due to cross-linking, internalization, and degradation (2), functional AChR block (3), and activation of complement with formation of membrane-attack complexes that cause focal lysis (4). Blinding of anti-AChR antibodies also include muscle production of paracrine factors, microvesicles and exosomes, as well as cytokines (5) with potential effects over neighboring structures (satellite cells, muscle cells and nerve terminal). Pro-inflammatory environment can be enhanced during MG acute phase by infiltrating macrophages release of cytokines (6). Compensatory mechanism at molecular (7,8) and cellular levels (9) preserve MG muscle fibers from the AChR autoantibodies induced damage. Ach, Acetylcholine; AChR, Acetylcholine receptor; LRP-4, low-density lipoprotein receptor-related protein; MAC, membrane attack complex; MuSK, muscle specific kinase; VGSC, voltage-gated sodium channel.

scaffolding, and AChR stabilization (20). MuSK-MG is often characterized by muscle atrophy and excellent response to plasma exchanges. Experimentally, animals that received repeated daily injections of patient IgG (42) or actively immunized with MuSK (43) show impaired neuromuscular transmission, with reductions in endplate AChR and EPP amplitudes [Review in (44)]. *In vitro*, anti-MuSK Ab induce inhibition of proliferation of a cell line, an effect correlated with disease severity and anti-MuSK Ab titer, that could explain the muscle atrophy in MuSK+ MG patients (45). The isotype of anti-MuSK Ab is generally IgG4 that lacks complement-activating properties and is considered functionally monovalent and is thus unable to induce antigenic modulation (46). Anti-MuSK Ab bind to a structural epitope in the first Ig-like domain of MuSK, prevent binding between MuSK and LRP_4 and inhibit agrin-stimulated MuSK phosphorylation resulting in defects of AChR clustering (**Figure 1B**, number 1) (47). In addition, anti-MuSK Ab block binding of ColQ to the NMJ, that may lead to compromised agrin-mediated AChR clustering and AChR deficiency in MuSK-MG patients (48). Finally, some anti-MuSK Ab are directed against the Cysteine-rich domain of MuSK that mediates the Wnt-MuSK interactions (49). In summary, by contrast with anti-AChR Ab, anti-MuSK Ab induce a functional effect by interfering with MuSK signaling and AChR clustering.

Anti-LRP_4 Ab

Mice immunized with the extracellular domain of LRP_4 exhibit MG-associated symptoms, including muscle weakness, reduced compound muscle action potentials, and compromised neuromuscular transmission (50, 51). Additionally, fragmented and distorted NMJs are evident at both the light and electron microscopic levels suggesting that LRP_4 contributes to NMJ maintenance in adulthood. In nerve terminals, a reduction in synaptic vesicle density and ACh release is observed, while on the postsynaptic side, AChR density is significantly reduced, with flattened junctional folds (50). Interestingly, injection in mice of neural agrin (N-agrin) that binds to LRP_4 leads to MG-associated symptoms, suggesting that agrin Ab may also play a role in MG pathogenesis (52).

MOLECULAR AND CELLULAR CHANGES IN MG MUSCLE

Several changes have been described inside and outside the giant syncytial muscle cell, and the importance of the local environment is increasingly considered (**Figure 1B**).

Inflammation and Cytokines

It is generally admitted that diffuse signs of inflammation are not evident in the muscle of MG patients. First of all, immune cells are scarcely found (29) [Review in (53)]. Second, the transcriptome analysis did not reveal an inflammatory signature (54).

However, increased expression of cytokines (TNF-α, IL-1, and IL-6) due to infiltrating macrophages has been described in the muscle of models of experimental autoimmune MG (EAMG), during the early phase of the disease (55) (**Figure 1B**, number 6). In addition, muscle tissues can also produce immunologically relevant factors. Rat skeletal muscle exposed to anti-AChR Ab synthesizes MCP-1, IL-15, and NO, that promote the generation of disease symptoms (56–58) (**Figure 1B**, number 5). Besides, myotubes in MG and EAMG overexpress IP-10 and CXCR3, two molecules regulated by interferon-γ (59). Interestingly, the skeletal muscle also upregulates the PD-L1 in MG, which may participate in the control of the local immune-mediated damage through the function of a checkpoint inhibitor (60).

Some cytokines and inflammatory proteins are increased in the sera of MG patients and constitute an inflammatory environment (61–64), then direct effects of these molecules on muscles could be suspected. As a proof of concept, muscle cells are responsive to IL-4, IL-6, IFN-γ, and LPS, by producing immunologically relevant molecules and may become antigen-presenting cells (65, 66). The expression of Toll-like receptors by the skeletal muscle could favor the sensitization of the muscle to the environment [reviewed in (67)].

Molecular Changes and Mechanisms of Compensation

Whether the molecular and cellular changes observed in and around the NMJ participate in the pathogenesis of MG disease or provide a mechanism of compensation are still an open question. Here, we will focus on two of these compensatory mechanisms.

First, the decreased expression in AChR is compensated by the release of an increased number of vesicles containing ACh, that has been shown in both muscles of MG patients and experimental rat models (**Figure 1B**, number 8) (27, 31, 68). The mechanism of this compensation may reside in several elements of the NMJ [Review in (27)]. At the presynaptic level, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) act through activation by Ca^{2+} (69), and this mechanism has been shown to be involved in the model of rats treated with alpha-bungarotoxin (70). Although not directly demonstrated in MG models, neuroligin (71), and Munc18 would act through the modulation of the number of docked release-ready vesicles (72). From the post-synaptic side,

LRRK2 would trigger the increase of the size of the release-ready pool of vesicles (73). It has also been suggested that a specific pool of ACh vesicles, with a slower turn-over, would be used for transient increase of quantal content (74). LRP_4 may be considered as a retrograde factor acting from muscle toward the presynaptic side (75). Clinically, the compensatory mechanism mediated by increased quantal content would be especially important during phases of intense, repetitive stimulation of the NMJ as it would counterbalance the natural rundown of quantal contents partially. Importantly, it should be noted that in MuSK-MG, this compensatory mechanism is not present, or it is blocked by the Ab, and these patients develop more severe disease (25, 27, 76).

Second, as a consequence of the attack of the AChR by Ab (**Figure 1B**, number 7), the degradation of the AChR is followed up by increased mRNA level expression of AChR subunits in muscles of myasthenic rats, rabbits, and mice compared with control animals (77–79). In MG patient muscle, the increase in AChR subunit transcripts correlates with the severity of the disease, indicating that this mechanism takes place only when the expression of AChR is significantly altered (80); *in vitro* studies show that the increase in AChR mRNA appears after a certain threshold loss of AChR (induced by monoclonal anti-AChR Ab) (80, 81). The expression of AChR is the resultant of loss and re-expression. Without such a mechanism of compensation, the AChR expression could be dramatically reduced, resulting in a fatal disease. Thus, this compensatory mechanism aims to balance the loss of AChR in human MG and is triggered above a certain degree of AChR loss (80).

Upregulation of AChR expression could also result from activation of neuregulin1/ErbB signaling pathway through overexpression of MuSK and rapsyn (82). Whether this pathway is implicated in MG has not been documented.

Other molecular alterations have been described in EAMG models and are likely to be secondary to the cross-reactive immune response. Notably, caveolin-3 shows aberrant overexpression. This muscle-specific membrane protein localized to the sarcolemma and T-tubule system is usually needed for muscle repair and skeletal muscle development (83). Also, the glucose-regulated protein 78 (GRP78) mRNA that is activated by ER stress is increased, suggesting that muscle weakness in MG might be caused by both NMJ disruption and ER stress (84). Another intriguing observation relates to the bone mineral density at skeletal sites, which is significantly decreased in the femur of EAMG mice compared to control animals, in parallel with the severity of the disease (85).

TRANSCRIPTOMIC ANALYSIS

A transcriptomics study was performed in 3 different muscles [EOM, diaphragm, and extensor digitorum longus (EDL)] in rats passively receiving anti-AChR Ab. Changes in 62 genes common among all muscle groups fall into four major categories (stress response, immune response, metabolism, and transcription factors). Interestingly, the EOM demonstrated a distinct RNA expression signature from EDL and diaphragm (86).

Transcriptome analyses were also performed on muscle biopsies from MG patients (compared with healthy controls) and on models of active EAMG in rats (compared with control rats). Similar changes in human and rat myasthenic muscles were found, highlighting the deregulation of genes included in the muscle fiber category. Also, genes related to cell metabolism and immune response were deregulated: Insulin-Like Growth Factor 1 (IGF-1) and Interleukin-6 (IL-6) pathways were identified. Indeed, increased IL-6 production was observed in human muscle cell cultures treated with MG sera or anti-AChR Ab. Besides, monoclonal anti-AChR Ab decrease Akt phosphorylation in response to insulin, indicating an effect of the Ab on cell metabolism (54). Since Akt plays a key role in multiple cellular processes such as growth and glucose metabolism, this reduced phosphorylation of Akt may have a significant impact on the muscle homeostasis, and fatigability observed in MG patients.

EFFECTS ON SATELLITE CELLS

Satellite cells (SCs) are quiescent muscular stem cells (**Figure 1**). After an injury, a process of muscle degeneration occurs, followed by the activation of the SCs that proliferate, become so-called myoblasts, differentiate, and fuse to give rise to new fibers (87).

Recently, the article by Attia et al. (88) unveiled an unexpected action of the anti-AChR Ab on these SCs. First, muscle sections from MG and EAMG contain an increased number of SCs identified by the Pax7 marker. Besides, SCs isolated from MG muscles proliferate as myoblasts and differentiate more actively than cells from control muscles. In addition, after a muscle injury induced in the EAMG mouse model, several changes were observed: a decrease in fiber size and MyoG mRNA expression and an increase in the number of fibers and embryonic myosin heavy-chain mRNA expression. These alterations suggest that as a result of the autoimmune attack, there is a delay in maturation of the muscle fibers.

A direct effect of the anti-AChR Ab on SC is unlikely since SCs do not express AChR. More likely, the binding of anti-AChR Ab to their antigens impairs the NMJ (see the mechanisms above) and alters the production of several paracrine factors, micro-vesicles, or exosomes by the muscle. These factors could then induce paracrine effects on the neighboring SCs associated with subtle modifications of the epigenetic signatures (**Figure 1B**, Number 9). This leads to the expression of MyoD and MyoG in MG SCs that will proliferate and differentiate more than in healthy ones.

Together, these data propose that MG muscles from EAMG mice regenerate worse than control ones. From a clinical perspective, symptom exacerbation upon sports practice or after a muscle injury could also be due to difficulties for MG patients to regenerate their muscle.

CONCLUSION AND PERSPECTIVES

In MG disease, the Ab to the different components of the NMJ have pathogenic consequences that are more extended than a focused effect on the target antigens. In other autoimmune

diseases, the attack by the Ab and by the MAC would have induced the death of the target cells. In the case of the muscle, this does not occur, but activation of molecular transcription and signaling pathways, mechanisms of compensation, and biological effects on local cell types such as satellite cells demonstrate that the muscle responds actively. Thus, the muscle is not a passive target in MG but interacts dynamically with its environment in several ways. However, the number of studies examining theses processes is still quite limited. A better appraisal of these processes would allow identifying new mechanisms and pathways, and new levels for symptomatic medical interventions. New approaches are rapidly developing to model MG and facilitate such studies. Indeed, with the advent of pluripotent stem cells differentiation, and the growth of bioengineering, cocultures of human myogenic and neurogenic cells are possible in two (89) or three dimensions (90, 91), so as to study the effect of MG Ab, and/or to provide organoid-like platforms for the study of pathologies and their drug design.

AUTHOR CONTRIBUTIONS

SB-A and J-TV wrote the manuscript with support from RL. AB conceived and designed the figure.

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Autoantibody Diagnostics in Neuroimmunology: Experience From the 2018 Italian Neuroimmunology Association External Quality Assessment Program

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Background: Neuroimmunology has impressively expanded in the past decade. Novel assays, especially cell-based assays (CBAs) can detect conformational antibodies (Abs) recognizing antigens in their native conformation. Generally, the availability of in-house and of commercial tests has improved the diagnostics, but introduced demanding laboratory tasks. Hence, standardization and quality controls represent a key step to promote accuracy. We report on the results of the 2018 external quality assessment program (EQAP) organized by the Italian Neuroimmunology Association.

Methods: EQAP regarded 10 schemes, including oligoclonal bands (OCBs), intracellular-neuronal (ICN)-Abs, neuronal-surface (NS)-Abs, aquaporin-4 (AQP4)-Abs, myelin oligodendrocyte glycoprotein (MOG)-Abs, myelin-associated glycoprotein (MAG)-Abs, ganglioside-Abs, acetylcholine-receptor (AChR)-Abs, and muscle-specific-kinase (MuSK)-Abs, and 34 laboratories. Assays were classified as tissue-based assays (TBAs), solid-phase assays (SPAs), liquid-phase assays (LPAs), and CBAs. Thirty-three samples were provided.

Results: Three-quarter of the tests were commercial. Median accuracy for the laboratories was 75% (range 50–100). In 8/10 schemes, at least one sample provided discrepant results. Inter-laboratory "substantial agreement" was found in 6/10 schemes (AChR, MuSK, MAG, AQP4, MOG, and NS-Abs), whereas the worst agreements regarded OCBs and ganglioside-Abs. Both commercial and in-house assays performed better in experienced laboratories.

Conclusions: Assays could be divided in (a) robust commercial tests with substantial inter-laboratory agreement (MAG-Abs; AChR- and MuSK-Abs); commercial/"in-house" tests with (b) partial inter-laboratory agreement (AQP4-Abs, MOG-Abs, NS-Abs,

ICN-Abs), and (c) with large inter-laboratory disagreement (OCBs, ganglioside-Abs). This real-life snapshot of the neuroimmunology test performances highlights shortcomings attributable to technician-dependent performances, assay structural limitations, and errors in test interpretations.

Keywords: external quality assessment scheme, standardization, neuroimmunology, antibodies, tissue-based assays, cell-based assays, radioimmunoassays, ELISA

INTRODUCTION

External quality assessment (EQA) testing is part of a wider educational approach aimed to improve and monitor quality in laboratory diagnostics. Since 2000, the Italian Association of Neuroimmunology (AINI) has espoused this commitment, which includes the production of standardizations of methods and of clinic-laboratory guidelines (1). Over these years, neuroimmunology diagnostics has been facing formidable challenges, especially after the discovery of autoantibodies to cell-surface neuroglial proteins, which associate with many potentially treatable neurological disorders (2, 3). Such autoantibodies preferentially bind antigens when their tertiary structure is preserved. This has revolutionized the neuroimmunology diagnostics, with the diffusion of "conformational" tests, such as cell-based assays (CBAs) and immunohistochemistry on lightly-fixed brain tissues for the diagnosis of autoimmune encephalitis (4), and for the differential diagnosis of the acquired demyelinating diseases of the CNS, including multiple sclerosis (5).

These new techniques have been developed as in-house protocols in specialized laboratories, thus requiring a proper expertise that often lacks in the large clinical chemistry laboratories using commercially available CBAs. In these laboratories, moreover, neuroimmunology diagnostics performed with automated or semi-automated systems is increasingly incorporated.

We herein report on the results of the 2018 EQA program that involved Italian laboratories of the AINI network, and that was extended to few European laboratories. These results provide a snapshot on how the participating laboratories perform, and useful information on the degree of reliability and accuracy characterizing each single test in real life.

MATERIALS AND METHODS

External Quality Assessment Program Design

The Neuroimmunology Laboratories in Pavia and in Milan were the program coordinators. The program was composed of 10 schemes, each addressing different areas of neuroimmunology diagnostics: oligoclonal IgG bands (OCBs) detection [with isoelectric focusing (IEF)] and pattern interpretation, intracellular neuronal antibodies (ICN-Abs), neuronal surface antibodies (NS-Abs), aquaporin-4 antibodies (AQP4-Abs), myelin oligodendrocyte glycoprotein antibodies (MOG-Abs), myelin associated glycoprotein antibodies (MAG-Abs), ganglioside-Abs, acetylcholine receptor antibodies (AChR-Abs), and muscle specific kinase antibodies (MuSK-Abs). Twenty-nine Italian and five European laboratories participated to the EQA program (**Supplementary Table 1** and **Supplementary Figure 1**). Each laboratory chose to take part to any number of the proposed schemes. The procedures for sample handling are described in **Supplementary Figure 2**.

A total number of 25 serum samples and 4 serum-CSF pairs were used (**Table 1**). The clinical diagnosis associated to each sample was established by trained neurologists (MG, DF, and FB). The results obtained by the coordinating centers (Pavia and Milan) were considered as the reference results. The participating laboratories were requested to test the samples according to their own routine standard operating procedures, and results were reported to the coordinating team using a result form. Report forms asked to classify the tested sample as "positive" or "negative" and to report the specific antibody type detected. Quantitative results from enzyme-linked immunosorbent assay (ELISA) and radioimmunoassays (RIAs) were collected, when appropriate.

All the results of the present EQA program will be presented anonymized, to preserve the confidential nature of the single laboratory performance.

Assays

Assays were classified as: (a) solid-phase assays (SPAs), including blots and ELISA; (b) tissue-based assays (TBAs), including immunohistochemistry/immunofluorescence on rodent and primate brain, or peripheral nerve; (c) cell-based assays (CBAs), including live and fixed CBA; (d) liquid-phase assays (LPAs), namely RIAs.

Commercial assays were performed according to manufacturer's instructions. In house CBAs and TBAs were performed according to published protocols, but adapted to each laboratory routine (6–10).

Statistical Analysis

Test results were considered as "concordant" or "discordant" when they matched/did not match the reference result, and "partially concordant" when they either reported incompletely what provided as reference, or when an additional positivity not included in the reference result was reported.

Qualitative variables were summarized as percentages, and quantitative variables as median with ranges.

Accuracy was calculated for each laboratory (frequency of tests concordant with the reference result among all the tests performed by the single laboratory). Between-laboratory

TABLE 1 | Samples used in the AINI EQA program.

Test	Sample N	Code	Material	Titer*	Clinical Diagnosis	Sent as
Isolectric focusing	1	S1L1	Serum-CSF pair	–	Post-infectious encephalomyelitis	Mirror (pattern #4)
	2	S2L2	Serum-CSF pair	–	Hydrocephalus and MGUS	Monclonal gammopathy (pattern#5)
	3	S3L3	Serum-CSF pair	–	Multiple Sclerosis	Mixed (pattern#3)
	4	S4L4	Serum-CSF pair	–	Clinically Isolated Syndrome	OCB (pattern#2)
Onconeural antibodies	5	O1	Serum	NA	Paraneoplastic cerebellar degeneration (ovarian tumor)	Yo pos
	6	O2	Serum	NA	Stiff person syndrome	GAD pos
	7	O3	Serum	NA	Healthy control	Neg
Neuronal Surface antibodies	8	C1	Serum	1:1200	Limbic encephalitis	LGI1 pos
	9	C2	Serum	1:400	NMDAR encephalitis	NMDAR pos
	10	C3	Serum	–	Healthy control	Neg
AQP4 antibodies	11	Q1	Serum	–	Healthy control	Neg
	12	Q2	Serum	1:10	NMOSD	Pos
	13	Q3	Serum	1:100	NMOSD	Pos
MOG antibodies	14	G1	Serum	1:160	Optic neuritis	Pos
	15	G2	Serum	–	Healthy control	Neg
	16	G3	Serum	1:640	Transverse myelitis	Pos
MAG antibodies	17	MAG1	Serum	40000_{BTU}	DADS neuropathy	Pos
	18	MAG2	Serum	25000_{BTU}	DADS neuropathy	Pos
	19	MAG3	Serum	17000_{BTU}	DADS neuropathy	Pos
Ganglioside antibodies	20	P1	Serum	NA	Miller-Fisher syndrome	Gq1b IgG pos
	21	P2	Serum	–	Healthy control	Neg
	22	P3	Serum	NA	CANOMAD	GD1b IgM and GQ1b IgM pos
	23	P4	Serum	NA	Motor Multifocal Neuropathy	GM1 IgM pos
AChR antibodies	24	A1	Serum	3.2 nmol/L	Myasthenia gravis	Pos
	25	A2	Serum	7.8 nmol/L	Myasthenia gravis	Pos
	26	A3	Serum	–	Healthy control	Neg
MuSK antibodies	27	M1	Serum	1.2 nmol/L	Myasthenia gravis	Pos
	28	M2	Serum	–	Healthy control	Neg
	29	M3	Serum	1.4 nmol/L	Myasthenia gravis	Pos

CSF, cerebrospinal fluid; MGUS, monoclonal gammopathy of uncertain significance; OCB, oligoclonal bands; NA, not available; GAD, glutamic acid decarboxylase; LGI1, leucine rich glioma inactivated protein 1; NMDAR, N-methyl-D-aspartate receptor; AQP4, aquaporin 4; NMOSD, neuromyelitis optica spectrum disorder; MOG, myelin oligodendrocyte glycoprotein; MAG, myelin associated glycoprotein; BTU, Bühlmann Titer Units; DADS, distally acquired demyelinating sensory neuropathy; CANOMAD, Chronic Ataxic Neuropathy, Ophthalmoplegia, IgM paraprotein, cold Agglutinin, Disialosyl antibodies; AChR, acetylcholine receptor; MuSK, muscle specific kinase.

**Titres are reported as endpoint titrations unless otherwise specified according to the coordinating centers results.*

agreement for each scheme was calculated using Fleiss' Kappa test with 95% confidence intervals (CI). Agreement was classified as following: "poor," kappa = 0.0; "slight," 0.00 ≤ kappa ≤ 0.20; "fair," 0.21 ≤ kappa ≤ 0.40; "moderate," 0.41 ≤ kappa ≤ 0.60; "substantial," 0.61 ≤ kappa ≤ 0.80; "almost perfect," 0.81 ≤ kappa ≤ 1.00 (11).

RESULTS

Overall Results

Twelve/34 laboratories participating to the EQA program took part to 1–2 schemes, 10/34 to 3–5 schemes and 12/24 to >5 schemes (**Supplementary Figure 1** and **Table 1**). The OCB scheme was the most attended (24/34 laboratories), followed by AQP4-Abs (20/34 laboratories).

Considering the total number of assays used by each laboratory in the EQA program, the most common assay type was SPAs (48.3%), followed by CBAs (32.4%) (**Figure 1**). Commercial assays were more common, and accounted for 76.7% of the total. The remaining 23.3% of the assays were made "in-house" (**Figure 1**).

The overall performance of all laboratories is showed in **Figure 2**. Twelve/34 (67.6%) laboratories had an accuracy >80% (**Figure 2A**). Overall median accuracy was 75% (range 50–100) (**Figure 2B**; **Supplementary Table 2**).

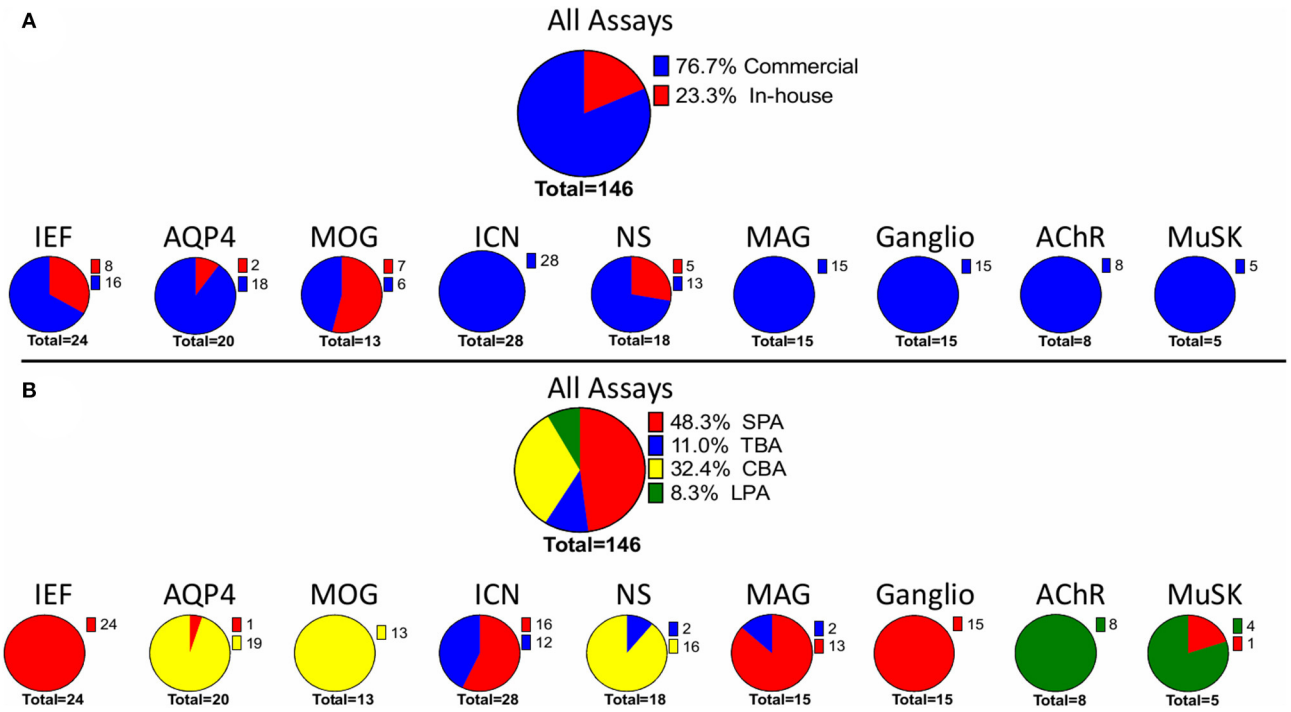

FIGURE 1 | Assays used in the AINI-EQA program. The figure considers the number laboratory using **(A)** either in house or commercial assays or **(B)** a specific assay type. A single laboratory could use more than one assay. MOG, myelin oligodendrocyte glycoprotein; NS, neuronal surface; AQP4, aquaporin 4; MuSK, muscle specific kinase; ICN, intracellular neuronal; IEF, isoelectric focusing. SPA, solid phase assay; CBA, cell-based assay; LPA, liquid-phase assay; TBA, tissue-based assay.

In 8/10 schemes at least one sample was critical, providing at least one discordant result among laboratories. The highest number of discordant results was found in OCB pattern interpretation (39.6%), ICN-Abs (23.4%), and NS-Abs (23.4%) (**Figure 2A**). A "substantial agreement" between laboratories was found in 6/10 schemes.

Detailed results from each scheme are depicted in **Supplementary Figures 3–5**.

Oligoclonal IgG Bands

Background of the Assay

The detection of the intrathecal production of oligoclonal immunoglobulins, which can be revealed in form of "discrete bands" on IEF, has high diagnostic relevance in multiple sclerosis (12), and in other inflammatory neurological diseases (13). Difficult-to-control factors, such as room temperature and humidity, gel conductivity, electroendosmosis, and ampholytes lot-to-lot differences, can affect the IEF technique making between-laboratory agreements very difficult to achieve (13). Interpretative issues of the IEF runs add complexity to the picture (14, 15). The introduction of semi-automated systems for IEF has simplified the test, but there is no comparison study on test performance vs. "in-house" assembled systems.

Results of AINI EQAS

The IEF scheme was split in two separate tasks. The first one required to establish presence or absence of OCBs in each of the four paired serum and CSF controls (8 samples), whilst the second required to interpret each of the ensuing IEF run as a whole, on the basis of the five patterns established by the 1994 consensus report on the topic (14).

S4L4 was the most critical sample, as it showed a few faint unique-to-CSF bands.

In this sample, bands were identified by 6/24 laboratories, and only 2/24 provided a correct interpretation of pattern #2 (14).

The serum-CSF pair S2L2, sent as pattern #5 (monoclonal gammopathy), was misinterpreted as a mirror, or a mixed pattern, by 10/24 laboratories.

As for the methods, 8/24 laboratories used "in-house" assembled IEF systems (where optimal run conditions were established in each laboratory), six using home-made agarose gels, two using commercial gels; the other 16 laboratories exploited semi-automated IEF systems. Overall accuracy of "in-house" assembled systems, which were used in the most experienced laboratories, was slightly, but not statistically significant superior than that of semi-automated systems, in both the task of band detection (85.9 and 81.3%, respectively), and of pattern interpretation (62.5 and 48.4%, respectively)

Overall agreement was "moderate" for bands detection (Fleiss' kappa = 0.51), and only "fair" for pattern interpretation (Fleiss' kappa = 0.31) (**Figure 2C**).

Conclusions

In line with the other previous programs on OCBs promoted by AINI, this EQA revealed the difficulties in detecting OCBs in critical samples. Even when recognized, OCBs can be

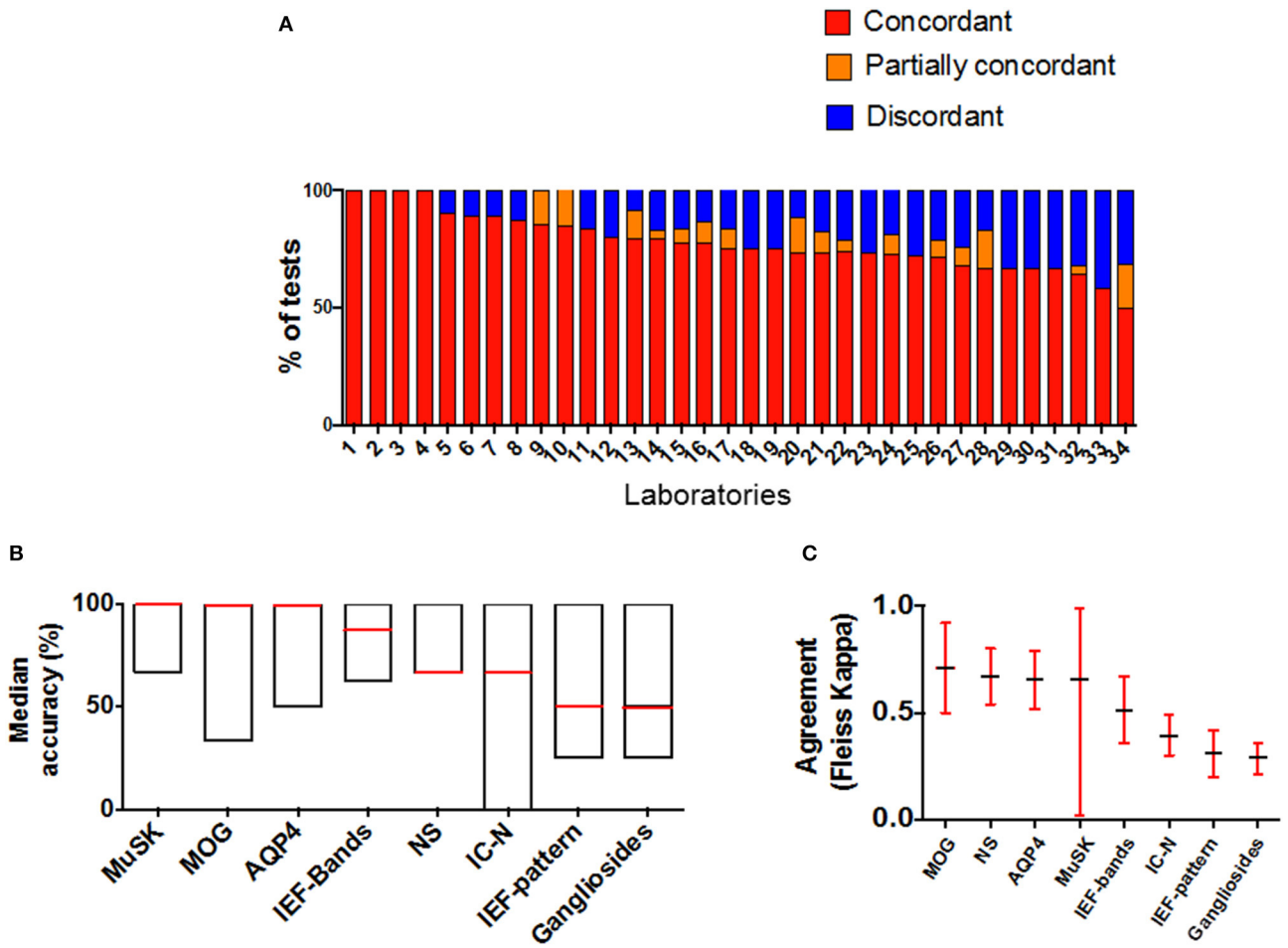

FIGURE 2 | Laboratory performances and schemes results in AINI-EQA program. **(A)** Accuracy is represented by the number of concordant results obtained by each lab in all the schemes joined. Each lab participated to a variable number of schemes; **(B,C)** represent the performance **(B)** and the concordance of results **(C)** in each scheme (MAG and AChR schemes are not represented since all results were concordant). MOG, myelin oligodendrocyte glycoprotein; NS, neuronal surface; AQP4, aquaporin 4; MuSK, muscle specific kinase; ICN, intracellular neuronal; IEF, isoelectric focusing.

misinterpreted as wrong patterns, with risks of wrong messages to the clinicians.

AQP4 Antibodies

Background of the Assay

The presence of serum AQP4-Abs identifies acquired demyelinating syndromes of the CNS mainly affecting the optic nerves and spinal cord, collectively defined as Neuromyelitis Optica Spectrum Disorders (NMOSD) (16), which are in differential diagnosis with MS. Initially, AQP4-Abs were detected with immunohistochemistry on rodent brain, but currently CBAs are the gold standard (17, 18). When compared with ELISAs, CBAs offer the advantage of being conformational (19). The AQP4 protein arranges on the cell surface in tetramers, associated in the orthogonal particle arrays (OPAs) that are relevant for AQP4-Ab binding (20–22). In a multicenter comparison of AQP4-Abs detection assays, CBAs resulted the most sensitive assays (6). Both live and fixed CBAs showed good analytical performances, although live CBAs performed with slightly higher accuracy (6). The use of ELISAs is progressively decreasing due to inferior performances compared to CBAs (6, 17, 21).

Results of AINI EQAS

CBAs were used by 18/19 laboratories ("in-house" live CBAs for two of them), and only one used a commercial ELISA. The overall agreement was "substantial" (Fleiss' kappa: 0.66, 95%CI: 0.52–0.79). Fifteen/20 laboratories reached 100% accuracy. Sample Q2, a low AQP4-Ab-positive serum from an NMOSD patient (titer 1:10 on the commercial CBA) was reported as negative by 5/19 laboratories. Only one laboratory, using the commercial CBA, reported the reference negative sample Q3 as AQP4-Ab positive.

Conclusions

The interpretation of fluorescence in samples with low titers of AQP4-Abs can be challenging, and could lead to false

negative results in the routine practice. The comparison between in-house and commercial CBA performances suggests that erroneous output evaluations mainly explained the relatively low concordance.

MOG Antibodies

Background of the Assay

Using non-conformational methods, MOG-Abs had been associated with MS for decades (23). Subsequently, these antibodies, when detected with appropriate conformational methods, have been increasingly associated with non-MS acquired demyelinating syndromes, such as optic neuritis and transverse myelitis (7, 24–26). Since only conformational MOG-Abs are considered clinically relevant, CBAs are the gold standard for their detection (27). CBAs are performed on live cells transfected with human full-length MOG; bound IgG can then be detected with either an anti-total human-IgG (9), or an anti-human-IgG_1, as secondary antibodies. (7) The output readout can be performed either by fluorescence microscopy, or flow-cytometry (28, 29). Recently, a commercial CBA for MOG-Abs detection relying on fixed cells has become available. In a three-center comparison, the fixed CBA showed rather good concordance with the live CBAs, with slightly lower specificity (30).

Results of AINI EQAS

Given the recent identification of MOG-Abs, this was the first year that the scheme was included in the AINI EQA program. Only laboratories using CBAs participated to this scheme, seven with "in-house" protocols with different characteristics of the secondary antibodies, which recognized total IgG ($n = 3$), IgG_1 ($n = 1$), or both ($n = 1$). The remaining six laboratories used the commercial fixed CBA.

The two positive samples had medium to high titers (1:320–1:640), and were positive for IgG_1 antibodies. The overall agreement was substantial (Fleiss' kappa: 0.71, 95%CI: 0.5–0.92). Eleven/13 laboratories correctly identified MOG-Abs in sample G1 and G2, and 13/13 recognized G3 as negative.

Conclusions

The participation of experienced laboratories only to this EQAS, using both live and/or fixed CBAs, likely accounted for overall good performances.

Neuronal Surface Antibodies

Background of the Assay

NS-Abs represent an expanding group of autoantibodies targeting key proteins implicated in synaptic function (3, 31). These antibodies associate with a wide spectrum of disorders variably presenting with cognitive impairment, seizures, movement disorders, and autonomic dysfunction, defined as "autoimmune encephalitis" (2, 32). After the identification of antibodies against the N-methyl-D-aspartate receptor (NMDAR), many other NS-Abs have been discovered in the last years (33, 34), including those against leucine rich glioma inactivated-1 (LGI1) and contactin-associated protein-like 2 (CASPR2), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1 and 2 (AMPAR), and γ-aminobutyric acid A or B receptor ($GABA_{A/B}R$).

The use of conformational assays is crucial for NS-Ab detection (35), and includes CBAs and/or TBAs on rodent brain optimized with light fixation procedures (4). TBAs can be, at least for some Abs, more sensitive than CBAs, although CBAs are necessary to identify antigenic targets (36). The combination of TBAs and CBAs can improve diagnostic accuracy (37).

A commercial fixed CBA is currently available for the most frequently detectable NS-Abs. Rarer NS-Ab reactivities require appropriate "in-house" diagnostics (2).

Results of AINI EQAS

As most laboratories used the commercial test that includes only the most frequent NS-Abs (NMDAR-Abs, LGI1-Abs, CASPR2-Abs, AMPAR-Abs, and $GABA_BR$-Abs), the EQA scheme was restricted to these Abs. Eleven/fourteen laboratories used the commercial CBA, whilst two used a strategy combining "in-house" TBAs and "in-house," or commercial CBA. One laboratory used "in-house" live CBAs only (**Table 2**).

Sample C2 [from a patient with definite NMDAR encephalitis (32)] was the only one providing conflicting results, as 9/14 laboratories failed to detect NMDAR antibodies. This sample tested at the coordinating center showed 1:200 positive titer using a TBA, and 1:10 positive titer using the commercial CBA (weak positivity) (42).

Conclusions

Discrepancies were mainly due to difficulties in detecting low titer NMDAR-Abs. This supports the message that, in autoimmune encephalitis, testing for both serum and CSF can increase diagnostic accuracy (42). Indeed, the paired CSF sample of the C2 control was positive at high titer.

Intracellular Neuronal Antibodies

Background of the Assay

ICN-Abs target nuclear or cytoplasmic antigens, and associate with a wide range of neurological syndromes often occurring in association with a tumor (paraneoplastic neurological syndromes, PNS). Classic PNS include, among others, limbic encephalitis, paraneoplastic cerebellar degeneration, and subacute sensory neuronopathy (43, 44). Although their association with cancer is much rarer, GAD-Abs are often included in this group, and are associated with stiff person syndrome, epilepsy, or cerebellar ataxia, but also type-1 diabetes (45–47).

ICN-Abs are usually identified with screening TBAs on murine or primate cerebellum, followed by confirmatory SPAs (commercial line/dot blots). Blots include the most common antibody targets, with some differences on the panel according to the manufacturer (**Table 2**). Although blots can be more sensitive than TBA in rare cases (48), their use without TBAs can lead to false positive results, and is therefore discouraged (4, 49, 50). In-house CBAs have been used with selected antigens, such as CV2 and SOX1, showing a higher sensitivity compared to commercial blots (51, 52). GAD antibodies can be quantified using ELISAs,

TABLE 2 | Assays used in the AINI EQA program.

Test	Assay	N of labs/total*	Main features
Oligoclonal IgG bands	Semi-automated systems	15/23	Precast agarose gels (small-medium size); manufacturer's recommended run conditions; direct immunofixation
	In-house assembled systems	8/23	In-house or commercial precast agarose gels (large size); run conditions optimized in each laboratory; capillary blotting and immunofixation
Intracellular neuronal antibodies	Immunohistochemistry on fixed primate brain + blot A	6/16	Commercial (Euroimmun) chip + line-blot (Ravo), antigens: HuD, Yo, Ri, CV2 (CRMP5), Amphiphysin, Ma1, Ma2
	Immunohistochemistry on fixed primate brain + blot B	6/16	Commercial (Euroimmun) chip + line-blot (Euroimmun), antigens: HuD, Yo, Ri, CV2 (CRMP5), Amphiphysin, Ma, PCA-2, Tr, SOX1, titin, recoverin
	blot A only	4/16	Line-blot (Ravo or Euroimmun), antigens: see above
Neuronal Cell Surface antibodies	Immunohistochemistry on rat brain + in-house CBA	2/16	In-house obtained slices from lightly fixed rat brain + in-house fixed (Euroimmun), or live CBA designed according to the staining pattern on tissue (10, 36)#
	In-house CBA	1/16	Live CBAs for specific antigens (38, 39)#
	Commercial CBA	13/16	Fixed CBA mosaic chip (Euroimmun); antigens: NMDAR, LGI1, CASPR2, AMPAR 1/2, $GABA_BR$
AQP4 antibodies	In-house CBA	2/20	Live CBA, transfection with M23 AQP4 isoform
	Commercial CBA	17/20	Fixed CBA (Euroimmun), transfection with M23 AQP4 isoform
	Commercial ELISA	1/20	RSR Limited, no information on AQP4 isoform used
MOG antibodies	In-house CBA A	3/13	Live CBA, transfection with full-length MOG, total IgG secondary antibody, titration cut-off (1:160) (8, 9)#
	In-house CBA B	1/13	Live CBA, transfection with full-length MOG, IgG1 secondary antibody (7)#
	In-house CBA C	1/13	Live CBA, transfection with full-length MOG, total IgG secondary antibody, titration cut-off 1:160 + IgG1 secondary antibody (7, 9)#
	In-house live CBA D	2/13	Like CBA A, cytofluorimetric analysis (40)#
	Commercial CBA	6/13	Live CBA, transfection with full-length MOG, total IgG secondary antibody, titration cut-off (1:10)
MAG antibodies	Commercial ELISA	10/14	Bühlmann
	Immunohistochemistry	1/14	Commercial, Immco Diagnostics
	Immunohistochemistry+blot	1/14	Commercial, not specified
	Commercial blot	1/14	Ravo
	Commercial blot	1/14	Euroimmun
Antibodies to Gangliosides	In-house ELISA	5/15	In accordance with INCAT (41)
	Commercial blot	3/15	Line blot (Euroimmun)
	Commercial ELISA	4/15	Bühlmann
	Commercial blot	3/15	Dot blot (Generic Assay)
AChR antibodies	Commercial RIA	5/8	IBL International; RSR Limited
	Commercial ELISA	3/8	RSR Limited
MuSK antibodies	Commercial RIA	4/5	RSR Limited
	Commercial ELISA	1/5	RSR Limited

*NMDAR, N-methyl-D-aspartate receptor; LGI1, leucine rich glioma inactivated 1; CASPR2, contactin-associated protein-like 2; $GABA_BR$, γ-aminobutyric acid B receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AQP4, aquaporin 4; ELISA, enzyme-linked immunosorbent assay; MOG, myelin oligodendrocyte glycoprotein; MAG, myelin associated glycoprotein; INCAT, Inflammatory Neuropathy Cause and Treatment; RIA, radioimmunosorbent assay; AChR, acetylcholine receptor; MuSK, muscle specific kinase. *Single laboratories can use more than one test; CBA, cell-based assay; #In-house assays were performed according to published protocols, but adapted to each laboratory routine.*

RIAs, and luciferase immunoprecipitation system (LIPS) (47, 53), which are more sensitive than TBAs and line/dot blots (4).

Results of AINI EQAS

Among the participating laboratories, the ICN-Ab detection was characterized by some heterogeneity of laboratory assays. Twelve/sixteen laboratories used a combination of TBAs and confirmatory SPAs, with two different commercial line blots (each used by six laboratories). Four laboratories did not perform a screening with TBA. No laboratory performed ELISAs, or LPAs for GAD antibodies.

Overall agreement for this scheme was "fair" (Fleiss' Kappa: 0.39, 95%CI: 0.3–0.49).

Sample O2 was wrongly identified as negative by 4/16 laboratories. One of the laboratories detected a compatible staining pattern with TBA, not confirmed on line blots, and the three remaining laboratories performed the line blot only. In addition, for the same sample 8/16 laboratories additionally reported a positivity for titin-Abs detected with line blots. The

same reactivity was reported by 7/16 laboratories with the sample O3 (sent as negative). Titin-Abs are variably used in patients with myasthenia gravis (MG) as biomarker of thymoma (54). O2 and O3 patients did not show any clinical manifestation of MG, and had no thymoma.

Conclusions

The poor performances of many laboratories in this EQA scheme could have the following main reasons: (a) TBAs are mandatory screening tests (42), so that using only line/dot blots, based on recombinant proteins, can yield false positive and false negative results (49); (b) the recognition of particular ICN-Abs patterns on TBAs is challenging (42); (c) faint antibody reactivities on line/bot blots should be interpreted as negative results.

The introduction of the titin antigen in the commercial dot/line blots for ICN-Abs is questionable, as MG had been considered an "independent disease," and thus excluded by the diagnostic criteria for PNS (42).

Ganglioside Antibodies

Background of the Assay

Ganglioside-Abs are associated with a wide spectrum of inflammatory peripheral neuropathies (55). However, only few of them have actual diagnostic meaning and associate with well-defined clinical phenotypes. These include: (a) antibodies against a dialosyl epitope, a sequence contained in GD1b, GD3, GT1b, and GQ1b molecules in patients with a paraproteinemic neuropathy defined as CANOMAD (Chronic Ataxic Neuropathy, Ophthalmoplegia, IgM paraprotein, cold Agglutinin, Disialosyl antibodies) (56, 57); (b) GM1 IgM-Abs in patients with motor multifocal neuropathy with conduction blocks (MMN) (58); (c) GQ1b (with/without GT1a) IgG antibodies in patients with Fisher syndrome, a variant of Guillain-Barrè syndrome (GBS) with ophthalmoplegia and ataxic neuropathy (59, 60). GD1a, GM1a, GM1b, and GalNAc-GD1a-Abs (IgG isotype) characterize the acute motor axonal neuropathy (AMAN), and GM1 and GD1a-antibodies (IgG isotype) characterize acute motor and sensory axonal neuropathy (AMSAN), but these axonal forms of GBS are more common in Asia and Central and South America than in North America and Europe.

Thin layer chromatography is considered the gold standard, but it is often unavailable for routine diagnostics, for which available options include line/dot blots and ELISAs, with suboptimal performances (56). In order to improve standardization, in 1999 an ELISA for ganglioside-Abs has been proposed by an experts panel (INCAT-ELISA) (41), and is still considered a valid assay notwithstanding the documented inter-laboratory variability (61). Limiting the tests to the above-mentioned autoantibodies, and considering positive results only when high titers are detected represent useful recommendations for clinicians (61).

Results of AINI EQAS

Five/fifteen laboratories performed ELISAs according to the INCAT guidelines, 4/15 used commercial ELISAs, and 6/15 commercial blot from two different manufacturers (**Table 2**). The EQA scheme for ganglioside-Abs had the lowest agreement (Fleiss' kappa: 0.29, 95%CI: 0.21–0.36), and the lowest accuracy (median: 50; range: 25–100) within the EQA program. Twelve/fifteen laboratories performed suboptimally, showing an accuracy $\leq$50%. Sample P1 (from a patient with Fisher syndrome) was correctly reported as GQ1b-IgG positive by 14/15 laboratories, but three laboratories additionally identified other ganglioside-Abs, such as GM1-IgM, or GT1a IgG, which, however, can coexist with GQ1b-IgG. Sample P3 (from a patient with CANOMAD) was classified as positive for both GD1b and GQ1b-IgM. Only three laboratories showed agreement with the reference value. Four/fifteen laboratories reported only one of the two ganglioside-Abs, whilst four reported additional ganglioside-Abs, possibly compatible with the clinical syndrome (such as, GD1b-IgM), or unrelated (such as, sulfatide IgM). Similarly, in sample P4 (from a patient with MMN, sent as GM1-IgM-positive) 9/15 laboratories reported additional reactivities including GM2 and GD1b-IgM. The interpretation of this scheme thus needed the arbitrary setting up the category of "partially concordant" results, when antibody reactivities compatible with the established clinical phenotypes were reported in addition to the reference reactivities. However, the statistical analysis of this EQAS was calculated including "partially concordant" results into the category of "discordant" results.

Conclusions

This scheme was the most critical of our EQA program, likely due to the relatively high heterogeneity of the tests employed by the various laboratories, and the technical drawbacks that intrinsically affect the current methods for ganglioside-Abs testing (62). The poorest performances still remain even if the category of "partially concordant" joins that of "concordant" results.

MAG Antibodies

Background of the Assay

MAG neuropathy is a rare disease typically associated with monoclonal IgM that recognize the glycoprotein (63). A slowly progressing neuropathy characterizes the disease (Distal acquired demyelinating symmetric neuropathy, DADS). MAG-Abs detection is preferentially performed with ELISA, which produces quantitative results useful for monitoring the disease. Other tests, including Western or line blot, and TBAs are available, but they show lower accuracy (63, 64).

Results of AINI EQAS

Despite the heterogeneity of the assays used (**Table 2**), all laboratories correctly identified MAG-Abs in the three reference samples, all from patients with DADS.

Conclusions

MAG scheme was not critical. However, among the laboratories that used the Bühlmann ELISA, large differences in quantitative values were detected, thus suggesting between-laboratory difference in performing the test.

AChR and MuSK Antibodies

Background of the Assay

MG is an autoimmune disorder of the neuromuscular junction characterized by muscle fatigue and reduced endurance upon repetitive use (65, 66). AChR-Abs are highly specific for MG, and are found in 85–90% of patients with generalized MG and in 40–70% with ocular MG (66, 67). More recently discovered, MuSK-Abs are present in serum samples of about one third of AChR-Abs-negative MG patients (68, 69).

LPA, and particularly RIA, either "in-house" or commercially available, are considered the gold standard for both AChR- and MuSK-Ab detection (69). Recently, novel tests using CBAs have been implemented, showing high sensitivity in detecting AChR- and MuSK-Abs in LPA antibody-negative patients (70, 71). This advantage is likely linked to the antigen clustering at the cell surface, thus improving the binding of divalent low-affinity AChR-Abs. However, such tests are performed on live cells, and thus they are necessarily "in-house" and non-standardized. Alternatively, commercial ELISAs are available for the detection of both AChR- and MuSK-Abs, but their performances are inferior to those of RIAs (69).

Results of AINI EQAS

The number of laboratories participating to AChR- and MuSK-Ab schemes was limited (8 and 5, respectively). Three laboratories in the AChR-Ab scheme, and one in the MuSK-Ab scheme, used a recently released commercial ELISAs, whilst the remaining laboratories used the consolidated commercial RIAs. Accuracy was high, but one laboratory using the ELISA identified MuSK-Abs in a negative sample.

Conclusions

RIAs remain the gold standard for AChR- and MuSK-Ab detection. CBAs for their detection are showing promising preliminary results (38), and forthcoming EQA programs will evaluate their performances.

GENERAL CONCLUSIONS

The Holy Grail of precision medicine requires endless efforts toward the production of biomarker data for accurate stratifications of patients, and, to determine the best approach to prevent, diagnose, or treat diseases. These efforts are exploiting the impressive technological advancements to identify new biomarkers. On the other hand, the contribute of well-established biomarkers should not be overlooked.

The here reported data from the 2018 AINI EQA program depict a complex picture on how currently used neuroimmunology biomarkers work in real life. The evidence derives from a single EQA evaluation, but we found similar performances in our previous AINI EQA programs [personal communication].

Briefly, the neuroimmunology tests here evaluated can fall into three categories:

(a) standardized and robust commercial tests with substantial inter-laboratory agreement (MAG-Abs; AChR- and MuSK-Abs); (b) commercial and "in-house" tests with partial inter-laboratory agreement (AQP4-Abs, MOG-Abs, NS-Abs, ICN-Abs); (c) commercial and in-house tests with large inter-laboratory disagreement (OCBs, ganglioside-Abs).

The CBAs used for AQP4 and MOG-Ab detection are of relatively recent introduction. Both in-house and commercial tests seem to perform suboptimally in low-titer sample controls. Accordingly, a large multicenter comparison of various tests for AQP4-Abs suggests that technical accuracy improves when tests are carried out in specialist laboratories (18).

As a whole, technical inaccuracy and shortcomings in results interpretations are likely the main reasons underlying the suboptimal performance put in evidence by our EQA program for NS- and ICN-Abs too. However, there are two tests that carry well-known "structural" limits, namely the IEF for OCB detection (8), and ELISA, or dot/line blot tests for ganglioside-Abs (60), which are very difficult to overcome. As for OCBs, such limits were one of the main points supporting their exclusion from MS diagnostic criteria (72). Exploiting the expertise of specialized laboratory, with a centralization of OCB testing, could minimize the above-mentioned shortcomings. The limits of the available tests for ganglioside-Abs, once recognized, should lead to a consensus including experts and the main manufacturers, to find the best compromise on the best single method to use and on interpretative rules for positive results.

The commercial fixed CBA for MOG-Abs seemed to perform as well as the in-house live CBAs, but only three samples were tested, not allowing the due statistical evaluations. The in-house live CBA for MOG-Abs yielded better results than a fixed CBA in a three-center comparison study (29).

The main limitation of this study is the low number (3 or 4) of samples sent for each assay. On the other hand, high volumes of control samples from patients with a given disease, that are necessary when many centers are involved in EQA programs, are not easily obtainable, and evaluations on single assay performances should better imply high numbers of samples tested by a few selected centers.

In conclusion, our findings give clinicians a panorama of what they can expect when they ask for neuroimmunology tests. Although restricted to Italian and a few European laboratories, the data of this EQA program are indeed in line with other similar surveys promoted for single tests (18, 29, 50). It is conceivable that in countries where neuroimmunology diagnostics is centralized in laboratories with specific expertise the quality of the service could be higher. Further efforts for standardizations are still needed, as well as the promotion of EQA programs, which are fundamental even for expert laboratories.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MG analyzed the data, drafted the manuscript, and cooperated to the AINI-EQAP planning and coordination. DF drafted the manuscript and was the main planner and coordinator of the AINI EQAP. AU, FB, and FA contributed to plan the AINI-EQAP

and revised the manuscript for intellectual content. SS and EZ contributed to sample preparation and shipping in the EQAP and to data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01385/full#supplementary-material

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REVIEW
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In vivo Mechanisms of Antibody-Mediated Neurological Disorders: Animal Models and Potential Implications

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Over the last two decades, the discovery of antibodies directed against neuronal surface antigens (NSA-Abs) in patients with different forms of encephalitis has provided a basis for immunotherapies in previously undefined disorders. Nevertheless, despite the circumstantial clinical evidence of the pathogenic role of these antibodies in classical autoimmune encephalitis, specific criteria need to be applied in order to establish the autoimmune nature of a disease. A growing number of studies have begun to provide proof of the pathogenicity of NSA-Abs and insights into their pathogenic mechanisms through passive transfer or, more rarely, through active immunization animal models. Moreover, the increasing evidence that NSA-Abs in the maternal circulation can reach the fetal brain parenchyma during gestation, causing long-term effects, has led to models of antibody-induced neurodevelopmental disorders. This review summarizes different methodological approaches and the results of the animal models of *N*-methyl-d-aspartate receptor (NMDAR), leucine-rich glioma-inactivated 1 (LGI1), contactin-associated protein 2 (CASPR2), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antibody-mediated disorders and discuss the results and the limitations. We also summarize recent experiments that demonstrate that maternal antibodies to NMDAR and CASPR2 can alter development in the offspring with potential lifelong susceptibility to neurological or psychiatric disorders.

Keywords: animal models, neuronal surface antibodies, passive transfer, maternal transfer, active immunization

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INTRODUCTION

Over the last two decades, it has become clear that antibodies against neuronal surface antigens, particularly receptor-gated ion channels of ion-channel-associated proteins, can reach the brain to cause a group of disorders referred to as antibody-mediated or autoimmune encephalitis (AE) (1). These are immune disorders of the central nervous system (CNS) characterized by a wide range of neurological and psychiatric clinical features and associated with antibodies against different proteins expressed on the neuronal surface, mainly at excitatory, and inhibitory synapses (**Figure 1**). Distinct from classical paraneoplastic syndromes that are associated with onconeural antibodies (3), in AE, the neuronal surface antibodies (NSAbs) are considered to be pathogenic, and patients respond substantially to immunotherapies that reduce antibody levels (4).

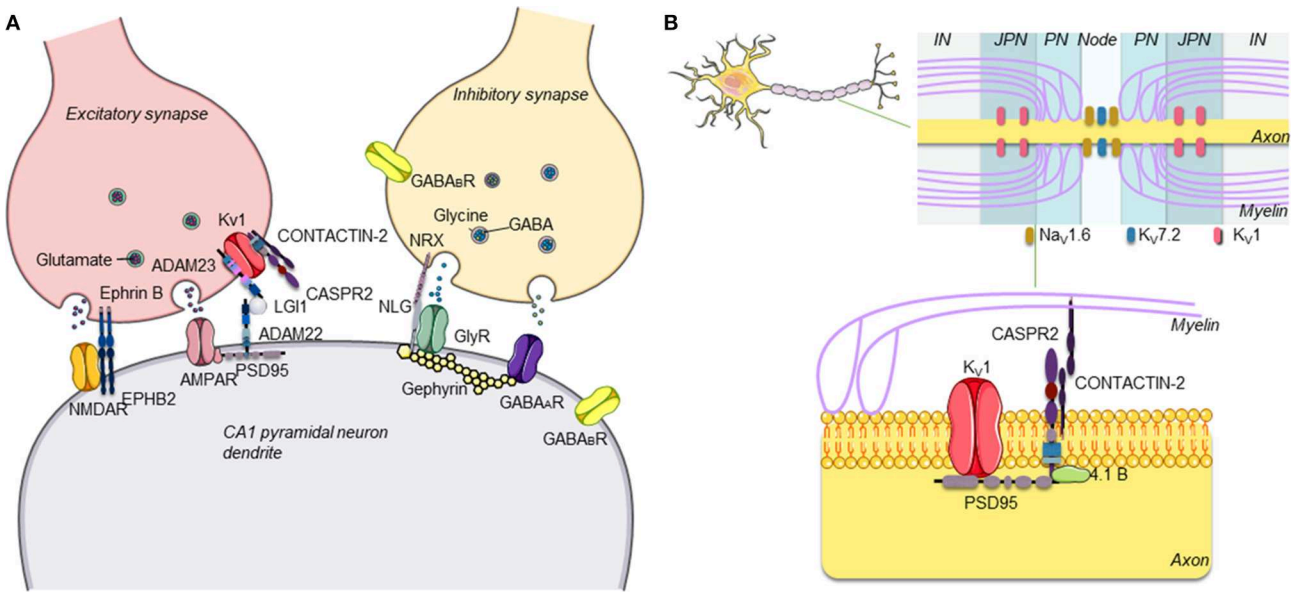

FIGURE 1 | (A) Schematic representation of central excitatory and inhibitory synapses and main antibody targets. The proteins targeted by antibodies associated with autoimmune encephalitis are proteins expressed on the neuronal surface, often at both presynaptic and postsynaptic levels on inhibitory (GABAergic) and/or excitatory (glutamatergic) neurons in the central nervous system (CNS). **(B)** Schematic representation of CASPR2. CASPR2 localizes at the juxtaparanode of myelinated axons. CASPR2 binds to contactin-2/TAG-1 via its extracellular domain and links to PDZ-binding proteins and to the cytoskeleton via protein 4.1B, stabilizing Kv1 channels [adapted with permission from Giannoccaro et al. (2)].

Interestingly, these pathogenic antibodies can be either predominantly immunoglobulin G1 (IgG1) or IgG4, depending on the target antigen. *In vitro* studies have helped to decipher the mechanisms by which they lead to neuronal dysfunction: in many cases, divalent antibodies (IgG1 > IgG3, IgG2) cause internalization of adjacent surface proteins, leading to their loss from the membrane; complement activation by these antibodies can be demonstrated *in vitro* but may not always occur *in vivo*. By contrast, in some disorders, IgG4 antibodies predominate and act principally or exclusively by direct inhibition of the function of the target antigen [see (5) and **Figure 2**].

However, an effect of the antibodies *in vitro* does not necessarily reflect a pathogenic role *in vivo*. For instance, IgG, IgA, and IgM *N*-methyl-D-aspartate receptor (NMDAR) antibodies (NMDAR-Abs) have been identified in a small proportion of healthy humans and mammals (6–9) and cause internalization of the NMDAR in cultured neurons (9, 10), similar to the antibodies found in patients with the IgG NMDAR-Ab encephalitis (NMDARE) (11). This suggests that other factors are likely required to induce the clinical syndrome, factors that may be difficult to model *in vitro* alone.

Indeed, according to the modified Witebsky criteria (12), direct and indirect evidence of pathogenicity requires the reproduction of the disease in a recipient through direct transfer of the antibodies (passive transfer) or through active immunization, respectively. Animal models not only provide evidence of pathogenicity but can also offer insight into sites of action, pathogenic mechanisms, and therapeutic approaches.

Accordingly, over the last few years, animal models, usually in mice, have been established for the most commonly encountered NSAbs in clinical practice. Below, we describe the approaches used and the results of these models and discuss their advantages and limitations. We also summarize recent experiments that demonstrate that maternal antibodies to these or other NSAbs can alter development in the offspring with potential lifelong susceptibility to neurological or psychiatric diseases.

DIFFERENT MODELS OF ANTIBODY-MEDIATED DISORDERS

Animal models of autoimmune disorders can be divided into two main categories: (1) spontaneous models where, comparably to humans, animals develop an autoimmune disease spontaneously and (2) induced models where an autoimmune disease is artificially provoked. Spontaneous forms of AE have been reported in different species, but they are uncommon (13, 14). Most of the models of AE have been obtained through induction by passive or active immunization. Passive immunization is based on the reproduction of the disease in a healthy recipient by transfer of serum, purified immunoglobulins, monoclonal antibodies, or, more rarely, antibody-producing cells isolated from an affected human or animal donor. Active immunization is based on the exposure to an antigen, often in association with adjuvants, to generate an adaptive immune response. The antigen can be in the form of purified proteins, recombinant or synthesized peptides (15).

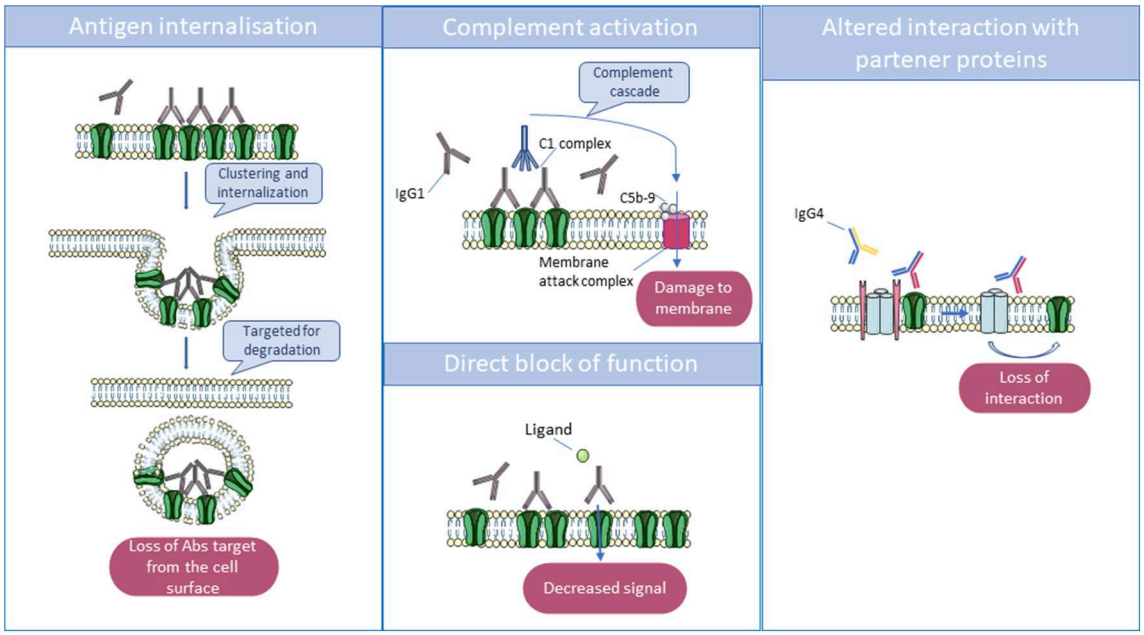

FIGURE 2 | Main mechanisms by which antibodies act to reduce the function of their targets. Immunoglobulin G1 (IgG1) and IgG3 can cross-link antigenic targets, leading to internalization, and degradation of the antigen in lysosomes. Also, IgG1 and IgG3 can activate the complement cascade via their Fc domains, which interact with complement proteins C1 and C1q. The complement cascade culminates in the formation of the membrane attack complex which disrupts the phospholipid bilayer, resulting in cell damage. Finally, some autoantibodies can directly block receptors by binding to an essential transmitter or regulatory binding site, but monovalent IgG4 can only act by disrupting the function of the target or the interaction between their target and partner proteins.

Work on myasthenia gravis (MG) provides examples of both active and passive immunization and has helped to shape our understanding of antibody-mediated diseases (**Table 1**). Passive transfer is the best way to assess the acute effects of human autoantibody-mediated diseases and has been used extensively to study patients' derived antibodies in MG [(16); see a brief review by Phillips and Vincent (17). By contrast, active immunization (see (18)] has been particularly useful to investigate more broadly the immunological factors underlying the disease, though with the limitations of possible differences between the function of the human and rodent immune systems and between different strains of mice. For instance, C57B1/6 mice were very susceptible to active immunization with acetylcholine receptor (AChR), whereas AKR/J mice were resistant (19, 20). Moreover, the use of the target antigen as a whole protein often induces high titers of antibodies, but if the protein is from a different species, not all of the antibodies will necessarily cross-react with the mouse antigen or be directed against the disease-causing epitope(s). Therefore, active immunization models are not always relevant to the human pathology but, when successful in producing an appropriate clinical and physiological phenotype, provide a long-term model of the disease that is suitable for testing experimental therapies.

In contrast to conditions such as MG, where the target antigens of the antibodies are peripheral and thereby easily accessible from the systemic circulation, the blood–brain barrier (BBB) limits the access of immune molecules to the brain. One way to overcome this limitation, in models of CNS antibody-mediated diseases, is to infuse the antibodies directly into the cerebrospinal fluid (CSF) within the cerebral ventricle(s) (intracerebroventricular, icv) or to inject them into the brain parenchyma. However, in the majority of autoimmune forms of encephalitis, the antibody levels are higher in the serum than in the CSF, suggesting that the antibodies could initiate the disease by diffusion through an incomplete or temporarily disrupted BBB (32) or at sites of limited BBB protection such as the choroid plexus. Therefore, another approach is to administer the antibodies in the periphery, using the intravenous (iv) or intraperitoneal (ip) route and if necessary to induce artificially a breach in the BBB to allow the antibodies to reach their targets. Classically, the latter is obtained by one or two ip injections of lipopolysaccharide (LPS), which induces a transient disruption of the BBB, particularly in the frontal cortex, thalamus, pons–medulla, and cerebellum (33). It is not yet clear whether the icv or ip route of administration is most appropriate and whether they could lead to different CNS changes.

Finally, there is a possibility of transfer from a mouse dam to developing embryos. Although the BBB interfaces are formed early in development (34), maternal IgG antibodies can cross into the fetal brain parenchyma during gestation (32). It is long established that a neonatal form of MG can result from the transfer of IgG antibodies from an affected mother to her fetus *in utero* (27, 35). Human MG AChR antibodies injected intraperitoneally into pregnant mice were shown to cross efficiently from the mouse dam to her fetuses and to cause neuromuscular changes *in*

TABLE 1 | Example of antibody-mediated diseases: clinical and experimental evidence for MG.

IN HUMANS:
• Clinical features (weakness and fatigue) can be reversed by plasma exchange and other immunotherapies (21).
• IgG1 and IgG3 antibodies to the AChR are present in the majority of patients (22, 23).
• IgG and complement deposition are found at the neuromuscular junction (24).
• The thymus gland contains germinal centers and produces some of the AChR antibodies (25).
• Thymectomy leads to long-term clinical benefit, reducing the need for immunotherapies (26).
• Mothers can transfer pathogenic antibodies to the fetus or neonate, causing a transient form of MG (27) or rarely a severe neurodevelopmental disorder (arthrogryposis multiplex congenital) (28).
GENETIC CONDITIONS:
• Genetic conditions caused by mutations in genes encoding AChRs cause similar clinical features but without evidence of autoimmunity.
• Genetic conditions can be modeled in transgenic mice [see (29)].
IN EXPERIMENTAL ANIMALS:
• Injection of patient IgG into mice or other species leads to short-term clinical or electrophysiological evidence of the disease (16).
• Active immunization against purified AChRs leads to a more severe and prolonged model (30).

For a brief review of the history of research into myasthenia gravis, see Vincent (31). MG, myasthenia gravis; IgG, immunoglobulin G; AChR, acetylcholine receptor.

utero (36); this model has since been used to study the effects of human serum antibodies on brain development (as described below).

MODELS OF NEURONAL ANTIBODY-MEDIATED DISORDERS

The clinical and investigative features of the patients with antibodies to neuronal surface proteins, and the results of the existing models, are summarized in **Table 2**.

NMDAR-AB ENCEPHALITIS

Clinical Disease and *in vitro* Mechanisms

NMDARE, the classical syndrome associated with IgG1 NMDAR-Abs, is the most commonly recognized AE in clinical practice. It is characterized by psychiatric symptoms, such as confusion, abnormal behavior, paranoia, and hallucinations, in addition to memory problems, seizures, dyskinesia, autonomic instability, catatonia, hypoventilation, lethargy, and language deficits (56). *In vitro*, pathogenic NSAbs bind and cause clustering (57), cross-linking, and internalization of NMDAR, leading to a loss of functional receptors on the cell surface (NMDAR hypofunction), which is reversible on removal of the NMDAR-Abs (11). Moreover, NMDAR-Abs induce dispersal of GluN2A-NMDAR, through the blockade of the interaction between the extracellular domains of GluN1/GluN2 subunits and ephrin-B2 receptors (EPHB2R) (58).

In a high proportion of younger women, the disease is caused by the presence in an ovarian teratoma of neuronal tissue expressing NMDARs and inducing an immune response (59, 60). In others, particularly young children, the disease can follow herpes simplex virus encephalitis (HSVE), probably as a secondary response to the neuronal damage caused by the virus (61).

Spontaneous or Genetic Disease

NMDAR-Abs have been described in other mammals (9) and are present at a low percentage (around 1%) in healthy individuals. In 2014, a retrospective study showed that Knut, the polar bear of the Berlin Zoological Garden who drowned in 2011 following seizures, had high levels of NMDAR-Abs in his serum and CSF, making him the first non-human case of NMDARE and reaffirming the epileptogenicity of these antibodies in mammals. Pathological examination showed a patchy distribution of infiltrating immune cells, with numerous plasma cells around vessels and within the parenchymal infiltrates, in the absence of marked neuronal abnormalities (14).

Mutations in GRIN1 [which encodes the GluN1 (NR1) subunit of NMDAR] have been associated with a phenotype consisting of severe intellectual disability, seizures, hyperkinetic and stereotyped movement disorders, and dysmorphic features (62–64). In mice, selective deletion of GluN1 in CA1 and CA3 pyramidal neurons abolished long-term potentiation (LTP) and induced memory impairment (65, 66).

Passive Transfer Models

Animal models of NMDARE have been published recently with results that recapitulate some of the specific features of the human disease. In rats, stereotactic parenchymal injection of CSF or purified IgGs from patients with NMDARE produced different outcomes. Infusion in the CA1 and premotor cortex increased the levels of extracellular glutamate and, consequently, neuronal excitability (46). On the other hand, several studies showed that a single injection of CSF from patients with NMDARE into the hippocampus produced a reduction of LTP in the CA1, CA3, and dentate gyrus (47–49). Behaviorally, effects ranging from impaired Morris water maze memory performance (47) to a lack of novel object recognition (49) were reported, in the absence of significant changes in locomotor activity or anxiety-like behavior (49).

Continuous icv infusions of CSFs pooled from individuals with NMDARE into mice over 14 days reproduced some of the

TABLE 2 | Summary of main features of NSAb diseases and the models.

Clinical features	Investigations	Main mechanisms identified *in vitro*	Active or PT	Animals, route, duration	Material	Behavior and other observations	Pathology	*Ex vivo* physiological studies	References to *in vitro* and *in vivo* models
NMDAR (IgG1 PREDOMINANTLY)									
NMDAR encephalitis: psychiatric syndrome, seizures, amnesia, movement disorders catatonia, autonomic instability	EEG variable MRI often normal CSF cellular, intrathecal synthesis		Active	C57BL/6 mice (12 months old) WT and ApoE$^{-/-}$; single injection of a mixture of GluN1 extracellular peptides and/or chicken ovalbumin + complete Freund's adjuvant	NMDAR1 peptides	Hyperactivity only after MK-801 in APOE$^{-/-}$ mice 4 weeks after immunization	No CD3 infiltrates, no microglia activation	NA	(9)
		After 24-h incubation with serum from proteoliposome-treated mice, cultured hippocampal neurons showed reduced NMDAR-mediated currents and a decrease of >50% in GluN1 immunoreactivity	Active	C57BL/6 adult mice; subcutaneous injection of NMDARs in proteoliposomes (or liposomes or saline) followed by a booster 2 weeks later	Purified GluN1/GluN2B NMDA fully assembled tetrameric receptors (holoreceptors) embedded in liposomes	Hyperactivity, stereotypied, and anxiety-like behavior 4 weeks after immunization; overt seizures (21%), and hunched back/lethargy (11%)	Perivascular cuffing; patchy areas of cell death; microgliosis; immune cell infiltrates in the brain	Reduced NMDAR-mediated currents in cultured hippocampal neurons incubated with serum of immunized mice	(37)
		Internalization of NMDARs Loss of NMDARs Disruption of ephrin interaction	PT	Male C57BL/6J mice (8–10 weeks old); icv infusion over 14 days	Pooled CSF	Cognitive and depressive-like	IgG bound, NMDAR loss	NA	(38) (11)
			PT	icv, single bolus	Purified serum IgG	Increased seizure susceptibility	IgG, no NMDAR loss	Seizures after PTZ	(39)
			PT	Male C57BL/6 mice (age 8 weeks); icv infusion over 18 days	CSF from patients with NMDARE	Impaired spatial memory as detected with the Morris water maze test	Decreased content of NMDAR in the hippocampus; no neuronal loss or inflammatory cell infiltrates; increased CXCL10 expression in the brain	NA	(40)
			PT	Male C57BL/6J mice (8–10 weeks old); icv infusion over 14 days	CSF from patients with NMDARE with or without ephrin-B2	Memory deficit and depressive-like behavior. EphB2 prevented antibody effects	Decrease of the density of cell surface and synaptic NMDAR and EphB2	Impairment of long-term synaptic plasticity	(41)

(Continued)

TABLE 2 | Continued

Clinical features	Investigations	Main mechanisms identified *in vitro*	Active or PT	Animals, route, duration	Material	Behavior and other observations	Pathology	*Ex vivo* physiological studies	References to *in vitro* and *in vivo* models
			PT	Male C57BL/6 mice (8–10 w old); ICV infusion over 14 days	CSF or IgGs purified from CSF of patients with NMDARE	Absence of overt changes in memory (NOR), anxiety, and locomotor activity (OF, RT). However, reduced preference for novel object at NOR	No neuronal loss; astrocytic hypertrophy but not proliferation in the hippocampus	Increased frequency of seizures; reduced excitability and membrane resistance of CA1 pyramidal neurons in mice hippocampal slices	(42)
		Patient-derived rhuMAb, specifically synaptic NMDAR clusters in cultured hippocampal neurons and NMDAR-mediated currents in NMDAR transfected cells	PT	Mice; icv infusion over 14 days	Recombinant human antibodies from clonally expanded intrathecal plasma cells	Memory impairment at NOR test	Human IgG bound; NMDAR loss in the hippocampus	NA	(43)
		mAb caused internalization of NMDAR	PT	Female Swiss Webster mice, 6–8 weeks old; single iv injection + LPS; 4 days' observation after 3 days' recovery	mAb from a patient with NMDARE	increased spontaneous locomotor activity	NA	NA	(44)
			PT	Female BALB/c mice (8–10 weeks old)	Intranasal inoculation of HSV-1 + ACV	NA	4/6 mice developed serum NMDAR-Abs and showed decreased brain NMDAR expression	NA	(45)
			PT	Males Wistar rats; single stereotactic injection in the hippocampus (CA1) and premotor cortex	CSF or IgGs purified from CSF of patients with NMDARE	Increased glutamate	NA	NA	(46)
			PT	Female Wistar rats (2 months old); single stereotactic injection in the hippocampus (dentate gyrus)	CSF of patients with NMDARE or commercial anti-NMDAR1-Ab	Impaired memory at Morris water maze	NA	Reduced LTP in the dentate gyrus; absence of increased frequency of recurrent epileptiform discharges induced by gabazine compared with controls	(47)
			PT	Female Wistar rats (60–90 days old); single stereotactic injection in the hippocampus (CA3)	CSF of patients with NMDARE	NA	NA	Reduced LTP magnitude at A/C fiber-CA3 synapses compared with controls; increased frequency of epileptiform after potentials following the fEPSP	(48)

(Continued)

TABLE 2 | Continued

Clinical features	Investigations	Main mechanisms identified *in vitro*	Active or PT	Animals, route, duration	Material	Behavior and other observations	Pathology	*Ex vivo* physiological studies	References to *in vitro* and *in vivo* models
			PT	Female Wistar rats (8–10 weeks old); single stereotactic injection in the hippocampus (CA1)	CSF of patients with NMDARE	Absence of overt alteration at NOR, locomotor activity, and anxiety. However, reduced preference for NO at NOR	NA	Schaffer collateral–CA1 LTP reduced in hippocampal slices	(49)
CASPR2 IGg4 > IGg1									
Peripheral (pain, neuromyotonia, autonomic dysfunction)	EMG evidence of peripheral nerve hyperexcitability	Loss of Kv1 expression on the surface of cultured DRG neurons incubated with CASPR2-IgG	PT	Male C57BL/6J mice (8–10 weeks old); ip daily injections for 14–18 days	Purified plasmapheresis IgG	Evidence of lowered thresholds for mechanical pain	IgG bound in DRG, small increase of microglia in spinal cord	Decreased Kv currents with increased excitability of DRG neurons	(50)
Central: limbic encephalitis, Morvan's syndrome	MRI FLAIR hippocampal hyperintensity, CSF bland, little intrathecal synthesis	Some internalization of CASPR2 but no loss of surface CASPR2	PT	Male C57BL/6J mice (8–10 weeks old); ip daily injections for 8 days + 1 ip LPS injection	Purified plasmapheresis IgG	Modest loss of working memory, abnormal behaviors in the presence of novel mouse	No loss of CASPR2 but extensive microglial activation and astrocyte activation with complement expression	NA	(51)
		CASPR2 internalization with reduction of CASPR2 surface expression and decreased intensity of surface GluA1 total and synaptic clusters	PT	C57BL/6J mice; single stereotactic injection; primary visual cortex (V1)	Purified IgG from PLEX	NA	NA	Reduced amplitude of AMPAR-mediated mEPSCs in V1-layer 2/3 pyramidal neurons incubated with patient IgG	(52)
LGI1 IGg4 > IGg1									
Central: LE with or without FBDS and or hyponatremia	MRI FLAIR hippocampal hyperintensity, usually normal CSF, rare OBs; Abs can be absent	Antibodies prevent the binding of LGI1 with ADAM22 and ADAM23	PT	Male C57BL/6J mice (8–10 weeks old); icv infusion over 14 days	Purified IgG from serum	IgG bound; reduced Kv1.1 and AMPAR	Memory deficit at NOR	Increased presynaptic excitability and glutamatergic synaptic transmission and impaired LTP in acute hippocampal slices from LGI1-IgG-injected mice	(53)
AMPAR									
Central: LE	Lymphocytosis; OBs; Abs usually present	Internalization of AMPARs; depletion of heteromeric synaptic AMPARs containing GluA2 most likely followed by a synaptic incorporation of GluA1 homomeric AMPARs; decreased mEPSC amplitudes and frequency in neurons treated with a-GluA2 IgG	PT	C57BL/6 mice (WT and GluA1-KO); icv infusion over 14 days or single stereotactic intrahippocampal (CA1) injection	IgG purified from serum	Memory impairment at NOR and anxiety-like behavior (maximum effect after 18 days during pump infusion)	IgG bound to hippocampus; unchanged spine density and morphology; downregulation of GluA2	Reduced mEPSC amplitudes and impairment of LTP in the SC- CA1 pathway in acute hippocampal slides	(54)

(Continued)

TABLE 2 | Continued

Clinical features	Investigations	Main mechanisms identified *in vitro*	Active or PT	Animals, route, duration	Material	Behavior and other observations	Pathology	*Ex vivo* physiological studies	References to *in vitro* and *in vivo* models
GlyR MAINLY IgG1									
Brain stem and spinal cord: PERM or SPS	Often no evident MRI or EEG findings. Pleocytosis in half of the cases, OBs (20%)	Cause internalization of GlyRs in transfected HEK cells. Inhibit GlyR function acutely	PT	ip daily injections of >10 mg/day for 11 days with 2 LPS injections	Purified plasmapheresis IgG	Modest motor phenotype with poor performance on rotarod and on narrow rods	IgG bound to brain stem and ventral horns IgG detected inside large brain stem neurons GlyRs persisted on surface of neurons	NA	(55) (Carvajal-Gonzalez et al., unpublished data)

neuropsychiatric features observed in patients such as memory deficits, anhedonia, and depressive-like behaviors. Seizures or movement disorders were not observed. IgG deposition and a decrease in NMDAR clusters on hippocampal neurons was observed in NMDAR-Ab-injected mice, which resolved within days after discontinuing the infusion (38). Further studies have also shown disruption of the normal interaction with other synaptic proteins, in particular EphrinB2R. Administration of ephrin-B2 (the ligand of the EphrinB2 receptor) in the 14-day infusion animal model prevented the pathogenic effects of NMDAR-Abs on memory and behavior, levels of cell-surface NMDAR, and synaptic plasticity (41). Recently developed human-derived monoclonal antibodies to the NMDAR have produced similar pathogenic effects *in vivo* and *in vitro* and offer a promising less-limited resource (compared to human CSF and IgG) for future experimental studies (43).

In another mouse model, icv injection of purified plasmapheresis IgG from individuals with NMDARE induced, in association with a subthreshold dose of the chemo-convulsant pentylenetetrazol (PTZ), more frequent and severe seizures than a single injection of IgG from control individuals [(39); see **Figure 3**]; cognitive and other features were not examined in these mice. Continuous wireless electroencephalogram (EEG) recording did not identify any spontaneous seizure activity. However, there was IgG bound to the hippocampus at 48 h post icv infusion, particularly to the CA3 region, and it correlated with the number and severity of seizures seen in the mice, but there was no apparent loss of NMDARs (**Figure 3**). In a more recent study, EEG recordings of mice infused intraventricularly for 14 days with CSF NMDAR-Abs showed a higher frequency of seizures compared with control mice, associated with variable behavior ranging from sleeping or normal exploratory activity to freezing and myoclonic jerks (42). Two main seizure patterns were observed, one, more frequent, characterized by high-amplitude rhythmic spikes that occurred at relatively constant rates or at irregular intervals and another, less common, characterized by high-amplitude fast rhythmic activity that fluctuated in amplitude in a spindle-like fashion (42). Continuous EEG recordings may be necessary to detect reliably spontaneous non-motor seizures in models of antibody-mediated encephalitis. Neuropathology showed absence of neuronal death and only mild astrocytic activation (42).

In another study using continuous icv infusion, mice receiving patients' CSF showed memory impairment in the Morris water maze, but not in the novel object recognition test, and a tendency to a reduced expression of NMDAR in the mouse brains. No overt inflammatory changes were observed, but an increase of the chemokine CXCL10 was detected (40), a finding that has been observed also in patients with NMDARE (67). Intravenous infusion of monoclonal NMDAR-Abs followed by LPS increased mouse voluntary locomotor activity at the mouse wheel-running test, similarly to that observed in mice treated with low doses of the NMDAR inhibitor MK-801 (44).

Overall, the passive-transfer animal models support the proposed mechanisms of cross-linking and internalization as well as the relevant role of altered NMDAR trafficking in the

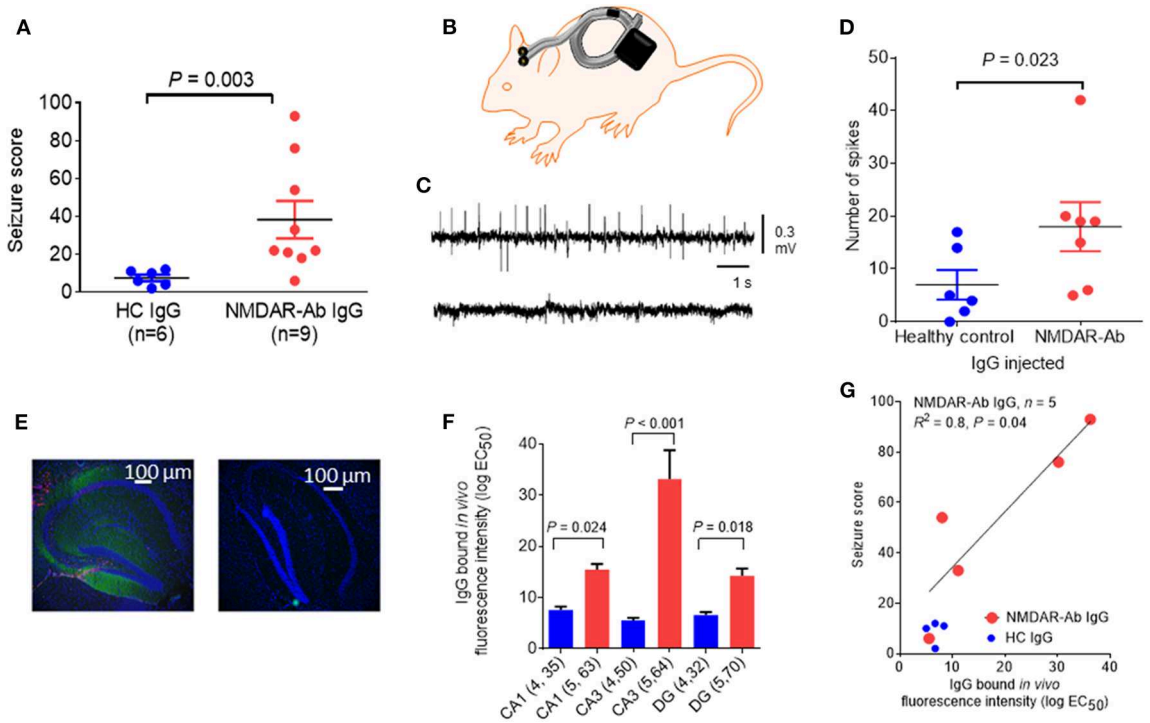

FIGURE 3 | Epileptogenic effects of a single intracerebroventricular (icv) injection of *N*-methyl-D-aspartate receptor antibody (NMDAR-Ab)-positive immunoglobulin G (IgG). **(A)** The seizure score of mice injected with NMDAR-Ab IgG was higher than that of those injected with control IgG following exposure to a subthreshold dose of PTZ. **(B)** Diagram showing placement of subcutaneous wireless electroencephalogram (EEG) transmitter that allows continuous EEG recording in injected mice with no need for tethering (Open Source, Hashemi Instruments, USA). **(C)** A representative EEG of an NMDAR-Ab-injected mouse post-PTZ shows a number of "spikes" corresponding to convulsive seizures (upper trace), compared with the EEG of a healthy control IgG-injected mouse, which has minimal spike activity (lower trace). **(D)** When analyzed using the computer-based event detection program and blinded observer verification, the number of spikes seen in the hour following PTZ injection was greater in the NMDAR-Ab ($n = 7$) compared with the healthy control IgG ($n = 6$) injected mice ($P = 0.023$, Mann–Whitney). Results are mean ± SEM. **(E)** Human IgG injected *in vivo* was detected postmortem in NMDAR-Ab IgG-injected mice with antihuman IgG (green) merged with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) (blue). The typical pattern of NMDAR-Ab in the molecular cell layer with sparing of the granule cell layer was found (left image). Control IgG-injected mice had no detectable IgG (right image). **(F)** Bound human IgG in the hippocampi, as determined by the mean fluorescence intensity analysis of brain sections, was higher in the NMDAR-Ab IgG-injected mice than in healthy control IgG-injected mice in the CA1, CA3, and dentate gyrus (DG). **(G)** For the NMDAR-Ab animals ($n = 5$), there was a linear correlation between IgG binding and seizure score ($R^2 = 0.8$; $P = 0.04$). The contents of this figure are taken from Wright et al. (39) with permission from Oxford University Press.

pathogenesis. However, these models have not demonstrated all the clinical features; for example, none have reproduced the (often-striking) movement disorders or shown long-term cognitive deficits and structural hippocampal damage as seen in some patients (68). A possibility is that some inflammatory changes are not reproduced by passive transfer. The discrepancies observed between different models might also relate to different protocols, to the use of different species and strains, and to different effects of the antibodies in relation to acute or chronic exposure.

Active Immunization

In a recent mice active immunization model, Pan et al. (9) showed that mice immunized against NMDAR1 peptides did not show behavioral changes at the open-field test. Even in the presence of high titers of NMDAR-Ab, an increase of locomotor activity, a psychosis-like behavior, was obtained only upon MK-801 challenge in ApoE$^{-/-}$ mice, which present a disrupted BBB. No lymphocyte (CD3) infiltrates nor microglial activation was detected on immunopathology. On the contrary, immunization with purified GluN1/GluN2B fully assembled tetrameric NMDARs (holoreceptors) embedded in liposomes induced a phenotype characterized by hyperactivity, stereotyped motor features (tight curling), and seizures in association with neuroinflammation and immune cell infiltrates (37). Distinct from the passive-transfer models, these immunized mice produced GluN1 and GluN2 antibodies that reacted with the linear epitopes of the NMDAR protein, and not the amino-terminal domain of GluN1 as seen in the human-derived antibodies (69). Nevertheless, this model may prove useful for testing novel treatments acting on the cellular inflammatory component of the disease.

Finally, a recent small study investigated the mechanisms involved in the pathogenesis of post-HSV-1 NMDARE (45). Following intranasal inoculation of HSV-1, 67% (four out of six) of mice developed serum NMDAR-Abs. The same mice showed reduced hippocampal NMDAR compared with mice without antibodies, inferring IgG-mediated loss, but the

authors did not demonstrate IgG antibodies bound to the hippocampus. This model could be a useful platform to further explore the mechanisms of post-HSV encephalitis with secondary NMDARE.

CASPR2-AB ENCEPHALITIS

Clinical Disease and *in vitro* Mechanisms

CASPR2 is a neurexin-related cell adhesion molecule expressed in the CNS and peripheral nervous system, and CASPR2 antibodies (CASPR2-Abs) react with both the brain and peripheral nerve tissues [(70); see **Figure 1**]. This expression pattern well-explains why CASPR2-Abs have been associated not only with peripheral nerve hyperexcitability (often called neuromyotonia) but also with CNS symptoms including cognitive impairment, memory loss, hallucinations, delusions, cerebellar symptoms, and epilepsy. Some patients present with Morvan syndrome (MoS), characterized by the combination of neuromyotonia, neuropathic pain, encephalopathy with hallucinations, and a sleep disorder, described as agrypnia excitata (71, 72); the latter is characterized by severe insomnia, dream-like stupor (hallucinations and enacted dreams), sympathetic hyperactivity (hyperthermia, perspiration, tachypnea, tachycardia, and hypertension), and motor agitation. CASPR2-Abs are mainly IgG4, but most patients have IgG1 antibodies as well.

CASPR2 is essential for clustering Kv1.1 and Kv1.2 channels at the juxtaparanodes of myelinated axons, where the channels are important for repolarization of the nerve axon, avoiding repetitive firing and helping to maintain the internodal resting potential. Their functions at CNS synapses are not well-defined.

The *in vitro* effects of CASPR2-Abs are complex. In one study, the antibodies inhibited CASPR2 interaction with contactin-2 but did not lead to CASPR2 internalization (73). However, in two others, *in vitro* exposure induced CASPR2 internalization *in vitro* (51, 52) with variable effects on CASPR2 expression, ranging from absent (51) to significant (52) loss of surface expression.

Spontaneous or Genetic Disorders

Interestingly, mutations in the *CNTNAP2* gene, encoding CASPR2, are associated with focal epilepsy, schizophrenia, and autism spectrum disorder (ASD) (74). CNTNAP2-knockout (KO) mice were shown to have social deficits, abnormal motor activity, cognitive deficits, and seizures (75).

Passive Transfer Models

Intraperitoneal injection of purified IgG from two CASPR2-Ab-positive patients to mice over 14–18 days, without attempt to breach the BBB, reduced the thresholds for mechanical stimuli, a signature of pain (50). The effects induced by the antibodies on pain sensitivity were also observed in KO mice lacking CASPR2 ($CNTNAP2^{-/-}$). These mice demonstrated enhanced pain-related hypersensitivity to noxious mechanical stimuli, although more severe than that obtained with the antibodies, and also to heat and algogens. Nevertheless, either immune or genetic-mediated ablation of CASPR2 enhanced the excitability of dorsal root ganglia (DRG) neurons through regulation of Kv1 channel expression at the soma membrane (50). CASPR2-IgG did not cause neuronal loss nor overt inflammation, although a modest increase in microglial cell count was observed in the spinal cord (50).

To explore the effects of CASPR2-Ab in the CNS, a similar protocol was used with eight daily injections of IgG purified from one patient with AE and from one healthy control (**Figure 4**). A single dose of LPS was added at day 3 to disrupt the BBB (51). Mice injected with CASPR2-IgG showed less alternation in the continuous spontaneous alternation tests, suggestive of memory impairment, and longer latency to interact and increased immobility during the social interaction test (**Figure 4**). These changes had not been seen during isolated open-field or other tests, suggesting that the effects could be indicative of anxiety in the context of a novel mouse, rather than an effect on normal exploratory activity. At neuropathology, CASPR2-IgG injected mice showed human IgG deposition, particularly in the cortex, hippocampus, and thalamus; mild loss of Purkinje cells and c-Fos activation as well as microglial and astrocyte activation without B- or T-cell infiltration (**Figure 4**). Microglial activation has been reported in neuropathological cases of patients with CASPR2-Ab encephalitis (76, 77).

Although this model showed evidence of pathogenicity of CASPR2-IgG, it failed to recapitulate the wide range of defects found in the patients (e.g., autonomic, sleep disturbance, and hormonal/neuropeptide abnormalities) who would require substantial additional tests. Moreover, it does not explain how CASPR2-Abs cause their effects. Indeed, IgG deposition was not associated with a reduction of CASPR2 expression on immunohistology. On the contrary, a trend toward higher levels of mouse CASPR2 was seen in the brain extracts of CASPR2-IgG-injected mice, suggesting some compensatory upregulation.

Injection of a mixture of CASPR2-Abs in mouse visual cortex produced impaired localization of mouse Caspr2 to excitatory synapses and significantly decreased AMPAR-mediated currents in layer 2/3 pyramidal neurons; this implied a dysfunction of glutamatergic transmission in the pathogenesis of CASPR2-Ab encephalitis (52). Future studies should evaluate in parallel the effects of CASPR2-Abs on its partner protein network and on neuronal activity.

LGI1-AB ENCEPHALITIS

Clinical Disease and *in vitro* Mechanisms

Autoantibodies to LGI1 (LGI1-Abs) are the most common autoantibody in patients with limbic encephalitis (LE), a clinical syndrome characterized by the acute development of mood changes, anxiety, short-term memory deficit, and seizures due to an inflammatory process involving the limbic system that includes the medial temporal lobes, hippocampus, amygdala, and frontobasal and cingulate cortices (1). In patients with LGI1-Abs, the onset of an overt limbic dysfunction can be preceded by episodes of faciobrachial or crural seizures that last a few seconds and occur many times during the day; these episodes have been described as faciobrachial dystonic seizures (FBDS) (78).

LGI1 is a protein secreted by the presynaptic terminals of neurons that bind to ADAM22 and ADAM23, two proteins

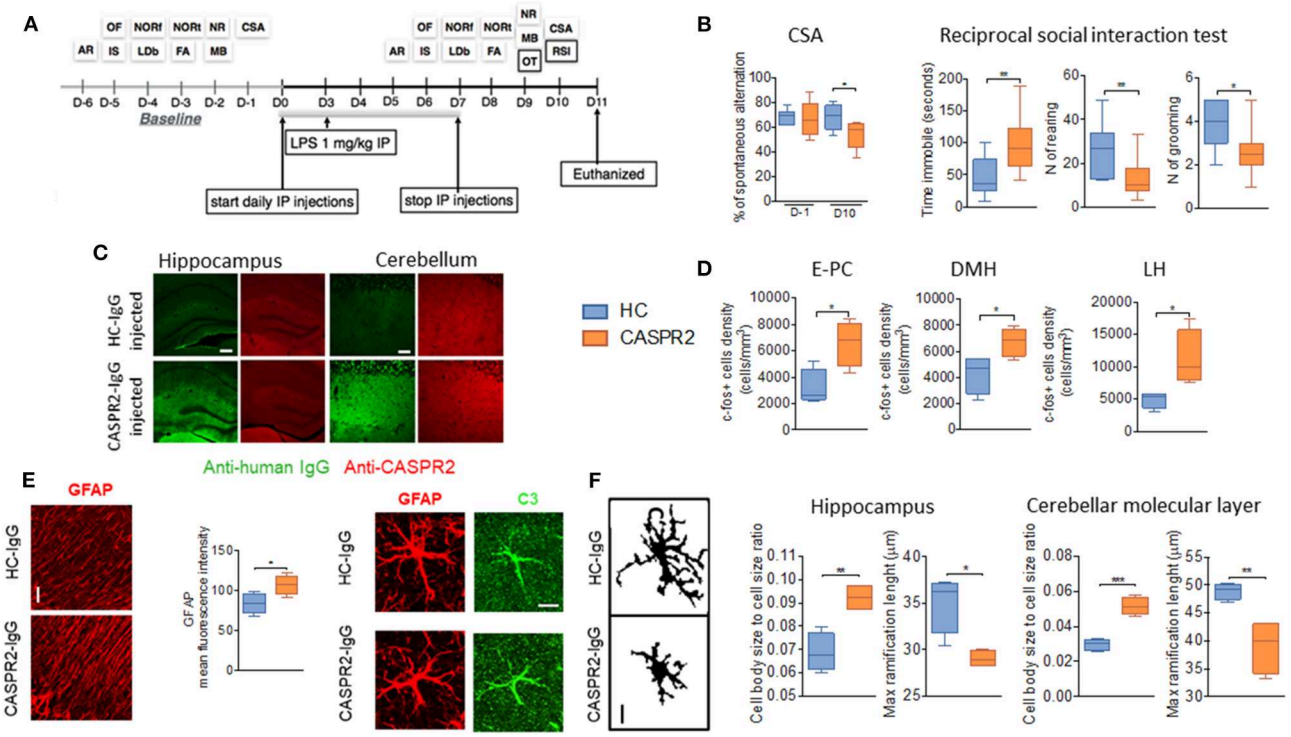

FIGURE 4 | Intraperitoneal (ip) injection of CASPR2 immunoglobulin G (IgG) with lipopolysaccharide (LPS) causes behavioral and neuropathological changes in mice. **(A)** Experimental design and selected behavioral tests. The behavioral tasks assessed locomotion (open field, OF), strength (inverted screen, IS), coordination (accelerating rotarod, AR; and narrow beam, NB), working memory (continuous spontaneous alternation, CSA), short- (forced alternation, FA) and long-term memory (novel object recognition, NOR—NORf, familiarization phase; NORt, test phase), anxiety (light-dark box, LDb), compulsive-like behavior (marble burying test, MB), social behavior (reciprocal social interaction tests, RSI), and olfaction (olfaction test, OT). **(B)** Continuous spontaneous alternations were reduced in CASPR2-IgG-injected mice compared with HC-IgG-injected mice ($P = 0.044$). In the RSI test, there was reduced latency to interact ($P = 0.04$; Mann–Whitney test) but no differences in the interaction time or number of interactions. However, in the non-social aspects of the test, there was increased time spent immobile ($U = 0.008$), reduced rearing ($U = 0.02$), and reduced grooming ($U = 0.018$). **(C)** Bound human IgG in the hippocampi and cerebellum of CASPR2- and HC-IgG-injected mice. CASPR2-IgG-injected animals had higher levels of IgG in the cortex (Cx) ($P = 0.03$), hippocampus (Hip) ($P = 0.023$), and thalamus (Th) ($P = 0.0004$) compared with HC-IgG-injected mice. No differences were observed in the levels of CASPR2 expression in the same areas ($n = 4$ per group). **(D)** c-Fos expression in the entorhinal–piriform cortex ($P = 0.020$), dorsomedial hypothalamus (DMH) ($P = 0.037$), and lateral hypothalamus (LH) ($P = 0.031$) was higher in the CASPR2-IgG-injected mice than in the HC-IgG-injected mice ($n = 4$ per group). **(E)** Representative images of glial fibrillary acidic protein (GFAP) staining in the molecular layer of the cerebellum and quantification of the mean fluorescence intensity in the same area showing higher GFAP expression in the CASPR2-IgG-injected mice ($P = 0.043$) ($n = 4$ per group; 40X, 10 µm). On the right, representative images of complement C3 expression on GFAP-positive cells. Percentage of C3/GFAP area ratio per cell showed increased C3 expression of astrocytes in CASPR2-IgG-injected mice. **(F)** Representative images of the z-stack projected IBA1 staining used for morphological analysis (40X, 10 µm). Quantification of morphological data in the hippocampus and molecular layer of the cerebellum showed that microglia from CASPR2-IgG-injected mice had a higher cell soma/cell total body size ratio [$t_{(6)} = 4.74$, $P = 0.0032$] and shorter [$t_{(6)} = 3.68$] ramifications than HC-IgG-injected mice, compatible with an activated phenotype in both the hippocampus ($P = 0.017$ and $P = 0.010$, respectively) and the cerebellum ($P = 0.0003$ and $P = 0.008$, respectively). ***The contents of this figure are taken from Giannoccaro et al. (51) with permission from Oxford University Press. * < 0.05, ** ≤ 0.01, *** ≤ 0.001.

involved in cell–cell adhesion and located presynaptically and postsynaptically, respectively (**Figure 1**). Binding to ADAM22, LGI1 regulates AMPAR-mediated synaptic currents in the hippocampus (79). Binding to ADAM23, LGI1 selectively prevents inactivation of the presynaptic voltage-gated potassium channel Kv1.1 (80) mediated by a cytoplasmic regulatory protein, Kvβ (81).

In cultured hippocampal neurons, LGI1-Abs disrupt the ligand–receptor interaction of LGI1 with ADAM22, resulting in reversible reduction in synaptic AMPARs [(82); see **Figure 1**]; these antibodies could be IgG4. However, in the few postmortem studies on patients who have died unexpectedly, there appears to be IgG deposition, some complement deposition, and loss of neurons. These findings would be compatible with the presence of IgG1 antibodies; although they are in the minority compared with IgG4, they tend to be much higher in patients with cognitive impairment (70, 83). IgG1-induced neuronal loss would explain why, despite a good response to immunotherapy, many patients are left with hippocampal atrophy (84), and only 35% of patients return to their baseline cognitive function (85).

Spontaneous or Genetic Disorders

LGI1 mutations have been associated with an autosomal dominant lateral temporal lobe epilepsy (ADLTLE) manifesting with focal seizures often with auditory features (86). The majority of mutations prevent LGI1 secretion, whereas others alter its interactions with ADAM22/ADAM23 (87). Animal models of LGI1 depletion all present spontaneous seizures

(88–92), although the mechanisms behind this increased epileptic susceptibility have not been fully elucidated and both enhanced excitatory transmission (90, 91, 93) and reduced AMPAR function (79, 80, 89) have been reported.

Intriguingly, a spontaneous model of LGI1-Ab encephalitis has been observed in cats with feline complex partial seizures with orofacial involvement (FEPSO) (13, 94–96). Clinically, they presented with acute onset of complex partial seizures with orofacial involvement (salivation, facial twitching, lip smacking, chewing, licking, or swallowing), motor arrest (motionless starring), and behavioral changes associated with bilateral hyperintensities at brain MRI (13, 94, 95). Postmortem analysis of three cases showed IgG and complement deposition associated with neuronal loss, consistent with the findings in the few available postmortem examinations from patients with LGI1-related encephalitis (3, 95). Subsequent neuropathological studies in cats showed also that, whereas T-cell infiltrates were present brainwide, BBB leakage was more restricted to limbic areas (96). This observation suggests that a local BBB vulnerability might be responsible for the selective involvement of the limbic system, even though LGI1 is expressed throughout the brain.

Passive Transfer Model

More recently, the pathogenicity of LGI1-Abs has been confirmed by a passive transfer mouse model based on cerebroventricular transfer of patient- or control-derived IgG (53). LGI1-Ab-injected mice showed memory impairment which slowly reversed after stopping the infusion. However, in contrast to the spontaneous feline model and LGI1-KO animals, no epileptic seizures were observed. Nevertheless, LGI1-Ab caused a significant decrease of the density of total and synaptic Kv1.1 and AMPAR clusters due to the disruption of LGI1 interactions with presynaptic ADAM23 and postsynaptic ADAM22. Consistent with decreased Kv1.1 expression and previous *in vitro* studies (97), increased presynaptic excitability and glutamatergic transmission were observed in acute brain slice preparations, resulting in increased evoked excitatory postsynaptic currents (eEPSCs) and reduced failure rate of synaptic transmission after minimal-stimulation excitatory postsynaptic currents (msEPSCs). Exposure to LGI1-Ab was also associated with impaired LTP, which was however independent of Kv1.1 blockade and possibly related to reduced availability of AMPAR during LTP. However, these changes were not sufficient to cause seizures in this model. It is likely that the changes induced by the antibodies are not as severe as those induced by genetic mutation or ablation of the LGI1 gene. On the other hand, complement activation and neuronal loss may play a major role in the human and feline diseases and mouse serum has a low intrinsic complement activity (98). Further studies are needed to investigate this aspect and its relevance to the clinical phenotype.

AMPAR-AB ENCEPHALITIS

Clinical Disease and *in vitro* Mechanisms

AMPAR antibodies (AMPAR-Abs) are usually associated with a typical LE, sometimes associated with extra limbic manifestations, although they can rarely present with rapidly progressive dementia or psychosis (99, 100).

AMPAR is a heterotetrameric ionotropic glutamate receptor that mediates most of the fast-excitatory transmission in the brain (101). AMPAR-Abs can be directed against the GluA1 or GluA2 subunits or both (100). Incubation of cultured rodent neurons with patients' IgG to GluA2 led to a decrease of synaptic AMPAR clusters, resulting in reduced frequency and peak amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) (100, 102).

Spontaneous or Genetic Disorders

Mutations in the GluA1 or GluA2 subunits have been associated with neurodevelopmental disorders (NDs) including intellectual disability and autism (103, 104). GluA1-KO mice present impaired hippocampal synaptic plasticity (105, 106) and working memory (107–109), whereas GluA2-KO mice are hypomorphs with poor motor coordination and low explorative activity (110, 111). Conditional ablation of GluA1 or GluA2 in mice causes memory deficits and remodeling of AMPAR subunit distribution (112–115).

Passive Transfer Models

In accordance with these findings, *in vitro* studies and *in vivo* hippocampal injection of human antibodies against the GluA2 subunit in mice was associated with synaptic downregulation of GluA2 and increased single-channel conductance in recordings of the GluA2 IgG-injected mouse, suggestive of GluA2 endocytosis and compensatory synaptic incorporation of GluA1-containing AMPARs, which have higher channel permeability (54), as observed in conditional KO models (113–115). Consistently, this compensatory increase in single-channel conductance was abrogated in KO mice deficient for GluA1 stereotactically injected with GluA2 antibodies (GluA2-Abs). Despite these compensatory mechanisms, injection of GluA2-Abs was associated with impaired LTP in the region of GluA2-IgG deposition. Both continuous icv infusion of GluA2-Abs over a 2-week period and stereotactic bilateral injections of patient IgG directly into the DG, CA1 and CA3 regions of the hippocampus, were associated with impaired memory and increased anxiety-like behavior in mice (54). Despite the observed AMPAR subunit rearrangement, mice did not show seizures. Therefore, future studies have to evaluate if these changes are associated with neuronal hyperactivity and how they are related to seizures in patients. Moreover, the pathogenicity and mechanisms associated with antibodies directed against the GluA1 subunit of AMPAR remain to be established.

ANIMAL MODELS OF NDS INDUCED BY *IN UTERO* EXPOSURE TO NSABS

There has been growing interest in the possibility that maternal antibodies to neuronal antigens could cause neurodevelopmental diseases, presenting neonatally or later in life. This sprung initially from studies in mothers with MG whose babies

developed arthrogryposis. The maternal antibodies were found to inhibit the function of the fetal AChR and, when crossing the placenta in the second trimester, paralyzed the babies *in utero*; consecutive pregnancies were affected (28, 116). A mouse model of maternal antibody transfer to the mouse fetus was developed to show that the maternal serum antibodies were pathogenic (36), and the model was then used to study a mother who had two consecutive children with NDs (one healthy, one with autism, and one with language disorder). The serum contained antibodies that bound to fetal cerebellar neurons in rat tissue sections and impaired motor behavior in the adult mouse offspring of injected dams (117). Since then, many studies looking for maternal antibodies in autism and testing their effects in mouse or non-human primates have been performed [see (118)], but until recently, none had defined a specific neuronal antigen that was likely to be the target of fetopathogenic antibodies.

As mentioned above, mutations in the gene encoding CASPR2 are not common but can be associated with a variety of neurological and psychiatric disorders, ranging from ASD or mental retardation and epilepsy to learning disability, schizophrenia, and Tourette syndrome (119). Mutations in the GluN genes that encode the *N*-methyl-D-aspartate (NMDA) subunits are found in children with a variety of NDs and epileptic syndromes (120). Both these proteins could be targets for antibodies that, during development, altered neurodevelopment. **Table 3** summarizes the most recent work in this field.

EVIDENCE FOR ANTIBODIES TO NSABS IN PREGNANCY

CASPR2-Abs

Only one study to our knowledge has looked for antibodies to these proteins in gestational samples from women whose children have subsequently been diagnosed with specific or non-specific neurodevelopmental conditions, comparing with mothers with no such history in their children. Coutinho et al. (121) measured a range of neuronal antibodies in Danish cohorts of early or mid-gestational sera. LGI1-Abs, AMPAR-Abs, and GABAB receptor antibodies were not found. NMDAR-Abs were not uncommon (overall 5.8%) and more common in mothers who developed psychosis at some time after the pregnancy. By contrast, CASPR2-Abs were present (4.9%) in mothers of children with a diagnosis of mental retardation or other disorders of psychological development in their children compared with only 0.9% of coded age-matched mothers with no such history. This supported the possibility that CASPR2-Abs could be a cause or contributor to neurodevelopmental diseases in the offspring. Surprisingly, CASPR2-Abs were low in mothers of autistic children and not different from the appropriate controls.

A maternal-to-fetal transfer of disease was performed in mice. The offspring of CASPR2-injected dams were normal postnatally but as adults showed changes in social interaction tests, and after termination, there was clear evidence of microglial activation and reduced glutamatergic synapses, suggesting that microglia activated by CASPR2-Abs induced changes that resulted in persistent synaptic loss (122).

A similar model was undertaken using a monoclonal CASPR2-Ab cloned from a mother of an autistic child (123). In this study, male mice exposed *in utero* to CASPR2-Abs showed an ASD-like phenotype, abnormal cortical development, and altered hippocampal neurons. Postnatal samples from selected mothers of autistic children were more often positive for CASPR2-Abs than from mothers of children with typical development or women of childbearing age. These sera were not gestational and in many cases obtained from mothers years after the affected birth.

NMDAR-Abs

In Coutinho et al. (121), NMDAR-Abs were relatively frequent (5.8%) during pregnancy. Although NMDAR-Abs were more frequent in mothers with NDs in their children (ND mothers) than coded age- and gestation-matched mothers with no such histories (HC mothers), this difference was not significant (7.7 vs. 4.6%). Indeed, among the few reported cases of NMDARE during pregnancy, the majority of newborns were healthy, except for three cases with neurological sequelae, including neurodevelopmental delay, movement disorders, and seizures, and three cases of miscarriages and abortion (125–127). Whether these complications are due to the antibodies or to the mothers' condition severity and related pharmacological treatments during gestation is not yet clear.

Jurek et al. (124) showed a marginal increase in NMDAR-Ab titers in postnatal sera from mothers of a mixed population of neuropsychiatric disorders in a recent study, compared with mothers of unaffected children. These authors preformed a similar model of *in utero* exposure to human NMDAR-Abs, but in this case using recombinant human monoclonal NR1-reactive IgG antibodies (124). The placentally transferred antibodies bound to synaptic structures in the fetal brain, and the pups demonstrated increased mortality and transiently reduced NMDAR brain density with impaired excitatory neurotransmission. The animals displayed hyperactivity, lower anxiety, and impaired sensorimotor gaiting during adolescence and adulthood. In aged mice (10 months), the volumes of the cerebellum, midbrain, and brain stem were all reduced (124). This study suggests that prenatal exposure to NMDAR-Abs may result in children's lifelong neurodevelopmental changes that are potentially treatable and preventable, if identified in the mothers during pregnancy, although there is no evidence of that so far. Such changes might predispose to specific NDs such as autism or schizophrenia.

DISCUSSION AND CONCLUSIONS

Animal models have helped to elucidate pathogenic mechanisms of several NSAbs. However, they often fail to recapitulate the entire phenotypic spectrum associated with human diseases. In particular, no movement disorders have been found in the models of NMDARE, and no seizures were detected in mice injected with LGI1-Abs. This could be related to several factors. Firstly, the choice of the species and strains is relevant. Nowadays, mice

TABLE 3 | Neurodevelopmental antigens and models.

Protein	Presence of antibodies in mothers of children	Antibodies injected and effects of antibodies on offspring of maternal-to-fetal transfer model: behavior	Effects of antibodies on offspring of maternal-to-fetal transfer model: neuropathology	References
Acetylcholine receptor	Rare mothers with antibodies that inhibit fetal AChR, paralyze baby *in utero*, and cause multiple fixed joints, with paralysis and death *ex utero*	Maternal plasma antibodies injected into dams during E13–18 of pregnancy. Proportion of offspring who died at birth or shortly after probably due to lack of respiration	Antibodies present in mouse offspring, offspring showed fixed joints mirroring changes in human babies	(36)
CASPR2	4.9% of mothers with children diagnosed with range of motor and psychological disorders, not autism. HC 0.9%	IgG purified from plasmapheresis samples of two CASPR2-Ab-positive patients. Mice showed changes in cognition and impaired social interactions	Long-term neuropathological changes with activated microglia and glutamatergic synaptic loss	(121, 122)
CASPR2	37% of selected (brain reactive Abs) mothers of children with autism spectrum disorder; 12% of unselected women of childbearing age	MAb binding CASPR2 cloned from the mother of an autistic child. Mice showed impairments in sociability, flexible learning, and repetitive behaviors	Abnormal cortical development, decreased dendritic complexity of excitatory neurons, and reduced numbers of inhibitory neurons in the hippocampus	(123)
NMDAR (NR1 subunit)	Marginal evidence for NMDAR antibodies in mothers of children with any psychiatric/neuropsychiatric disorders	mAbs from NMDAR-Ab-positive women. Mice showed early postnatal mortality (27.2%), altered blood pH, and impaired neurodevelopmental reflexes. *Ex vivo*, NMDAR reduced in brain, with altered spontaneous excitatory postsynaptic currents. When adult, persistent hyperactivity, lower anxiety, and impaired sensorimotor gating	NMDAR was reduced (up to 49.2%), and electrophysiological properties were altered, reflected by decreased amplitudes of spontaneous excitatory postsynaptic currents in young neonates (−34.4%). Cerebellum, midbrain, brain stem volumes reduced	(124)

are the preferred animals for the majority of immune models; however, certain strains used can be resistant to development of diseases, as shown by MG models of active immunization. The gender is another potentially relevant factor, as hormones can significantly impact several immunological and neuronal aspects.

Different immunization models have different advantages and disadvantages. Intraventricular or intraparenchymal administration routes are useful in exposing the antibodies to their targets, but they may be misleading when peripheral antibodies play a major role as appears to be the case for CASPR2-Abs and LGI1-Abs. On the other hand, peripheral injection of the antibodies often requires "opening" the BBB by some method, and these methods may bias the results, allowing the antibodies to access certain brain areas and not others that are more relevant to the human disease (128, 129).

Passive transfer of antibodies is ideal to investigate the downstream mechanisms by which the patient antibodies affect their targets with possible secondary effects, but by itself, it does not appear to enlist cellular mechanisms that might be important in the human condition. Thus, it does not provide insight into the immunological mechanisms behind the generation of the antibodies nor the immunological effectors. For instance, the poor ability of human IgG to fix mouse complement is a limitation if complement activation plays a relevant part in the disease. Overall, the immune cells and the Fc receptors relevant for the human immune response might be different in animal models due to the use of alternative pathways, different effectors, and different cellular receptor affinities (130–132). Future passive transfer studies of patient-derived immune cells into humanized models or studies in non-human primates might help define the involvement of specific immune cells in the pathogenesis of these disorders.

Active immunization models could be helpful in overcoming some of these limitations and could also be more helpful in studying the effector immune mechanisms, but few studies have used this approach to date. Moreover, using peptide sequences for immunization is unlikely to generate the most appropriate pathogenic antibodies if the natural disease recognizes the native membrane protein rather than peptide or polypeptide sequences.

It is also important to note that the failure to reproduce some clinical features observed in patients might be related to the experimental approach or timing of protocols. For example, as shown for NMDAR-Ab, the presence of spontaneous seizures could be overlooked in the absence of continuous EEG monitoring (42). Similarly, antibodies may manifest their maximum effects up to 18 days after CSF infusion (38). Behavioral testing has to be carefully tailored and should take into account the effects of habituation and test repetition.

Future research and refinement of these animal models require a collaborative approach and sharing of optimal methods. Effective and reliable preclinical testing of novel treatments demands rigorous and reproducible protocols that not only allow study of the underlying neurobiology but also facilitate therapeutic studies with rapid translation to the clinic.

AUTHOR CONTRIBUTIONS

MG: conception and drafting of the manuscript. SW: drafting, editing, and review of the manuscript. AV: conception, drafting, editing, and review of the manuscript.

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Effects of the Positive Threshold and Data Analysis on Human MOG Antibody Detection by Live Flow Cytometry

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Human autoantibodies targeting myelin oligodendrocyte glycoprotein (MOG Ab) have become a useful clinical biomarker for the diagnosis of a spectrum of inflammatory demyelinating disorders. Live cell-based assays that detect MOG Ab against conformational MOG are currently the gold standard. Flow cytometry, in which serum binding to MOG-expressing cells and control cells are quantitively evaluated, is a widely used observer-independent, precise, and reliable detection method. However, there is currently no consensus on data analysis; for example, seropositive thresholds have been reported using varying standard deviations above a control cohort. Herein, we used a large cohort of 482 sera including samples from patients with monophasic or relapsing demyelination phenotypes consistent with MOG antibody-associated demyelination and other neurological diseases, as well as healthy controls, and applied a series of published analyses involving a background subtraction (delta) or a division (ratio). Loss of seropositivity and reduced detection sensitivity were observed when MOG ratio analyses or when 10 standard deviation (SD) or an arbitrary number was used to establish the threshold. Background binding and MOG ratio value were negatively correlated, in which patients seronegative by MOG ratio had high non-specific binding, a characteristic of serum that must be acknowledged. Most MOG Ab serostatuses were similar across analyses when optimal thresholds obtained by ROC analyses were used, demonstrating the robust nature and high discriminatory power of flow cytometry cell-based assays. With increased demand to identify MOG Ab-positive patients, a consensus on analysis is vital to improve patient diagnosis and for cross-study comparisons to ultimately define MOG Ab-associated disorders.

Keywords: demyelination, optic neuritis (ON), myelitis, MOG antibody, flow cytometry analysis, antibody detection, patient diagnosis

INTRODUCTION

Detection of human autoantibodies targeting myelin oligodendrocyte glycoprotein (MOG Ab) is now a relevant and important diagnostic biomarker in the field of central nervous system (CNS) demyelination. MOG Ab-associated disorders encompass a disease entity involving the brain, optic nerve, and spinal cord that is distinct from multiple sclerosis (MS) and aquaporin-4 Ab-positive neuromyelitis optica spectrum disorder (NMOSD) (1–15). The reemergence of MOG Ab in the field of autoimmune diagnostics has sparked wide interest, and with ongoing advances in our understanding of MOG Ab-associated disease, requests for MOG Ab testing have risen dramatically, as treatment regimens and prognosis for MOG Ab-positive patients are divergent from MS and aquaporin-4 Ab-positive NMOSD patients (11, 16, 17). Moreover, some MOG Ab-positive patients, particularly those with relapsing disease or delayed immunotherapy, may accrue residual disability (11, 12, 15–19). As such, early and accurate identification of MOG Ab-seropositivity is crucial.

Detection of human MOG Ab against full-length native conformational MOG using live cell-based assays by flow cytometry or microscopy has been established as the diagnostic gold standard and is superior to assays utilizing fixatives (20–22). Flow cytometry provides an investigator-independent quantitative measure of MOG Ab titers and has been validated and proven reliable, with high sensitivity and specificity (20, 21). Due to the data complexity and non-specific binding in human sera, different analyses of flow cytometry data have been reported. For example, when serum binding to MOG-expressing cells is compared to control cells, quantification of MOG Ab titers has been reported by subtraction (delta) or division (binding ratio). Additionally, there are disparities in calculating the positive threshold. A comparison of published analyses using the same dataset is required to observe whether these variations can influence the assessment of MOG Ab serostatus and patient diagnosis.

Herein, we have used our extensive flow cytometry and clinical published datasets (11, 15, 21) of 482 sera to address the influence of data analysis on the interpretation of MOG Ab serostatus. Furthermore, we make recommendations for the international standardization of flow cytometry-based MOG Ab analysis.

MATERIALS AND METHODS

Patient and Control Samples

In the absence of consensus clinical diagnostic criteria for MOG Ab-associated disorders, sensitivity and specificity were determined from 482 sera divided into two groups: Group A, sera from monophasic and relapsing disorders with reported MOG Ab-association (ADEM, ON, BON, LETM, etc.), and Group B, sera from healthy controls, general medicine, non-inflammatory neurological disorders, demyelinating disorders not associated with MOG Ab (MS, CIS other than ON), and demyelinating disorders not yet associated with MOG Ab (21). Overall, using our own analysis (Analysis 2, **Table 1**), the dataset included 48 healthy or other neurological disorder patients (24 children and 24 adults, Group B), 47 MOG Ab-negative (MOG Ab-) patients (24 children, 14 in Group A, 10 in Group B, and 23 adults, 8 in Group A, 15 in Group B), 74 adult MS patients (Group B), and 313 MOG Ab-positive (MOG Ab+) sera (151 sera from 123 children, 150 in Group A, 1 in Group B, and 162 sera from 125 adults, 161 in Group A, 1 in Group B). All patient serostatuses have been published, and clinical phenotypes were retrospectively obtained and detailed in (6, 15, 21, 35, 36). The phenotypes of the 25 MOG Ab- patients in Group B ($n = 10$ children, $n = 15$ adults) were included in **Supplementary Table 1**.

Detection of Human MOG Ab by Flow Cytometry

A flow cytometry live cell-based assay was used to detect human serum MOG Ab, as previously described (6, 21, 37). In brief, patient serum (1:50) was incubated with a transduced cell line expressing full-length human MOG, followed by fluorochrome-conjugated anti-human IgG (H+L). Dilution of serum at 1:50 was standard and was most frequently used across studies (2, 3, 6, 8, 23, 24, 30, 32, 38) (**Supplementary Table 2**). Samples were reported positive if they were above the positive threshold in at least two of three quality-controlled experiments, a feature that may not have been implemented in other studies but ensures a reliable serostatus report and provides insight into serostatus reproducibility (21). MOG-expressing (MOG+) and empty vector control (MOG-) cells incubated with serum in two independent wells were compared in the "separate wells" analysis, and MOG+ cells (∼80% transduction rate) were compared to the untransduced cells from the same single well in the "mixed" analysis (**Table 1**).

Comparison of Analyses in Determining MOG Ab Positivity

Assessment of a patient MOG Ab serostatus by flow cytometry can be separated into four stages (**Figure 1**). (1) Gating of empty vector or untransduced/untransfected MOG- control cells, indicative of serum background binding, and MOG+ cells. Serum can be incubated with MOG- and MOG+ cells seeded together (mixed) or in independent wells (separate). (2) Quantification of sera binding to MOG- and MOG+ cells can be quantified by the median, mean, or geometric mean fluorescence intensity. (3) Determination of MOG Ab binding to MOG by subtraction (ΔMOG, delta); [MOG = Fluorescence of MOG+ cells – Fluorescence of MOG- cells], or division (MOG ratio); $\left[\text{MOG ratio} = \frac{\text{Fluorescence of MOG+ cells}}{\text{Fluorescence of MOG− cells}}\right]$ between MOG+ and MOG- cells. (4) Establishing the positive threshold by 3, 4, 6, or 10 standard deviations (SD) above the mean of a control cohort or above an absolute value (**Figure 1**). Raw flow cytometry datasets were obtained from all patients ($n = 3$ experiments per patient) and reanalyzed using published analyses detailed in **Table 1**. An age-matched control cohort ($n = 24$), which included patients with general medical and non-inflammatory neurological disorders (and healthy controls in adults), was run concurrently with MOG Ab testing to generate the positive threshold. Published analyses are detailed in **Table 1** and were

TABLE 1 | MOG Ab positivity status across different published flow cytometry analyses.

	Flow cytometry MOG Ab analysis				Pediatric serum, $n = 151$[a] *n (% total)*		Adult serum, $n = 162$[a] *n (% total)*	
	Quantification of MOG Ab ***(Seeding of MOG+ and MOG- cells for serum incubation)***[b]	**Positive threshold or cut-off**[c] ***Standard deviations above the mean of controls***	**Controls** ***n (study)***	**Publications**[d]	**MOG Ab-**	**MOG Ab+**	**MOG Ab-**	**MOG Ab+**
Analysis 1	ΔMOG Mean (Separate wells)	3 SD	24 HC/OND (6) 52 HC/OND (11)	(6, 11)	0 (0)	151 (100)	0 (0)	162 (100)
Analysis 2	ΔMOG Median (Separate wells)	3 SD	28 HC/OND (2) 24 HC/OND (21)	(2, 21)	0 (0)	151 (100)	0 (0)	162 (100)
Analysis 3	ΔMOG Median (Mixed)	(a) 3 SD	8 OND	(23, 24)	0 (0)	151 (100)	0 (0)	162 (100)
		(b) 6 SD	5 HC	(25)	5 (3)	146 (97)	4 (2)	158 (98)
		(c) 10SD	8 OND	(26, 27)	18 (12)	133 (88)	8 (5)	154 (95)
Analysis 4	Ratio median (Mixed)	>2.5[e]	–	(28, 29)	43 (28)	108 (72)	23 (14)	139 (86)
Analysis 5	Ratio geometric mean (Separate wells)	(a) 4 SD	39 HC	(4)	4 (3)	147 (97)	5 (3)	157 (97)
		(b) 6 SD	89 OND	(30)	7 (5)	144 (95)	10 (6)	152 (94)
Analysis 6	Ratio Mean (Separate wells)	(a) 3 SD	71 OND (3) 23 HC (8)	(3, 8)	10 (7)	141 (93)	25 (15)	137 (85)
		(b) >3[e]	–	(31)	53 (35)	98 (65)	29 (18)	133 (82)
Analysis 7	Ratio Median (Separate wells)	(a) 4 SD	14 HC, 19 OND	(32)	14 (9)	137 (91)	20 (12)	142 (88)
		(b) 10 SD	30 HC	(33)	64 (42)	87 (58)	57 (35)	105 (65)
Analysis 8	ΔMOG Ratio Mean[f]	>1	–	(34)	17 (11)	134 (89)	7 (4)	155 (96)
Analysis 9	ΔMOG Median (Mixed)	4 SD	24 HC/OND	Recommended	0 (0)	151 (100)	1 (1)	161 (99)
Analysis 10	Ratio Geometric mean (Separate wells)	(a) >2.5	–		40 (26)	111 (74)	25 (15)	137 (85)
		(b) >3	–		66 (44)	85 (56)	34 (21)	128 (79)

[a] *151 pediatric and 162 adult sera with reported clinical phenotype were included from Tea et al. (21).*
[b] *Serum was incubated with MOG-expressing (MOG+) and control (MOG-) cells in independent wells (separate wells) or untransduced MOG+ cells were gated and compared to the MOG+ cells from the same well (mixed).*
[c] *Positive threshold calculated using 24 age-matched controls according to published analysis.* [d] *Analyses were only included if >10 MOG Ab+ patients were reported and detailed flow cytometry analyses were provided.*
[e] *Positive threshold determined by an arbitrary number.*
[f] *ΔMOG/MOG- cells. Seropositivity was reported if a patient is above threshold at least two times in three experiments.*
HC, Healthy controls; ΔMOG, MOG+ – MOG-; OND, other neurological diseases; Ratio, MOG+/MOG-; SD, standard deviation.

included if the study detailed selection of MOG+ and MOG– cells, quantification of MOG Ab, and threshold calculation and reported at least 10 MOG Ab-positive patients.

In the absence of diagnostic criteria for MOG Ab-associated disorders, sensitivity and specificity analyses were determined using Groups A and B described above (21). Receiver operating characteristic (ROC) curves were generated to evaluate the optimal diagnostic performance of each analysis between these two groups of patients.

Statistics

Correlation analyses and R^2 values were generated using a linear regression model. Youden's Index, which maximizes sensitivity and specificity, was used to determine the optimal threshold from each ROC curve analysis (39, 40). McNemar's Chi-squared test was used to compare the similarity of the seropositive and seronegative results obtained in the different analyses. McNemar's test compared analyses from the same flow cytometry live dataset. Flow cytometry data were analyzed using FlowJo v10 (TreeStar) software and Microsoft Excel. Figures and schematics were generated using Prism v7.0a (GraphPad Software) and Adobe Illustrator CC 2015 (Adobe Systems).

RESULTS

Reduced MOG Ab Seropositivity and Detection Sensitivity by MOG Ratio Analysis

We first compared the serostatus across different published analyses using a flow cytometry live dataset obtained from our previous publications (6, 11, 15, 21) (**Table 1**). Using a threshold obtained from the control cohort, all patients were determined to be MOG Ab+ when MOG+ and MOG- cells were

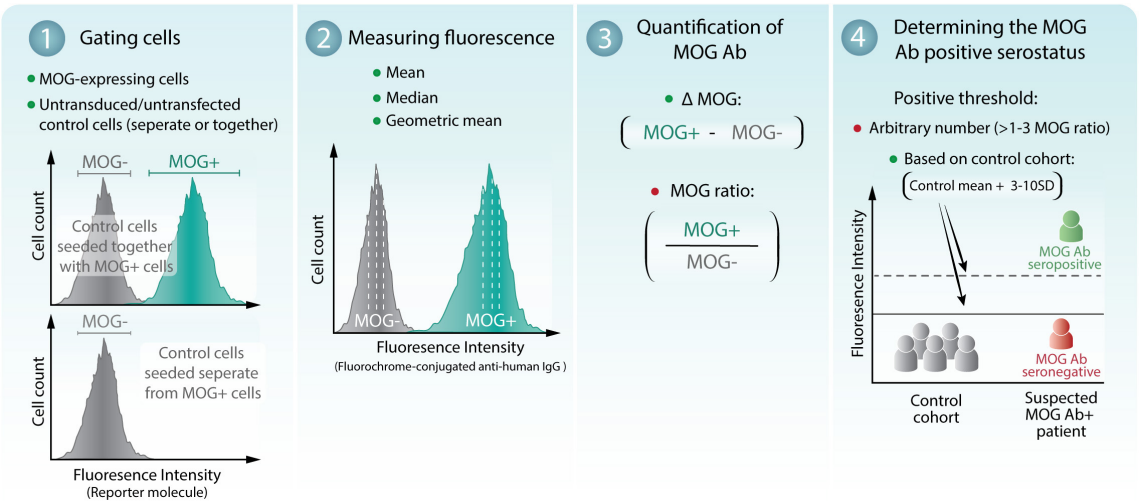

FIGURE 1 | Assessment of patient MOG Ab serostatus by flow cytometry live cell-based assay. (1) MOG-expressing cells (MOG+) and empty vector or untransduced/untransfected control cells (MOG-) were gated. MOG- cells can be either seeded together with or separate from MOG+ cells. (2) The mean, median, or geometric mean fluorescence intensity of the MOG+ and MOG- cells can be determined. (3) MOG Ab binding to MOG is quantified by subtraction (ΔMOG) or division (MOG ratio) of MOG+ and MOG- cells. (4) The threshold of seropositivity can be determined by an arbitrary number or calculated at 3–10 standard deviations (SD) above a control cohort. A breakdown of the analyses is shown in **Table 1**. Recommended methods of analysis are indicated by green dots. Analyses that demonstrated reduced seropositive outcomes and detection sensitivity are indicated by a red dot.

analyzed from two independent wells (Analyses 1 and 2) or in a single well (Analysis 3a) (151 pediatric and 162 adult samples) (**Table 1**). There was low intra-assay variability across repeated experiments (**Supplementary Table 3**), and these analyses led to similar seropositivity results, with high detection sensitivity and specificity (**Table 2**, **Supplementary Table 4**). Furthermore, using a ΔMOG and 3SD threshold, MOG Ab positivity serostatus was similar when MOG Ab titers were determined by the mean (Analysis 1), median (Analysis 2), or geometric mean (data not shown) of MOG+ and MOG- cell populations (**Tables 1**, **2**). These results suggest that quantification of MOG Ab binding by flow cytometry is reliable and reproducible and that the practicality of incubating serum with MOG+ and MOG- cells in a single well, rather than two independent wells, could be considered.

Among ΔMOG analyses, the seropositivity by a control cohort-based threshold using 3SD (Analysis 1–3a) was not significantly different from 6SD (Analysis 3b) (**Table 2**). Across analyses that utilized thresholds from 3 to 6 SD above a control cohort-based threshold, MOG ratio analyses (Analysis 5a, 5b, 6a, 7a) showed reduced MOG Ab detection sensitivity (average sensitivity, children 87% ± 2, and adults 87% ± 6) compared to ΔMOG analyses (Analysis 1, 2, 3a, 3b, average sensitivity, children 91% ± 2 and adults 94% ± 1; **Table 2**, **Supplementary Table 4**). There was an average seropositive loss of 6% ± 3 in children (average $n = 10 \pm 5$, range 5–15) and 11% ± 6 among adults (average $n = 20 \pm 12$, range 7–29) across all MOG ratio analyses, which increased with higher SD thresholds (**Table 1**). When the ratio was determined between ΔMOG and MOG- cells (Analysis 8), detection sensitivity and specificity were high (**Table 2**); however, the serostatus remained significantly different from that in a ΔMOG analysis (**Tables 1**, **2**). Notably, the MOG ratio determination by geometric mean (Analysis 5) performed the best out of all MOG ratio analyses (**Table 1**) and performed significantly better than the MOG ratio median with the same 4SD threshold (Analysis 5a vs. 7a, children, $P = 0.027$, adults $P = 0.001$, data not shown; **Table 2**).

An increasing loss of MOG Ab seropositivity was observed with higher thresholds across all analyses (**Table 1**). Indeed, when the positive threshold was set 10 SD above the control cohort (Analysis 3c, 7b), there was a ∼29% reduced detection sensitivity (**Table 2**, **Supplementary Table 4**), and significant loss of seropositivity, which was more pronounced in the MOG ratio (Analysis 7b, $n = 64$ children and $n = 57$ adults reported negative) than ΔMOG analysis (Analysis 3c, $n = 18$ children and $n = 8$ adults reported negative; **Table 1**). Across all flow analyses, seropositivity loss was the greatest and significantly different from Analysis 2 when an arbitrary threshold was used (MOG ratio > 2.5 or 3, Analysis 4 and 6b), even when a geometric mean was used to quantify MOG Ab binding (Analysis 10; **Tables 1**, **2**). Although it may be hard to compare absolute values directly due to variability in experimental conditions, this suggests that an arbitrary threshold may be difficult to translate across studies.

Overall in children and adults, when using a 3 or 4 SD threshold, the confidence intervals were narrower (**Table 2**) and sensitivity was higher in the ΔMOG analyses (Analysis 1, 2, and 3a, average sensitivity; 93%) than in the MOG ratio analysis (Analysis 5a, average sensitivity: 91%, 6a: 83%, and 7a: 83%) (**Table 2**, **Supplementary Table 4**). Therefore, the ΔMOG value, rather than the MOG ratio, may be a more reliable measure to determine MOG Ab seropositivity. Although

TABLE 2 | Comparison of sensitivity and specificity of MOG Ab detection across different published flow cytometry analyses.

Flow cytometry live analysis[a]		Children, $n = 199$[b]			Adults, $n = 283$[c]		
		Sensitivity % (CI)	Specificity % (CI)	*P*-value[d]	Sensitivity % (CI)	Specificity % (CI)	*P*-value[d]
Analysis 1		91.5 (85.8–95.1)	97.1 (83.4–99.9)	1.0	95.3 (90.6–97.8)	95.6 (89.6–98.4)	1.0
Analysis 2		91.5 (85.8–95.1)	97.1 (83.4–99.9)	–	95.3 (90.6–97.8)	95.6 (89.6–98.4)	–
Analysis 3	(a)	91.5 (85.8–95.1)	97.1 (83.4–99.9)	1.0	95.3 (90.6–97.8)	94.7 (87.3–97.3)	1.0
	(b)	88.4 (82.3–92.7)	97.1 (83.4–99.9)	0.073	92.9 (87.6–96.1)	98.2 (93.2–99.7)	1.0
	(c)	80.5 (73.4–86.1)	97.1 (83.4–99.9)	<0.001*	90.5 (84.8–94.3)	99.1 (94.5–100)	0.387
Analysis 4		65.9 (58–73)	100 (87.7–100)	<0.001*	81.7 (74.8–87)	99.1 (94.5–100)	<0.001*
Analysis 5	(a)	89 (83–93.2)	97.1 (83.4–99.9)	0.133	92.3 (86.9–95.7)	98.2 (93.2–99.7)	0.723
	(b)	87.8 (81.6–92.2)	100 (87.7–100)	0.131	89.3 (83.5–93.4)	99.1 (94.5–100)	0.181
Analysis 6	(a)	86 (79.5–90.7)	97.1 (83.4–99.9)	0.03*	80.5 (73.5–86)	97.4 (91.9–99.3)	<0.001*
	(b)	59.8 (51.8–67.2)	100 (87.7–100)	<0.001*	78.1 (71–83.9)	99.1 (94.5–100)	<0.001*
Analysis 7	(a)	83.5 (76.8–88.7)	97.1 (83.4–99.9)	0.003*	83.4 (76.8–88.5)	99.1 (94.5–100)	0.002*
	(b)	53 (45.1–60.8)	100 (87.7–100)	<0.001*	58 (50.2–65.5)	99.1 (94.5–100)	<0.001*
Analysis 8		81.7 (74.8–87.1)	100 (87.7–100)	<0.001*	91.1 (85.5–94.8)	95.6 (89.6–1)	0.023*
Analysis 9		91.5 (85.8–95.1)	97.1 (83.4–99.9)	1.0	94.7 (89.8–97.4)	97.4 (91.9–99.3)	1.0
Analysis 10	(a)	67.7 (59.9–74.6)	100 (83.4–100)	<0.001*	80.5 (73.5–86)	99.1 (94.5–100)	<0.001*
	(b)	51.8 (43.9–59.6)	100 (83.4–100)	<0.001*	75.1 (67.8–81.3)	99.1 (94.5–100)	<0.001*

[a]*Seropositivity determined by the threshold using 24 age-matched controls according to analyses detailed in* ***Table 1****. Cohorts included 164 pediatric*[b] *and 169 adult*[c] *sera from patients with monophasic and relapsing disorders with reported MOG Ab-association and 35 pediatric*[b] *and 114 adult*[c] *sera from disorders with no MOG Ab-association yet reported and disorders not associated with MOG Ab (****Supplementary Table 3****).* [d]*P-values determined by McNemar's Chi-squared test with Analysis 2 as the comparator (*P < 0.05). CI = 95% confidence interval.*

sensitivity can be compromised, specificity could be improved by increasing the SDs used to calculate the threshold. Indeed, a ΔMOG median (mixed) analysis using a 4 SD threshold (Analysis 9) showed the highest combined detection sensitivity and specificity (**Table 2**) and the lowest intra-assay variability (**Supplementary Table 3**). Furthermore, seropositive results in Analysis 9 were not statistically different from those of Analysis 1 or 2 (**Tables 1**, **2**), but specificity was increased by reducing seropositivity in two MS patients (**Supplementary Table 3**).

High Background Binding in Patient Serum Reduces MOG Ab Detection Sensitivity in a Ratio Analysis

Non-specific background serum binding to MOG- cells varied among patients. Analysis by ΔMFI was superior to a ratio analysis, as the MOG ratio was greatly influenced by background binding (**Figures 2A,B**). Indeed, by MOG ratio analyses, the background binding detected from seronegative samples was significantly higher compared to seropositive samples (children and adults, $P < 0.0001$, **Figures 2A,B**). There was a negative correlation between background binding and MOG ratio mean, ΔMOG ratio mean, MOG ratio median, and MOG ratio geometric mean, i.e., sera with higher background had lower MOG ratio ($P < 0.001$, **Figures 2A–D**), further supporting the influence of background binding on reducing detection sensitivity. MOG Ab-positive patients negative by MOG ratio analysis exhibited a wide range of MOG Ab levels when determined by ΔMFI (**Figure 2E**, red and orange dots), suggesting that MOG Ab titers might not be accurately represented in a MOG ratio in patients with high background binding. Furthermore, among patients of known phenotype, most children and adults negative according to MOG ratio analysis presented with typical MOG Ab-associated phenotypes (**Figure 2F**). There was no clinical distinction between MOG Ab-positive patients reported to be negative or positive by MOG ratio analyses. Interestingly, although seropositivity results between ΔMOG (Analysis 2) and ratio analyses (Analysis 6–8, 10) were significantly different ($P < 0.05$), Analysis 2 performed similarly to MOG ratio analyses using the geometric mean (Analysis 5, $P = 0.133$ and $P = 0.723$, **Table 2**).

Comparisons Against an Optimized Threshold by ROC Analysis

A ROC curve was generated for each analysis, and an optimal threshold with the highest sensitivity and specificity was determined (**Supplementary Figure 1**, **Supplementary Table 5**). When the performances of each analysis using the optimal threshold were compared to one another, MOG Ab serostatuses were similar for all analyses with the exception of the MOG ratio Analysis 7 in children and Analysis 6 in adults (data not shown). This demonstrates the high discriminatory power of the flow cytometry dataset when an appropriate positive threshold is used.

DISCUSSION

Within the expanding field of MOG Ab-associated demyelinating disorders, there are variations in the analysis of flow cytometry

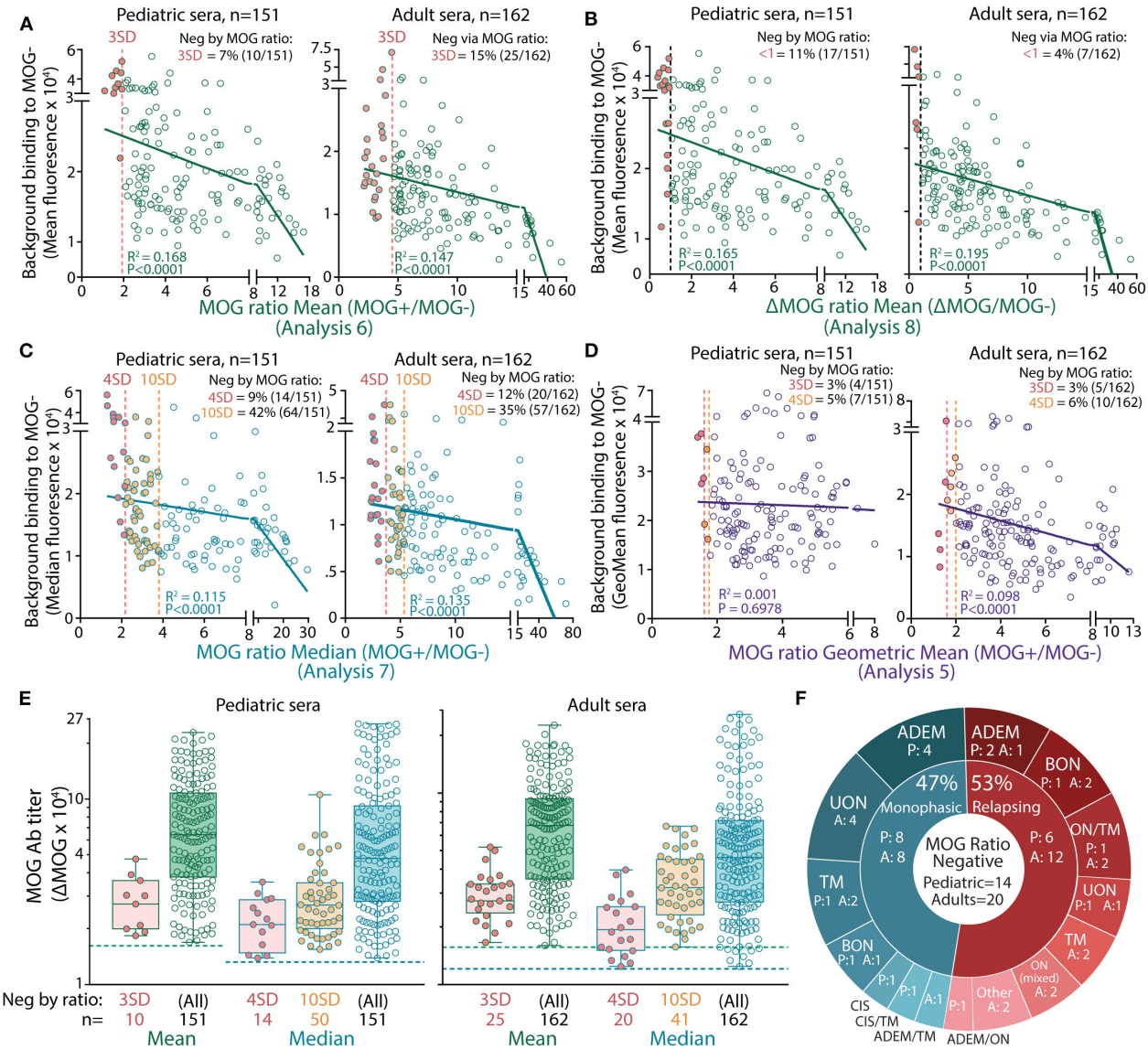

FIGURE 2 | High serum background binding reduced seropositivity detection in MOG ratio analysis. Patients negative (filled red) by mean **(A,B)**, median **(C)**, and geometric mean **(D)** MOG ratio analysis had high background binding. There was a negative correlation between background binding and mean or median MOG ratio values ($P < 0.0001$). **(E)** ΔMOG values of MOG Ab+ patients reported negative in MOG ratio analysis by 3 or 4 SD (red, Analysis 6a and 7a, respectively) or 10 SD (orange, Analysis 7b). Children (left) and adults (right) negative by MOG ratio analysis had a broad range of MOG Ab titers and fell within the range of ΔMOG values of patients who were positive by MOG ratio analysis. Dotted lines represent the ΔMOG positive threshold 3 SD above controls. Representative data from three experiments are shown. **(F)** Patients reported negative by MOG ratio median analysis (4 SD, Analysis 7a) clinically presented with MOG Ab-associated phenotypes. P, pediatric patients; A, adults; ADEM, acute disseminated encephalomyelitis; BON, bilateral optic neuritis; CIS, clinically isolated syndrome; LETM, longitudinally extensive transverse myelitis; ON mixed, combination of BON and UON; ON/TM, simultaneous ON and transverse myelitis; relapsing ADEM, multiphasic ADEM (41); TM, transverse myelitis; UON, unilateral optic neuritis.

data. Precise detection of disease-relevant MOG Ab is essential to advance our understanding of human MOG Ab-associated disorders and implement immunotherapies. Here, we examined the differences across published analyses and demonstrated that high serum background binding in ratio analysis and seropositivity thresholds determined by high SD and arbitrary values reduced detection sensitivity. Furthermore, we showed that the flow cytometry cell-based assay is a robust method with high discriminatory power once appropriate thresholds are utilized.

The human relevance of MOG Ab has been controversial for decades. Fortunately, with a better understanding of the binding characteristics of human MOG Ab, methods of detecting disease-relevant MOG Ab have improved immensely. Microscopy live cell-based assays are widely used (12) but are semi-quantitative and observer-dependent, whereas flow

cytometry allows quantification of a broad range of MOG Ab titers, permitting an in-depth comparison between MOG Ab seropositivity analyses. Although other secondary antibodies specific to IgG Fc or IgG1 have been used in the literature, the secondary antibody utilized to generate the flow cytometry dataset in the current study targeted heavy and light IgG chains. However, most of the seropositive patients in our cohort had MOG IgG1 Ab (21), and only a small proportion of patients harbored MOG Ab of the IgM isotype (21, 42). ROC curve analysis and generation of the optimal threshold was used to evaluate the performance of the assay to distinguish disorders with reported MOG Ab association from disorders for which MOG Ab association is not yet reported and disorders not associated with MOG Ab. Flow cytometry analysis demonstrated high specificity and sensitivity among most published analyses with similar seropositive and seronegative reports.

MOG Ab have been demonstrated to be highly sensitive to conformational changes and therefore require the native surface antigen. Once the protein is fixed, in the case of in-house fixatives or commercial kits, assay sensitivity is reduced (20, 21). It is recommended that cells remain live to ensure that conformational MOG epitopes are available for binding. As the assay performance was reliable when MOG-expressing and control cells were incubated together rather than in two independent wells, a pragmatic consideration would be to combine both control and MOG-expressing lines into a single well for acquisition and analysis. Furthermore, although seropositivity between mean, median, and geometric mean was similar, the median or geometric mean value, being more resistant to outliers, represents a truer central value.

We demonstrated reduced MOG Ab seropositivity in a MOG ratio analysis, when the signal from MOG-expressing cells was divided by control cells. This was largely due to the high level of background fluorescence detected on control cells after incubation with some sera. Human serum contains a plethora of proteins and exogenous antigens that could non-specifically bind to cells. As flow cytometry is a highly sensitive method of detection, a broad range of background binding levels can be detected, which will affect the MOG ratio. Although these observations were determined by flow cytometry, these insights can be extended to microscopy, where serum background binding should be critically considered before determining a patient's MOG Ab serostatus.

A common threshold determination across the field is necessary to allow reliable study comparisons. The stringency of the positive cut-off is important in optimizing the sensitivity and specificity of an assay. We showed that an increase in the number of SD values, for example, to 10 SD, changed the performance of the assay, with a notable reduction in sensitivity. Three SDs above a control mean representing the 99th percentile, commonly used in a diagnostic setting, presented with high detection sensitivity and specificity, but 4 SD demonstrated the highest discriminatory power in a ΔMOG median analysis. Although there is a broad range of MOG Ab titers, the data are not normally distributed (21) and are "bottom-heavy;" therefore, the serostatus of patients with MOG Ab titers close to threshold are more susceptible to threshold changes. If a control cohort was tested alongside the patient samples, which was the case for many flow cytometry analyses, a ΔMOG analysis is recommended. As arbitrary thresholds may not be accurately translated across studies, an independent threshold should be generated for each experiment to account for inter-assay variability. However, a ratio analysis can be advantageous when a control cohort is not available. The geometric mean normalizes skewed data and is most appropriate in the quantification of ratios. We showed that the geometric mean MOG ratio analysis was similar to the ΔMOG median analysis and demonstrated that ratio values could discriminate disease from non-disease when an optimal threshold by ROC analysis was used. Once this threshold is validated in several cohorts, the geometric mean MOG ratio could be an alternative if a control cohort is not available.

A limitation of this study was that the threshold for all analyses was generated with 24 controls, although the number of control samples used to establish the threshold in the published analyses varied. However, the 24 controls in the current study generated a stringent threshold for all analyses. Furthermore, the most frequent dilution across studies was similar to the one used to generate our dataset, but other studies have tested a range of different dilutions. Although assessing the effect of different serum dilutions was outside the scope of this study, the influence of high serum background in flow cytometry remains useful.

As MOG Ab are becoming a prevalent diagnostic biomarker, these results highlight caveats in using a binding ratio and prompt an international agreement on data analysis, which will permit direct comparisons between studies and streamline diagnosis of MOG Ab-associated disease.

Recommendations for MOG Ab Analysis by Flow Cytometry

1. MOG-expressing cells can be incubated in the same well as control cells. Fluorescence intensity of control or MOG-expressing cells can be calculated by the mean, median, or geometric mean.
2. A positive threshold determined by a control threshold generated in each experimental run is ideal. MOG Ab binding to MOG should be calculated using ΔMOG instead of a MOG ratio if a control cohort is available.
3. Ratio analysis using the geometric mean could be utilized if a control cohort is unavailable. The optimal threshold by ROC curve analysis should be validated before implementation.
4. Additional parameters may vary, such as serum dilution, secondary antibody, and flow cytometry experimental conditions. Although these recommendations are based on a serum dilution of 1:50 and detection of IgG (H+L), these concepts can be applicable to all flow cytometry analyses.

DATA AVAILABILITY STATEMENT

The datasets presented in the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sydney Children's Hospitals Network (NEAF 12/SCHN/395). Informed consent to participate in this study was provided by the participants or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FT and FB designed the study. The Australasian and New Zealand MOG Study Group enrolled and managed patients. FT, SR, and DP conducted experiments and analyses. FT wrote the first draft of the manuscript and prepared figures and tables. FB designed and coordinated research and verified results. All authors reviewed the draft before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00119/full#supplementary-material

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BRIEF RESEARCH REPORT
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Novel Surrogate Markers of CNS Inflammation in CSF in the Diagnosis of Autoimmune Encephalitis

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Background: Autoimmune encephalitis (AE) is an important cause of refractory epilepsy, rapidly progressive cognitive decline, and unexplained movement disorders in adults. Whilst there is identification of an increasing number of associated autoantibodies, patients remain with a high clinical probability of autoimmune encephalitis but no associated characterized autoantibody. These patients represent a diagnostic and treatment dilemma.

Objective: To evaluate routine and novel diagnostic tests of cerebrospinal fluid (CSF) in patients with a high probability of AE to attempt to identify better biomarkers of neuroinflammation.

Methods: Over 18 months (2016–2018), adult patients with a high clinical probability of AE were recruited for a pilot cross-sectional explorative study. We also included viral polymerase-chain-reaction (PCR) positive CSF samples and CSF from neurology patients with "non-inflammatory" (NI) diagnoses for comparison. CSF was examined with standard investigations for encephalitis and novel markers (CSF light chains, and cytokines).

Results and Conclusions: Thirty-two AE patients were recruited over 18 months. Twenty-one viral controls, 10 NI controls, and five other autoimmune neurological disease controls (AOND) were also included in the analysis. Our study found that conventional markers: presence of CSF monocytosis, oligoclonal bands, anti-neuronal immunofluorescence, and magnetic resonance imaging (MRI) changes could be suggestive of AE, but these investigations were neither sensitive nor specific. Promising novel makers of autoimmune encephalitis were the CSF cytokines IL-21 and IP10 which may provide better delineation between viral infections and autoimmune encephalitis than conventional markers, potentially leading to more immediate diagnosis and management of these patients.

Keywords: autoimmune encephalitis, cytokines, inflammation, surrogate markers, cerebrospinal fluid, CSF, diagnostic investigations

INTRODUCTION

Autoimmune encephalitis (AE) is an important cause of unexplained movement disorders, rapidly progressive cognitive decline and refractory epilepsy (1, 2). Whilst a proportion of these patients have associated detectable anti-neuronal antibodies, there is currently no gold standard investigation to confirm the diagnosis (2).

A further clinical challenge are patients who clinically appear to have AE, but no associated autoantibody is identified (3, 4). Supportive findings indicating AE include the following conventional surrogate markers of neuroinflammation on CSF: oligoclonal bands, raised protein level, and monocytosis (2, 5). Patients with antibody-negative or positive AE may not have elevations of these markers but still respond to a trial of immunomodulatory treatment (2, 6). Earlier initiation of treatment may result in better outcomes (3, 4). However, treatment of AE often involves significant immunosuppression (1, 6) and the decision to subject an individual to therapy is challenging if convincing objective evidence of an autoimmune etiology is lacking (1).

Novel surrogate markers including CSF light chains and CSF cytokines have been associated with CNS inflammation (7–13). Indirect immunofluorescence (IIF) on primate brain using CSF for anti-neuronal antibodies often reveals staining patterns not associated with a known antigen (non-specific IIF) and it is unclear whether these patterns are indicative of CNS autoimmunity.

A superior biomarker which more reliably differentiates CNS autoinflammation from other causes will assist clinicians commence treatment earlier. Our study examines a cohort of patients with high clinical suspicion of AE to identify biomarkers that might indicate this disease.

MATERIALS AND METHODS

Recruitment of Patients

This is an exploratory study aiming to identify conventional or novel surrogate biomarkers of neurological inflammation associated with AE which provides class III evidence for the potential of the CSF cytokines IL21 and IP10 as biomarkers for AE. This study was approved by the Ethics Committee of Westmead Hospital (LNR/16/WMED/192) and written informed consent was obtained by all participants.

Patients at a single quaternary referral center in Western Sydney, Australia were prospectively recruited over 18 months between 2016 and 2018 (**Figure e-1**). Previously proposed diagnostic criteria for antibody negative AE (2) includes subacute onset of working memory deficits; altered memory status or psychiatric symptoms with new focal CNS findings; and seizures not explained by previous known seizure disorder paired with investigation findings of CSF pleocytosis (2); magnetic resonance imaging (MRI) features suggestive of encephalitis; and exclusion of alternative causes. Therefore, our inclusion criteria was based on the clinical grounds for suspicion of AE: refractory or multiregional seizures/epilepsy; rapidly progressive cognitive decline and unexplained movement disorders (1–6). Adult patients (16 years or older) with a high clinical suspicion of AE, as assessed by a neurologist, were enrolled in the study.

The decision for recruitment was based on clinical grounds, prior to lumbar puncture and before knowledge of subsequent results of investigations. If investigations results revealed an alternative diagnosis, patients were reclassified to the appropriate group i.e., infectious. Patients with an identified autoantibody associated with AE were classified as antibody positive (AbPAE) while those without antibodies were classified as antibody negative (AbNAE). To prevent information bias, negative results from CSF analysis did not exclude enrolled patients.

Recruitment of Controls

Any enrolled patients diagnosed with a CNS viral infection through polymerase-chain-reaction (PCR) were included in the infectious control cohort. In addition, stored CSF samples classified as viral infection based on positive PCR results were included as viral controls. These samples were supplied as deidentified aliquots.

Non-inflammatory (NI) control CSF samples were obtained from patients undergoing large-volume lumbar puncture for "non-inflammatory" neurological disease (NIND) and from patients undergoing routine spinal anesthesia. Patients in the NIND group had a diagnoses of simple headache, idiopathic intracranial hypertension (IIH), or normal pressure hydrocephalus (NPH).

A disease control group consisting of patients with neuropsychiatric lupus, cerebral vasculitis, and multiple sclerosis were also included (OAND group).

Sample Collection and Storage

CSF samples for AE and NI controls were collected in standard 10 mL CSF tubes. CSF for light chains and cytokine analysis were aliquoted from these samples and frozen at −80 degrees Celsius. Assays for CSF light chains and CSF cytokines were batched for analysis to minimize analytical variation.

Assays

All investigations unless otherwise stated, were performed at ICPMR (NSW Health Pathology, Australia).

Patients underwent conventional investigations for AE including blood tests and lumbar puncture for collection of CSF (**Figure e-1**). Conventional serum and CSF studies were: isoelectric focussing for oligoclonal bands (Sebia Paris, France), indirect immunofluorescence (IIF) on primate brain (Inova San Diego, USA) and line blot (PCA-1, PCA-2, ANNA-1, ANNA-2, Ma-1, Ma2, Amphiphysin, CV2, CRMP5) for onconeural antibodies (Ravo Bettlach Switzerland) and a limbic encephalitis panel [NMDAR, LGI-1, CASPR2, GABA (B), AMPA1 and AMPA2] on HEK2 transfected cells (Euroimmun Lubeck, Germany), as well as voltage-gated potassium antibodies (VGKC) (performed by radioimmunoassay; Queensland Pathology, Royal Brisbane Hospital, Australia; kits from RSR Cardiff, United Kingdom). Confirmation of IgLON5 antibody was performed at Euroimmun, Lubeck Germany based on staining pattern on primate brain IIF in our laboratory.

The following tests were also performed on serum: anti-thyroid antibody (Siemens Munich, Germany) and thyroglobulin antibody (Siemens Munich, Germany); and CSF: microscopy and culture; protein (Siemens Vista Erlangen, Germany), anti-glutamic acid decarboxylase (GAD) antibodies (ELISA, SEALS Pathology, Prince of Wales Hospital, NSW Australia; RSR Cardiff, UK), and polymerase chain reaction (PCR) for viral infections: HSV (Artus Hamburg, Germany), VZV (in-house PCR assay), ENTV (in house PCR assay), and EBV by PCR (Ellitech Paris, France).

All conventional investigations were collected according to current practice and performed according to the usual procedures available at the receiving diagnostic laboratory.

CSF studies performed purely on a research basis were: CSF light chains (Freelite assay; Binding Site, Birmingham United Kingdom) and a broad panel of CSF cytokines (Milliplex; Merk Millipore Darmstadt Germany) using the magnetic multi bead array kits (MPHSCTMAG28SK17; MPHCYP3MAG63K01; MPHCYTOMAG60K03; MPHCYTP2MAG62K02. Cytokines tested were: IFN-γ, ITAC/CXCL11, IL-12p70, TNFα, CXCL9, IP-10/CXCL10 (Th1 cytokines); IL-13, IL4, IL5, TARC/CCL17, Eotaxin/CCL11 (Th2 cytokines); IL17a, IL-6, IL-8 (Th17 cytokines); and IL-1β, IL-21, IL-2, IL-23, IL-7, IL-10, BCA-1/CXCL13, GMCSF, GCSF (other cytokines). Kits were chosen based on maximum sensitivity for cytokine detection. Lower limits of detection of the cytokine assay are detailed in **Table e-3**. Samples for CSF light chains and cytokines were run as per assay kit instructions. CSF cytokines were run by two operators and in duplicate except when the sample amount was insufficient when it was run in singlicate (six AE patients).

Any additional clinically necessary investigations for diagnosis or management including MRI was performed. MRI reports included in our data as suggestive of neuroinflammation had features of hyperintensity, hippocampal swelling, or other signs of oedema. Reports including cortical dysplasia, mild involutional change or atrophy, and bleeding were not considered positive. MRI results were not available for NI and viral controls.

TABLE 1 | Patient Demographic Details.

	NI controls	OAND controls	AbPAE		AbNAE		
				P (vs. NI)		*P* (vs. NI)	*P* (vs. antibody positive)
Number of patients	10	4	9	n/a	23	n/a	n/a
Median age	54	46.5	37	0.98	44	0.4	0.2
Age range	17–81	19–60	15–58	n/a	18–73	n/a	n/a
Gender (M:F)	4:6	1:3	3:6	0.4	16:7	0.1	0.02

Clinical Details

Clinical details for AE patients, NI, and OAND cohorts were collected by interviewing treating clinicians and verified through medical records. Clinical data for viral samples were not available.

Statistical Analysis

Analysis of the surrogate markers examined in this study was performed using StataMP 13 and scatterplot figures of results were prepared using GraphPad Prism.

For continuous independent variables, univariate analysis using the Mann-Whitney *U*-test or Kruskal-Wallis test were performed to compare the various disease groups. Univariate logistic regression was performed for binomial and categorical variables. Heat map analysis of cytokines was performed using Morpheus (Broad Institute) to find cytokines of potential interest.

Significant findings were then combined in a multivariate logistic model to determine markers that were significantly and independently associated with disease group classification (AE vs. viral, NI, and OAND groups). These markers were then fitted to a predictive model and a receiver operating characteristic (ROC) curve created.

RESULTS

A total of 32 patients with a high clinical probability of AE were recruited. These were subdivided into nine AbPAE patients and 23 AbNAE patients. Ten NI controls, 24 viral controls, and five OAND were also included in the analysis (**Figure e-1**). Demographic details of recruited patients are described in **Table 1**. Demographic details of the viral controls were unavailable. Clinical and diagnostic details for the AbPAE and AbNAE groups are summarized in **Table e-1**. The antibodies detected in the AbPAE group were NMDA-R (3), GFAP (1), IgLon5 (1), LGI-1 (1), CASPR2 (1), Anti-ANNA1(Hu) (1), and Anti-GAD (1).

Most investigations were analyzed in over 90% of samples from the AE groups. Exceptions were CSF GAD (78%) and serum VGKC (59%).

TABLE 2 | Conventional Markers of CNS inflammation.

	NI (*N* = 5)	OAND (*N* = 5)	AbPAE (*N* = 9)		AbNAE N = 23	
	n (%)	*n* (%)	*n* (%)	*p*	*n* (%)	*P*
CSF monocytosis > 5 (2)	0 (0%)	1 (20%)	2 (22%)	n/a	4 (17%)	n/a
CSF oligoclonal bands	0 (0%)	3 (60%)	6 (67%)	n/a	5 (22%)	n/a
CSF protein > 0.45 g/L	3 (60%)	2 (40%)	4 (44%)	0.5	9 (32%)	0.6
Neuronal IIF (any staining)	1 (20%)	2 (40%)	n/a	n/a	8 (35%)	0.4
MRI changes	n/a	4 (80%)	3 (33%)	n/a	5 (22%)	n/a

P-values are calculated as AbAE or AbNAE vs. all other control groups (NI, viral and OAND).

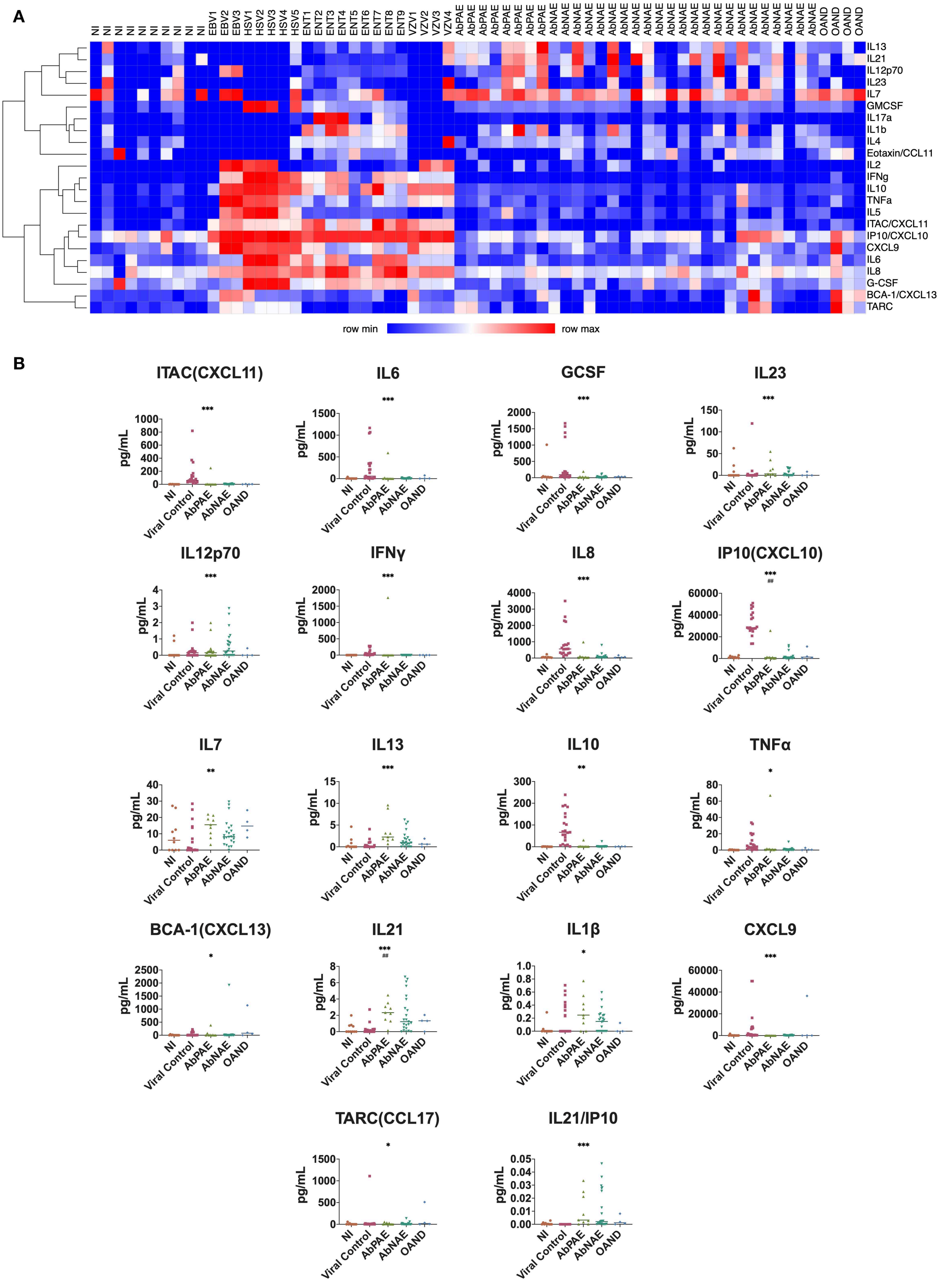

FIGURE 1 | CSF cytokines. **(A)** Heat map cluster distinguishes viral from AbPAE and AbNAE encephalitis. Each column represents a participant. The X-axis identifies the cohort each participant belongs to, while the right-handed Y axis describes the corresponding cytokine. Increasing expression is depicted as increasing shades of

(Continued)

FIGURE 1 | red. Th1 and proinflammatory cytokines appear to be associated with viral infections, with the addition of IL7a, IL1b, and IL4 associated with enterovirus infections in this group. **(B)** Cytokines where a significant difference was found in univariate analysis of the autoimmune encephalitis group compared with a combined pool of NI, viral, and other autoimmune disease controls. Cytokines where the statistical significance was also seen in a univariate analysis are indicated with asterisks (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001). Statistical significance seen in multivariate analysis are indicated with hatches (# < 0.05, ## < 0.01, ### < 0.001, #### < 0.0001). Lines indicate medians. **Table e-2** details the p-values of individual group comparisons. AbPAE, patients clinically high risk for autoimmune encephalitis who had identified associated antibodies; AbNAE, patients clinically high risk for autoimmune encephalitis without identified associated antibodies; NI, samples from patients either undergoing perioperative anesthesia or diagnosed with non-inflammatory neurological diseases; OAND, patients with other inflammatory neurological disease; EBV, Epstein Barr virus; VZV, varicella zoster virus; HSV, herpes simplex virus; ENT, enterovirus.

Of the 10 NI controls, five were perioperative patients where only 1 mL of CSF was able to be collected. These samples were reserved for assessment of novel markers.

Two of the OAND controls had commenced immunosuppression at time of lumbar puncture: one cerebral lupus (methotrexate and mycophenolate) and one with cerebral vasculitis (pulsed methylprednisolone). However, both these patients required intensification of immunosuppression prior to remission.

Twenty-five PCR positive viral controls were included in this study. One was a recruited patient diagnosed with herpes simplex virus (HSV) positive on PCR. Twenty-four others were obtained from aliquoted stored samples and consisted of nine enterovirus (ENTV) positive samples, four HSV positive, three Epstein-Barr virus (EBV) positive, and eight varicella-zoster virus (VZV) positive samples. All ENTV samples were 500 μL in volume and were used for both CSF cytokine and CSF light chain analysis. There were only 200 μL of CSF for VZV, EBV, and HSV samples. Therefore, 5 HSV, 3 EBV, and 4 VZV samples were used for cytokine analysis and a further 4 VZV samples were used CSF light chain analysis. Thirteen viral samples also underwent IIF on primate brain.

Conventional Markers

There was a trend for increased proportions of positive results in some conventional CSF markers of CNS inflammation in patients with AE and OAND compared to NI controls (**Table 2**) but this was not seen in all patients. Comparison of these markers with viral controls was not available.

Two of 9 (22%) of AbPAE and 4/23 (17%) of AbNAE patients had evidence of CSF monocytosis >5 monocytes. Six of 9 (67%) of the APAE and 4/23 (17%) of AbNAE high-risk patients had evidence of CSF oligoclonal bands. None of the NI group had CSF monocytosis >5 or oligoclonal bands and these markers were not able to be statistically analyzed.

Raised CSF protein (>0.45 g/L) was seen in 4/9 (44%) of the AbPAE and 9/23 (32%) of AbNAE groups but also in 3/5 (60%) of the NI group.

CSF neuronal IIF staining was observed in 8/23 (35%) of the AbNAE group but was also observed in 1/5 of the NI group, 1/13 (8%) of the viral controls with sufficient sample for testing (enterovirus only) and 2/5 (40%) of OAND controls. The viral sample with non-specific IIF staining was EBV positive on PCR however this was supplied as a deidentified aliquot and further clinical details could not be verified.

Three of 9 (33%) of the AbPAE group and 5/23 (22%) of the AbNAE group had non-specific changes on MRI indicative of neuroinflammation. No MRI results were available for viral or NI controls.

Therefore, whilst markers such as CSF oligoclonal bands, monocytosis or presence of MRI changes may indicate an autoimmune process, these are not sensitive or specific (14, 15) enough for a reliable diagnosis.

CSF Cytokines

Heatmap cluster analysis revealed differential profiles of cytokine concentrations in patients with viral infections and NI controls compared to the combined AbPAE, AbNAE group, and OAND groups (**Figure 1A**).

There were no significant differences in cytokine levels between the AbPAE and AbNAE groups. Therefore, for statistical analysis, the AE patients were analyzed as one group when compared to NI and viral controls. The cytokines IL1b and IL12p70 were raised in the AE group when compared to the OAND group. Results of univariate analysis between individual groups are detailed in **Table e-2**.

Univariate analysis found that levels of IL21 ($p = 0.0001$), IL13 ($p = 0.0002$), IL12p70 ($p = 0.0008$) and IL7 ($p = 0.009$) were increased in the AE patients (**Figure 1b**) when compared to a combined cohort of normal, viral and OAND controls. As expected, Th1 related cytokines and other proinflammatory cytokines were elevated in viral controls.

A multivariate logistic regression model was used to compare the combined cohort with a combined group of normal, viral, and OAND controls. Only IL-21 ($p = 0.002$) and CXCL10/IP-10 ($p = 0.003$) independently contributed to the model. A ROC curve constructed using this multivariate logistic regression model had an area under the curve (AUC) of 0.90 (**Figure 2A**). A ratio of IL21/IP10, in a univariate logistic regression model was also significant when compared a combined group of normal and viral controls ($p = 0.01$) with a ROC curve of 0.84 (**Figure 1B**).

Other Novel CSF Markers

Only CSF lambda light chains were higher in both AE ($p = 0.03$) and viral control ($p = 0.03$) groups compared to NI controls. Kappa and lambda were both significant raised in OAND controls ($p = 0.03$ and 0.003, respectively). However, when the AE groups were compared to viral controls there was no statistical difference in CSF (kappa or lambda) light chains. Therefore, whilst CSF lambda may be a non-specific marker of neuroinflammation, it cannot be relied on to differentiate between AE from other differentials, such as viral infection.

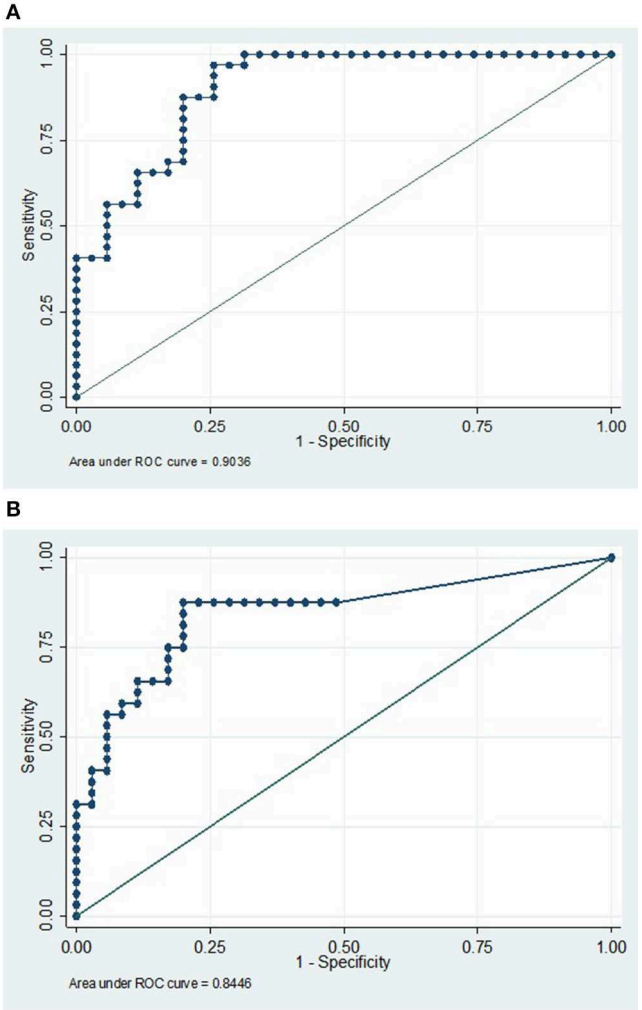

FIGURE 2 | (A) IP10 and IL21 has a high sensitivity and specificity in discriminating AE from a combined control cohort. ROC curve analysis using a multivariate model with CXCL10/IP10 and IL21 in comparing a pooled AE cohort (compromising of both antibody positive and antibody negative groups) vs. a pooled viral control, NI cohorts, and OAND controls. AUC 0.90. **(B)** An IP10/IL21 ratio is a reasonably sensitive and specific single result that may differentiate the autoimmune encephalitis group from normal and viral controls. This ROC curve analysis uses a univariate logistic model with IL21/IP10 in comparing a pooled high risk for autoimmune encephalitis cohort (compromising of both antibody positive and antibody negative groups) vs. a pooled viral control and NI cohorts. AUC 0.84 AE, autoimmune encephalitis; NI, non-inflammatory controls; OAND, patients with other inflammatory neurological disease.

DISCUSSION

The diagnosis of antibody-negative AE remains largely one of exclusion (2) and better biomarkers are required to assist with diagnosis to limit the potentially severe sequelae associated with treatment delays. Our study has demonstrated that selected CSF cytokines are promising biomarkers of AE, with or without characterized antibodies being present.

The most promising surrogate marker for AE is IL21 which was raised in both AbPAE and AbNAE groups. While the detected levels of IL-21 in the CSF were in the low range of the assay (0–6 pg/ml) they were significantly increased compared to viral and normal controls. Considering the short serum half-life of IL-21 (1–3 h) (16), this may represent sustained IL-21 production.

IL21 has many roles in B, CD8 T, and NK cell activation. In B cells, IL-21 acts as both an inhibitor and activator (17, 18). It stimulates apoptosis of B cells that become activated in the absence of T cell help (17, 18) but also stimulates B cell proliferation in the setting of T cell help. In combination with IL-4, IL21 has a significant role in switching B cells to IgG1 and IgG3 production (17–19). IL-21 also stimulates B lymphocyte-induced maturation protein transcription 6 (BLIMP6), which induces differentiation of B cells into long-lived plasma cells (17, 18). Therefore, IL-21, may contribute to autoantibody production in AE.

Antibodies associated with AE are continually being described. It is possible that patients diagnosed with antibody-negative AE may have an antibody that is yet to be discovered. Another consideration is that the finding of higher IL21 indicates a role in non-antibody mediated inflammation. IL21 down regulates FOXP3+ regulatory T cells leading to enhanced autoimmunity (17–19). In addition to being a T and NK cell activator, IL21 also critically regulates Th17 cell development, expansion, and function. With IL-7 or IL-15, IL-21 further enhances CD8+ T cell proliferation (17–19). It stimulates the proliferation of NK and NKT cells and enhances NK cytolytic function. These effects are demonstrated in anti-tumor models (17, 18) and may contribute to a predominantly cell-mediated autoinflammatory encephalitis.

The main mimic of AE is viral encephalitis which is an important consideration in the context of potential immunosuppression. The main cytokine indicative of viral infection in our study was IP10/CXCL10. IP10/CXCL10 is secreted in response to interferon gamma (20) which is produced as part of the Th1 response to viral infection. It is a chemoattractant for T cells, monocytes, natural killer (NK) cells and dendritic cells (20, 21). IP10/CXCL10 was raised in all viral infections included in our study. Other reports have associated IP10/CXCL10 with herpes and flaviviruses (22). However, this needs to be validated across a greater range of infections before it can be definitively used as a surrogate marker of infection.

Translating these findings into routine clinical practice, IL-21 and IP10 may contribute to the diagnostic armamentarium in the investigation of encephalitis, possibly helping to differentiate AE from conditions presenting in a similar fashion where immunosuppression may be harmful. A pragmatic way of comparing these values may be through an IL-21/IP10 ratio. In our cohort, this ratio had an excellent AUC on ROC curve analysis when AE was compared to NIND and viral controls, but this needs to be further validated.

Data available on CSF cytokines in this disease setting are limited and comparisons between studies are difficult because

of heterogeneity in disease definitions and differences between cytokine detection platforms and their lower limit of detection, as well as kit manufacturers.

There is only one other study to our knowledge that examines CSF cytokine profiles in adults. This study examined CSF cytokines in 78 patients using a different platform manufacturer (Bio-Rad), including 20 with an autoimmune neurological disease (10). This study differed to ours in cohort with a significant proportion of patients with demyelinating disease (9/20) in autoimmune cohorts (excluded from our AE group) and patients with bacterial or tuberculosis CNS infections in their infectious cohort (9/38). They observed that MPO and IL8 was increased in cohorts with infectious and unknown etiology but did not find CXCL10/IP10 a significant marker of infection. They found IL-4, IL-10, IL-1R α, and IL1- β were higher in CSF of patients with immune-mediated disease (10). They did not examine IL21.

There is more literature available about CSF cytokines associated with AE in children, but it is unclear if these data are applicable to an adult population. One study (of children aged 28 days-14 years old) examined CSF cytokines (Bio-Rad kit) in viral encephalitis compared with NMDAR (four patients) encephalitis and found significant elevations in IL-6, IL7, and IL13 in the viral encephalitis group compared to the NMDAR encephalitis group (13), but did not examine IP10/CXCL10 or IL21. A second study examined the CSF of children (aged 2–14 years) with enterovirus encephalitis or NMDAR encephalitis and ADEM but found no significant differences in cytokine concentrations between these groups of patients. This study used cytokine kits from the same manufacturer as our study (Milliplex) but used kits under different catalog numbers to what we have used. Therefore, differences in findings for cytokine levels between this study and ours may reflect differences in children vs. adults or varying analytical sensitivities across different cytokine detection kits (11). A published review of CSF cytokines in children also found Th1 cytokines to be associated with viral encephalitis and CXCL13 and IL6 to be associated with NMDAR-antibody associated encephalitis and non-herpetic limbic encephalitis, respectively (12).

Our study did not find other novel potential novel markers useful in differentiating AE from NI or viral controls. In examining the literature, raised CSF free light chains (FLC) levels have been associated with neuroinflammation, however, the normal range is not well-established (7–9). Our results suggest it may be a better indication of general neuroinflammation rather than identifying a specific cause.

Currently utilized conventional markers are neither sensitive (1, 2, 23) nor specific (14, 15, 24, 25) enough for the diagnosis of AE (26). Whilst there were increased proportions of positive results in CSF monocytosis and oligoclonal bands in our study in some AE patients, the presence of these markers have been described in infectious and/or neuroinflammatory states (14, 15, 24, 25). Similarly, the majority of cases of AE did not have detectable abnormalities on MRI (2). Elevated CSF protein was not a good indicator of neuroinflammation. There was a trend for an increased proportion of patients (35%) with any positive staining in IIF in the AbNAE group compared to one patient in the viral and NI groups, respectively. This may indicate that this finding may be useful but need to be examined further.

This study was limited by small patient numbers and limited CSF volumes, reflecting the rarity of this disease. There was difficulty in obtaining sufficient normal and viral control samples of CSF and there were pre-analytical collection issues in this study. The use of deidentified viral PCR positive CSF aliquots for controls meant clinical correlation was not possible. We did not check serum cytokines for patients in our cohort and comparison between CSF and serum cytokine levels in these patients should be a focus of further study.

Nevertheless, this is the first study, to our knowledge, to prospectively examine both conventional and novel markers of neuroinflammation in these groups of adult patients prior to immunosuppression. We have demonstrated the CSF cytokines CXCL10/IP-10 and IL-21 are potential differentiators of AE from viral encephalitis, particularly when there is no CNS specific autoantibody detected. These novel markers could have a future role to help expediate the decision to commence immunosuppression in this group of patients warranting their prospective validation in separate cohorts of AE patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was approved by the Ethics Committee of Westmead Hospital (LNR/16/WMED/192) and written informed consent was obtained by all participants.

AUTHOR CONTRIBUTIONS

JJ: study design, patient recruitment, data extraction, cytokine assays, statistical analysis, drafting, and editing of manuscript. NF: cytokine assays, heatmap production, and editing of manuscript. SD: cytokine assays and editing of manuscript. AF, SRD, and MS: patient recruitment and editing of manuscript. SR and AB: initial study design and editing of manuscript. SB: assistance with assays and editing of manuscript. BJ: recruitment of controls and editing of manuscript. DB and M-WL: study design, patient recruitment, and editing of 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/fneur.2019.01390/full#supplementary-material

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Demeclocycline Reduces the Growth of Human Brain Tumor-Initiating Cells: Direct Activity and Through Monocytes

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Myeloid cells that infiltrate into brain tumors are deactivated or exploited by the tumor cells. We previously demonstrated that compromised microglia, monocytes, and macrophages in malignant gliomas could be reactivated by amphotericin-B to contain the growth of brain tumorinitiating cells (BTICs). We identified meclocycline as another activator of microglia, so we sought to test whether its better-tolerated derivative, demeclocycline, also stimulates monocytes to restrict BTIC growth. Monocytes were selected for study as they would be exposed to demeclocycline in the circulation prior to entry into brain tumors to become macrophages. We found that demeclocycline increased the activity of monocytes in culture, as determined by tumor necrosis factor-α production and chemotactic capacity. The conditioned medium of demeclocycline-stimulated monocytes attenuated the growth of BTICs generated from human glioblastoma resections, as evaluated using neurosphere and alamarBlue assays, and cell counts. Demeclocycline also had direct effects in reducing BTIC growth. A global gene expression screen identified several genes, such as DNA damage inducible transcript 4, frizzled class receptor 5 and reactive oxygen species modulator 1, as potential regulators of demeclocycline-mediated BTIC growth reduction. Amongst several tetracycline derivatives, only demeclocycline directly reduced BTIC growth. In summary, we have identified demeclocycline as a novel inhibitor of the growth of BTICs, through direct effect and through indirect stimulation of monocytes. Demeclocycline is a candidate to reactivate compromised immune cells to improve the prognosis of patients with gliomas.

Keywords: glioma, innate immunity, monocytes, macrophages, microglia, stem cells

INTRODUCTION

Malignant gliomas are brain tumors that arise from within the central nervous system (CNS). The most aggressive form, glioblastoma multiforme, has a dismal prognosis with a median survival of 15 months; <10% of patients survive beyond 5 years (1–3). The poor prognosis of malignant gliomas is attributed in part to the existence of glioma stem cells, also called brain tumor-initiating cells (BTICs) (4–9). BTICs are resistant to therapies as they continue to seed and form new tumor foci in the brain. BTICs have been shown to contribute to the tumourigenesis and recurrence of gliomas (10), particularly due to their high chemo- and radio-resistance (11, 12).

Surrounding BTICs *in situ* are microglia, which are innate immune cells of the CNS and macrophages that have infiltrated as monocytes from the circulation (13–16). These cells are thought to be initially recruited to eradicate the tumor by stimulating apoptosis of glioma cells (17) and by secreting inflammatory factors that prevent glioma growth and invasiveness (18). However, glioma cells have been shown to induce an immunosuppressive phenotype that in turn enhances tumor growth. For example, glioma cells have been shown to secrete periostin, which selectively recruits macrophages with an immunosuppressive profile (19). Furthermore, interactions between glioma and macrophages/microglia can lead to promotion of tumor growth (20–22). These immune cells have been shown to enhance tumor CCL21 expression, which facilitates tumor immune escape (23). Notably, BTICs also interact with macrophages and microglia within the tumor microenvironment, inducing an immunosuppressive macrophage/microglia cell profile that leads to promotion of tumor invasion (24, 25). We made the discovery that microglia, monocytes, and macrophages derived from glioma patients are deficient in their capacity to reduce the growth of BTICs (26).

Based on the above discussion, activating or reprogramming immune cells may represent an approach to curb BTIC growth (27–29). Kees et al. (30) demonstrated that stimulation of microglia with toll-like receptor-3 agonist, poly(I:C), prior to co-culture with tumor cells promotes microglia tumouricidal activity *in vitro*. However, direct poly (I:C) treatment was ineffective in glioma patients (30).

A recent study has shown that manipulation of RNA regulator in tumor-associated microglia and macrophages stimulates anti-tumor immunity and reduces glioma growth (31). More recently, we found that the compromised monocyte, macrophages and microglia from patients with glioma could be reactivated by amphotericin B to reduce BTIC growth in culture and to prolong the lifespan of mice with intracranial patient-derived BTIC xenografts (26). Despite these promising results, amphotericin B may not find clinical utility in gliomas as it has significant acute and chronic toxic side effects ranging from hypoxia to nephrotoxicity (32).

We discovered the pro-activation capacity of amphotericin B on microglia during a screen of a 1,040-drug library (33). During that screen, we discovered another stimulator of the activity of microglia in culture, as measured by TNF-α production: meclocycline, a tetracycline antibiotic. Meclocycline has significant toxicity and is limited to topical use, but a derivative, demeclocycline, can be administered systemically (34) (www.drugs.com). Demeclocycline is used clinically as a prescription medication to treat susceptible bacterial infections, as well as off-label to manage chronic syndrome of inappropriate secretion of anti-diuretic hormone (SIADH).

Here, we have evaluated the effects of demeclocycline on BTIC growth either through direct mechanisms or indirectly through the stimulation of monocytes. We chose monocytes for study as systemic monocytes would be exposed to demeclocycline prior to their infiltration into brain tumors as macrophages to influence BTIC properties. Our results suggest the potential utility of demeclocycline in glioblastoma.

MATERIALS AND METHODS

Isolation and Culture of Monocytes and Macrophages

Human monocytes were isolated from the venous blood of adult healthy individuals as described elsewhere (26). Briefly, monocytes (100,000 cells/well/100 μl) following isolation were plated in RPMI medium supplemented with 20% human serum in 96 well plates. After 24 h, cells were switched to BTIC medium. Cells were transferred to BTIC medium an hour prior to treatment. Treatment involved administering each drug at different concentrations with or without LPS (100 ng/ml). Briefly, monocytes were treated with demeclocycline (10 or 1 μM) for 48 h in BTIC medium and conditioned media were collected. Bone marrow-derived macrophages (BMDM) were isolated from mice as described elsewhere (35). Unless otherwise stated, BMDM cells were plated at 30,000 cells in AIMV medium for collection of conditioned media (see below), or for assessment of their activity.

Evaluation of Activity of Monocytes and Macrophages in Response to Demeclocycline Treatment

We utilized the level of tumor necrosis factor-α (TNF-α) as a first indicator of cellular activity. Following 24 h treatment with demeclocycline (10 μM, Sigma) with or without IFNγ (100 ng/ml)/IL-1β (100 ng/ml) (Peprotech) or LPS (100 ng/ml), the conditioned medium was collected for TNF-α ELISA following manufacturer's instructions (Life Technologies Invitrogen).

Chemotaxis Assay

Human monocytes were treated with demeclocycline (10 μM). After 1 h of incubation at 37°C with 5% CO_2, IFNγ (100 ng/ml)/IL-1β (100 ng/ml) was added. After 24 h, human monocytes were harvested and resuspended in RPMI 1640 media supplemented with 2% penicillin/streptomycin, 10% fetal bovine serum, L-glutamine, and 1 mM sodium pyruvate. Two hundred thousand cells were plated onto the filters of 5 μm pore size ChemoTx plates (NeuroProbe). Recombinant human CCL2 (Peprotech) (10 ng/ml) was diluted in supplemented RPMI 1640 media and 300 μl/well was added into wells below

the filter so as to provide a chemotactic stimulus. Two controls were used in this assay. The first control was medium only. The second one was chemokinetic control where the cells plated onto the filter contained the 10 ng/ml of CCL2 as in the underlying well. To obtain a standard curve, halving numbers of cells were plated ranging from 0 to 200,000. Cells were incubated at 37°C in humidified air with 5% CO_2 for 16 h. They were then washed off the top of the filter and the plate spun at 1,400 rpm for 10 min at room temperature. One hundred and fifty microliter of the media was discarded in the microplate and replaced with 15 µl of alamarBlue® (Invitrogen). The plate was then placed at 37°C in humidified air with 5% CO_2 for 4 h and signal was read at 570 nm. This assay was also conducted with mouse BMDM.

Human Neuron Toxicity Assay

Brain tissue from fetuses legally aborted at 15–20 weeks was used to isolate human neurons. The use of the brain cells was conducted with ethics approval from the University of Calgary human ethics committee. The neurons were isolated by removal of the meninges followed by mechanical dissociation of the tissue. Tissue was then digested in DNase (6–8 mL of 1mg/mL; Roche), 4 mL 2.5% trypsin and 40 mL PBS (37°C, 25 min). Digestion was quenched by the addition of 4 mL of fetal bovine serum (FBS) after which the solution was filtered through a 132 µm filter. The solution was then centrifuged three times at 1,200 rpm for 10 minutes. Cells were then cultured in medium supplemented with 10% FBS, 1 µM sodium pyruvate, 10 µM glutamine, 1x non-essential amino acids, 0.1% dextrose, and 1% penicillin/streptomycin (Invitrogen). Cells were plated in poly-L-ornithine-coated T75 flasks and cultured for two cycles in medium consisting of 25 µM cytosine arabinoside (Sigma-Aldrich). The inclusion of cytosine arabinoside inhibits astrocyte proliferation. To complete experiments, cultures enriched (∼80%) for neurons were re-trypsinized and plated in poly-L-ornithine coated 96-well plates at a density of 100,000 cells/well. After 24 h, medium was changed to serum-free AIM V medium. After another 24 h, demeclocycline (10 µM) was added to the neurons. Cells were fixed 24 h after with 4% paraformaldehyde and stained for MAP-2 (mouse anti-MAP-2 antibody; clone HM-2; Sigma; M4403; 1:1,000) and Hoeschst S769121. Cells were imaged with an ImageXpress® imaging system (Molecular Devices) and quantified using MetaXpress® (Molecular Devices).

Culture of Human BTICs

BTICs were isolated from resected specimens of patients with glioblastoma (7, 9, 26, 36). We used three BTIC lines derived from glioma patients designated BT012, BT025, and BT048. These lines had different genetic mutations (26) including BT012: EGFR wildtype (wt), p53 wt, PTEN mutant (mt, frameshift in codon 328); BT025: EGFR wt, p53 mt (T125R), PTEN mt (G129R); and BT048: EGFR mt (K294R, G598V), p53 wt, PTEN wt. BT025 and BT048 were employed in the majority of experiments since they were characterized extensively in our previous study (6–9, 26). To propagate the lines, BTICs were dissociated and plated into T25 flasks at regular intervals and grown in serum free culture medium supplemented with epidermal growth factor and fibroblast growth factor-2 in 5% CO_2 as described elsewhere (36, 37); we refer to this as BTIC medium. The lines with higher passage numbers were checked for stemness and self-renewal property (data not shown). All experiments with human cells or resected brain specimens were conducted with approval from the Conjoint Health Research Ethics Board, University of Calgary, with informed consent from the human subjects.

We documented passage number (denoted by P) after thawing a new vial of cells from liquid nitrogen and kept a record of the number of subsequent passaging. Frozen stocks of BTIC cells were made as soon as possible from previously thawed BTIC lines, to avoid cell changes, contamination, etc. for the next set of experiments. Every time a vial (of BTIC line) was thawed, new stocks were made within a week or two of the growing culture. Thus, the BTICs were usually frozen between P2-P3 of a newly thawed culture. We used BTICs (after thawing) from the expanding and/or growing cultures for experiments between P2 and P10. The lines with higher passage numbers (P8–P10) were checked for stemness using stemness markers such as nestin, SOX-2 and Musashi-1 by FACS analysis, and by the ability of dissociated single cells to form spheres. Importantly, BTICs were also sent for sequencing at regular intervals to verify the identity of lines (to ascertain their genetic background with the parental line).

Evaluation of BTIC Growth

For neurosphere assay, BTIC cells (10,000 cells/well/100µl of serum free BTIC medium) were plated into 96-well plates (7, 26, 36). The resultant number of spheres above the 60 µm diameter cutoff, a convenient parameter to describe growth characteristics, was monitored after 72 h by photographing multiple fields per well with subsequent image analyses. Where total cell number was documented, the medium containing the BTIC spheres was collected, centrifuged, resuspended in 25 µL of Accumax™, mixed with Trypan Blue (1:1) and counted using a TC20™ automated cell counter (Bio-Rad).

An alamarBlue® assay was also used to evaluate growth. At predetermined times, alamarBlue® dye (1:10, Life Technologies) was added to each well of cells for 4–6 h after which readings were taken with a Spectra Max Gemini XS (emission wavelength = 590 nm, excitation wavelength = 544 nm; Molecular Devices). Finally, annexin V–propidium iodide staining was carried out and analyzed using FACS as described before (26).

Drugs Used

Demeclocycline hydrochloride, tetracycline hydrochloride, and oxytetracycline hydrochloride stock solutions were prepared (10 mM in DMSO; all chemicals from SigmaAldrich) and diluted immediately prior to treatment of cells. While, demeclocycline was used at 1, 5, and 10 µM final concentrations, tetracycline and oxytetracycline were used at 10 µM only. All dilutions from stock were done in BTIC medium.

Microarray and Bioinformatics

BTICs (BT012, BT025, and BT048) were treated with 10 µM demeclocycline for 6 h. RNA was then extracted using a mirVana

miRNA Isolation Kit (Ambion, Austin, USA) according to the manufacturer's protocol. Total RNA was purified with RNeasy Plus Micro Kit (Qiagen, Valencia, USA) to remove genomic DNA. The RNA quality of integrity number (RIN) was measured with Agilent RNA Nano Chips on 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The total of 250 ng of RNA for each sample with RIN higher than 9 was labeled with WT Express Kit (Ambion) and hybridized to Affymetrix GeneChip Human Gene 2.0 ST Array at 45°C for 16 h. Arrays were stained and washed on Affymetrix GeneChip Fluidics_450 following manufacturer's protocol and scanned with Affymetrix GeneChip Scanner 3,000 7G System.

For data analysis, array data files were generated with GeneChip® Command Console® Software (AGCC) and statistical analyses were carried out in GeneSpring™ (Agilent Technologies). The fold change between treatment and control was based on the $p < 0.05$ from Ttest of unpaired samples.

Statistical Analyses

The one-way ANOVA with *post-hoc* Tukey's comparisons was used for multiple group comparisons unless otherwise mentioned, while the *t*-test was used for comparisons of two groups. We used GraphPad Prism software for statistical analysis.

RESULTS

Demeclocycline Promotes the Activity of Monocytes and Macrophages and Is Not Toxic to Neurons

While we identified meclocycline as an activator of microglia (33), it was necessary to confirm that its better tolerated derivative, demeclocycline, also has such activity. We first carried out some preliminary studies with mouse bone marrow derived macrophages (BMDM). Notably, demeclocycline alone did not induce mouse macrophages to increase TNF-α production; however, when combined with LPS, demeclocycline further promoted the production of TNF-α (**Figure 1A**). Moreover, as another index of activity, demeclocycline promoted the chemotaxis (**Figure 1B**) of stimulated macrophages when combined with LPS.

To corroborate the above findings of mouse cells, we evaluated whether human cells were responsive to demeclocycline. We investigated monocytes isolated from healthy human donors, as these cells would be exposed to demeclocycline in the circulation after systemic administration and could then traffic into the glioma microenvironment as macrophages. We found that human monocytes under basal conditions did not elevate their production of TNF-α, an index of

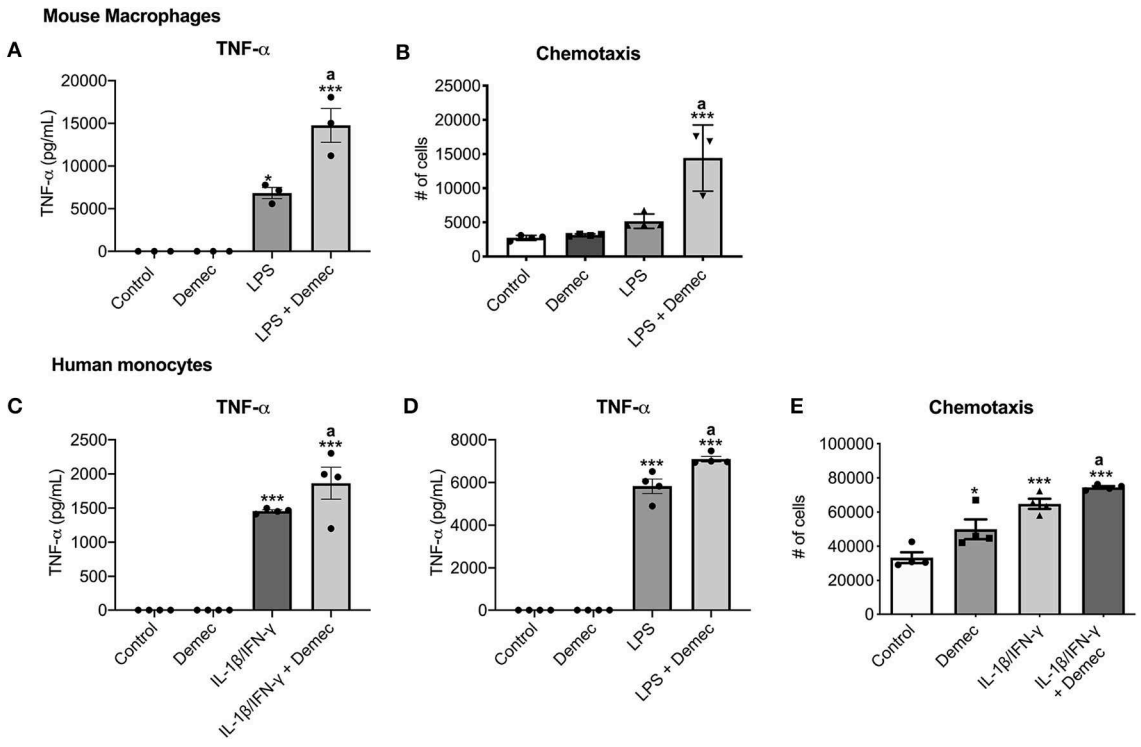


FIGURE 1 | Demeclocycline enhances TNF-α production and modulates monocyte functions. **(A,B)** For mouse BMDM, demeclocycline increases TNF-α level and CCL2-directed chemotaxis in LPS-stimulated condition. **(C,D)** ELISA for TNF-α in human cells shows a further elevation of cytokine in IL-1β/IFN-γ **(C)** or LPS **(D)** stimulated monocytes by demeclocycline. **(E)** The chemotaxis of human monocytes toward a CCL2 gradient is promoted by demeclocycline in IL-1β/IFN-γ-stimulated condition. In all cases, demeclocycline was used at 10 μM. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to control; [a]significantly different from IL-1β/IFN-γ or LPS in their respective panels (1-way ANOVA with Tukey's multiple comparisons test). Error bars represent s.e.m.

activity, in the presence of demeclocycline alone. However, when human monocytes were exposed to IL1β/IFN-γ (**Figure 1C**), cytokines that are elevated in glioma subjects (38), or to the toll-like receptor-4 ligand LPS (**Figure 1D**), demeclocycline elicited a further increase in TNF-α levels in activated cells.

The migration of monocytes to a chemokine source, chemotaxis, constitutes another index of cellular activity. In IL-1β/IFN-γ-primed conditions, we noted that demeclocycline promoted the chemotaxis of human monocytes to CCL2 (**Figure 1E**); alone, demeclocycline had some enhancing activity.

Demeclocycline-Treated Monocytes Reduce BTIC Growth

Our previous study (26) found that the capacity of microglia, monocytes and macrophages to reduce BTIC growth could be elicited through the conditioned medium of these cells. Thus, monocytes were isolated from the peripheral venous blood of healthy volunteers and conditioned media were generated (**Figure 2A**). To determine the capacity of demeclocycline as a novel stimulator of innate immunity to reduce BTIC growth, BTIC lines plated at 10,000 cells per well in 96-well plates were exposed to conditioned medium from untreated monocytes (MonoCM) or to the conditioned medium of monocytes

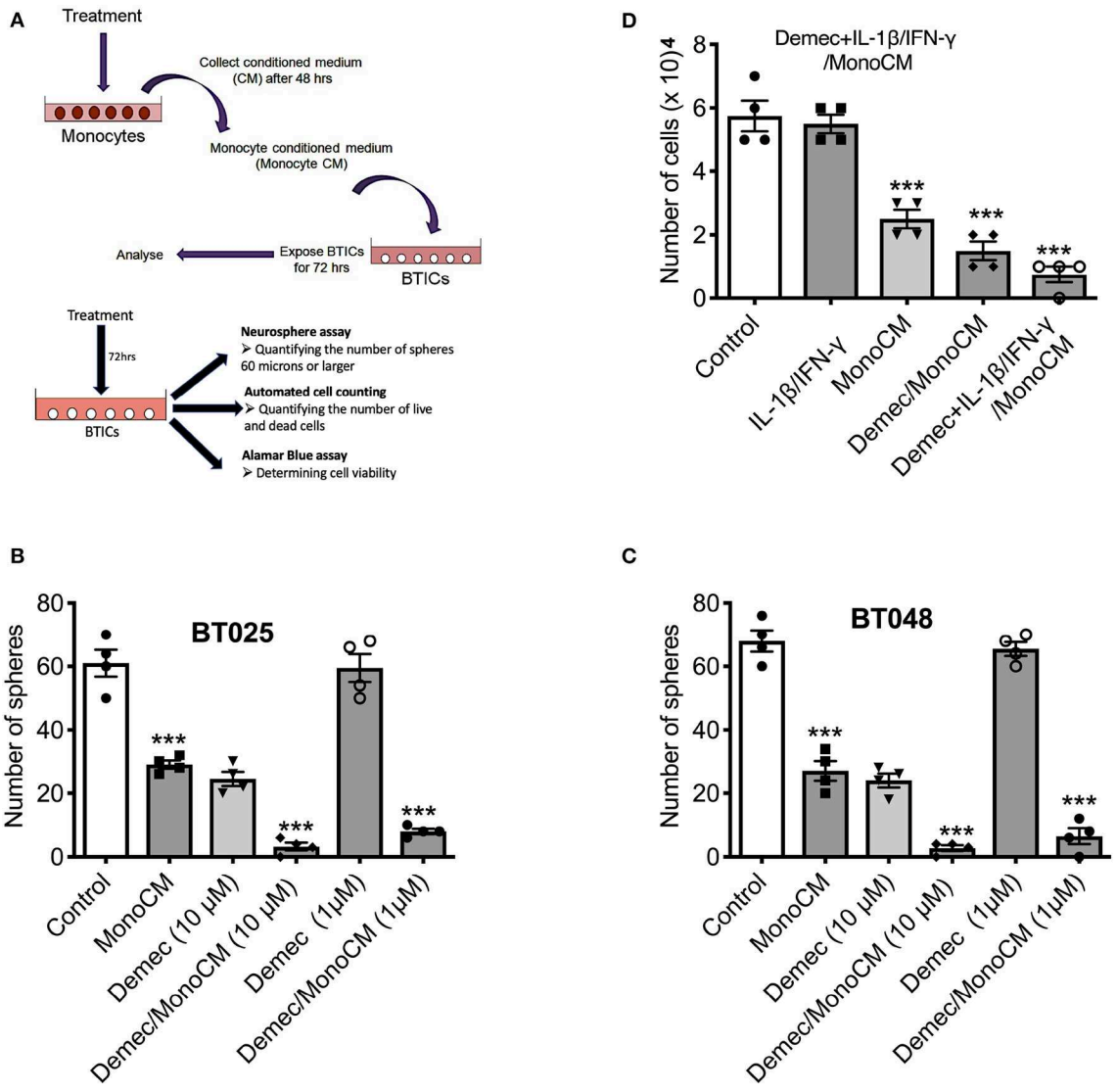


FIGURE 2 | Demeclocycline activated monocytes reduces BTIC growth in culture. **(A)** Generation of monocyte-conditioned media. **(B,C)** Untreated human monocytes (MonoCM) or monocytes exposed to demeclocycline (Demec/MonoCM) attenuate BTIC growth compared to basal control after 72 h as measured through neurosphere assay. Although demeclocycline alone reduced the BTIC growth at 10 μM, it was ineffective at 1 μM in both lines. Notably, Demec/MonoCM had robust effect on BT025 and BT048 cells, and was significantly greater than that of either MonoCM or demeclocycline alone. **(D)** Demec/MonoCM also reduced the total cell counts in BT048 line, and this BTIC growth inhibitory effect was promoted in IL-1β/IFN-γ treated monocyte cultures. ***$p < 0.001$ compared to control (1-way ANOVA with Tukey's multiple comparisons); $n = 4$ for all groups. Error bars represent s.e.m.

exposed for 48 h to 10 μM demeclocycline (Demec/MonoCM). Reproducing previous results (26), MonoCM reduced the growth of the BT025 and BT048 lines (**Figures 2B,C**) in sphere-forming assays; importantly, Demec/MonoCM decreased the growth of BTICs further (**Figures 2B,C**) and there was an additional effect on reducing BTIC growth when conditioned medium from monocytes exposed to both demeclocycline and IL-1β/IFN-γ (Demec + IL-1β/IFN-γ/MonoCM) was used (**Figure 2D**).

In these experiments, we noted that demeclocycline (10 μM) added directly to BTICs in the absence of monocyte intermediary was sufficient to reduce BTIC growth, suggesting that the medication may affect BTICs in 2 ways: through monocyte intermediary and directly on BTICs.

Demeclocycline Directly Affects the Growth of BTICs

Because the above results suggest that demeclocycline alone reduced BTIC growth, we sought to investigate its direct role further. We subjected BTICs to different concentrations of demeclocycline and found that 5 and 10 μM decreased sphere formation and cell number (**Figures 3A,B**); an effect on BTIC could be documented for 1 μM demeclocycline using the alamarBlue® assay (**Figure 3C**). Notably, demeclocycline at 10 μM concentration had selective efficacy on BTICs as it was without obvious toxicity to non-transformed CNS cells such as microtubule associated protein-2 (MAP-2) labeled neurons (**Figures 3D,E**).

As the above experiments involved the treatment of freshly dissociated BTIC lines with demeclocycline to determine whether the medication reduced sphere formation and other indices of BTIC growth, we next addressed whether demeclocycline affected BTIC spheres that were already well-formed. We found that when demeclocycline (10 μM) was added to growing spheres 3 days after their formation from singly dissociated cells, the drug still reduced the further growth of spheres of the BT025 and BT048 lines (**Figures 3F–H**).

Overall, our results suggest that demeclocycline can control BTIC growth in two ways: using monocytes as an intermediary, and directly by affecting the proliferation and sphere-forming capacity of BTICs.

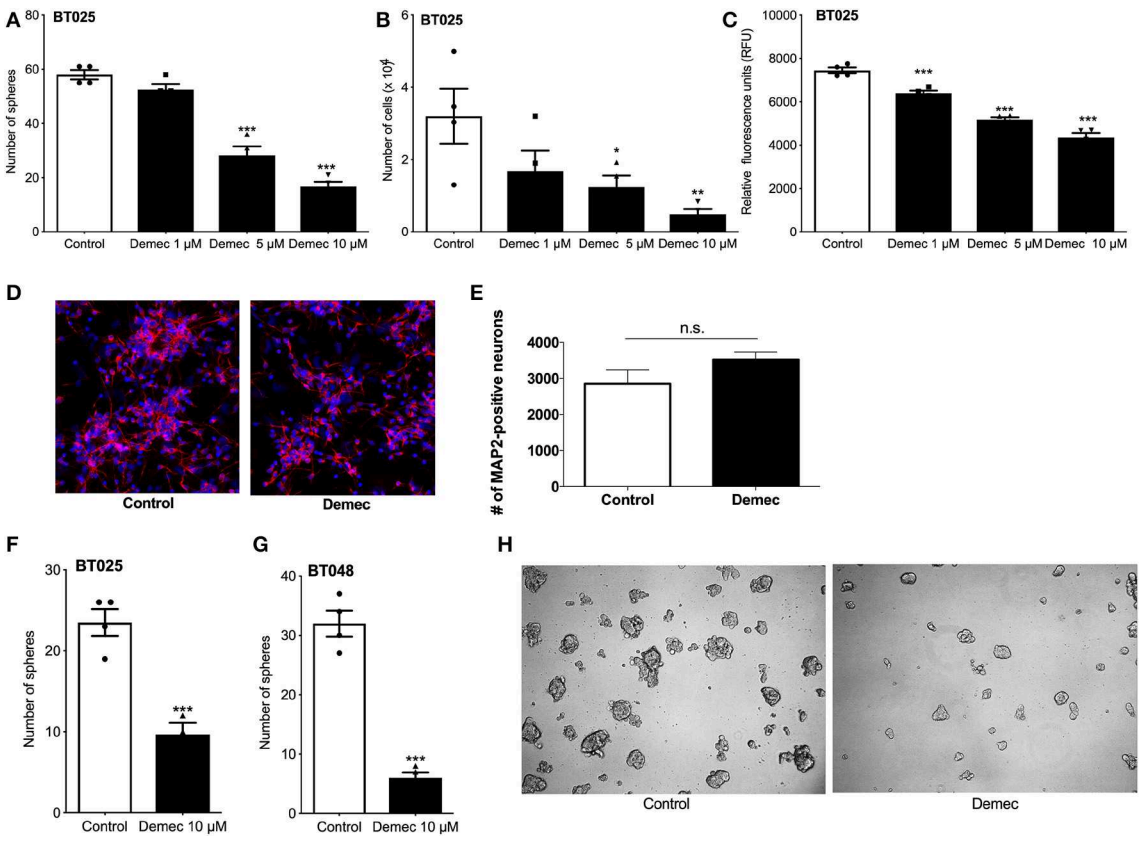


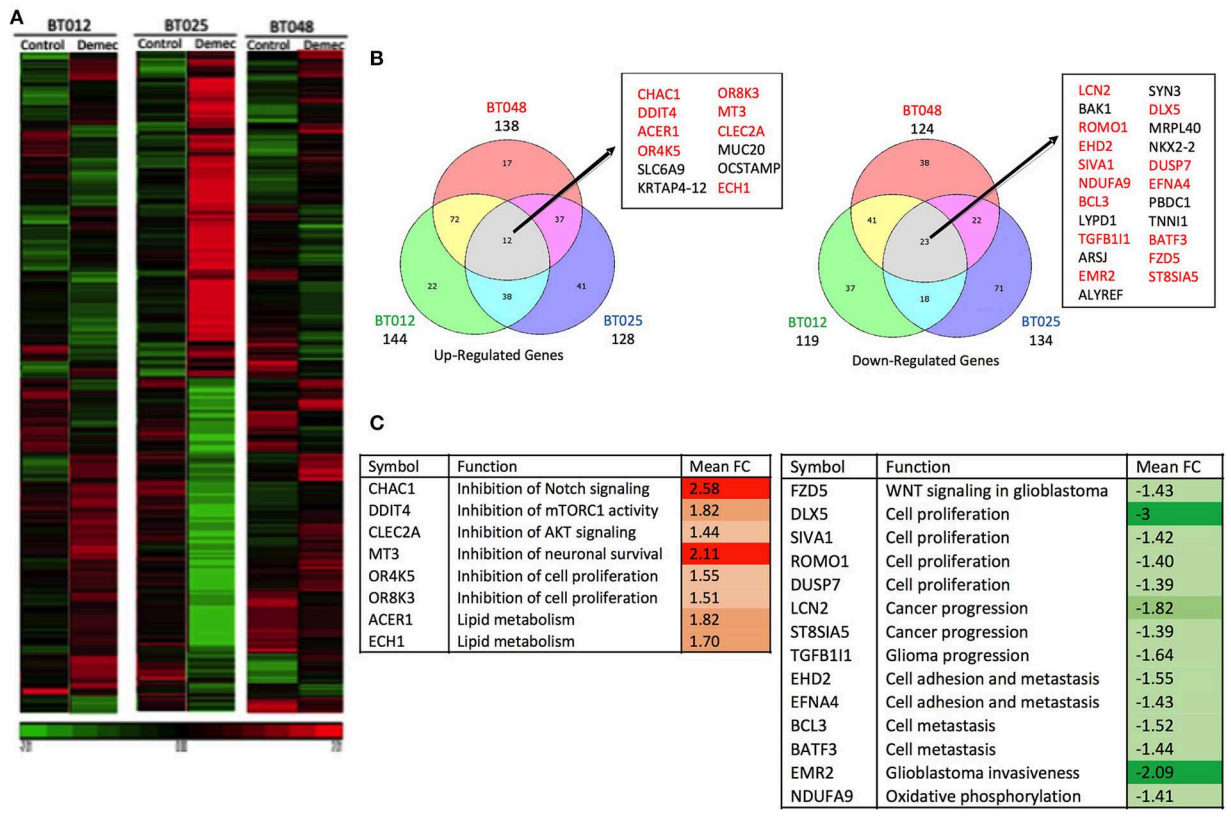
FIGURE 3 | Demeclocycline has a direct impact on BTIC growth reduction. **(A–C)** Treatment of BT025 with varying concentrations of demeclocycline reduced BTIC growth in culture as evident by reduced sphere formation **(A)**, total number of cells **(B)**, and alamarBlue® assay **(C)**. Results are reproduced with BT048 (data not shown). Error bars represent s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to controls (1-way ANOVA with Tukey's multiple comparisons test). **(D,E)** Representative images of MAP2-positive neurons untreated or treated with demeclocycline, and quantification of MAP2-positive neurons demonstrates that demeclocycline was not toxic to human neurons. **(F,G)** Demeclocycline reduced the number of spheres above the 60 μm cutoff when applied to growing spheres 3 days after their formation from singly dissociated cells. Error bars represent s.e.m (*n* of 4). $^{***}p < 0.001$ (two-tailed Student's *t*-test). **(H)** Representative images showing reduced sphere size in demeclocycline-treated BTICs (BT048) compared to control at 5 days.

Mechanisms of Demeclocycline-Mediated BTIC Growth Reduction

We sought to obtain insights into the mechanisms by which demeclocycline directly reduces BTIC growth. We subjected 3 BTIC lines to microarray analyses and identified 301 genes (with a cutoff fold change = 1.3) amongst the three lines that were commonly affected by demeclocycline treatment compared to controls (**Figure 4A** and **Supplementary Table 1**) (GEO accession number GSE81515). Analysis of the array data using gene-ontology criteria with Panther Classification System bioinformatics software identified several genes that were up or down regulated with demeclocycline treatment (**Figure 4B** and **Supplementary Figure 1**). Notably, we found a number of genes known to be involved in glioma progression, invasiveness, signaling or cancer progression that were down regulated by demeclocycline. These include transforming growth factor β1 induced transcript 1 protein (TGFB1I1) (39), frizzled class receptor 5 (FZD5) (40), epidermal growth factor module-containing mucin-like receptor 2 (EMR2) (41), reactive oxygen species modulator 1 (ROMO1) (42) and B cell lymphoma 3 protein (BCL3) (43) (**Figure 4C**). In contrast, genes that negatively regulate notch signaling, mTOR activity or AKT such as ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1 (CHAC1), DNA Damage Inducible Transcript 4 (DDIT4) and C-Type Lectin Domain Family 2 Member A (CLEC2A) were up regulated. With demeclocycline treatment in three BTIC lines (**Figure 4C**). Notably, a recent study has identified that temozolomide and radiotherapy could induce DDIT4 and repressed mTORC1 activity in some glioblastoma cell lines (44). Thus, overexpression of DDIT4 by demeclocycline in BTIC could be beneficial for glioblastoma patients. Interestingly, when we interrogated glioblastoma databases we found that elevated level of DDIT4 expression was associated with the longevity of glioma patients (**Figure 5**).

Comparisons of Demeclocycline With Other Tetracyclines on BTIC Growth *in vitro*

We compared two other tetracyclines (tetracycline and oxytetracycline) to demeclocycline. We subjected three BTIC lines generated from glioblastoma patients with divergent genetic



Symbol	Function	Mean FC
CHAC1	Inhibition of Notch signaling	2.58
DDIT4	Inhibition of mTORC1 activity	1.82
CLEC2A	Inhibition of AKT signaling	1.44
MT3	Inhibition of neuronal survival	2.11
OR4K5	Inhibition of cell proliferation	1.55
OR8K3	Inhibition of cell proliferation	1.51
ACER1	Lipid metabolism	1.82
ECH1	Lipid metabolism	1.70

Symbol	Function	Mean FC
FZD5	WNT signaling in glioblastoma	-1.43
DLX5	Cell proliferation	-3
SIVA1	Cell proliferation	-1.42
ROMO1	Cell proliferation	-1.40
DUSP7	Cell proliferation	-1.39
LCN2	Cancer progression	-1.82
ST8SIA5	Cancer progression	-1.39
TGFB1I1	Glioma progression	-1.64
EHD2	Cell adhesion and metastasis	-1.55
EFNA4	Cell adhesion and metastasis	-1.43
BCL3	Cell metastasis	-1.52
BATF3	Cell metastasis	-1.44
EMR2	Glioblastoma invasiveness	-2.09
NDUFA9	Oxidative phosphorylation	-1.41

FIGURE 4 | Microarray analyses of BTICs exposed to demeclocycline. **(A)** Heat map depicting patterns of changing gene expression in three genetically divergent glioblastoma patient-derived BTIC lines after 6 h of demeclocycline treatment, compared to respective controls; red represents elevation while green displays genes that are reduced by demeclocycline (GEO accession number GSE81515). **(B)** Venn diagrams comparing up-regulated (fold change, FC ≥ 1.3) and downregulated (FC≤ −1.3) genes in the three BTIC lines following treatment with demeclocycline. Genes intersecting in all 3 sets are noted in box; genes which may be involved in tumor progression are represented in red. Diagrams are generated by Bioinformatics.lu software. **(C)** Genes with possible role in tumor progression are shown. Up-regulated genes (relative to untreated cells) are represented in red; down-regulated genes (relative to untreated cells) are represented in green. Mean fold change (FC) is calculated for the three BTIC lines following treatment with demeclocycline vs. no treatment. The intensity of the colors represents the strength of the gene deregulation.

mutations, using demeclocycline as a positive control. Growth was assessed at 72 h after plating 10,000 cells/well in 96-well plate, using 10 μM of each drug. **Figure 6A** shows that while tetracycline and oxytetracycline reduced the sphere-forming capacity of BTICs to varying extents across different lines, demeclocycline inhibited the sphere-forming capacity of BTICs consistently across all lines. These results were corroborated by alamarBlue® assays (**Figure 6B**) and cell counts (data not shown).

DISCUSSION

Tumorigenesis not only alters the surrounding microenvironment, but is regulated by it (45, 46). Unfortunately, immune cells in the high grade glioblastoma (GBM) microenvironment generally assume tumor-promoting roles (47–51). Under the influence of GBM, microglia/macrophages are immunosuppressed and may even contribute to GBM invasion (52–54). BTICs are thought to help enforce immunosuppression (11, 12, 55–58). Thus, we have sought to sway the microglia/macrophage interaction with BTICs toward an anti-tumor phenotype. Via a drug screen of currently available medications, we discovered that amphotericin B could activate blood-derived monocytes to suppress BTIC proliferation and induce differentiation (26). However, amphotericin B is unlikely to be used as an immunostimulator for intracranial disease because of its substantial toxicity at high doses (59). Our attention turned to another drug found on the screen, meclocycline, and its more clinically attractive derivative, demeclocycline.

Demeclocycline is clinically attractive for several reasons. It has already been used for the treatment of bacterial infections and as a treatment for the syndrome of inappropriate antidiuretic hormone (SIADH) in humans (60, 61). A recent study showed that demeclocycline was also a promising contrast agent for the intraoperative detection of brain tumors (62). Moreover, when we exposed neural cells to demeclocycline, no significant toxicity was noted. Hence, its application as a drug to treat intracranial disease such as glioblastoma is more conceivable than with amphotericin B or meclocycline.

We first characterized the *in vitro* ability of demeclocycline to activate monocytes as these cells in the circulation could enter a glioma tumor to become macrophages (63). Thus, exposure of monocytes to an immunostimulator could theoretically result from systemic administration of a drug such as demeclocycline. Alone, demeclocycline did not increase TNF-α secretion by monocytes, a measure of monocyte activity. However, in the presence of primers such as IL-1β and IFN-γ, cytokines that are

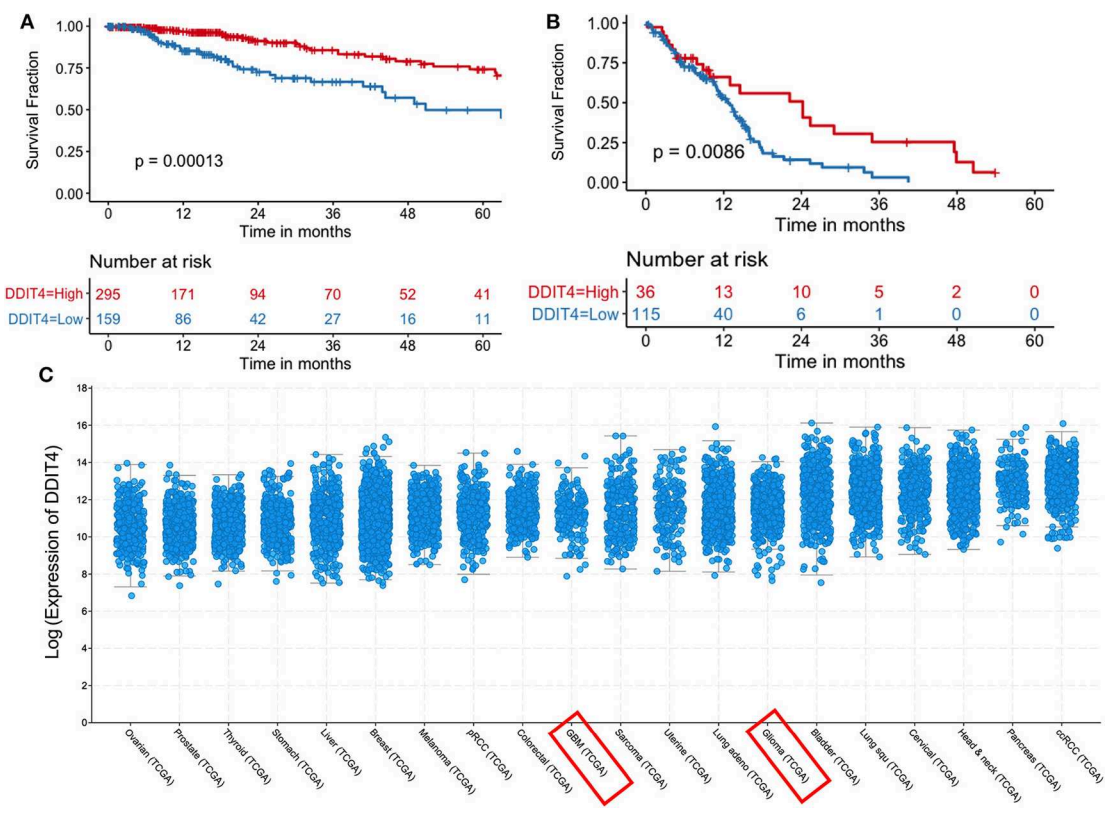


FIGURE 5 | Increased DDIT4 expression is associated with improved survival in gliomas. **(A,B)** Kaplan–Meier curves showing the association between *DDIT4* mRNA expression and overall survival in TCGA **(A)** low-grade glioma and **(B)** glioblastoma patients. **(C)** *DDIT4* mRNA expression was plotted across 20 major solid tumor types in TCGA database arranged by increasing median expression (pRCC, Papillary Renal Cell Carcinoma; ccRCC, clear cell renal cell carcinoma; Lung squ, lung squamous cell carcinoma; GBM, glioblastoma).

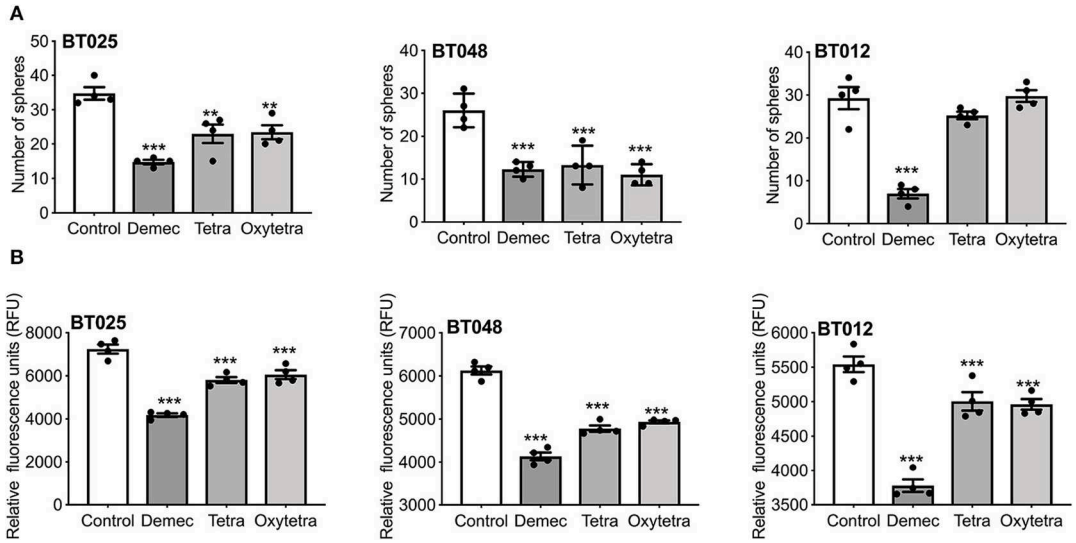


FIGURE 6 | Effects of various tetracyclines on BTIC growth in culture. **(A)** Three glioblastoma patient-derived BTIC lines were exposed to 10 μM each of demeclocycline (demec), tetracycline (tetra) or oxytetracycline (oxytetra). **(B)** AlamarBlue® assay shows effect of demeclocycline, tetracycline and oxytetracycline on different BTIC lines with divergent genetic mutations, corroborating the reduced sphere-forming capacity of BTICs to varying extents across different lines. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control (1-way ANOVA with Tukey's multiple comparisons). Error bars represent s.e.m. (*n* of 4).

commonly elevated in glioma patients (38, 64), demeclocycline had stimulatory properties beyond that of when either IL1β or IFN-γ were administered alone. As confirmation of the immune-stimulatory effect of demeclocycline in the presence of a priming condition, similar results were seen when a conventional stimulator, LPS, was added. Also, the promotion of migration in demeclocyclineexposed monocytes supported the notion that this drug was an activator. To verify these results, the experiments were recapitulated with mouse macrophages. As another measure of the immunestimulatory capacity of demeclocycline, it was shown that conditioned medium collected from monocytes exposed to demeclocycline even in the absence of stimulators could decrease BTIC sphere formation. Given that temozolomide is the frontline chemotherapy for glioblastoma, this adds to the promise of demeclocycline as an additional treatment modality in glioblastoma.

Importantly, demeclocycline is not only an immune-stimulator, but can independently decrease BTIC viability, as indicated by alamarBlue and sphere formation assays. As with present treatments, subgroups of glioblastoma patients will be more sensitive to certain treatments based on factors such as genetics and previous treatments (65, 66), which may be true of the cell lines derived from those tumors.

To elucidate the mechanisms behind BTIC inhibition by demeclocycline, we employed a microarray analysis. This analysis has implicated several genes known to be involved in glioma or cancer progression (i.e., proliferation, invasion, metastasis) such as TGFB1II (39), FZD5 (40), EMR2 (41), ROMO1 (42), and BCL3 (43) which were significantly down-regulated with demeclocycline treatment in three BTIC lines (**Figure 4**). On the contrary, genes that negatively regulate Notch signaling, ATK signaling or mTOR activity, such as CHAC1, DDIT4, CLEC2A, were up regulated with demeclocycline treatment across all three BTIC lines (**Figure 4**). Taken together, these results support the use of demeclocycline as an anti-GBM treatment alone, or as an immunostimulatory agent acting on monocytes and macrophages, and potentially microglia if demeclocycline gains entry into the CNS.

In summary, we have identified a novel, potentially clinically compatible stimulator of monocytes that also has direct inhibitory actions on BTICs. This study has served as the basis for future work in which we will determine the safety and efficacy of demeclocycline in preclinical investigations, with the hopes to expand its use into humans.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO accession number GSE81515.

ETHICS STATEMENT

All experiments with human cells or resected brain specimens were conducted with approval from the Conjoint Health Research Ethics Board, University of Calgary, with written informed consent from the human subjects.

AUTHOR CONTRIBUTIONS

SS, YL, and RM provided data for the response of BTICs to demeclocycline, other tetracyclines, and monocyte conditioned media. KR, CP, and JW provided data of the response of myeloid cells to demeclocycline. MK and PB conducted the bioinformatics and interrogation of databases. SS and VY oversaw the entire project and completed the editing of the manuscript after input from all co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00272/full#supplementary-material

Table S1 | Dysregulated genes after treatment BTICs with demeclocycline.

Figure S1 | Clustering of dysregulated genes in BTICs after treatment with demeclocycline. Clusters of up-regulated **(A)** or down-regulated **(B)** genes were generated by the PANTHER classification system based on their functions. Up-regulated **(C)** or down-regulated **(D)** genes in corresponding categories are listed in the tables. Mean fold change (FC) is calculated for the three BTIC lines following treatment with demeclocycline versus no treatment.

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REVIEW
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Beyond Metabolism: The Complex Interplay Between Dietary Phytoestrogens, Gut Bacteria, and Cells of Nervous and Immune Systems

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The human body has a large, diverse community of microorganisms which not only coexist with us, but also perform many important physiological functions, including metabolism of dietary compounds that we are unable to process ourselves. Furthermore, these bacterial derived/induced metabolites have the potential to interact and influence not only the local gut environment, but the periphery via interaction with and modulation of cells of the immune and nervous system. This relationship is being further appreciated every day as the gut microbiome is researched as a potential target for immunomodulation. A common feature among inflammatory diseases including relapsing-remitting multiple sclerosis (RRMS) is the presence of gut microbiota dysbiosis when compared to healthy controls. However, the specifics of these microbiota-neuro-immune system interactions remain unclear. Among all factors, diet has emerged as a strongest factor regulating structure and function of gut microbial community. Phytoestrogens are one class of dietary compounds emerging as potentially being of interest in this interaction as numerous studies have identified depletion of phytoestrogen-metabolizing bacteria such as Adlercreutzia, Parabacteroides and Prevotella in RRMS patients. Additionally, phytoestrogens or their metabolites have been reported to show protective effects when compounds are administered in the animal model of MS, Experimental Autoimmune Encephalomyelitis (EAE). In this review, we will illustrate the link between MS and phytoestrogen metabolizing bacteria, characterize the importance of gut bacteria and their mechanisms of action in the production of phytoestrogen metabolites, and discuss what is known about the interactions of specific compounds with cells immune and nervous system. A better understanding of gut bacteria-mediated phytoestrogen metabolism and mechanisms through which these metabolites facilitate their biological actions will help in development of novel therapeutic options for MS as well as other inflammatory diseases.

Keywords: multiple sclerosis and neuroimmunology, phytoestrogen, gut microbiome, immune system, nervous system, diet

MULTIPLE SCLEROSIS AND GUT MICROBIOTA

Relapsing-remitting multiple sclerosis (RRMS) is a chronic inflammatory disease of the central nervous system (CNS), affecting ∼1 million people in the US and over 2.3 million worldwide (1). Collective evidence suggests that disease results from an aberrant T-cell mediated response to myelin-derived antigens in genetically susceptible individuals. Multiple genetic and environmental factors have been implicated in the predisposition to RRMS (2). Among a number of environmental risk factors linked with MS, the gut microbiota appear to be particularly important, as highlighted by a number of recent studies reporting gut dysbiosis in RRMS patients (3–8). Although the mechanism through which the gut microbiota influences RRMS pathogenesis is unknown, diet has emerged as the strongest factor influencing the gut microbiome.

The adult human gut is colonized by a large number of microorganisms ($\sim 10^{13}$ bacteria). The majority of which (∼90%) belong to the Firmicutes and Bacteroidetes phyla. The remainder represent Actinobacteria, Proteobacteria, and few other phyla present at very low abundance (9). The fact that only a few bacterial phyla are present in the human gut suggests that they were actively selected during human evolution. As human evolution is nutrition centric, it is hypothesized that gut bacteria capable of efficiently extracting energy from ingested plants and animal meat would provide a survival advantage. Gut microbiota composition is heavily influenced by dietary habits, with unindustrialized rural communities showing higher abundance of bacteria enriched in enzymes capable of digesting plant-based complex polysaccharides. At the same time, individuals from industrialized nations and eating a western diet rich in animal protein, fats, and simple sugars are enriched in gut bacteria containing enzymes responsible for metabolism of simple sugars, amino acids and bile acids (10). Even within a population, gut microbiota composition can be altered due to seasonal change in the food source (11). A study of Hadza hunter-gatherers of Tanzania showed this seasonal change in gut microbiome based on dietary sources, as this population rely mostly on plant based foods during rainy season but shift to plant plus meat-based diet during dry season (11). These seasonal changes in gut microbiota confirm an important role of diet (plant and meat both) in influencing the bacterial community; however, it is unclear whether one diet has advantage over other. Specifically it is unknown whether proposed pathogenic effect of meat based and/or Western diet (12) is due to meat itself or due to factors associated with industrialization/modernization such as processing of foods, increased use of antibiotics etc. (13). However, evaluation of the microbiome-mediated benefits or drawbacks of plant- vs. meat-based diets, western diet, or any other dietary interventions of interest in the scientific community are beyond the scope of this review.

Breakdown of these foods might generate key metabolites necessary for various physiologic functions of the host including development and regulation of nervous and immune systems (14). It has been clearly established that change in diet changes the composition of the gut microbiota; however the mechanisms by which this affects host physiology are slowly being understood. Non-redundant bacterial metabolites which are dependent on ingestion of certain dietary components, such as the phytoestrogens and their metabolites discussed here, are of increasing interest. As the gut microbiome plays an important role in the energy harvesting for the host, therefore any changes in the composition of the gut microbiota could have widespread effects on physiologic homeostasis and overall human health (15–18).

Our recent summary of RRMS microbiome studies across different geographical regions (USA, Japan, UK and Italy) shows enrichment or depletion of specific bacterial genera when compared to healthy controls (HC) (19, 20). One observation was loss of Bacteroidetes species, especially those of the genera *Prevotella* and *Parabacteroides* suggesting a role of these bacteria in RRMS [**Table 1;** (3–8)]. Our group (19) and others (3–5) reported a lower abundance of *Prevotella* in fecal samples of RRMS patients compared to HC. Additionally, treatment with disease modifying therapies (DMT) led to a higher abundance of *Prevotella* in RRMS patients than in untreated patients (5, 20). Further, Cosorich et al. also reported lower level of *Prevotella* when analyzing duodenal biopsies from RRMS patients with active disease compared to HC (7). Another Bacteroidetes genus, *Parabacteroides* has been reported to be at lower abundance in adult RRMS patients when compared to HC (4, 8). We observed reduced abundance of *Parabacteroides* in RRMS patients vs. HC from the Midwestern United States (4). Similarly, Cekanaviciute et al. reported that *Parabacteroides distasonis* is at lower

TABLE 1 | Comparison of adult MS microbiome studies.

MS Microbiome Study # samples Tissue (Country) *[Reference]*	Lower abundance in MS patients vs. HC	Increased abundance in MS patients after treatment
RRMS ($n = 31$) HC ($n = 36$) Fecal (USA) Chen et al. (4)	***Prevotella***, ***Parabacteroides***, ***Adlercreutzia,*** *Collinsella*, *Lactobacillus*	
RRMS ($n = 60$) HC ($n = 43$) Fecal (USA) Jangi et al. (5)	*Butyricimona, Prevotella, Parabacteroides*	***Prevotella Sutterella***
RRMS ($n = 20$) HC ($n = 40$) Fecal (Japan) Miyake et al. (3)	*Bacteroides*, *Fecalibacterium*, ***Prevotella,*** *Anaerostipes, Clostridium, Sutterella*	
RRMS ($n = 30$) HC ($n = 14$) Fecal (UK) Castillo-Alvarez et al. (20)		***Prevotella***
RRMS ($n = 71$) Fecal (USA) Cekanaviciute et al. (8)	***Parabacteroides distasonis***	
RRMS ($n = 19$) HC ($n = 17$) Mucosa (Italy) Cosorich et al. (7)	***Prevotella***	

Bolded microbes are phytoestrogen metabolizing bacteria.

abundance in treatment naive RRMS patients from the US west coast than HC, suggesting that higher level of *P. distasonis* may protect against RRMS (8). Further, we also observed a lower abundance of the phytoestrogen metabolizing bacteria *Adlercreutzia (equolifaciens)* in RRMS patients compared to HC (4) and *Adlercreutzia* was also reported to be increased in germ-free (GF) mice transplanted with fecal matters from HC compared to mice receiving fecal transplant from RRMS patients (21).

Thus, these studies indicate that loss of Bacteroidetes genera *Prevotella* and *Parabacteroides* might play a role in the predisposition and/or exacerbation in RRMS. As MS is an inflammatory disease where balance between pro- and anti-inflammatory responses are shifted toward inflammatory responses, it is reasonable to hypothesize that bacteria depleted in MS were involved in induction/maintenance of anti-inflammatory responses. More discussion relating to the possible mechanism of this protective role through induction of immunoregulatory cells will be discussed in this review under Phytoestrogens and Immune Cells.

Conversely, Firmicutes such as *Akkermansia, Dorea,* and *Archaea-Methanobrevibacter* were more abundant in stool from RRMS patients (4, 5, 8), suggesting that these gut microbes might have pro-inflammatory effects. This increased abundance could reasonably contribute to the induction and/or maintenance of pro-inflammatory cells in the gut, thus influencing or contributing to a systemic inflammatory state consistent with RRMS. However, *Akkermansia* had been shown to have anti-inflammatory effects in obesity and diabetes due to their ability to produce short-chain fatty acids (SCFA) (22). Similarly, *Dorea* has been suggested to be anti-inflammatory based on the observation that patients with pouchitis and Crohn's disease-like have lower abundance of *Dorea* (23) Overall, the mechanisms through which these bacteria might induce inflammation and the factors which may influence this are not well-understood and beyond the scope of this review.

Phytoestrogens are compounds produced naturally in plant foods such as legumes, soybeans, beans, nuts, flax seeds, sesame seeds, hops, and other plants (**Figure 1**). They are known to have estrogenic/antiestrogenic, antioxidant, and anti-inflammatory effects, among others (24). It is important to highlight that the role of phytoestrogens in the cancer field has been studied extensively; however, their significance in inflammatory autoimmune diseases is less understood. *Prevotella*, *Parabacteroides, and Adlercreutzia* are known to metabolize phytoestrogens and produce secondary molecules such as equol, enterolactone, and secoisolariciresinol (**Table 2**). These bacteria can also metabolize fibers to produce SCFAs(as reviewed in Freedman et al. (62). Importance of the gut microbiota in MS has been studied extensively in its animal model experimental autoimmune encephalomyelitis (EAE). The

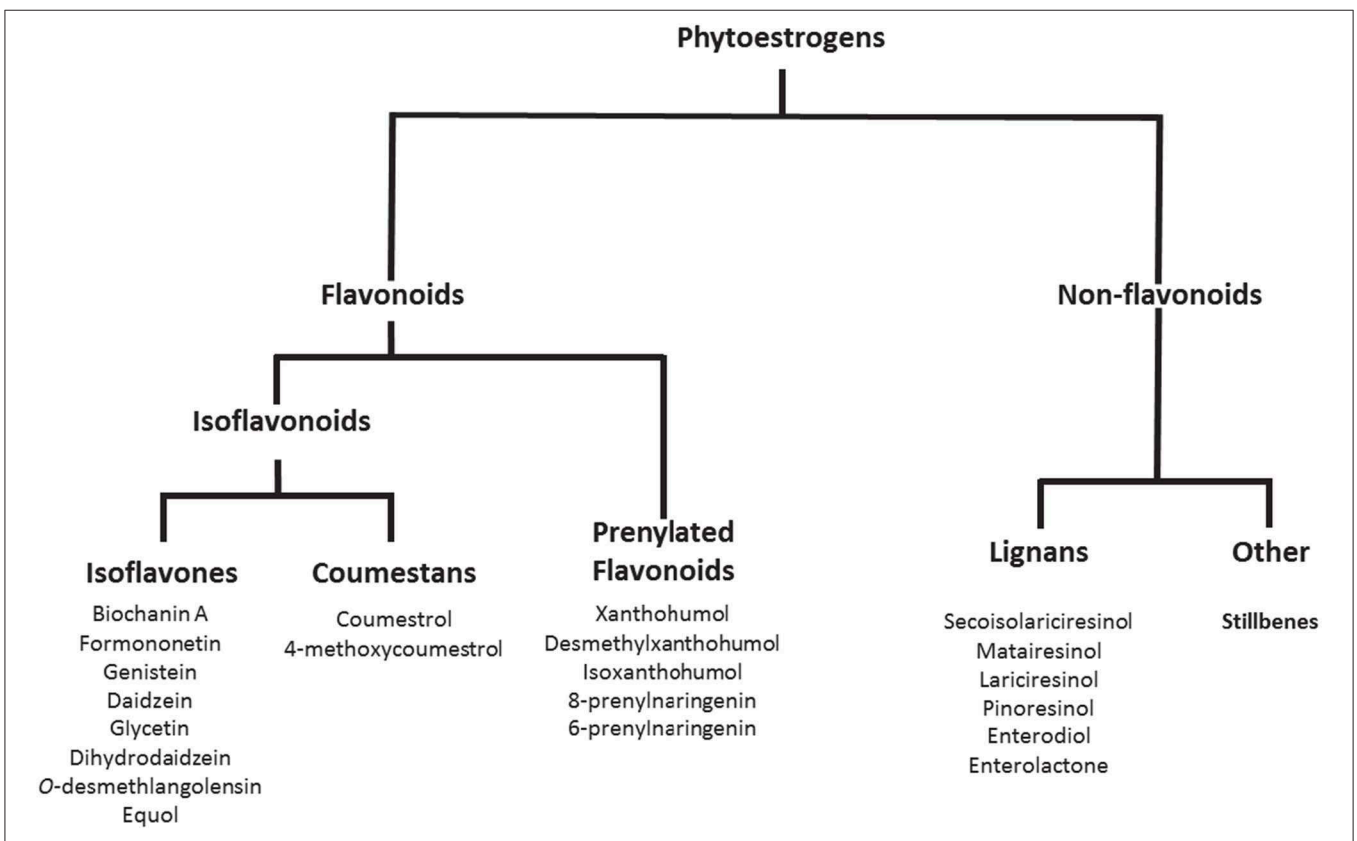

FIGURE 1 | Phytoestrogens and their metabolites generated with the help of gut bacteria. Copyright © 2019 American Chemical Society. All Rights Reserved.

TABLE 2 | Phytoestrogen metabolizing gut bacteria.

Metabolite	Strain	Reference
Daidzein	Eubacterium limosum	(25)
	E. coli strain HGH21	(26)
	Bifidobacterium animalis	(27)
	Bifidobacterium longum-a	(27)
	Bifidobacterium pseudolongum	(27)
	Strain HGH6	(26)
Genistein	*Eubacterium limosum*	(25)
	HGH21	(26)
	Strain HGH6	(26)
DHD	*Asaccharobacter celatus*	(28)
	Coprobacillus sp. *MRG-1*	(29)
	Lactococcus garvieae (Lactococcus sp. *20–92)*	(30)
	Strain HGH6	(31)
	Strain TM-40	(32)
Equol	*Adlercreutzia equolifaciens FJC-B9T*	(33)
	Asaccharobacter celatus	(28)
	Asaccharobacter celatus AHU1763	(34)
	Bacteroides (parabacteroides) distasonis#	(35)
	Bacteroide ovatus spp.	(36)
	Bifidobacterium spp.	(37)
	Bifidobacterium spp.	(27)
	Coriobacteriaceae sp *MT1B9*	(38)
	Eggerthella sp. *YY7918*	(39)
	Enterococcus faecium EPI1	(40)
	Finegoldia magna EPI3	(41)
	Lactobacillus mucosae EPI2	(34)
	Lactobacillus sp. *Niu-O16*	(42)
	Lactobacillus rhamnosus JCM 2771	(43)
	Prevotella veroralis	(35)
	Ruminococcus productus	(44)
	Ruminococcus productus spp.	(36)
	Slackia equolifaciens DEZ	(45)
	Slackia isoflavoniconvertens HE8	(46)
	Slackia sp. *NATTS*	(47)
	Strain Julong 732	(41)
	Streptococcus intermedius spp.	(36)
	Veillonella spp. EP	(31)
	Veillonella spp. EP	(48)
5-hydroxy-equol	Coriobacteriaceae sp MT1B9	(38)
	Slackia sp HE9	(46)
ODMA	*Clostridium* spp. HGHA136	(31)
	Eubacterium ramulus	(48)
	Eubacterium ramulus wK1	(48)
	Strain SY8519	(39)
Secoisolariciresinol	*Bacteroides fragilis*	(49)
	Bacteroides (Parabacteroides) distasonis[#]	(49)
	Bacteroides ovatus	(49)
	Bifidobacterium bifidum WC 418 and WC 421	(50)
	Bifidobacterium catenulatum ATCC 27539	(50)
	Bifidobacterium longum subsp. *infantis ATCC 15697*	(50)
	Bifidobacterium longum subsp. *longum WC 436 and WC 439*	(50)
	Bifidobacterium pseudocatenulatum WC 401, WC402, WC402 and WC 407	(50)
	Butyrivibrio fibrosolvens	(51)
	Butyrivibrio proteoclasticus	(51)
	Clostridium ramosum	(50)
	Clostridium cocleatum	(49)
	Clostridium sp. *SDG-Mt85-3Db*	(49)
	Eggerthella lenta	(52)
	Prevotella albensi	(51)
	Prevotella brevis	(51)
	Prevotella breyantii	(51)
	Prevotella ruminicola	(51)
Dihydroxyenterodiol	*Butyribacterium methylotrophicum*	(49)
	Eubacterium callanderi	(49)
	Eubacterium limosum	(49)
	Peptostreptococcus productus	(49)
	Clostridiaceae bacterium END-2	(53)
Enterodiol	*Clostridium scindens*	(49)
	Eggerthella lenta	(49)
	Eubacterium sp. *ARC-1*	(54)
	Enterococcus faecalis	(55)
Enterolactone	*Strain ED-Mt61/PYG-s6*	(49)
	Eubacterium sp. *ARC-1*	(54)
	Eggerthella sp. *SDG-2*	(54)
	Enterococcus faecalis	(55)
	Ruminococcus sp. *END-1*	(54)
	ClostridiaceaebacteriumEND-2	(54)
8-prenylnaringenin	*Eubacterium limosum*	(56)

Adapted from Lopes et al. (57), Yoder et al. (58), Sánchez-Calvo et al. (59), Setchell et al. (60), Rafii et al. (61) [#]*Parabacteroides was classified as Bacteroides in old nomenclature.*

suppression of EAE disease in GF mice on fecal transfer from HC and exacerbation of disease on fecal transfer from MS patients supports a critical role of gut microbiota in MS (8, 21). Gut bacteria have been shown to influence disease through modulation of multiple metabolic pathways such as short-chain fatty acid (63–65), tryptophan (62, 66), and phytoestrogen metabolism (66). As discussed before, we and others have reported the loss of bacteria involved in phytoestrogen metabolism (62). Through metabolism of phytoestrogens, these microbes may play an important role in the regulation of inflammation; thus, we hypothesize that reduced abundance of phytoestrogen metabolizing bacteria in the gut would influence the inflammation and demyelination in RRMS (4). Therefore, in this review we will focus on the importance of phytoestrogen metabolism by the gut microbiota, as well as on the effect of this phenomenon on the host physiology. We will discuss in detail: the mechanisms whereby gut bacteria metabolize phytoestrogens into structurally and functionally distinct metabolites; the

ability of such metabolites to modulate various physiological processes, such as immune and neuronal/glial cell activity; and the ability of the metabolites to modulate disease in animal models of MS.

PHYTOESTROGEN METABOLITES IN THE GUT

Phytoestrogens can be categorized, based on their structures, as flavonoids or nonflavonoids (**Figure 1**). Flavonoids are phenolic compounds with a basic structure consisting of 3 rings (denoted A, B, and C) comprised of 15 carbon atoms arranged in two aromatic rings connected by a 3-carbon bridge (67). These rings give them structural similarity to estradiol and the ability to mimic the function of estrogen. Further subclassification such as those discussed in this review (coumestans, prenylflavonoids, and isoflavones) are distinguished according to structural differences in the connection between the B and C rings, as well as the degrees of saturation, oxidation, and hydroxylation of the C ring [**Figure 2**; (67)]. Non-flavonoids consist of phenolic acids in either C6-C1 (benzoic acid) or C6-C3 (cinnamic acid) conformations, stilbenes, and lignans, the latter being the primary class implicated in microbiota-induced influence of human health (68).

Coumestans

The primary molecule studied in the coumestan family is coumestrol. In addition to the classical flavonoid structure, coumestrol has a furan ring in the junction between the C and B rings (69) and one hydroxyl group each at the C4 and C7 carbons, similar to the structure of estradiol (57). These moieties confer the ability to bind mammalian estrogen receptors (70) and provide free radical scavenging properties (69, 71), thus suggesting that coumestans may provide protection against breast, prostate, and ovarian cancers (72–74).

In vitro treatment of MCF7:WS8 (estrogen sensitive) and MCF7:5C (estrogen deprived) breast cancer cells with coumestrol had anti-proliferative and pro-apoptotic effects, respectively, which depended on estrogen receptor alpha (ERα) signaling (75). Similar observations were made in triple-negative breast cancer cells, but through the estrogen receptor (ER)-independent Bax/Bcl-2 pathway (76). Other coumestans have not been well-characterized in relation to their estrogenic activity. Furthermore, as coumestrol has an affinity for mammalian estrogen receptors reportedly only 10–20 times lower than 17β-estradiol (77), it is one of the more potent phytoestrogen compounds. Taken together, coumestrol and potentially novel coumestans or coumestan metabolites may prove to be an interesting target for study in neurological diseases such as MS.

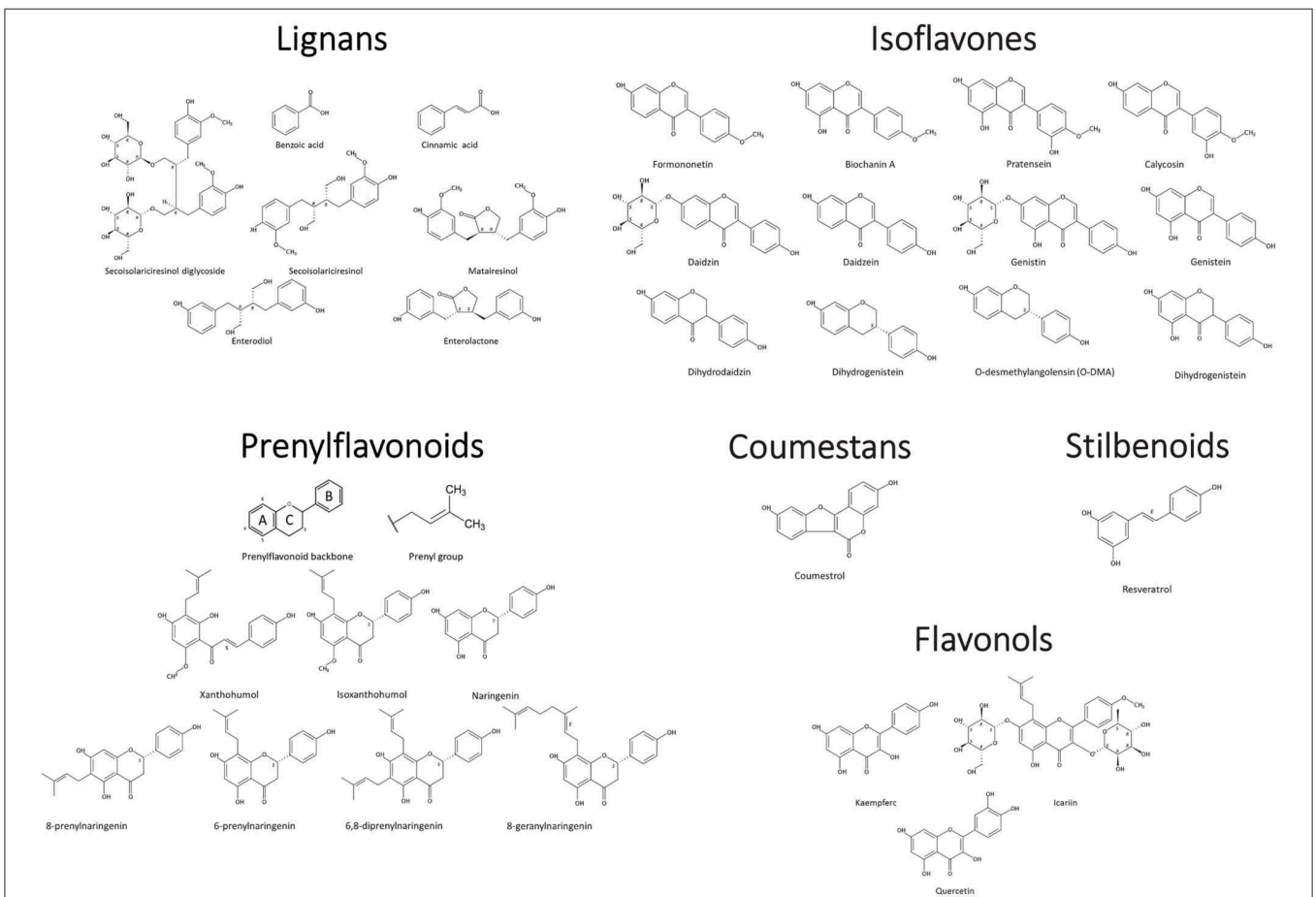

FIGURE 2 | Phytoestrogen structure and classification. Chemical Structures Copyright © 2019 American Chemical Society. All Rights Reserved.

Prenylflavonoids

Prenylflavonoids are characterized by prenylated side chains on the flavonoid backbone, generally at the 6, 8, 3, and/or 5 positions (78). Prenyl chains come in numerous forms, notably 3,3-dimethylallyl substituent, geranyl, 1,1-dimethylallyl, and is moieties (79). The most common prenylflavonoids in the human diet are xanthohumol and isoxanthohumol, both of which are found in hops, beer, and an increasing number of dietary supplements (80, 81). A systematic review of the prenylflavonoid literature in 2014 revealed an association of these molecules with a variety of biological effects (including cytotoxicity, particularly against tumor cells), as well as antibacterial effects (their primary function in the plant species in which they were originally identified) (79). Prenylflavonoids also modulate the functions of many enzymes; e.g., they inhibit the activities of cholinesterase and aldose reductase (AR), and they enhance those of alcohol- and aldehyde dehydrogenases (79). Like coumestans, they have also been shown to have anti-oxidant activity, including strong radical scavenging properties. Finally, xanthohumol metabolite 8-prenylnaringenin's (8PN) *in vitro* estrogenic activity was shown to be stronger than that of other phytoestrogens, including coumestrol, genistein, daidzein; as well as its precursors xanthohumol, isoxanthohumol, naringenin, and related compounds 6-prenylnaringenin, 6,8-diprenylnaringenin and 8-geranylnaringenin (82–85).

Numerous studies have described the processing of prenylflavonoid compounds, xanthohumol and isoxanthohumol, by human liver microsomes (81, 86–88). Nikolic et al. also characterized the processing of 8PN in human liver microsomes (80), which is thought to be more potent ligands of ER than original plant compounds. Recently, however, *Eubacterium limosum* was also shown to convert isoxanthohumol into 8PN and to demethylate some isoflavonoids (89). Furthermore, both the yeast species *Pichia membranifaciens* (ATCC 2254) and the fungal species *Cunninghamella echinulata* (NRRL 3655) were found to metabolize 8PN (90, 91). The additional metabolism by gut microbiota gives potential for more estrogenic compounds to be bioavailable. Thus, biological relevance of this metabolite may be greatly altered when considering the actions of commensal species, especially if prenylflavonoids follow similar patterns of absorption to other dietary polyphenols and 80–90% of consumed compound is potentially available to colonic bacteria (92). Furthermore, additional metabolites of 8PN produced with the help of gut bacteria, could exist and have various biological effects (80).

Lignans

Although isoflavones are the best studied phytoestrogens, lignans are significantly more prominent in the Western diet (93–95). Dietary lignans are found at high levels in seeds such as flax and sesame seeds, and more broadly in cereals, fruits, and vegetables at moderate to high levels (58, 96). These non-flavonoid compounds are distinguished by a unique coupling of phenylpropanoid units at their 8' positions. The two major dietary lignans, secoisolariciresinol (or secoisolariciresinol-diglycoside) and matairesinol, give rise to the metabolites enterodiol and enterolactone. When first discovered, these metabolites were thought to originate from the ovaries, but further study in antibiotic-treated and germ-free rats indicated that intestinal bacteria are required for the production of both enterodiol and enterolactone (97). This was later confirmed in humans as well (98). The mechanism by which gut microbiota enzymes metabolize lignans is well-established (52, 99–101). From secoisolariciresinol-diglycoside, hydrolysis of the sugar moiety takes place first, followed by subsequent dehydroxylation, and demethylation to produce enterodiol (99, 102). Enterodiol can be further oxidized to enterolactone (99, 102). Similarly, matairesinol is dehydroxylated and demethylated to form enterolactone directly (102).

Both of these lignan metabolites have far greater biological effects than their precursors. The identification of both compounds during pregnancy and the cyclic pattern of excretion in females during menstrual cycle (established in both humans and monkeys) have physiological implications possibly related to interactions with ER, though whether these are estrogenic or antiestrogenic is unclear (103). Furthermore, both compounds are inhibitors of enzymes involved in steroid metabolism such as aromatase, 5 α-reductase, and 7β-hydroxysteroid dehydrogenase (103–106). Potential anticancer activity via antiestrogenic or antioxidant activity has been long proposed and well-studied (107–112). While parent compounds certainly have some physiological effects (113–117), the metabolism by microbiota serves to greatly alter and/or enhance the function of lignans.

Isoflavones

Isoflavones are low molecular-weight compounds derived from plants with hydroxyl groups in the C4 and C7 positions, similar to coumestans discussed above, and estradiol (57). Isoflavones are among the most studied dietary phytoestrogens, and they are abundant in soybeans and soybean products, as well as in several other legumes (96, 118). Formononetin and biochanin A are processed, via either intestinal glucosidases (96) or enzymes in hepatic microsomes (118), to the more estrogenic compounds daidzein and genistein. Furthermore, daidzein, and genistein can be found directly in foods, generally in their glycoside forms bound to a sugar moiety. For gut absorption, isoflavone glycosides (e.g., daidzin and genistin), must be further processed into the aglycone form which lacks this sugar moiety (119, 120). Once hydrolyzed, they are readily absorbed and are detectable in plasma, urine, and feces (120). In rats, the aglycone forms of genistein and daidzein were detectable in plasma as soon as 3 min after an oral dose was administered (121). In a separate experiment in which glycoside forms of the same isoflavones were administered, detection was much slower because processing by intestinal β-glucosidases (expressed at highest levels in the duodenum) was required for absorption (121).

Isoflavones genistein, dihydrogenistein, and equol are proposed to bind ERβ with nearly the same, or slightly lower, affinity as 17β-estradiol, while affinity for ERα is generally weaker (122, 123). Isoflavones provide several benefits, including: antioxidant and antiangiogenic effects (57, 124–126); protection against breast cancer (127); and prevention of several menopause-related conditions (128–131). These

effects are all thought to be mediated through the activation of ERα and/or ERβ, though in many cases the exact mechanisms are unknown.

The metabolism of daidzein to equol or *O*-desmethylangolensin (*O*-DMA) is entirely dependent on one or more bacterial strains in the gut, including but not limited to *Adlercreutzia equolifaciens, Eggerthella* sp., and *Slackia isoflavoniconvertens* (33, 132) isolated from humans, A*saccharobacter calatus* and *Enterorhabdus musicola* identified in mice [**Table 2**; (133)]. These bacteria contain a specific set of enzymes, including daidzein reductase, dihydrodaidzein reductase, and tetrahydrodaidzein reductase required to metabolize daidzein into equol and/or *O*-desmethylangolensin (ODMA) (134). Biologically, it is *S*-equol (S-EQL) that is found in mammals and therefore has been the target of most research, whereas *R*-equol (R-EQL) could only be synthetically produced. However, more recently racemic mixture has been detected following synthesis by Lactococcus strain 20–92 and Eggerthella strain Julong 732 (135). The importance of *O*-DMA in human physiology is not well-understood and further research is needed to determine its significance to human health.

PHYTOESTROGENS AND PROCESSING IN THE GUT

Gut microbes are thought to play an essential, non-redundant role in the metabolism of phytoestrogens in humans. This notion is supported by the fact that both GF mice on a soy-based diet and newborn infants up to 4 months of age (both of which lack diverse microbiota) lack equol (97, 136, 137). Additionally, culturing of human fecal matter from equol-producing individuals with soy or daidzein resulted in the formation of S-EQL (138, 139), and the inclusion of antibiotics in these cultures resulted in inhibition of equol production (139). Although the importance of intestinal bacteria in S-EQL production is well-established, the bacterial enzymes required and the microbes which contain them are slowly being characterized. Further research in this area and characterization of these bacteria in diseases vs. healthy states may provide important insight into mechanisms behind negative correlations observed with numerous diseases (e.g., obesity, breast cancer) in populations which consume high amounts of soy.

The majority of S-EQL is produced by conversion of daidzein, via enzymes derived from gut bacteria. However, daidzin, which is present in plant-based foods, must first be hydrolyzed into the bioactive aglycon form, daidzein. This hydrolysis step is catalyzed by β-glucosidase in the brush border membrane of the proximal intestine (140). As conjugated forms (glucosides) cannot cross intestinal epithelial cells, hydrolysis is a critical step in the formation of bioactive isoflavone metabolites. Three enzymes are required to metabolize daidzein into *S*-EQL and ODMA: daidzein reductase (DHNR), dihydrodaidzein reductase (DHDR), and tetrahydrodaidzein reductase (THDR) (46). Similar mechanisms might be involved in the digestion of other phytoestrogenic compounds and in the production of small metabolites.

PHYTOESTROGEN RECEPTORS

The downstream effects of phytoestrogens are thought to be mediated, in part, through estrogen receptors, which are expressed widely, including in the cells of the immune and nervous systems (141, 142). Phytoestrogens and their metabolites can interact with the prototypic estrogen receptors, ERα and ERβ, effecting changes in cell physiology through modulation of transcription and gene expression (**Table 3**). Alternatively, phytoestrogen can also signal through the G-protein coupled estrogen receptor (GPER), which allows for more rapid and dynamic regulation of cell processes because the mechanisms are predominantly non-genomic (143–145). However, the majority of research in phytoestrogen signaling has focused on signaling through ER receptors and their ability to activate ER receptors compared to the natural ligand 17β- estradiol. Individual phytoestrogen metabolites have been proposed to have higher affinity for one ER over the other. For example, genistein and daidzein have significantly higher affinity for ERβ than for ERα (141). As ERα is the predominant estrogen receptor on the cells of the immune system (142) either the signaling pathways used by internal estrogens and phytoestrogens/phytoestrogen metabolites differ slightly; or ER-independent pathways such as GPER signaling might play a significant role in phytoestrogen-mediated modulation of immune cells. This may mean that greater shifts in phytoestrogen availability are needed to potentiate a change in signaling; however, additional research is needed to better understand the mechanisms of action of phytoestrogens and their metabolites in regard to receptor binding and the signaling pathways required for their biological activities, especially in the context of cells of immune and nervous systems.

PHYTOESTROGENS AND NEURONS

After phytoestrogens are metabolized in the gut and transported to the liver, they may have systemic effects (52). With regard to the CNS, an organ system in which ERs are widely expressed, phytoestrogen metabolites have been found to have direct neuroprotective effects, based on both *in vitro* and

TABLE 3 | Estrogen Receptor Expression on Immune Cells.

Cell Type	Human		Mouse			
	ERα	ERβ	ERα	ERβ	GPER	Reference
CD4+ T-cell	Yes	Yes	Yes		Yes	(142, 143)
CD8+ T-cell	Yes	Yes			Yes	(142, 143)
B-cell	Yes	Yes	Yes	Yes	Yes	(142, 143)
NK Cell	Yes	Yes	Yes	Yes		(142)
Macrophages			Yes	No	Yes	(142, 143)
Monocyte-derived DC	Yes	Yes				(142)
Bone marrow-derived DC			Yes	Yes		(142)
Splenic DC			Yes	No		(142)
Plasmacytoid DC	Yes	Yes	Yes			(142)
CNS inflammatory DC				Yes		(142)

in vivo studies (in animal models). Phytoestrogens can exert neuroprotective effects by attenuating toxic insults to neurons. For example, several studies showed that toxin-induced plasma-membrane damage was reduced in neurons treated *in vitro* with genistein and daidzein (146). In another study, such treatment resulted in neural-cell proliferation and improved cell viability (147). Furthermore, quercetin and kaempferol (phytoestrogen flavonoids) prevented neuronal cell death in the context of oxidative stress (148). Phytoestrogens can also exert neuroprotective effects by attenuating microglial mediated inflammatory responses; one study found that formononetin, daidzein, pratensein, calycosin, and irilone attenuated LPS-induced proinflammatory cytokine production by microglia (149).

Several *in vivo* studies have documented neuroprotective effects of phytoestrogens in the diet. The synaptic density was much greater in rats fed either a daidzein- plus genistein-based diet or a soy-enriched diet, compared to rats on either a standard chow or diet lacking soy (150). Furthermore, rats fed a diet containing soy-derived phytoestrogens exhibited improved learning and memory compared to rats on a control diet (151). Additionally, pretreatment with phytoestrogens protected mice from neurotoxicity in the CNS following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's Disease (PD) (152). These studies suggest that phytoestrogens might alter the structure and/or the function of both healthy and diseased neurons.

The mechanism underlying phytoestrogen-induced neuroprotective effects might be related to estrogen receptor agonistic activity. Estrogen replacement therapy (ERT) has been shown to improve CNS function, especially in Alzheimer's disease, by preventing oxidative stress and the formation of amyloid plaques (153). Indeed, genistein, daidzein, and zearalenone stimulate ERα- and ERβ-dependent transcription of genes that contain estrogen response elements (EREs) in their promoters (154). However, phytoestrogens have been shown to be 100–1,000 × less potent than 17β-estradiol, but they can also target GPR-30, a GPER on the plasma membrane of number of cells in a variety of tissues (155). However, further research is needed to determine the significance of the GPER receptor in phytoestrogen mediated signaling.

PHYTOESTROGENS AND IMMUNE CELLS

Phytoestrogens have varied effects on immune system function which have been summarized in **Table 4**. These effects are most often anti-inflammatory and protective in nature. In the case of the adaptive immune system, studies have shown that genistein and other isoflavones can suppress lymphocyte proliferation, allergic responses, and antigen-specific immune responses in both T- and B-cells (156, 158, 162–165). However, genistein has also been shown to enhance both the cytotoxic activity of CD8 T-cells, and the production of cytokines by T-cells more generally (156–161). Notably, this largely mirrors the effects of estrogens on these cells. One study by Kojima et al. showed that various phytoestrogens enhance gene expression mediated by retinoic-acid-receptor-related orphan receptor (ROR) γ and α in T-lymphoma cells, leading to increased expression of IL-17. Others have shown that when activated T-cells are treated with formononetin, daidzein, or equol, the levels of IL-4 expression increase (159). Collectively these studies suggest that phytoestrogens can interact with the T-cell compartment to induce various responses that may improve disease outcomes. Although the mechanisms leading to these actions are not well-understood, it has been suggested that either enhancement or inhibition of the NF-kB pathway could contribute, especially to cytokine responses.

Interactions of the B-cell compartment with phytoestrogens are less well-characterized, but limited studies have reported that phytoestrogens can induce an anti-inflammatory, anti-allergic phenotype that could be beneficial for the host. Specifically, multiple studies have shown that isoflavones and coumestrol can lower serum titers of immunoglobulin G2a (IgG2a) antibodies (162–165). One such study also showed that low-dose coumestrol can decrease the titers of antigen-specific IgG1 and IgG3 during experimental autoimmune thyroiditis (165). Further isoflavones can suppress the expression of IgE, possibly thereby contributing to the overall anti-allergic phenotype that has been reported in response to phytoestrogen treatment in several animal models, including but not limited to airway allergy and peanut-sensitization models (162).

Phytoestrogens have also been shown to modulate the innate immune system and the majority of studies suggest an anti-inflammatory role in this context. Combination of isoflavones genistein and daidzein alone, or these plus glycitein have been shown to inhibit the ability of dendritic cells (DCs) to induce the production of IFN-γ, TNF-α, IL-9, and IL-13 from CD4+ T-cells (162, 163). These phytoestrogens have also been shown to inhibit direct cytokine secretion from activated DCs (163). Phytoestrogens also suppress DC maturation and the expression of MHCI, but not MHCII, in an intra-nasal allergic response model. These data suggest that phytoestrogens might slow the inflammatory immune response by inhibiting the antigen-presentation and effector-cell priming functions of DCs (162, 163). Genistein and daidzein, in particular, can suppress allergic inflammation by significantly reducing (by 25–30%) mast cell degranulation (162, 164). However, treatment of activated DCs with genistein or daidzein led to increased NK-cell degranulation and cytotoxicity, outcomes that have not been studied in a disease (163). Phytoestrogens can also modulate NK cell activity by specifically reducing expression of IL-18 receptor α (IL-18Rα), and inhibiting IFN-γ production in response to IL-12 and IL-18 (167). These actions of phytoestrogens have not been found to reduce NK cell cytotoxicity (156, 157, 163, 166). Thus, it remains unclear why phytoestrogens might have anti-inflammatory functions in DC populations, but potentially mixed effects in NK cell populations.

In macrophages, phytoestrogens have been shown to induce overall anti-inflammatory responses. Dia et al. showed that genistein and daidzein can decrease the production of nitric oxide and the expression of iNOS (inducible nitric oxide synthase), as well as inducing the activities of super oxide dismutase and catalase (168). Another study found that genistein treatment can skew macrophage polarization toward an M2, anti-inflammatory

TABLE 4 | Summary of effects of phytoestrogen compounds on various cell types.

Cell Type	Results	Compound	Model	References
T-cell	Enhanced CD8+ T-cell cytotoxicity	Genistein	Genistein treatment of mice with B16 melanoma	(156, 157)
	Increased cytokine expression and production	Genistein, formononetin, daidzein, equol	*Ex vivo* stimulation of cells from treated mice; *In vitro* gene expression studies	(158–161)
	Enhanced RORγ and RORα expression	Genistein, formononetin, daidzein, biochanin A	*In vitro* gene expression in Jurkat and CHO-K1 cells	(160)
	Suppressed proliferation	Genistein	*Ex vivo* stimulation of cells from genistein treated mice	(156, 158)
	Suppressed antigen-specific responses	Genistein	*In vivo* in immunized/sensitized mice	(158, 162–164)
B-cell	Decreased antigen specific IgE, IgG2a, IgG3, and IgG1	Isoflavones, coumestrol	*In vivo* in peanut antigen sensitization model and experimental autoimmune thryoiditis	(162–165)
NK cell	Enhanced cytotoxicity	Genistein	*Ex vivo* stimulation of NK cells from treated mice or rats	(156, 157, 163, 166)
	Increased degranulation	Genistein, daidzein	*In vitro* co-culture with treated, activated DCs	(163)
	Reduced IL-18Rα expression	Genistein	*In vitro* human NK cell treatment/analysis	(167)
	Reduced IL-12/IL-18 dependent IFN-γ production	Genistein, daidzein, equol	*In vitro* pretreatment of cells, *in vivo* plasma cytokine measurement after treatment of mice	(167)
Macrophage	Decreased nitric oxide production	Genistein, daidzein	*In vitro* treatment of LPS-activated RAW 264.7 macrophages	(164, 168)
	Decreased iNOS expression	Genistein, diadzein	*In vitro* treatment of LPS-activated RAW 264.7 macrophages	(164, 168)
	Increased superoxide dismutase and catalase production	Genistein, daidzein	*In vitro* treatment of LPS-activated RAW 264.7 macrophages	(164, 169)
	Increased M2 polarization	Genistein	*In vivo* counting in various tissue after DSS-induced colitis and treatment in mice	(161)
	Increased ARG-1 and IL-10 expression in M2 macrophages	Genistein	*In vivo* characterization of mouse splenic M2 macrophages after DSS-induced colitis and treatment	(161)
Dendritic Cell	Inhibited cytokine secretion	Genistein, daidzein	*In vitro* treated LPS-activated monocyte-derived DCs	(163)
	Decreased TLR-dependent maturation marker expression	Genistein, daidzein	*In vitro* treated LPS-activated monocyte-derived DCs	(163)
	Decreased MHC class 1 expression	Genistein, daidzein	*In vitro* treated LPS-activated monocyte-derived DCs	(163)
	Inhibited CD4+ T-cell priming	Genistein, daidzein	*In vitro* co-culture of treated, activated monocyte derived DCs and naïve CD4 T-cells	(163)
	Increased ability to activate NK cells	Genistein, daidzein	*In vitro* co-cultures of treated, activated monocyte-derived DCs and autologous NK cells	(163)
Other granulocytes	Inhibited mast cell degranulation	Genistein, daidzein		(162, 164)

phenotype, while also reducing systemic concentrations of inflammatory cytokines (161). The same study also found that macrophages induced to take on the M2 phenotype when treated with genistein express ARG-1 and IL-10 at higher levels than those induced to take on this phenotype by other agents (161). Thus, genistein appears to push macrophages toward an actively anti-inflammatory phenotype, i.e., its actions are not solely non-inflammatory. The collective activities of the phytoestrogens in regard to the innate immune compartment may explain some of the systemic anti-inflammatory effects of phytoestrogens that have been described in the literature (e.g., decreased allergic responses and decreased autoreactive immune responses).

PHYTOESTROGENS AND THE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

EAE is a very well-studied model of MS in which myelin antigen in combination with pertussis toxin and complete Freud's adjuvant is used to induce an autoimmune response. It is characterized by spinal cord pathology, manifesting in ascending paralysis that can be scored on a standard 6 point scale (0–5) (170). Several groups have documented the therapeutic and disease-preventative potential of phytoestrogens using the murine EAE model of MS. These studies indicate that common phytoestrogens, especially isoflavones genistein and daidzein, have potential as therapeutics for autoimmunity affecting CNS components. One group showed that sub-cutaneous (s.c.) treatment with genistein post-EAE induction resulted in significantly ameliorated EAE (171). The genistein treatment group showed reduced production of pro-inflammatory cytokines (including TNFα, IFNγ, and IL-12p40) by splenocytes and/or CNS lymphocytes (171). Similarly, 7-O-tetradecanoyl (TDG), a lipophilic genistein analog, suppressed disease when administered s.c. 14-days post-EAE induction. In these studies, disease amelioration correlated with a decrease in the number of IL-17 producing CD4+ T cells, and an increase in the number of FoxP3+CD4+ T cells, in the brain (172). Another study reported that daily oral treatment with high-dose daidzein

starting 10 days after EAE induction ameliorated disease and simultaneously reduced IFNγ levels in the brain and splenocytes compared to controls with induced disease (173). These studies suggest that phytoestrogenic compounds or their analogs might have therapeutic potential in an animal model of MS.

Besides isoflavones, other phytoestrogens have also been shown to exert a protective effect in EAE. Wei et al. showed that therapeutic oral administration of high-dose Icariin (ICA), a phytoestrogen from flowering plants of the Epimedium genus, ameliorated EAE (comparison was to vehicle control). The effectiveness of ICA was similar to that of estrogen, and high-dose ICA or estrogen treatment increased expression of both ERα and ERβ in the white matter of the CNS (174). In a separate study, these researchers reported that therapeutic oral administration of high-dose ICA in combination with methylprednisolone (MP), a corticosteroid used therapeutically in MS, had a greater disease-ameliorating effect than either treatment alone. This combination treatment (ICA+MP) also had a synergistic effect, enhancing both the reduction of serum IL-17 and apoptotic cell death (Annexin V^+ cells) in the spinal cord (175). Taken together, these studies show the therapeutic potential of phytoestrogen compounds, both alone and in combination therapies, as a promising complementary and alternative therapy for further study.

Quercetin is a phytoestrogen flavonoid that is abundant in soybeans, vegetables, and fruits and has also been evaluated for its efficacy in the EAE model. Quercetin protects against EAE when injected intra-peritoneally (i.p.; comparison was to a vehicle control). Additionally, quercetin caused a dose dependent suppression of antigen specific proliferation and IL-12 in splenocytes in *ex-vivo* antigen-recall response (176). Thus, quercetin might be able to influence encephalitogenic T cells directly. Resveratrol, a phytoestrogen found in the skins of red grapes and berries, had been shown to ameliorate EAE by interfering with the miR-124/sphingosine kinase 1 (SK1) axis in encephalitogenic T cells, thereby resulting in cell-cycle arrest and apoptosis (177). These studies clearly indicate that phytoestrogen compounds protect against EAE and may have implications/therapeutic potential in MS as well.

However, several knowledge gaps remain to be addressed. For example, most of the studies described above introduced phytoestrogen compounds via a non-physiological route (s.c. or i.p.). Given that humans obtain phytoestrogens through diet, study of the effectiveness of oral delivery or consumption of these compounds would better reflect the mechanisms involved in a more physiological context. Furthermore, the studies using s.c. or i.p. routes of administration do not account for the importance of phytoestrogen-metabolizing gut bacteria, which humans rely on for proper breakdown of dietary phytoestrogens. This may explain why the studies that did provide oral phytoestrogens required a very high dose for protection.

Although the exact mechanism through which phytoestrogenic compounds suppress EAE is unknown, studies have suggested that their neuroprotective and immunomodulatory effects might play an important role in their ability to suppress disease as described above.

CONCLUDING REMARKS

In RRMS patients, the presence of gut dysbiosis and the depletion of bacteria with the ability to metabolize phytoestrogens highlights the importance of these compounds in maintaining a disease free-state of the host. As stated above, various phytoestrogen metabolites play important roles in a number of biological processes including neuroprotection and regulation of the immune system. However, further research is certainly needed to better understand the pathways through which gut bacteria induced phytoestrogens metabolites regulate the balance between pro- and anti-inflammatory responses and provide neuroprotection. For example, future studies determining the relationship between levels of phytoestrogen metabolites and the severity of RRMS disease are expected to shed light on the extent to which phytoestrogen metabolism correlates with the etiopathogenesis of RRMS. Also, dissection of the role of phytoestrogen metabolism in the development and regulation of the immune system in germ-free mice is expected to reveal the significance of specific phytoestrogen metabolites in regulating the function of various immune subsets. In the meantime, however, the existing literature provides a solid rationale for the selection and testing of the therapeutic potential of various phytoestrogen metabolizing bacteria, including *Prevotella, Parabacteroides, and Adlercreutzia* in a preclinical model of MS. A successful outcome from these studies will help in development of bacteria as drug (BRUG) based treatment options for MS patients.

AUTHOR CONTRIBUTIONS

AM conceptualized the review, helped with overall structure of the manuscript, and gave final approval of the manuscript to be published. NC wrote the phytoestrogen metabolism section of the manuscript. SF wrote role of phytoestrogen in EAE and nervous system section. SP helped with writing the section on phytoestrogen receptors and effect of phytoestrogen on immune cells. All authors commented on the manuscript.

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REVIEW
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Therapeutic Plasticity of Neural Stem Cells

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Neural stem cells (NSCs) have garnered significant scientific and commercial interest in the last 15 years. Given their plasticity, defined as the ability to develop into different phenotypes inside and outside of the nervous system, with a capacity of almost unlimited self-renewal, of releasing trophic and immunomodulatory factors, and of exploiting temporal and spatial dynamics, NSCs have been proposed for (i) neurotoxicity testing; (ii) cellular therapies to treat CNS diseases; (iii) neural tissue engineering and repair; (iv) drug target validation and testing; (v) personalized medicine. Moreover, given the growing interest in developing cell-based therapies to target neurodegenerative diseases, recent progress in developing NSCs from human-induced pluripotent stem cells has produced an analog of endogenous NSCs. Herein, we will review the current understanding on emerging conceptual and technological topics in the neural stem cell field, such as deep characterization of the human compartment, single-cell spatial-temporal dynamics, reprogramming from somatic cells, and NSC manipulation and monitoring. Together, these aspects contribute to further disentangling NSC plasticity to better exploit the potential of those cells, which, in the future, might offer new strategies for brain therapies.

Keywords: neural stem cell, transplant, repair, plasticity, cell engineering

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INTRODUCTION

The concept of the stem cell niche was officially used for the first time by Schofield (1) in 1978 to define local environments with specific molecular and cellular characteristics that are required for the maintenance of hematopoietic stem cells. Ten years previously, Smart (2) and Altman (3) identified tissue in the brain that was thought to be capable of self renewal, namely two specific regions with proliferative capacity one localized in the subventricular zone (SVZ) of the lateral ventricle and one in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. The assay to test *in vitro* neural stem/progenitor cell (NPC)-self-renewal and multi-potency consisted of assessing their ability to give rise to neurospheres (4). *In vivo,* in mice, their self-renewal capacity was proved using targeted ablation of dividing GFAP-positive cells and by genetic lineage tracing (5, 6). Similarly to the hematopoietic niche and obviously in addition to the intrinsic and specific characteristics of neural stem cells (i.e., their ability to originate neuro-glia cells), the fate of NPCs and their (lifelong) self-renewal and differentiation capacity are tightly regulated by complex interactions between intrinsic and extrinsic signals provided by surrounding cells in the niche and by distant sources (7). The microenvironment of the neurogenic niche includes multiple cell populations whose interplay, including that between stem cells themselves, is still largely unknown and under active exploration. Moreover, physical activity, stress, environmental enrichment, aging, and intrinsic factors, such as cytokines, growth factors, hormones, or neurotrophins, finely regulate the fate of neural stem cells.

These features are shared with all other stem niches, such as the originally identified hematopoietic niche (8). A thorough characterization of other niche components has recently been provided in Andreotti et al. (9) and in Bacigaluppi et al. (10).

Understanding the potential of endogenous or administered NPCs as well as the cross-talk between neural stem cells and their niche components is essential for identifying what can be modulated and how for the development of therapies against neurological disorders in which neural stem cell function is altered or in which its improvement might be of help.

In this review, we would like to focus on the intrinsic and comprehensive added value of neural stem cell plasticity. NSC plasticity indeed is *per se* fundamental for development but represents an important asset in a therapeutic perspective since the neurogenic niche remains an exception in the "static" brain and represents a possible unique source of new neurons useful for substantially incurable neurological disorders and brain aging problems which are a heavy social and economic burden.

We will first frame NSCs in the stem cell context and then illustrate their plasticity in a developmental perspective, summarizing the current understanding of NSC modes of division and their mechanisms of persistence in the adult. We will compare NSCs in the two neurogenic regions of the adult mammalian mouse and human brain and discuss recent controversies on neurogenesis in the adult human brain. Last, we will discuss the current therapeutic exploitation of NSC plasticity along with the technological advancements that are being implemented, to conclude with the pros and cons, the benefits and hurdles, linked to taking advantage of these assets.

STEM CELLS

Stem cells (SC) are unspecialized, immature cells with self-renewing capacity, namely the ability to produce nearly identical copies of themselves for a long period of time without differentiating and with the possibility to differentiate into various cell lineages (11).

Totipotent stem cells, such as zygote cells and the first few cells from their division, can differentiate into all possible cell types. Pluripotent stem cells can instead differentiate into cells of the three embryonic layers, i.e., mesoderm, endoderm, and ectoderm, and can give rise to tissue and organ specialized cells. Multipotent stem cells, such as adult hematopoietic or neural stem cells, can differentiate into closely related families of cells to renew tissue-specific cell populations in organs, such as liver, intestinal tract, and skin. Exceptionally, this does not occur by default for the brain. Last, unipotent stem cells can differentiate only into a single cell type, usually of a single specialized tissue or organ.

SCs can also be classified according to their source of origin. Embryonic Stem Cells (ESCs) are totipotent, derive from the inner cell mass of human blastocysts, and can potentially proliferate indefinitely, giving rise to all types of cells in the human body. Adult Stem Cells are undifferentiated, totipotent, or multipotent cells able to replenish dying cells and to regenerate damaged tissues (if possible). Induced Pluripotent Stem Cells (iPSCs), recently developed by genetic reprogramming of adult, non-pluripotent somatic cells, are comparable to human ES cells, having differentiation potential *in vitro* and a capability to generate *in vivo* teratomas. iPSCs can be generated by over-expression through retro- or lenti-viral vector transduction of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 [c-Myc is dispensable (12)]. These cells express human ES markers (such as OCT3/4, SOX2, and NANOG) at the same or higher level than ESCs and stain positive for markers of the three germ layers, confirming their pluripotency and differentiation potential (13). They can also be generated using small molecules that mimic the effect of transcription factors (14) or by miRNAs (15). Last, Cancer Stem Cells emerge from malignant transformation of adult stem cells or from somatic cells that acquire self-renewing potential. They have been proposed as the source of tumors and of metastases and have been isolated from various tissue types (16).

Stem cells gained value in the last 15 years for the development of cell-based therapies for many serious diseases and injuries. For, example, hematopoietic stem cell transplants became established therapeutics for leukemia and for burns and corneal disorders (11). For complex neurological diseases, unfortunately not all stem cells can be exploited. In principle, ESCs would be perfect for cell replacement therapy because they can proliferate indefinitely (17), but there is also a risk of tumor formation and immune rejection along with ethical, religious, and philosophical problems. To reduce the tumor-forming potential, human ESCs could be pre-differentiated *in vitro* in committed precursor cells or neural precursor cells (NPCs) (18), which maintain self-renewal capacity and at the same time are restricted to generate only neural cells (neurons and glia) *in vivo* upon transplant, but these still raise ethical concerns. ESCs might, in principle, be directed to differentiate into specialized neuronal subtypes (19) to further reduce the risk of tumorigenicity. But more than 200 distinct neuronal subtypes with regional specificity exist, and the applicability of transplanted differentiated cells is still far from realization.

An alternative strategy is to use neural stem/precursor cells from aborted human fetuses at the gestational age of 6–20 weeks. They can be maintained, expanded, and split without losing their self-renewing and neurogenic capacity for a long period of time *in vitro*. Their main drawback, however, is the limited availability and the unpredictability of when, where, or in what conditions the material will be obtained.

It is also possible to obtain NPCs from reprogrammed somatic cells, hiPSCs, differentiated to generate NPCs with very high neurogenic potential and virtually devoid of tumorigenicity if intracerebrally transplanted (20). Moreover, iPSC-derived NSCs, unlike adult fetal NPCs, which cannot be used as an autologous cell source, offer the possibility of autologous transplantation.

Further, NPCs could also be generated via transdifferentiation (iNPCs) of a cell type into another not following the "normal" re-programmed differentiation path, because transdifferentiated cells do not become pluripotent at any time (21–26). iNPCs, similarly to NPC-derived iPSCs, are useful for transplantation therapy, for establishing disease models, and for drug screening. In principle, they hold a low risk of tumorigenesis, maintain

the capacity of self-renewal, and give rise to multiple neuronal subtypes *in vitro* and *in vivo*. Indeed, specifically for the *in vivo* applicability, murine iNPCs transplanted into healthy adult mouse brain survived for 6 months without overgrowths, achieved functional integration (27), and could differentiate into neuronal cells, although they retained a mixed neuro-glia phenotype (M2+ and GFAP+) (28). In the context of spinal cord injury, iNPCs generated by transfection with four reprogramming factors and transplanted in rat spinal cord, differentiate into all neuronal lineages (29). Direct cell conversion has also been tested *in vivo* by transplanting human fibroblasts and human astrocytes engineered to express inducible neural reprogramming genes that converted fibroblasts and astrocyte cells into neurons directly in the adult rodent brain (30).

This field is still in its infancy, and before considering the development of personalized regenerative therapies with iNPCs (31), further investigation is required to better understand the detailed mechanisms occurring in the transdifferentiation processes to improve the efficiency and the maturation into desirable cells with neurotransmitter and region-specific phenotypes.

Lastly, glial-restricted progenitor cells (GRPs) represent another therapeutic alternative. They are self-renewing cells derived from CNS tissue of 19–22 gestational weeks that have a limited differentiation potential and are able to give rise to oligodendrocytes and astrocytes but not neurons, as assessed in the demyelinated shiver mouse model (32) and in transverse myelitis, an inflammatory condition of the spinal cord that leads to demyelination (33). GRPs have also been proposed for multiple sclerosis (MS) because endogenous OPCs in the lesions initially engage in remyelination (34), but with time, the number of OPCs declines and remyelination becomes inefficient (35). Isolation and expansion of GRPs were recently implemented by Q Company (36), which started a phase I clinical trial (37).

NEURAL STEM CELLS IN THE MAMMALIAN BRAIN: FETAL VS. ADULT COMPARTMENT, MOUSE VS. HUMAN

A detailed characterization of the neural niches for both mouse and human is now available (38). In the mouse, the central nervous system (CNS) originates at E7.5–E8 with the neural plate that folds into the neural tube and then divides along the rostro-caudal axis into the rostral forebrain, midbrain, and hindbrain vesicles, while the caudal vesicle gives rise to the spinal cord. The cortical layer, adjacent to the lateral ventricles (LV) and known as the ventricular zone (VZ) is made of highly proliferating progenitors with apical basal polarity (neuroepithelial cells, NECs) (38) that, before neurogenesis (E10.5–E12.5), undergo extensive symmetric divisions to expand (**Figures 1**, **2**). When neurogenesis starts (E12.5 onwards), NECs become radial glial cells (RGCs), express glial markers, assume an elongated morphology, and divide asymmetrically, originating one RGC and one neuron or one RGC and one intermediate progenitor (IP, Tbr2+) (43). IPs themselves migrate radially to give rise to two pyramidal neurons that establish connection and form synapses (44). The CNS builds up in ∼1 week during gestation, and NPCs (RGC/IPs) are retained in the two distinct and small proliferative areas of the SVZ and the SGZ (44, 45). During neurogenesis, first-born neurons populate the deeper cortical layers (V–VI), while later-born neurons progressively populate the more superficial layers (II–IV). These layers contain neuronal subtypes that are different in morphology, electrophysiological activity, axonal connectivity, and gene expression. During embryonic and late neurogenesis, RGCs, because of their elongated radial morphology, sense extrinsic cues from meninges, vasculature, newborn neurons, and cerebrospinal fluid, which regulate their cell fate decision. During late embryonic development and the first weeks after birth, radial glia also differentiate into astrocytes and oligodendrocytes, which populate the different brain structures, and ependymal cells will line on the ventricle surface. Thus, adult SVZ NSCs are regionally specified during the early embryonic stage and remain largely quiescent until, post-natally, they are re-activated (46, 47); they have intrinsic temporal programs linked to their positional characteristics (dorsal-ventral, rostral-caudal) (48) that guide differentiation in a cell-autonomous manner and cycle independently, but they also sense extrinsic cues that tune temporal programs and help indicate the 'right' time to progress (49, 50).

The functional integrity and behavior of the niche are maintained by the extracellular membranes (ECMs) (51) of both the basal and apical sides. They are rich in laminin, αβ integrin glycoproteins, and tenascin C. Similarly, a fundamental functional role for the VZ/SVZ is played by the CSF and by the blood vessels that form in early stages of CNS development (E9) (52, 53). Of note, neurogenesis and angiogenesis are regulated by the same molecules, such as vascular endothelial growth factor (VEGF), Notch, and Shh (54).

In humans, similarly to rodents, during early brain development, the inner part of the neural tube that then becomes the cerebrospinal fluid (CSF)-filled ventricular structure consists of a layer of proliferative cells that originally contributes to the expansion of the cerebral cortex along with their descendant radial glia (GFAP+) and their intermediate progenitor cells (**Figures 3**, **4**). Radial glia bodies are in tight contact with the monolayer of ependymal cells that covers the ventricles (57), which serves both as barrier and transport system between the interstitial fluid of the parenchyma and the CSF (58, 59). Studying the behavior of human NPCs is difficult; thus, to evaluate their properties, cells from 6.5- to 9-weeks-old aborted embryonic human forebrains were expanded in culture for up to 21 passages and were transplanted into the dentate gyrus, the rostral migratory stream (RMS), the striatum, or the SVZ of adult immunosuppressed rats. Migration was modest in the dentate gyrus or in the striatum if compared to rodent-in-rodent transplant and was considered a random dispersion process during the implantation. Larger migration was observed if transplant was into the SVZ or RMS. Cells did not show tumor formation 6 weeks post-transplantation and interestingly exclusively adopted a neuronal fate when in the olfactory bulb or in the SGZ of the hippocampus (60). It is worth mentioning also that both fetal/embryonic rodent and human NPCs display regional differences in terms of proliferation and

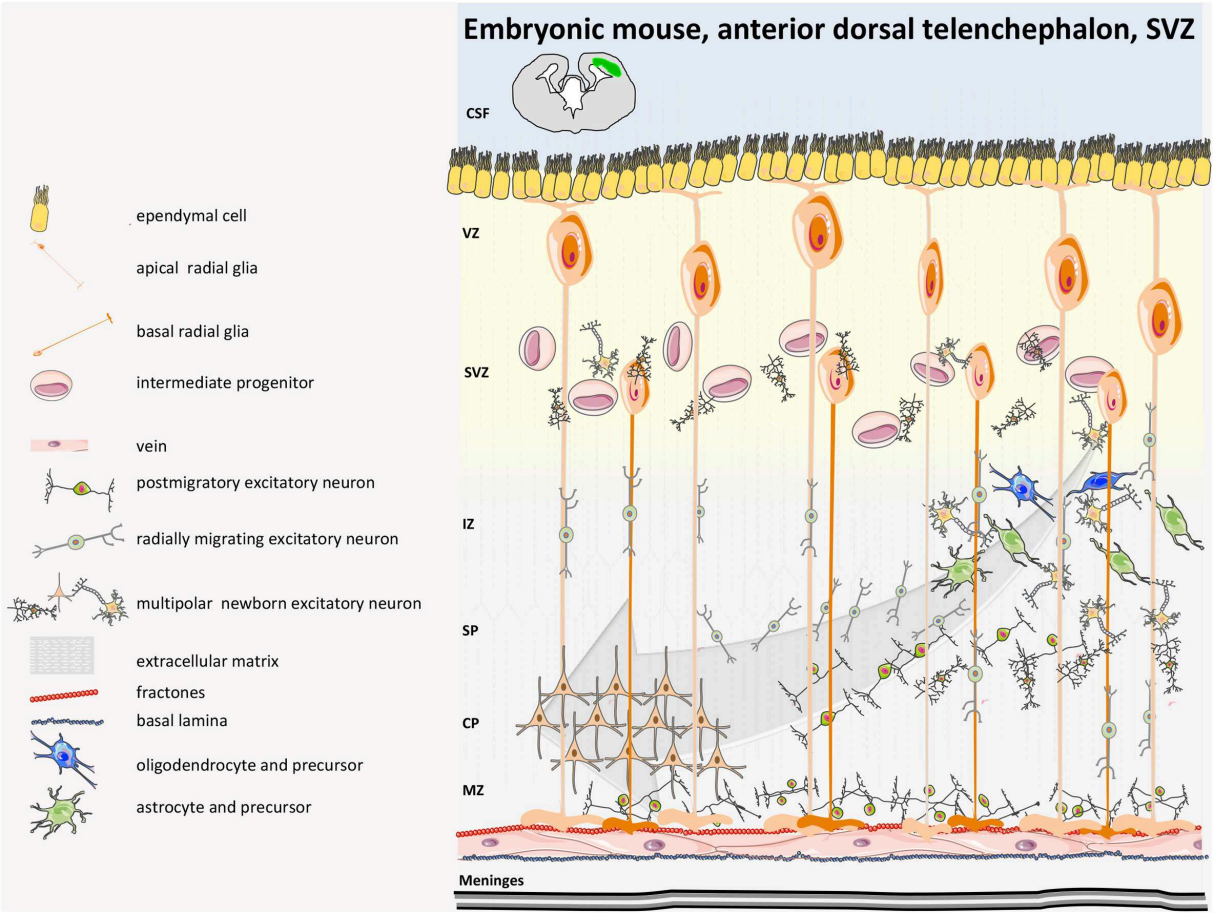

FIGURE 1 | Mouse developmental SVZ structure. Neurogenesis in humans begins with the expansion of the neuroepithelium and apical radial glia (aRG). Excitatory neurons are directly generated from apical radial glia (aRG) in the dorsal VZ or are derived from multipolar basal intermediate progenitors (bIPs) that have delaminated from the apical and basal surface and reside in the SVZ. At early stages of neurogenesis in mice, newborn deep-layer excitatory neurons move basally toward the marginal zone (MZ) by somal translocation. Once the developing cortex becomes thicker, newborn neurons reach the intermediate zone (IZ), where they undergo a multipolar-to-bipolar transition and pass through the IZ and CP. Neurons then migrate basally toward the pia, passing by earlier-born neurons; they then terminate their migration in the MZ. Inhibitory GABAergic interneurons are specified in the distant medial and caudal ganglionic eminences, where RGs, intermediate progenitors (IPs), and numerous subapical progenitors (SAPs) proliferate and migrate tangentially in two streams to integrate into the various cortical layers of the cerebral cortex (not depicted in the figure). CSF, cerebrospinal fluid; SP, subplate (39–41).

differentiation potential according to the region of the brain where they originate (cortex or striatum) (61, 62).

In comparison to the spatially and temporally regulated niches of the developing brain, in the post-natal and adult rodent brain, neurogenesis occurs and neural stem cells (NSCs) persist in the ventricular-subventricular zone (V-SVZ) of the lateral ventricle and in the SGZ of the dentate gyrus in the hippocampus.

Regarding the SVZ (**Figure 5**), the population of adult NSCs is quite complex and heterogeneous, as demonstrated by single-cell sequencing data (67) and by marker-specific analysis (GFAP, EGFR, CD133, Nestin, CD9, CD81, CD24, and VEGF). NSCs of embryonic origin are called B1 cells (68), and there are roughly 7,000 in each young lateral wall of lateral ventricle. Most of the B1 cells generated between days E13.5 and E15.5 remain almost quiescent until soon after birth, when they become reactivated and start proliferating (46) or dividing very slowly (63, 69). NSCs divide symmetrically to self-renew or to differentiate, which leads to a decline in NSC number over time (69). B1 that face the ventricle side give rise to B2 cells, a population of fusiform-stellate proliferating V-SVZ astrocytes, that are non-neurogenic and whose function is still unknown (70). They share many astroglial characteristics with B1 cells, including contacts with blood vessels (BV), but lack contact with the apical membrane. B1 cells also generate transient-amplifying cells (type C cells) that divide symmetrically three to four times (71) and ultimately give rise to migrating neuroblasts that become young neurons (type A cells) (72). In young adult mice, B1 cells produce around 10,000 young interneurons every day that migrate for 3–8 mm along the rostral migratory stream to the olfactory bulb (73). Ventral NSCs produce deep granule cells and calbindin-positive periglomerular cells, while dorsal NSCs produce superficial granule cells and tyrosine hydroxylase-expressing periglomerular cells (74). They

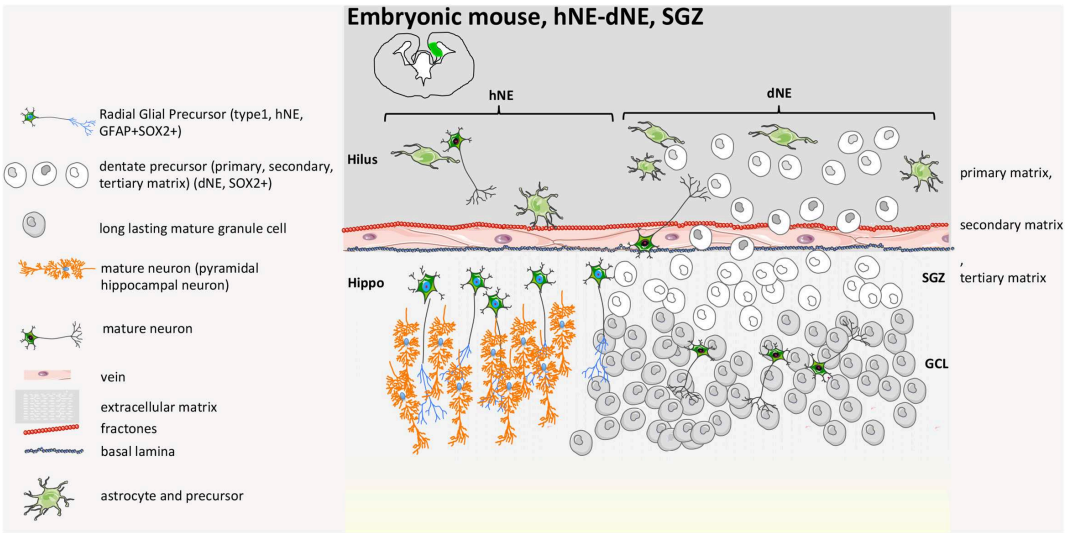

FIGURE 2 | Mouse developmental SGZ structure. At E14.5, in the VZ of the hippocampal neural epithelium (hNE), radial glial precursors give rise to hippocampal pyramidal neurons. The DG originates from the dentate neuroepithelium (dNE), called the primary matrix, a part of the ventricular zone (VZ). At late gestational stages, a heterogeneous mixture of stem cells and neuronal precursors at different stages of differentiation migrate from the VZ to the hippocampal fissure, constituting a new migratory progenitor population called the secondary matrix. The process is guided by hem-derived Cajal-Retzius cells. Neural progenitors reach the hippocampal fissure, where they accumulate and form a hub of proliferating cells called the tertiary matrix (SGZ). Granule cells generated during DG development from precursors of all three matrices form the GCL. By early post-natal stages, the tertiary matrix becomes the only source of dentate progenitors and granule cells (39, 40, 42).

integrate into the existing olfactory bulb network and influence the plasticity of olfactory-related behaviors (75). B1 cells on the apical ends, which are completely surrounded by multiciliated and biciliated ependymal cells (E1 and E2 cells, respectively), which form pinwheel-like structures around them, sense the cerebrospinal fluid of the ventricle through the apical primary cilium. The choroid plexus is also considered part of the niche, and its secreted factors into the CSF regulate B1 cells. Supra-ependymal axons on the surface of the ventricular wall contact both E and B1 cells. Mature neurons and astrocytes are found below the V-SVZ (63).

The number of B1 cells drastically decreases in the first year of life in mice, but the number of newborn neurons in the olfactory bulb (OB) is not significantly affected by age, suggesting that another population of NSCs that lack apical contact and that can differentiate might exist in the adult rodent brain (69). B1 cells primarily give rise to neuro-glia cells. As regards astrocytes, B1-cell ability to differentiate into astrocytes was, for example, demonstrated upon photothrombotic ischemic cortical injury (76) and upon chemical demyelination (77). Nonetheless, it has been reported that B1 cells can also give rise to oligodendrocytes (78) destined for the corpus callosum, where they myelinate axons in both healthy (77) and demyelinating conditions (77, 79). Although dispensable in this latter condition, they protect neurons from increased axonal loss (79). Notably, post-natal and adult neurogenesis in the SVZ is carefully controlled by microglial cells (80–82).

As regards the neural niche in the dentate gyrus (**Figure 6**), neurogenesis occurs on the side of the granule cell (GC) layer facing the hilum, in two or three-thin strata of the SGZ. NSCs originate in the ventral hippocampus during late gestation and then re-locate to the dorsal hippocampus. Here, quiescent NSCs, called radial glia-like (rRGL, or Type 1) cells become activated (aRGL) and divide to self-renew and to make intermediate proliferating progenitors (IPCs) that then differentiate into neuroblasts. About 25% of them survive and mature into granule neurons of the DG (85) or into mature astrocytes (86, 87) with a strategy that still needs to be fully elucidated (88–90).

While in the SVZ NSCs mainly give rise to inhibitory interneurons, in the DG, they generate new excitatory neurons that are involved in learning, memory, and pattern separation (91). New-born neurons of the SGZ are mainly located in the GC layer and do not migrate. Further, while in the SVZ, depending on the position, progenitors develop toward a different fate, the neural progenitors of the SGZ present only a bipotential fate (6). The two neurogenic niches face a 50–70% death rate during the first few days of birth, and short-living new-born neurons not only have different electrical properties than mature ones but may have their own functional role (39).

In both adult mouse neurogenic niches, it has also been reported that stem cells and differentiated daughter cells act to regulate their respective maintenance. For example, differentiated neurons release diffusible or contact-mediated signals, such as the neurotransmitter GABA and the Notch ligand Delta-like 1 (Dll1), which help to maintain NSC quiescence (92, 93). On the other hand, Tang C. and coworkers recently demonstrated a feed-forward mechanism between NSCs and newly generated neurons through pleiotrophin (PTN) ligand, whose release by NSCs supports the development of the

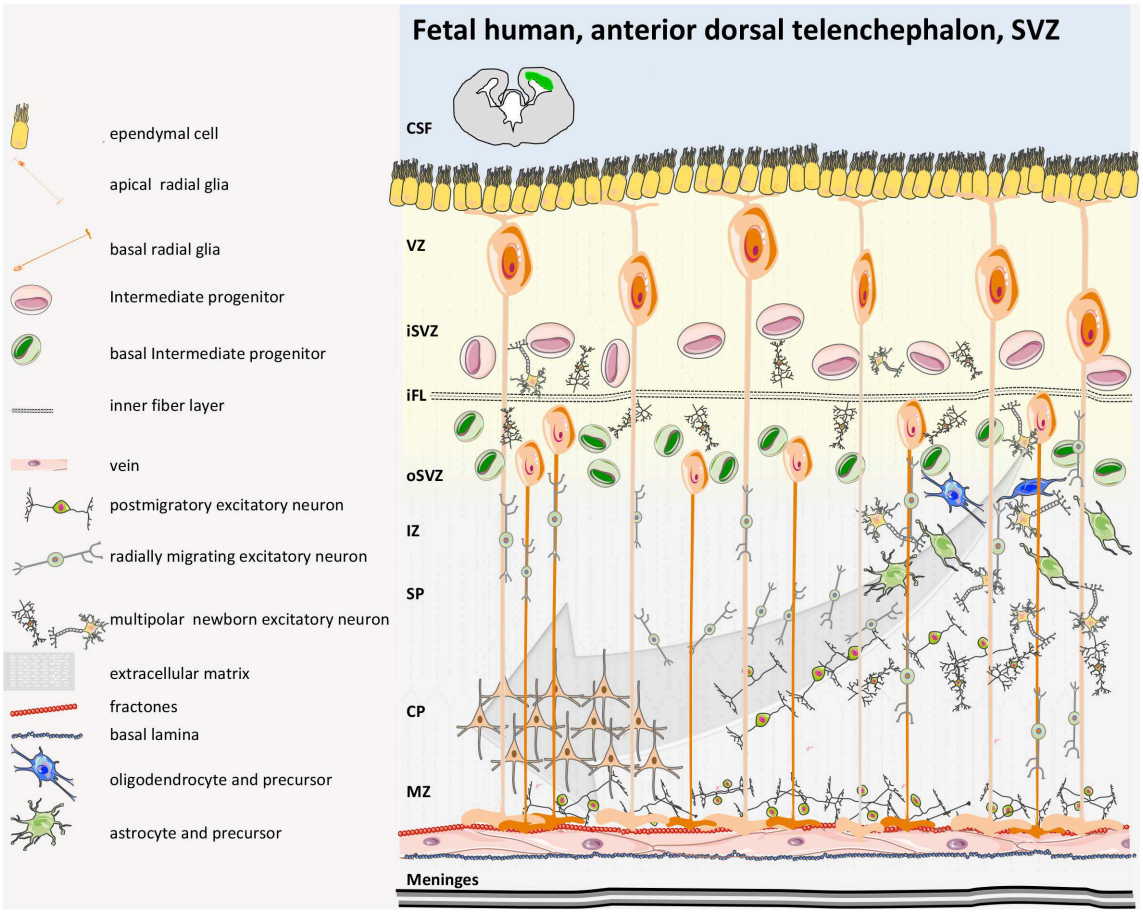

FIGURE 3 | Human fetal SVZ structure. In the developing human gyrencephalic cerebral cortex, the SVZ is subdivided by the inner fiber layer (iFL) into the inner SVZ (iSVZ) and the outer SVZ (oSVZ). Neurogenesis begins with expansion of the neuroepithelium and apical radial glia (aRG) *via* asymmetrical cell cycling. Human aRGs divide to give rise to basal RG (bRGs), which delaminate from the apical surface (retaining their basal process and attachment to the pial surface), migrate basally, and populate the oSVZ. The oSVZ is also populated by basal intermediate progenitors (bIPs) that proliferate and generate neurons. The oSVZ is the predominant germinal region in the human neocortex. The basal processes of bRG act as guides for migrating newborn neurons that disperse in the tangential axis to expand the surface area of the cerebral cortex (40, 41).

newly differentiated neurons (94). Down the line, this cross-talk might impact the important role of striatal neurons in cognitive functions and goal-directed behavior (the dorsomedial striatum, DMS) (95), as well the sensorimotor territory and habit formation [the dorsolateral striatum (DLS)] (96).

In humans, post-natal SVZ (**Figure 7**) is different from in other mammals because it consists of a smaller inner and expanded outer SVZ (iSVZ and oSVZ, respectively). The oSVZ contains radial glia that support neurogenesis and cortical expansion during fetal development (98). After corticogenesis, the neurogenic niche of the iSVZ and oSVZ remains proliferative in neonates along the wall of the lateral ventricle in the site of former lateral ganglionic eminence, generating new neurons that populate the pre-frontal cortex and, partially, the olfactory bulb (81, 99) for a few months after birth. Subsequently, however, this activity declines dramatically, and, within 2 years, there is almost no detectable neurogenesis or migration (100–103). Perinatally, SVZ stem cells differentiate and migrate along three specific pathways toward the anterior forebrain: (i) to the frontal lobe where they become interneurons (arc pathway) (103); (ii) to the medial pre-frontal cortex along the medial migratory stream (MMS); (iii) to the olfactory bulb along the RMS (104). Moreover, while in many mammals newly SVZ-generated neurons migrate specifically to the olfactory bulb to guarantee olfaction throughout life (105), in the human frontal cortex, only inhibitory neurons are born post-natally with unclear function or contribute to neurocognitive maturation and plasticity, important in infancy (103, 106).

Mature, adult human SVZ consists of four layers. Moving from the ventricle side, Layer I consists of ependymal cells in contact with the lumen, and this is followed by an almost acellular layer (Layer II), which originates post-natally as a consequence of neuroblast depletion. This layer includes a dense network of astrocytes and ependymal cell processes, where astrocytes and ependymal cells exchange signaling, and the few microglial cells influence communication among the

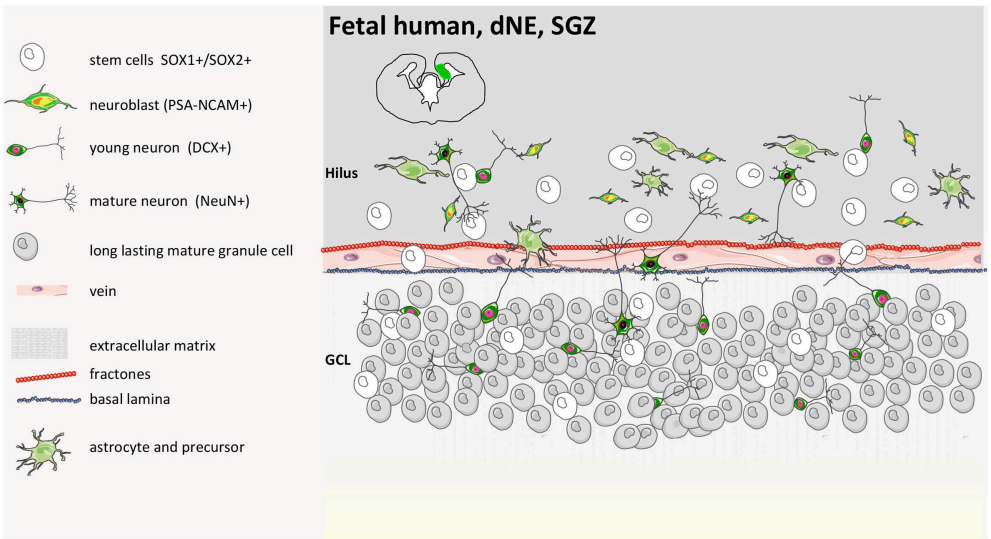

FIGURE 4 | Human fetal SGZ structure. Fetal development of the SGZ starts from the dentate neuroepithelium (dNE), which is located at the edge of the ammonic neuroepithelium (aNE) close to the fimbria. SOX1+/SOX2+ precursors are organized in ribbons between dNE and GCL (granule cell layer) already at 14 gestational weeks (GW) with PSA-NCAM and DCX positive cells. SOX1 and SOX2 cells are present in the GCL and hilus and between the GCL and the dNE. A coalesced proliferative SGZ does not form in the human DG. NeuN-positive cells are seen along with SOX1 and SOX2 at 22 GW. The cellular network reported in the illustration remains until soon after birth, when either hippocampal neurogenesis continues with aging (55) or completely disappears (9, 39, 40, 55, 56).

cell types (107). Adjacent to Layer II, there is a dense rim of astrocytic cell bodies (Layer III) with variable morphology. Finally, Layer IV consists of a transitional region with few cells, similar to the brain parenchyma. Although some astrocytes can proliferate (99, 108), neuroblasts are absent in the adult human SVZ niche and in the rostral migratory stream toward the olfactory bulb (101). Interestingly, in the adult human brain, newly generated cells are mainly oligodendrocytes, not neurons (109), suggesting that the oligodendrogenic process and myelin maintenance is more important in the human brain compared to other mammals.

Of note, comparing mouse and human adult SVZ, the proportion of type A:B:C cells in the mouse brain is 3:2:1, while in human, it is estimated at 1:3:1 (110).

Besides the differences between embryonic and adult niches, we have already anticipated that the niche changes during development. Indeed, a general comprehensive analysis of NPCs in mice from post-natal age P7 and P28 revealed not only that the number of NPCs decreases over the course of development but also that the genetic profile of the NPCs at the two ages was significantly different, suggesting early adulthood senescence (111).

Interestingly, it has been shown that neurons born during embryonic development (E19) and early adolescence (P21) (in mice) survived throughout adulthood (up to 2–6 months), while the cells generated at P6 displayed 15% cell death during adulthood, suggesting that early post-natal granule cells have an important unique function in terms of hippocampal plasticity (112). Early-life post-natal hippocampal neurogenesis is crucial to strengthen the ability to learn and to acquire new information via a rapid and continuous generation of new granule cells at the expense of existing memories and information storage.

Moreover, a recent report in mice showed that a population of NSCs exists in the DG that contributes to neurogenesis throughout development and adulthood and that the NSCs shift from a quiescent to an active state at different time points (84), suggesting that hippocampal neurogenesis is crucial for maintaining tissue plasticity. Indeed, the technology of single-cell RNA sequencing demonstrated that while there is an early post-natal transformation of radial glia cells from embryonic progenitors to adult quiescent stem cells maintained as such through adulthood, intermediate progenitor cells, neuroblasts, and immature granule cells are very similar at all ages (113).

Although the evidence that progenitor cells exist in the human brain is robust (114, 115) (**Figure 8**), controversies still persist about *in vivo* evidence that neurogenesis occurs in the adult hippocampus and about its functional relevance. A first landmark study on autoptic human brain tissue measured the concentration of ^{14}C in genomic deoxyribonucleic acid (DNA) and estimated that 700 new neurons are generated each day in the adult human hippocampus corresponding to an annual turnover of 1.75% (118), comparable to what is seen in middle-aged rodents.

In this process, aging, an altered immune-related molecular and cellular status, is the most critical environmental driver that can impair neurogenesis and contribute to its decline. Indeed, when heterochronic parabiosis was attempted by exposing aged animals to a young systemic environment, adult neurogenesis increased through a-yet unknown precise mechanisms (119, 120). Moreover, Spalding et al. showed that neurogenesis also

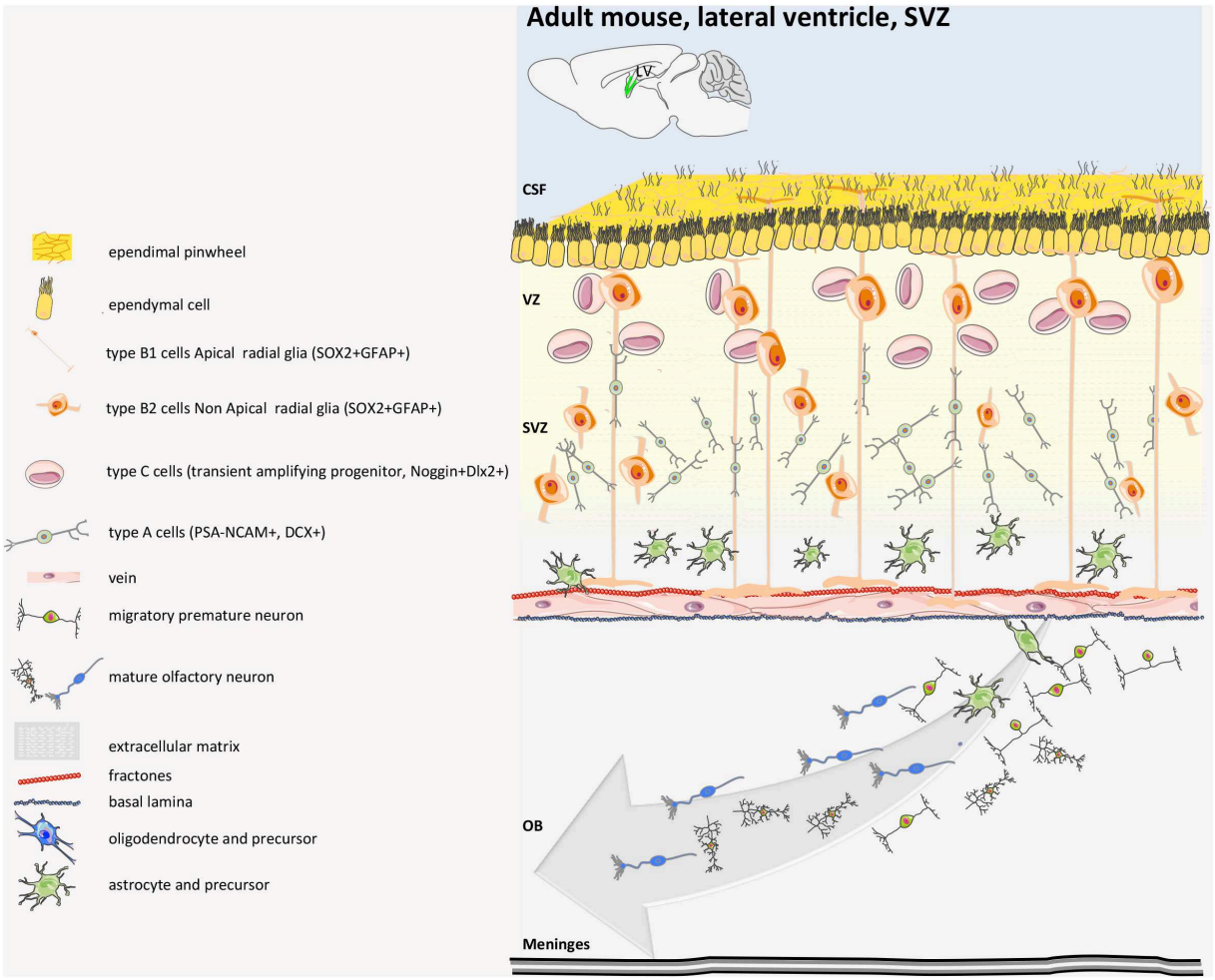

FIGURE 5 | Mouse adult SVZ structure. Adult NSCs (also called radial glia-like, RGL, pre-B1 cells) of SVZ derived from embryonic radial glial (RG) cells that make neurons of the embryonic brain. The adult SVZ NSCs at embryonic day 14 (E14) upregulate p57kip2 to enter quiescence (qRGL, B1 cells). The qRGLs become activated after birth to participate in adult neurogenesis in the SVZ. In the SVZ, the RGLs mostly undergo symmetric cell division. The SVZ RGL symmetric self-renewal could occasionally also result in another type of RGL cell that lacks the apical process, named non-apical B1 cells or B2 cells. Type B1 cells give rise to neuroblast type A cells (transient amplifying cells). These young neurons are surrounded by a glial sheet and migrate anteriorly toward the olfactory bulb (OB) and differentiate in granular and periglomerular GABAergic interneurons. The adult SVZ also generates oligodendrocytes, although in much lower numbers. CSF, cerebrospinal fluid (39, 40, 63–66).

occurs in the human hippocampus in older age, in contrast to the age-related decline previously described in rodents (121). Similarly, another group observed immature and mature adult-born neurons in hippocampal post-mortem samples of healthy adult individuals (55). This evidence has recently been replicated by Moreno-Jiménez et al. who described the presence of immature neurons in the DG of 90-years-old human subjects (122) and by Tobin et al. who demonstrated that hippocampal neurogenesis occurs in the tenth decade of life (123). In contrast, a study on peri- and post-natal human samples from subjects with a wide range of diseases reported few young neurons in young individuals (7–13 years of age) and no newborn neurons in the DG of adults. Immature neurons were found only in specimens of 1-year-old subjects (56). Divergences between mouse and human are likely due to differences in the rate of generation and maturation of newborn neurons (124), while divergences among human studies might depend on limitations when using human post-mortem tissues to study neurogenesis with variable time from death to fixation.

FEATURES OF PLASTICITY IN NEURAL STEM CELLS

NPCs, because of their intrinsic stem nature, exploit several plastic features. We have already mentioned their dual cell division capacity, symmetric and asymmetric, their capacity to stay quiescent in the niche for a long time and then to be activated/proliferate and to differentiate, and their

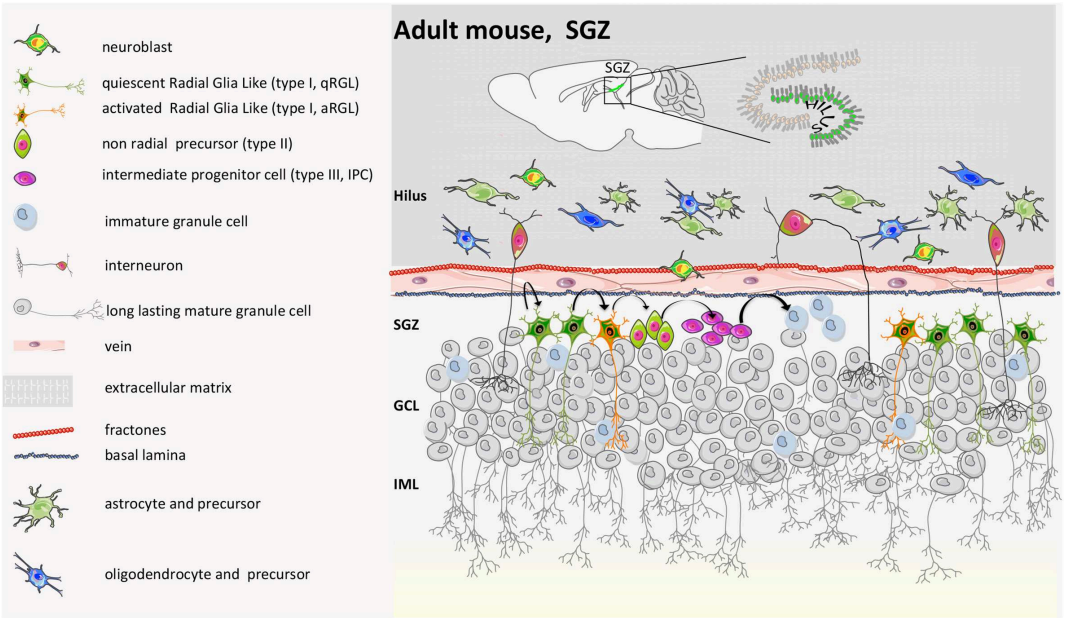

FIGURE 6 | Mouse adult SGZ structure. During the second post-natal week, proliferation in the DG becomes confined to the SGZ, where NSCs reside throughout adulthood. Genetic cell lineage tracing of Sonic Hedgehog (SHH)-responsive cells has revealed that adult NSCs are induced at peri-natal stages in a restricted region next to the most ventral side of the hippocampus in close proximity to the lateral ventricle. From there, they migrate to populate all regions of the DG. Thus, embryonic and adult NSCs in the DG have different origins. Indeed, the generation of new neurons in the DG starts from radial glia-like progenitor (type I). Type I cells become activated. Activated type I cells generate intermediate progenitors (type IIa, ab, and b and type III). Type III converts into immature granule cells and finally into mature long-lasting calbindin/calretinin-positive granule cells. IML, inner molecular layer; GCL, granule cell layer. Nicola et al. showed that a condensed germinal zone in SGZ only appears during post-natal days 7–14, likely because it depends on neural activity for adult neurogenesis established by the SVZ (83). A recent report suggests that a dentate-specific neural progenitor, arising in mice at ~E11.5 and marked by *Hopx* positivity, persists from embryonic development to adulthood. These progenitors give rise at E18.5 and P7 to the dentate region and then transition to quiescence early post-natally, to contribute to neurogenesis only during the adult lifespan. Those RGLs might have limited capacity for self-renewal, are skewed toward neurogenic differentiation, and rarely make astrocytes (40, 42, 65, 84).

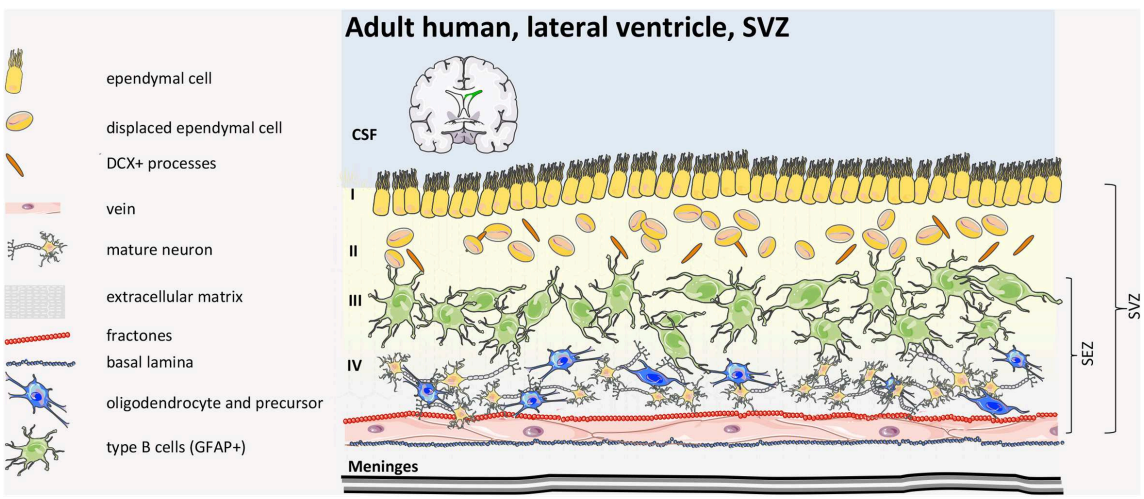

FIGURE 7 | Human adult SVZ structure. The human adult SVZ consists, from the ventricle side to the parenchima, of Layer I of multicyliated ependymal cells, with radial and tangential processes, followed by a hypocellular layer (Layer II) of astrocytic and neuronal cell bodies with a number of cytoplasmic expansions of ependymal cells inserted by astrocytic ramifications. Layer III consists of a ribbon of proliferative astrocytes (type B cells). Some oligodendrocyte-like precursors and misplaced ependymal cells are found. The inner layer (Layer IV) consists primarily of myelin tracts and neuronal bodies. SEZ, sub-ependymal zone; CSF, cerebrospinal fluid (39, 40, 97).

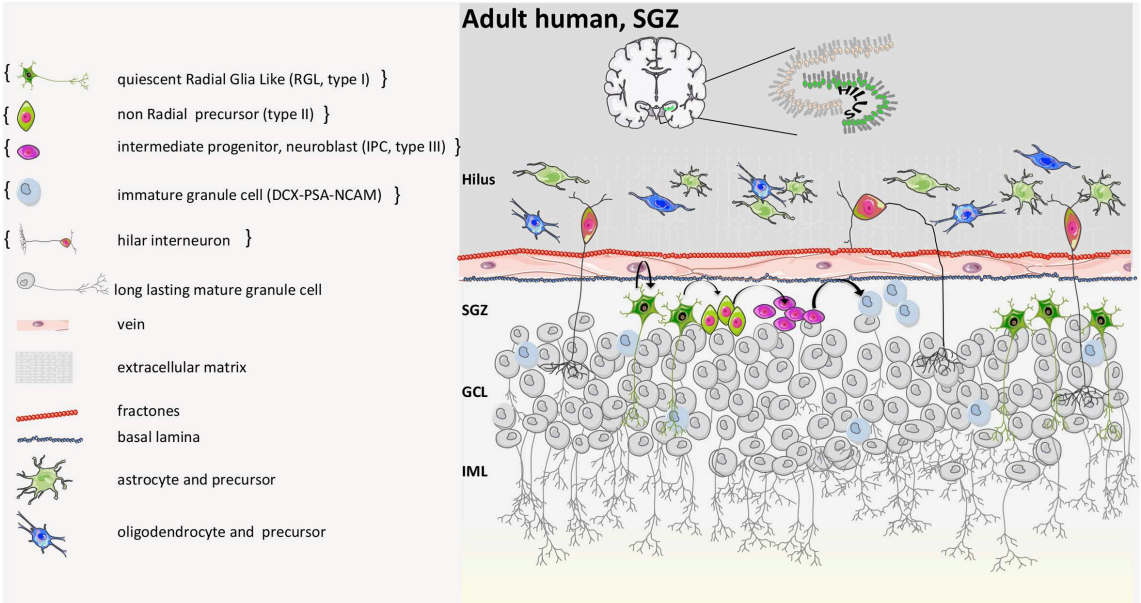

FIGURE 8 | Human adult SGZ structure. Based on the report by Boldrini et al. neurogenesis persists during adulthood. The generation of new neurons starts from quiescent radial glia-like progenitor cells (type I). Type I cells become activated and then, by asymmetric division, generate intermediate progenitors (type II). Type II become neuroblasts or intermediate neural progenitors (INP type III) that convert into immature granule cells and finally mature into long-lasting granule cells that send their apical processes to the CA3 part of the hippocampus. On the other hand, according to Sorrells et al. neurogenesis is not detected in adult. In the dentate gyrus, a proliferative subgranular zone (SGZ) is not formed near the granular cell layer, and proliferative progenitor stem cells are scattered in the hilus only. They disappear anyway after 7 years from birth, and young neurons are not found in adult individuals. Curly brackets define the condition described in Sorrells et al. (9, 39, 40, 55, 56, 64, 116, 117).

regional differences, as well as their capacity to sense the environment (125) and promote neuro-biochemical changes (94). The brain is substantially a "non-renewable organ," and brain cells slowly die with age, but neural stem cells guarantee to the CNS a certain ability to reorganize structurally and function according to intrinsic and environmental demands (126). Although proliferation and differentiation occur, it is still debated whether endogenous adult NSCs really exhibit long-term self-renewal capacity.

Encinas and collaborators reported that in the SGZ, NPCs can proliferate and then differentiate into astrocytes but that this causes progressive pauperization of the niche without returning to quiescence (127). Bonaguidi et al. instead showed, via clonal analysis, that NPCs face repetitive rounds of activation followed by astrocyte differentiation and quiescence (6). Unfortunately, different genetic labeling strategies target different populations of NSCs and different activation states of the same population, providing still complex and partial results.

Another plasticity feature for NPCs is related to the intrinsic tri-lineage potential of endogenous NSCs in the adult mammalian brain. However, although *in vitro* in culturing conditions, NSCs, both human and mouse, give rise to three different cell types, time-lapse analysis revealed generation of either neurons or oligodendrocytes, not both (128). Vice-versa, *in vivo*, different reports are available: population fate mapping has described that SVZ-NSCs generate neurons and astrocytes, not oligodendrocytes (6), while clonal analysis has recently indicated that only neuronal lineages are generated from individual NSCs (129). Cell commitment could derive from a progressive restricted lineage profile occurring in the adult (130). According to an alternative hypothesis, the trilineage potential is shaped and maintained by the niche environment (131).

The possibility that precursor cells can make new neurons is important for their therapeutic potential in neurodegenerative conditions. This potentiality has been exploited in preclinical models for stroke (132, 133). Unfortunately, after stroke, only few neuroblasts survive and differentiate, migrating from the SGZ into the granule cell layer to form novel neural circuits (134); these are not numerous enough to recover neurologic functions under ischemic conditions, and only 0.2% of lost neurons are replaced (135). Therefore, enhancing proliferation, survival, and neuronal maturation of endogenous or transplanted NSCs is important for brain disorders. Of note, while in animal models of neurodegenerative diseases like stroke, depression, epilepsy, Alzheimer's, Huntington's, and Parkinson's diseases, as well as in affected humans, progenitor cell proliferation and neurogenesis occur in the SGZ (at a lower rate in the latter and depending on the disease), in human, the SVZ is particularly sensitive to neurodegeneration and more responsive than SGZ via proliferation (136).

Under physiological conditions, the plasticity of NPCs is exploited for brain development, learning, and memory (137, 138) and is mediated by the release of trophic factors. This feature

is fundamental during neurogenesis; it is important to tune synapse connections and to modulate neuronal networks during healing processes after brain injuries. Synaptic connectivity is indeed mediated by released neurotrophins (NTs), such as brain-derived neurotrophic factor (BDNF), NT-3, and NT-4 (139, 140). Depending on the type and concentration, they strengthen or weaken synaptic morphology (141) and synaptic responses (142), leading to long-term potentiation (LTP) or long-term depression (LTD). Moreover, they can form new connections and pathways and can change the wiring of the circuits (143). In severe pathological conditions, physiological trophic effects will not provide sufficient tissue regeneration. However, engraftment of exogenous stem cells, via the release of neuroprotective, trophic, or immunomodulatory factors, may stimulate endogenous neurogenesis, angiogenesis, and neovascularization, helping in the healing processes (144) and promoting the formation of new pathways around damaged tissue. The mechanism is active in the perinatal period and early-childhood, but it is progressively reduced in the brains of older children and adult individuals (145).

Cell therapy could be more beneficial when stem cells are engineered (146, 147). For example, elevated NT-3 expression can provide a microenvironment favorable to the survival and differentiation of transplanted neural stem cells (148).

For many (if not all) of the features above mentioned, epigenetic regulation plays an important role in shaping the response to the environmental cues of NPCs, depending on their developmental stage (149, 150).

The plasticity of NPCs is also exploited by their capacity to interact with scaffolds, as detailed below in the Spinal Cord Injury (SCI) section.

THERAPEUTIC APPLICATIONS

Leveraging their plasticity, NPCs have been proposed for (i) neurotoxicity testing, (ii) cellular therapies to treat CNS conditions, (iii) neural tissue engineering and repair, (iv) drug target validation and testing, and (v) personalized medicine, as detailed below.

Since the developing CNS is more vulnerable to chemical exposure, *ad hoc* pharmacological testing is required (151). To address this goal, NSCs turned out to be useful for neurotoxicity testing. Developmental Neurotoxicity (DNT) is indeed a function not only of the type of exposure (dose, duration) but also of the developmental stage of the brain at the time of exposure (152). The blood–brain barrier (BBB) is not completely formed until at least 6 months after birth, facilitating the entrance of a chemical into the fetal/neonatal brain (153). Considering the increase of children's neurodevelopmental impairments [e.g., learning disabilities, autism, attention deficit hyperactivity disorder (ADHD)], likely due to exposures to chemicals with DNT potential, concerns have been raised about the need to identify suitable tools to properly ascertain drug toxicity. Assessment has been primarily based on animal studies, but the tests are very resource-intensive in terms of animals, time, and costs (154, 155), underlining the need to develop alternative approaches to identify DNT. *In vitro* work has been performed using rodent and human neuronal and glial cellular models (neuroblastoma cell lines) to evaluate (via dose-response relationships) the impact of a compound on various stages of brain development. Unfortunately, transformed/immortalized cell lines present limitations, such as the expression of proliferating genes that impact cellular response to chemical exposure (156, 157). On the other side, human *in vitro* neuronal cultures derived from neural progenitor cells (NPCs) or brain fetal NPCs grown as neurospheres can better mimic critical brain developmental processes, including proliferation, apoptosis, migration, and differentiation (158). However, as already mentioned, the ethical issues regulating the generation and use of human embryonic or fetal-derived tissues have been a matter of intense debate. Therefore, hiPSC-derived neuronal and glial models have been proposed for their applicability in *in vitro* pharmacological and toxicological studies. Indeed, human iPSC-derived cultures of mixed neuronal and glial cells are suitable for DNT, actually more so than for adult neurotoxic evaluation (159) because hiPSC-derived cells (and hESCs) reproduce in a more difficult way the terminal differentiation and the functional characteristics of adult brain physiology even after long term culture (160). Moreover, hiPSC-derived NPCs have an earlier neurodevelopmental phenotype because, instead of differentiating in culture into Nestin and GFAP+ like primary human NPCs, they express TubβIII (161).

Current efforts in the context of hiPSCs to optimize culturing and differentiation protocols are on-going to better mimic the brain context using defined factors and co-culture conditions. The generation of microglia-like cells from hiPSCs has helped to introduce the immune component into neuroglia culture (162–166). Further, hiPSCs have also been differentiated into brain endothelial cells that mimic the functionality of the BBB *in vitro,* adding further value to their use in DNT tests (167). Additionally, three dimensional (3D) culture and cerebral organoids have been developed and can recapitulate brain region connections occurring *in vivo* in the cerebral cortex (168). Assessing endpoints in 3D systems will be critical for guaranteeing the applicability of hiPSCs for DNT in complex assays. Combining *in vitro* DNT tests with *in vivo* epidemiological human data is crucial for developing Integrated Approaches to Testing and Assessment (IATA) for regulatory purposes (chemical screening, hazard identification/characterization, or risk assessment) (169).

NPCs surely play a crucial role in CNS tissue repair, and their intrinsic plastic nature gives them therapeutic potential in neurological diseases via two Modes of Action (MoA)L cell replacement and the bystander effect.

As regards cell replacement, NPCs are, in principle, a suitable therapeutic strategy for those diseases in which neurodegeneration and cellular loss are prevalent, not only because they mediate cell replacement but also because they can re-establish and/or support neuro-glial functional connections lost during the pathological process. This approach was tested decades ago, for example with fetal NSCs (170) transplanted into subjects with Parkinson's disease, and recently with hiPSC-derived cortical precursors transplanted into an Alzheimer's mouse model (171). However, in the first case, transplanted

stem cells or progenitors could not survive for long or form the desired cell types. In the second case, human cells reproduced the pathological phenotype of AD neurons, influenced by the genetic background they had been transplanted into, suggesting that the NPC cell replacement strategy is promising only when extrinsic inflammatory neurodegenerative factors have faded in the CNS site where transplant occurs or for cell-autonomous neurological disorders. In general, transplanted NPCs give rise to the atypical ectopic perivascular niche with intense cell-to-cell cross-talk between transplanted NPCs and resident cells. NPCs may either remain in the niche in an undifferentiated state or move to acquire a terminally differentiated phenotype (172, 173) and adapt their fate according to the region of engraftment, developing neuronal or glial markers (174–176). For example, transplanted NPCs have been shown to form functional gap junctions to rescue host neurons and their projections in an animal model of Purkinje neurodegeneration (177). Moreover, human iPSC-derived NPCs have been shown to engraft and establish long-distance connections in animal models (178, 179), although concerns on the approach are still under evaluation (see below). It is crucial to mention the importance of homotopic rather than heterotopic transplantation to avoid tumorigenic risk, since region-specific cues instruct the grafts of NSCs (180). So far, this has been the predominant strategy.

Besides this evidence, NPCs might protect the CNS through mechanisms alternative to direct cell replacement, which implies the interaction of NPCs with both resident neural and immune cells. Indeed, transplanted NPCs rather exert immunomodulatory or neuroprotective functions modulating the response of the pathological processes of astrocytes, microglia, and inflammatory blood-born cells through paracrine and endocrine mechanisms (bystander effects). NPCs, upon interaction with CNS-resident cells, start releasing neurotrophic factors, such as Nerve Growth Factor-NGF, BDNF, and Glial Derived Neurotrophic Factor-GDNF, along with reactive species, binding proteins, purines, or cytokines that might significantly reduce scar formation and/or increase the survival and function of endogenous glial and neuronal progenitors. This was originally demonstrated in mice with primary inflammatory disorders, including the animal model of MS (173, 181) or stroke (182, 183) and in mice with neurodegenerative diseases mediated by reactive inflammation, such as Parkinson's Disease (184). Those properties have been then described for other stem cells, such as mesenchymal stem cells (185). The concept that therapeutic effect derives from released molecules opened the possibility of using the "secretome" of stem cells, which implies a cell-free therapeutic approach (186–191). The cross-talk with the environment is fundamental for promoting the release by NPCs of a context-specific arsenal of biological weapons, and the impact of external cues on paracrine signaling has been widely described recently (186, 192–197). However, most of the environmental cues that trigger the production of bioactive and restorative factors and the mechanisms they elicit in a specific disease are still unknown. Therefore, triggering *in vitro* the production of biologics and collecting and using the secreted factors, although promising, remains a reductionist approach, and efforts to efficiently transplant cells that sense and respond *in situ*, *ad hoc* to the environment are still most appropriate.

The immunomodulatory function is a feature of human NPCs (198, 199) that enables them to inhibit T-lymphocyte proliferation as well as dendritic cell maturation *in vitro*, to ameliorate disease severity when transplanted systemically in non-human primates with EAE, and to persist long-term, not only in the host CNS but also in peripheral lymph nodes (200). NPCs show pathotropism for the pathological sites, thanks to the expression of chemokine receptors, cell adhesion molecules, and integrins. Once transplanted (intravenously, i.v., or intrathecally, i.t.) and after migration into inflamed CNS areas, NPCs do no significantly differentiate but survive in close proximity to blood vessels, where they interact with CNS-infiltrating blood-derived inflammatory cells, endothelial cells, and CNS-resident astrocytes and microglia, releasing therapeutic molecules (201). In diseases characterized by primary inflammation, such as MS, stroke, or spinal cord injury, a precise control of time and route of cell administration is important to gain the therapeutic effect because NPCs transplanted in immunocompetent mice can be rejected in animals with ongoing neuroinflammation (202), and the immunomodulatory and trophic support might have a limited effect. Nonetheless, early NPC transplantation is important because, immediately after CNS damage, genes supporting tissue growth predominate over genes promoting anti-plasticity and differentiation (203).

Examples of NPC cell replacement and bystander effects for some diseases are detailed below.

Ischemic Stroke

Stem cell transplantation for stroke has represented a valuable therapeutic strategy using various sources of NSCs. Human ESC-derived NPCs have been implanted in rodents after cerebral ischemia, and they have shown neural differentiation and improved functional recovery (204, 205). Moreover, transplanted and engrafted NSCs (i) reduced cell death and inflammation near the graft (182) and promoted angiogenesis (206); (ii) promoted proliferation and neuronal differentiation of endogenous NSCs of the subventricular and hippocampal subgranular zone in rodents (135), primates (207), and humans (208, 209); (iii) survived to intracerebral transplantation in lesioned brain and differentiated into mature neurons (178), integrating in host neuronal circuitry to promote post-stroke morphological and electrophysiological recovery (20), although several months later.

A key aspect for clinical applications of exogenous NPCs is the route of administration, which can be: (i) intraparenchymal, implying direct injection of the cell suspension close to the site of injury; this strategy achieved motor and cognitive improvements in grafted patients (210, 211), or (ii) intravascular, which is used in a limited number of trials because it is more suitable for mesenchymal stem cells. Although a greater number of cells can be administered, unfortunately, the majority does not migrate to the brain (212). Moreover, this approach retains a risk of tumorigenicity due to the possibility of heterotopic graft (180).

Since NSC transplantation in preclinical stroke models was able to promote the proliferation of endogenous NSCs and the migration of endogenous neuroblasts to the damaged

brain region where they differentiate into mature neurons (213), activation of endogenous NPCs for remodeling neural tissue after ischemic injury has also been considered as a therapeutic strategy because it would not require transplantation of exogenous cells and would avoid annexed risks of introducing exogenous pathogens and of enhancing CNS immune surveillance, inflammatory reactions, and tissue rejection, as well as bypassing political and ethical concerns. In this perspective, treatment of stroke conditions with growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) promoted the recruitment of endogenous NPCs and regenerated hippocampal circuitry, restoring synaptic function after ischemia (214).

The option of using NSCs from iPSC in stroke in human is a bit more complicated to implement, due to the advanced age of most stroke patients, making it very difficult to efficiently generate iPSCs from aged subjects that can be used to perform autologous transplant. Moreover, although in some stroke models they have shown efficacy (215), it remains questionable whether iPSCs derived from aged patients are beneficial for post-stroke functional recovery (216, 217).

A phase 1 study on stroke subjects using the CTX0E03 or ReN001 cell line (ReNeuron) derived from genetically modified human fetal neuroepithelium has been conducted (211). c-mycERT AM technology was used to drive the expression of an estrogen receptor under tamoxifen (4-OHT) (in culture conditions) to control cell proliferation. Cell division was indeed arrested, and differentiation into neuronal and glial lineages was induced by removal of tamoxifen and of growth factors from the medium. Eleven men were enrolled; they did not receive any immunosuppressive therapy and were followed for 2 years. While immunological or severe adverse effects were not recorded, modest improvements on the different motor scales were observed [NIHSS, Barthel index, Ashworth Spasticity Scale for the arm and leg, and a quality-of-life and health status questionnaire, EuroQoL Five Dimensions (EQ-5D)].

Spinal Cord Injury (SCI)

NPCs have also been intensively studied and their use proposed as a therapeutic strategy for traumatic spinal cord injury, despite the complexity of the pathology (218).

NPCs have the potential to repopulate severely injured spinal cord (197, 219), but their ability to survive and reconstitute neural tissue and neural connections remains limited by parenchyma loss and by the very toxic milieu (220). Moreover, the epicenter of the primary lesion site rapidly become necrotic, so NPCs may need an extracellular skeleton to support survival and guide tissue reorganization. Biomaterials represent a suitable support for cells, replacing the extracellular matrix to favor cell survival, differentiation, re-vascularization, and re-colonization of the tissue by glial and endothelial cells. Moreover, complex biomimetic materials that can be produced may guide axonal growth, restoring long-distance connections. More preclinical research in this innovative field is definitely required. Regenerative compounds, biomaterials, and tissue, along with cellular transplants, have been used for SCI to enhance neurite outgrowth and facilitate tissue regeneration (221). Indeed, three-dimensional highly porous "scaffolds" made of biodegradable copolymers have been tested and seeded with NPCs into the lesion to facilitate donor cell survival, migration, differentiation, functional structural repair, and neural circuit activation (222). Recently it has been reported that NPC-mediated functional recovery could depend on oligodendrocyte differentiation (223). Although NPCs have been quite extensively tested in SCI preclinical models, improvement for patients is still limited. Okano's team in Japan started a human clinical study using allogenic iPSC-derived NPCs because costs, quality testing, safety concerns, and time were not compatible with autologous transplants. Nonetheless, when immunosuppression was stopped, complications arose. Thus, so far, only autologous iPSC-derived NPCs hold promise for repair of the injured spinal cord (224).

Neurodegenerative Diseases

NSCs may be delivered by three different routes: intravenous, intraparenchymal, or intra-cerebroventricular via lumbar puncture injection. Preclinical data have shown that via intraparenchymal delivery, NSCs migrate and spread along the corpus callosum, driven by tissue-specific disease factors (225). Via intravenous injection, NPCs cross the inflamed BBB, reaching the demyelinating areas of the CNS in animal models of multiple sclerosis (EAE) and eliciting therapeutic actions (172, 200), although NPCs could exert their bystander immunomodulatory effect also systemically. NPCs represent an effective therapeutic tool in multifocal, primary inflammatory diseases, such as multiple sclerosis, being able to migrate and exploit their bystander effect. Currently, the preclinical results in the MS context have been translated to the clinic using fetal NPCs (NCT03269071) in primary progressive MS subjects. fNPCs are currently used as a therapeutic choice also for other neurodegenerative diseases (225), and several clinical trials are in progress for neurodegenerative diseases, such as Parkinson's disease, ALS, tumors, and various pediatric diseases (not reported).

Table 1 summarizes the ongoing non-pediatric clinical trials selected on clinicaltrials.gov by using the key terms "neural stem cells," and "neural progenitor cells." Around 30 clinical trials reporting on transplant of NSCs have been registered on clinicaltrial.gov.

The therapeutic plasticity of NSCs has been exploited in the specific context of neural tissue engineering and repair. The development of safe techniques to generate autologous NPCs (iPSC technology and direct reprogramming of somatic cells) opened up novel therapeutic opportunities in the regenerative field. In particular, directly reprogrammed Neural Precursor Cells (drNPCs) (226) are non-immunogenic and have a stable genome and minimal risk of malignant transformation, if compared to induced-pluripotent and embryonic stem cells, while exhibiting self-renewal and multipotency.

To increase the therapeutic potential of NSCs and analogs, combination therapy of cells with engineered and miniaturized scaffolds improved spinal motor functions, as reported in a meta-analysis of more than 70 preclinical studies (227), and transplantation with tissue-engineered constructs outperformed the efficiency of suspended cells alone (228). Similarly, there

TABLE 1 | List of clinical trials using NPC/NSC in adult subjects.

Disease	Title	Trial phase	N° patients	Age	Follow-up (months)	Cell type	Site and mode of administration	Sponsor	NCT Number	Status
Age-Related Macular Degeneration	Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Age-Related Macular Degeneration (AMD)	Phase 1 Phase 2	15	>50	12	Human neural stem cell	Subretinal space (injection)	StemCells, Inc.	NCT01632527	Completed
Amyotrophic Lateral Sclerosis	Human Neural Stem Cell Transplantation in Amyotrophic Lateral Sclerosis (hNSCALS)	Phase 1	18	20–75	36	Human fetal neural stem cell	Lumbar spinal cord (surgical device)	Azienda Ospedaliera Santa Maria, Terni, Italy	NCT01640067	Completed
Amyotrophic Lateral Sclerosis	CNS10-NPC-GDNF for the Treatment of ALS	Phase 1	18	>18	12	Human neural stem cell	Lumbar spinal cord (stereotactic device)	Cedars-Sinai Medical Center	NCT02943850	Active, not recruiting
Amyotrophic Lateral Sclerosis	Dose Escalation and Safety Study of Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis	Phase 2	18	>18	24	Human neural stem cell	Spinal cord (injection)	Neuralstem Inc.	NCT01730716	Unknown status
Amyotrophic Lateral Sclerosis	Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis (ALS)	Phase 1	18	>18	48	Human neural stem cell	Lumbar spinal cord (surgical implant)	Neuralstem Inc.	NCT01348451	Unknown status
Brain Tumors	Genetically Modified Neural Stem Cells, Flucytosine, and Leucovorin for Treating Patients with Recurrent High-Grade Gliomas	Phase 1	18	>18	always	Human neural stem cell	Intracranial	City of Hope Medical Center	NCT02015819	Active, not recruiting
Brain Tumors	A Pilot Feasibility Study of Oral 5-Fluorocytosine and Genetically-Modified Neural Stem Cells Expressing *E. coli* Cytosine Deaminase for Treatment of Recurrent High Grade Gliomas	Phase 1	15	>13	always	Human neural stem cell	Debulking craniotomy	City of Hope Medical Center	NCT01172964	Completed
Brain Tumors	Neural Stem Cell Based Virotherapy of Newly Diagnosed Malignant Glioma	Phase 1	36	>18	NA	Induced neural stem cells	Intracranially	Northwestern University	NCT03072134	Recruiting

(Continued)

TABLE 1 | Continued

Disease	Title	Trial phase	N° patients	Age	Follow-up (months)	Cell type	Site and mode of administration	Sponsor	NCT Number	Status
Brain Tumors	Carboxylesterase-Expressing Allogeneic Neural Stem Cells and Irinotecan Hydrochloride in Treating Patients with Recurrent High-Grade Gliomas	Phase 1	53	18–69	180	Human neural stem cell	Intracranial	City of Hope Medical Center	NCT02192359	Recruiting
Ischemic Stroke	Pilot Investigation of Stem Cells in Stroke Phase II Efficacy (PISCES-II)	Phase 2	23	>40	12	Human neural stem cell	Intracerebral	ReNeuron Limited	NCT02117635	Completed
Ischemic Stroke	Intracerebral Transplantation of Neural Stem Cells for the Treatment of Ischemic Stroke	Phase 1	18	30–65	24	Human neural stem cell	Intracranial injection	Suzhou Neuralstem Biopharmaceuticals	NCT03296618	Active, not recruiting
Ischemic Stroke	Investigation of Neural Stem Cells in Ischemic Stroke (PISCES III)	Phase 2	110	35–75	12	Human neural stem cell	Stereotactic injection	ReNeuron Limited	NCT03629275	Recruiting
Ischemic Stroke	A Clinical Study of iNSC Intervent Cerebral Hemorrhagic Stroke	Early Phase 1	12	30–65	12	Induced neural stem cells	Intracerebral Transplantation	Allife Medical Science and Technology Co., Ltd.	NCT03725865	Not yet recruiting
Parkinson's Disease	A Study to Evaluate the Safety and Efficacy of Human Neural Stem Cells for Parkinson's Disease Patient (hNSCPD)	Phase 2 Phase 3	12	35–70	6	Human fetal stem cell	Nasal injection	Second Affiliated Hospital of Soochow University	NCT03128450	Unknown status
Parkinson's Disease	A Study to Evaluate the Safety of Neural Stem Cells in Patients with Parkinson's Disease	Phase 1	12	30–70	12	Induced neural stem cells	Intracerebrally to the striatum and substantia nigra	Cyto Therapeutics Pty Limited	NCT02452723	Active, not recruiting
Parkinson's Disease	A Study on the Treatment of Parkinson's Disease with Autologous Neural Stem Cells	Early Phase 1	10	18–60	12	Induced neural stem cells	NA	Allife Medical Science and Technology Co., Ltd	NCT03815071	Not yet recruiting
Parkinson's Disease	Transplantation of Neural Stem Cell-Derived Neurons for Parkinson's Disease	Phase 1 Phase 2	12	35–85	6	Human neural stem cell	Basal ganglia	NeuroGeneration	NCT03309514	Not yet recruiting
Parkinson's Disease	Safety and Efficacy Study of Human ESC-derived Neural Precursor Cells in the Treatment of Parkinson's Disease	Phase 1 Phase 2	50	50–80	12	Human embryonic stem cell-derived neural precursor cells	Intra-striatal injection	Chinese Academy of Sciences	NCT03119636	Recruiting

(Continued)

TABLE 1 | Continued

Disease	Title	Trial phase	N° patients	Age	Follow-up (months)	Cell type	Site and mode of administration	Sponsor	NCT Number	Status
Pelizaeus-Merzbacher Disease (PMD)	Long-Term Follow-Up Study of Human Stem Cells Transplanted in Subjects with Connatal Pelizaeus-Merzbacher Disease (PMD)	Phase 1	4	Child, Adult, Older Adult	4	Human neural stem cell	Brain	StemCells, Inc.	NCT01391637	Completed
Peripheral Arterial Disease	Safety Trial of CTX Cells In Patients With Lower Limb Ischemia	Phase 1	5	>50	12	Human neural stem cell	Gastrocnemius muscle	ReNeuron Limited	NCT01916369	Completed
Progressive Multiple Sclerosis	Neural Stem Cell Transplantation in Multiple Sclerosis Patients (STEMS)	Phase 1	12	18–55	24	Human fetal-derived Neural Stem Cells	Intrathecal	IRCCS San Raffaele	NCT03269071	Enrolling by invitation
Secondary Progressive Multiple Sclerosis	Safety Study of Human Neural Stem Cells Injections for Secondary Progressive Multiple Sclerosis Patients (NSC-SPMS)	Phase 1	24	18–60	12	Human neural stem cell	Intraventricular	Casa Sollievo della Sofferenza IRCCS	NCT03282760	Active, not recruiting
Spinal Cord Injury	NeuroRegen Scaffold, Combined with Stem Cells for Chronic Spinal Cord Injury Repair	Phase 1 Phase 2	30	18–65	24	Human neural stem cell	Spinal cord (injection)	Chinese Academy of Sciences	NCT02688049	Enrolling by invitation
Spinal Cord Injury	Long-Term Follow-Up of Transplanted Human Central Nervous System Stem Cells (HuCNS-SC) in Spinal Cord Trauma Subjects	NA	12	18–65	NA	Human neural stem cell	Intramedullary spinal cord transplantation	StemCells, Inc.	NCT01725880	Terminated
Spinal Cord Injury	Safety Study of Human Spinal Cord-derived Neural Stem Cell Transplantation for the Treatment of Chronic SCI (SCI)	Phase 1	8	18–65	54	Human neural stem cell, spinal cord derived	N/A	Neuralstem Inc.	NCT01772810	Recruiting
Spinal Cord Injury	Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Patients with Thoracic Spinal Cord Injury	Phase 1 Phase 2	12	18–60	48	Human neural stem cell	Intramedullary transplantation	StemCells, Inc.	NCT01321333	Completed

has been recent testing of a "liquid matrix" strategy, which is based on platelet-rich plasma (PRP)-derived hydrogel on a solid anisotropic complex scaffold prepared using a mixture of recombinant analogs of the spider dragline silk proteins which significantly stimulated proliferation and neuronal differentiation (229). Moreover, self-assembling peptides (SAPs) have been used to generate hydrogel to support human NSC differentiation into neurons, *in vitro*, in 3D, and to test the neuroregenerative potential in rat spinal cord injuries (230). Further, graphene composites have been optimized to promote human NSC differentiation and to increase conductivity and electroactivity (231, 232), a useful strategy for peripheral nerve recovery (233).

Lastly, the therapeutic plasticity of NSCs can also be exploited in drug target validation and testing. Indeed, primary cells have the best physiological relevance, but they are limited in availability, expansion, and reproducibility, and for some diseases, they are not accessible at all. In contrast, stem cells can be propagated for a long period of time, can be cryopreserved, and can be differentiated *in vitro* into a particular lineage to model a specific disease. Moreover, research and developmental efforts have been put in place in biotech and pharmaceutical companies to generate cells for high-throughput screening (234, 235). Further, since iPSCs from patients can be differentiated into specific lineages, patient-specific derived cells have been proposed for personalized medicine. The technology is surely going to translate to the clinic for monogenic rare hereditary diseases, where iPSCs provide a model to compensate for the lack of predictive human samples or for *in-vivo* preclinical models, since CRISPR/Cas9 technology or genome manipulation can help to introduce mutations of interest (179, 236).

Moreover, the possibility of assessing the molecular consequences of drug testing at specific stages of differentiation will help to identify active pathways and possible mechanisms for target identification (237). Bioinformatics, machine-learning algorithms, and big data tools for pattern recognition can be efficiently used for data analysis, orthogonal target validation, and biomarker discovery.

TECHNOLOGICAL ADVANCES IN THE FIELD OF NSCs THAT LEVERAGE THEIR THERAPEUTIC PLASTICITY

Recent technological advances in the field of stem cells and molecular biology have helped to potentiate their therapeutic efficacy. For example, gene therapy through the over-expression of key genes that encode for proteins with bystander potential has recently been proposed (gene therapy). This strategy has been applied to exogenous NSCs for important growth factors like NGF and BDNF. Indeed, adult human olfactory bulb neural stem/progenitor cells expressing NGF increased their proliferation and oligodendrocytic differentiation potential (238), while ESC-derived NPC expressing BDNF presented enhanced neuronal and striatal *in vivo* differentiation and turned out to be useful in Huntington's disease (239). Similarly, transplant of PSA-NCAM neural progenitors expressing BDNF was therapeutically useful in a mouse model of spinal cord injury (240), while embryonic rat NSCs expressing BDNF stimulated synaptic protein expression and promoted functional recovery in a rat model of traumatic brain injury (241). Of note, tumor formation was completely absent (242). Overexpression of GDNF was instead effective in stroke (243). The strategy has been applied not only to growth factors but also to transcription factors, such as Nurr1 (244), a critical gene in the embryonic differentiation of dopaminergic neurons (245).

Similarly, recombinant adeno-associated virus rAAVr3.45-IL10-infected human NSCs (HFT13) have been transplanted to evaluate their potential in ischemic injuries. Overexpressed IL10 had immunomodulatory effects and accelerated the recovery of neurological deficits and the reduction of brain infarction volume (246).

Engineering strategies using genome editing via CRISPR/Cas9 are being deployed on NSCs to precisely insert a gene of interest in the safe harbor human and mouse *loci* of AAVS1 and Rosa26, to perform a biallelic knockout of neurodevelopmental transcription factor genes, and to knock-in tags and fluorescent reporters (247). More recently, gene targeting at multiple *loci* using Cas9 showed great promise for a wide range of neurodegenerative disorders and injuries of the CNS, including lysosomal storage disorders (248). More sophisticated technological advances in the genome-editing field are being developed (249). Leveraging CRISPR/Cas9 for genes with ascertained therapeutic potential and with a spatio-temporal control might be possible to further harness the therapeutic plasticity of NPCs (**Figure 9**).

The discovery of induced pluripotency, which forces terminally differentiated adult somatic (i.e., blood or fibroblasts) cells into the pluripotent state, has provided the possibility of modeling complex neurological disorders (250). Differentiated cells are useful for screening drug candidates that can rescue molecular, cellular, and functional abnormalities in disease-specific hiPSC-derived cell types and offer the possibility of performing personalized medicine (251). In this same context, reprogramming or direct conversion of somatic cells using a non-viral system (liposome or cationic polymers) represent an interesting alternative in the perspective of clinical applicability (due to the reduced risk of tumor formation). Similarly, several types of nanoparticles useful for reprogramming have been developed. Graphene oxide-polyethylenimine complexes represent an efficient and safe system for mRNA delivery for direct reprogramming of somatic cells to induce neurons (252). Overall, on one side, the possibility of expanding *in vitro* hiPSC-derived NPCs opens up the perspective of autologous transplant and, on the other, NPCs derived from cells obtained with the new reprogramming strategy might overcome current hurdles associated with NPCs of conventional origin (both primary and from reprogrammed somatic cells) (253, 254).

It is becoming more and more important to be able to image the behavior of adult NSCs *in vivo* to explore how and where activation and division occur (255). This might be achieved with powerful microscopic and technological advances.

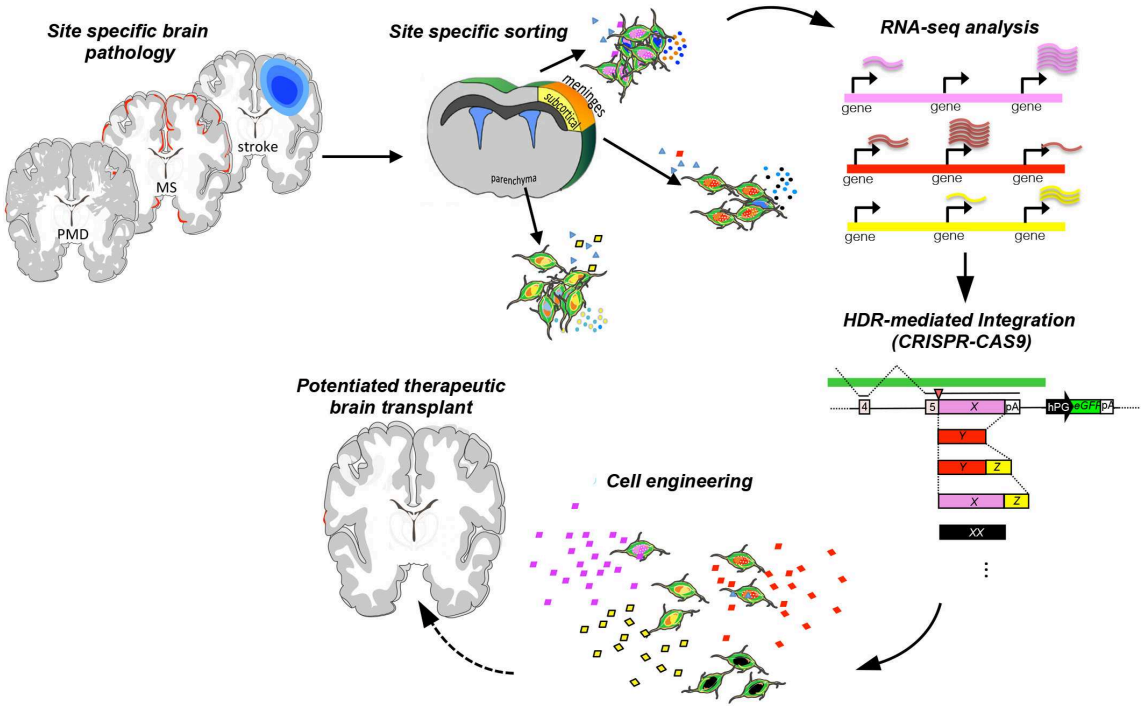

FIGURE 9 | Engineering strategy to potentiate the therapeutic plasticity of neural stem cells. Transplant of NPCs in suitable preclinical neurodegenerative inflammatory and demyelinating disease models [stroke, Pelizaeus-Merzbacher disease (PMD), or multiple sclerosis], cell recovery from the pathological tissue, and then sequencing allow the identification of key molecules that exert the therapeutic effects. Further, NPCs can be engineered to potentiate *ad hoc* the expression of therapeutic targets and rescue the brain-healthy phenotype.

With this aim, novel imaging sensors and tools have been developed for MRI technology, which provides excellent image quality, sensitivity, and 3D spatial resolution. Gadolinium (III) (Gd^{3+}) is the heavy metal contrast agent conventionally used in clinical and animal experimental MRI. Manganese (Mn^{2+}) is another useful positive T1 contrast agent that is widely used (256), similarly to iron oxide particles (SPIO), which have even higher sensitivity, better biocompatibility (function and phenotype), and increased paramagnetic power (257). Nonetheless, there are limitations in labeling stem cells with magnetic contrast agents because the label could be diluted due to stem cell proliferation after transplant. Moreover, particle loading allows stem cell tracing, but it is not informative regarding the survival state of stem cells and of possible changes induced in and by the microenvironment. Indeed, the signal could come from dead transplanted cells or cells phagocytized by microglia (258). MRI has also been improved using super-paramagnetic nanoparticles (MPI) (259) not present in biological samples, such as fluorine-19 (^{19}F), a strategy that is suitable for quantification and is devoid of the ambiguity of contrast tracking (260). In addition, the resolution has been augmented by increasing the number of coil receiver channels, the strength of the magnetic field, and the number of image acquisitions.

Nuclear medicine imaging techniques, such as positron emission tomography (PET) and Single-Photon Emission Computed Tomography (SPECT), represent other promising imaging modalities for tracking stem cells. SPECT has gamma camera detectors for gamma-ray emissions from the tracers (up to two different radioisotopes at the same time) injected into the patient. PET instead measures the decay effect of different radioisotopes that emit positrons, which interact with electrons from the body, are annihilated, and generate two gamma photons emitted in opposite directions.

^{111In}-oxyquinoline, ^{99}mTc-HMPAO, and, mainly for the CNS, ^{18}F-FDG or 2-deoxy-^{18}F-FDG, 3′-deoxy-3′^{18}F-FDG have been used for non-invasive imaging of NSC proliferation with PET (261, 262). It is still crucial to identify the safe dose of a radiotracer.

As an alternative to isotope cell loading and to overcome problems associated with particle loading, MRI reporter genes have been introduced for stable and robust tracking of implanted stem cells (263). The "imaging reporter genes" strategy consists of the production of a particular protein that interacts with a radioactive probe whose signal can be detected by PET/SPECT for a long time without being limited to the half-life of the tracer. With this approach, only living cells will be detected, excluding false signals (264). Cell labeling has been performed with green fluorescent protein (GFP) and red fluorescent protein (RFP), as well as with some fluorescent dyes, such as DiD, DiI, and indocyanine Green or semiconductor nanocrystals called quantum dots (QD). QDs emitting in the Near-infrared-(NIR) have been already used to track transplanted cells in the human brain (265).

Moreover, the introduction of a reporter gene that encodes for a special luciferase protein (bioluminescence imaging, BLI) has been widely applied in preclinical studies of stem cell imaging in the brain (266). Combining the high anatomical spatial resolution of MRI and the high sensitivity of PET with BLI was very useful for sensitivity and precise localization. Multimodality imaging can also be used, combining fluorescent QDs with magnetic nanoparticles (267).

Single-cell sequencing represents another fundamental technological advancement that enables the temporal and spatial dynamics of stem cells to be exploited. Since NPCs are significantly heterogeneous, each line maintained *in vitro* would need to be deeply characterized to assess the level of heterogeneity. Further, single-cell sequencing *ex vivo* on recovered transplanted cells will help develop an understanding of the therapeutic profile exploited in specific pathological conditions (**Figure 9**).

Obtaining data at the single-cell level helps with understanding how different types of brain cells develop and with identifying key genes to be used for cell engineering. Studies in drosophila represent an excellent model system with which to investigate how spatial and temporal factors are integrated during neurogenesis and can be translated to deep characterization in mammals (268).

PROS AND CONS OF HARNESSING THERAPEUTIC PLASTICITY

Harnessing neural plasticity is important due to its potential to support brain healing and rewiring to fight neurological and neurodegenerative diseases and, given the physical-chemical interaction between the SVZ and the striatum, to tune neuropsychological behavior that is often associated with neurodegenerative disorders. Indeed, the neural niche represents a reservoir of cues that influence proper brain cognitive functions and decisions. An altered concentration of released soluble factors by the stem niche may be responsible for unhealthy maintenance of striatal interneurons and for modified behavioral adaptation and striatum functions, ultimately leading, in extreme conditions, to obsessive–compulsive disorders. Of note, alterations in adult neurogenesis have been linked to psychiatric disease in humans (269, 270). According to the neurogenic hypothesis, major depressive disorder (MDD) is linked to impairments of adult neurogenesis in the hippocampal DG, and antidepressants are efficacious because they increase neurogenesis (271).

Harnessing therapeutic plasticity is tantalizing, not only to balance neuronal or neurodegenerative disorders but also to open up new learning opportunities in adulthood when conventional

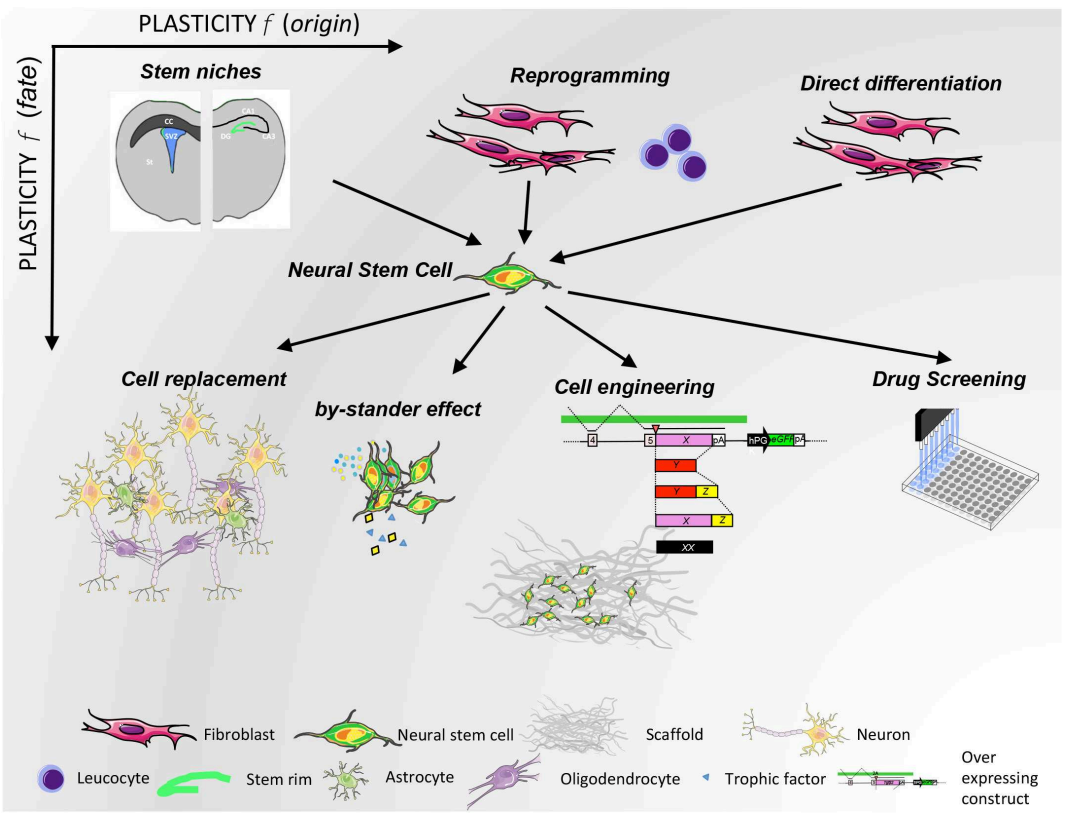

FIGURE 10 | NSC plasticity as a function of origin (x-axis) and therapeutic use (y-axis). NSCs originate both from physiological niches and from *in vitro* manipulation. Their therapeutic potential is exploited with different strategies, as depicted in the lower part of the illustration.

pruning, mediated by the environmental inputs in the early phase of brain development, has already occurred and connections have already been established, through a "use it or lose it" principle. Further, this approach can be translated to aging conditions and might be useful for preserving neuronal integrity.

Plasticity is important, but the brain also needs stability. Pharmacological or genetic modification can indeed increase plasticity, but a targeted and balanced (in time and amount) intervention is fundamental because excessive release of trophic factors might be detrimental. For example, brain overexposure to TGFβ2 as an anti-inflammatory approach (272) might cause a malignant TGFβ2 autocrine loop that leads to glioblastoma (273). Excessive plasticity could also be detrimental because massive memory capabilities [Savant abilities (274)], a reflection of over-plasticity, are linked to autistic profiles, because plasticity degenerates in chaos (275). For those reasons, engineered molecular tools should be responsive to and controlled by environmental signals.

Adult NSCs reside in restricted areas of the adult CNS and have limited capacity to proliferate (276). Thus, *in vitro* expansion is a limiting factor, and growth in suspension can be troublesome. Therefore, culture in adhesion has been developed using different coatings with the ultimate goal of maintaining stable expression of stem markers, such as Nestin and Sox2. Moreover, it is always important to consider that neurospheres may be heterogeneous because they are not derived from a single NSC. On the other hand, a limited proliferation capacity might be advantageous for ensuring that NSCs do not present tumorigenic potential, and genetic stability from one passage to another is likely to be maintained.

From the perspective of expanding neural precursors in culture at large scale, the iPSC technology has helped with the generation *in vitro* of expandable and freezable samples. However, although iPSCs are an important source of NPCs, caution is necessary because of the potential risks at the genomic and epigenomic level (277). Further, NSCs derived from iPSCs could cause rejection, so they might need to be combined with an immunosuppressant. The development of non-immunogenic iPSC-based therapies is very important to minimize the probabilities of patient rejection. Nonetheless, NSCs remain the best solution for neurological diseases, compared with other stem cell types, since recovery can be promoted not only by indirect paracrine effects but also by direct neural cell replacement, which is not supported by other sources of stem cells of another developmental origin, making the latter unable to properly differentiate in the CNS (**Figure 10**).

CONCLUSIONS

The discovery of neural stem cells and their potential has revived the field in terms of functional cell replacement, and concerns related to the risk of tumor formation have been dampened because the majority of NSC transplantation studies revealed no tumor formation. NSCs are a promising therapeutic approach for neurodegenerative disease. They can differentiate and replace the lost neural tissue as well as secreting neurotrophic factors that can protect or regenerate. Nonetheless, further studies are needed to quantify doses and administration periods and to define the most promising cellular NSC source considering also combined therapies to take NSCs/NPCs close to pharmacological prescription.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Protective Microglial Subset in Development, Aging, and Disease: Lessons From Transcriptomic Studies

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Microglial heterogeneity has been the topic of much discussion in the scientific community. Elucidation of their plasticity and adaptability to disease states triggered early efforts to characterize microglial subsets. Over time, their phenotypes, and later on their homeostatic signature, were revealed, through the use of increasingly advanced transcriptomic techniques. Recently, an increasing number of these "microglial signatures" have been reported in various homeostatic and disease contexts. Remarkably, many of these states show similar overlapping microglial gene expression patterns, both in homeostasis and in disease or injury. In this review, we integrate information from these studies, and we propose a unique subset, for which we introduce a core signature, based on our own research and reports from the literature. We describe that this subset is found in development and in normal aging as well as in diverse diseases. We discuss the functions of this subset as well as how it is induced.

Keywords: microglia, CD11c microglia, heterogeneity, CD11c, transcriptomics, subset, DAM, single cell

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INTRODUCTION

The term "microglia" was brought to the scientific community's attention a century ago with its first use by Pio del Rio-Hortega (1), who strived to distinguish them from oligodendrocytes. His early work also highlighted their phagocytic ability, as well as their potential to undergo morphological changes. This early description led the community to consider microglial cells as a homogeneous population, even though the first description of a microglial subset ("satellite microglia") appeared as early as 1919 (1).

Microglia originate from yolk-sac progenitors that start migrating toward the fetus around mid-pregnancy. These progenitors reach the embryonic brain around embryonic day (E) 9.5–E10.5 (2, 3) until the formation of the blood–brain barrier around E13.5–E14.5 in the mouse, and between the 4th gestational week to the 24th gestational week in the human (4, 5). As such, they are among the first cells to colonize the developing brain, and they participate in central nervous system (CNS) development. For instance, they contribute to refine brain wiring through enhancing both synapse formation (6, 7) and elimination (8, 9), they modulate axonal growth (10, 11), they secrete factors promoting neuronal progenitors survival (12) helping with neuronal positioning (11, 13), and they participate in the clearance of live and apoptotic cells during development (14). Microglia also take on physiological functions in the adult CNS, as they constantly sense their immediate environment,

in a so-called "never-resting state" (15, 16). Our knowledge of microglial physiology and process motility relies heavily on studies in anesthetized animals. Understanding of microglial functions in the steady state is challenged by a recent study showing that microglial process motility and morphology are affected by the wakefulness state of mice (17). Aside from this surveillance immune function, they are also fundamental for regulation of social behavior, learning, and memory, as these functions are impaired upon their depletion and restored after repopulation (18). Microglial roles in injury and disease contexts have been investigated extensively, with new advances contributing to deepen our understanding of Microglia and their effect on other glial cells [reviewed in Greenhalgh et al. (19)].

These physiological functions advanced our view of microglia, from being initially thought of as exclusively sentinel cells reacting in the context of injury. This dated view on microglia led to the superposition of macrophage M1/M2 phenotypes onto them (20), which was an early attempt to grasp the extent of microglial diversity. This classification is however mostly obsolete nowadays, as it was proved to be simplistic and disconnected from *in vivo* reality (21).

Indeed, the variety of functions microglia take on in space, time, and health states along with reports of sex differences in microglial function have led the community to infer a greater microglial heterogeneity than initially thought. With the progress of technology, investigating such diversity has become possible, notably through the development of high-throughput techniques such as mass cytometry and with the recent advances in transcriptomic studies with single-cell RNA-sequencing (RNA-seq). These technologies allowed the identification of microglial signatures linked to their "activation state." In 2014, Butovsky et al. described a "homeostatic" microglial signature, comparing microglia with monocytic populations and other CNS cells (22). This signature includes genes such as *P2ry12*, *Fclrs*, *Tmem119*, *Hexb*, *Mertk*, *Cx3cr1*, *Csf1r*, etc. that have been used in numerous studies thereafter to identify microglial cells. This was a fundamental step in distinguishing resident microglia from other tissue-resident macrophages and infiltrates in disease context. This "homeostatic" signature was more recently revised and extended to developmental stages in addition to adulthood by Matcovitch-Natan et al. (23). In this study, single-cell RNA-seq helped associate the microglial signature identified at each different age to the potential functions these cells take on during life. They pinpointed three different temporal stages of development, each linked to a particular signature: early microglia associated with proliferation and differentiation, pre-microglia related to neuronal development, and adult microglia.

It has recently been suggested that microglial heterogeneity peaks early during development and then reaches a minimum in the homeostatic adult brain, only to regain diversity in old age (24). In addition, some microglial subtypes have been based on surface markers and sometimes function [discussed in Stratoulias et al. (25)]. This has been mostly achieved through systematic transcriptional investigation of microglia in different contexts. However, because every study is done with different techniques (microarrays, bulk RNA-seq, single-cell RNA-seq, etc.), on different kinds of samples (whole brain, sorted microglia based on different gating strategies, microdissected microglia, sorted nuclei, etc.), and in different animal models, there is a risk for confusion of data. We believe that there is a need for an overview—by looking at the big picture, common patterns can be identified between studies that might otherwise have been overlooked.

In this review, we summarize and interpret transcriptomic studies on microglia from development, homeostasis, and disease states to bring to light a subpopulation common to all these different states. We discuss the factors inducing this subpopulation and its functional importance in all of the studied conditions. Finally, we provide a core signature for this subset and propose to systematize and unify the naming of this microglial subpopulation to clarify the literature and avoid redundancy in future studies. We propose to use a name already used in numerous studies and that accounts for these cells' expression signature: CD11c+ microglia.

CD11c+ MICROGLIA HISTORY, DISCOVERY, AND IDENTIFICATION

For long, microglia have been considered simply as macrophages, due to the belief that all macrophages emerged from the bone marrow. Consensus that a subset of microglia expressed CD11c was therefore at first difficult to achieve. CD11c was widely accepted as a marker for dendritic cells (DCs), to the extent that some studies have used it as the sole identifier for DCs. Added to this was the constant difficulty of discriminating CNS-resident parenchymal microglia from blood-derived myeloid cells, with which they share many markers [reviewed in Amici et al. (26)]. Until recently, it was indeed not possible to reliably discriminate microglia, especially activated microglia, from blood-derived monocytic myeloid cells, using morphology or routine myeloid markers. Panels of differentially expressed genes that can be used to distinguish microglia including TMEM119 (27) and the homeostatic marker P2RY12 (22) were however recently identified and validated in both homeostatic and disease conditions (28).

To our knowledge, the first observation of microglia expressing CD11c was made in human multiple sclerosis (MS) tissue by immunohistochemical analysis (29). One, however, cannot be completely certain of the exclusive microglial nature of the cells identified in this study based on the markers used and our current knowledge of myeloid cell marker expression patterns. The first report to explicitly identify CD11c+ cells in the CNS as microglia came from Butovsky et al. in 2006 (30). They identified populations of CD11c+ cells in a mouse model for Alzheimer's disease (AD) as microglia, based on their location and co-expression of isolectin B4 and CD11b, although these cells showed a dendritic morphology. The major point of interest in that study was the observation that all MHC-II+ microglia that engulfed amyloid β in the brain of glatiramer acetate (GA)-vaccinated transgenic (Tg)-AD mice co-expressed CD11c. Also, relevant to our subsequent studies, these cells could be stained with an antibody specific for insulin-like growth factor 1 (IGF1).

A "gold standard" for microglial identification remains their relatively low level of expression of CD45 in flow cytometry

analyses (31). In the course of study of glial responses in the dentate gyrus to axonal transection in the entorhinal cortex (the Perforant Path lesion model), we noted a subpopulation of CD45^{low} CD11b+ CD11c+ cells in flow-cytometry profiles of cells isolated from lesion-reactive hippocampus. Their functional significance and whether they derived intraparenchymally or by immigration from bone marrow were not determined (Babcock and Owens, unpublished). Exactly similar cells were then observed in cuprizone-demyelinated corpus callosum (32, 33). These were described to express slightly higher levels of CD45 than their CD11c− counterparts, while remaining within the CD45^{low} gate (33, 34). In addition, they did not express CCR2 characteristic for infiltrating leukocytes and expressed high levels of CX3CR1 supporting their microglial status (33). Further analysis showed that CD11c+ microglia were also induced in experimental autoimmune encephalomyelitis (EAE) (33–35) and a mouse model for neuromyelitis optica (NMO) (33), as well as during postnatal development (24, 35–37).

In older studies, ambiguity in assigning CD45 levels resulted in CD11b+ CD11c+ populations in CNS of mice with EAE or infected with *Toxoplasma gondii* being identified as DCs (38), although, with hindsight, consideration of bimodal CD45 profiles allows that at least some of them may have been microglia. The fact that CD11c+ microglia express slightly higher CD45 levels than resting microglia may have contributed to uncertainty, and claims that DCs derived from microglia (38, 39) may need re-evaluation.

Relative CD45 levels as detected by flow cytometry are not as useful for histological discrimination. Depending on the antibodies and staining protocols used, microglia may even not be detected as CD45+ cells, or else cannot be distinguished from other CD45^{hi} cells. Similarly, CD11c promoter-driven fluorescent reporter transgenic mice cannot discriminate between the many cell types that can express or upregulate CD11c without co-staining for lineage-specific markers. Identification of CD11c+ microglia in such mice relies on interpretation of sometimes fortuitous observations that include consideration of a cell's morphology and location. Using an EYFP-CD11c transgenic strain, Bulloch et al. identified a small fraction of CD11c+ microglia that were immunoreactive for Mac-1, IBA1, CD45, and F4/80 (40). The parenchymal juxtavascular IBA1+ CD11b+ GFP-CD11c+ cells described by Prodinger et al. in a CD11c-GFP reporter mouse likely included microglia, although in a non-diseased mouse, they would only account for around 2% of them (41). Flow-cytometric analysis confirmed CD45^{low} GFP-CD11c+ cells in the CNS of these mice (42). The fact that they were MHC II-negative likely reflects that they derived from non-diseased tissue, unlike the EAE-derived cells that we described (34). Typical microglia markers and their functions are listed in **Table 1**.

TABLE 1 | Microglia markers and their function.

	Marker	Main functions	References
Common in microglia	CD45	Pan-leukocyte protein with tyrosine phosphatase activity Controls adhesion in macrophages	(43)
	CD11b	Integrin family member Pairs with CD18 to form CR3, a receptor for complement C3bi, mediating complement-coated particle uptake Plays a role in synaptic pruning	(44)
	CX3CR1	Fractalkine receptor Controls microglia activation Mediates microglia–neuron interaction Participates in chemotaxis	(45)
	IBA1	Calcium-binding protein Key molecule in membrane ruffling and phagocytosis	(46)
	TMEM119	Surface protein Unknown function in the CNS	(27)
	FCRLS	Scavenger receptor Unknown function in the CNS	(22)
Specific to CD11c+ microglia	CD11c	Integrin family member Pairs with CD18 to form CR4, a receptor for complement C3bi, mediating complement-coated particle uptake Regulates the activation and proliferation of leucocytes	(47)
	CLEC7A	Pattern recognition receptor Regulates autophagy, phagocytosis, and the respiratory burst	(48)
	SPP1	Secreted glycophosphoprotein Plays a role in in cellular motility, adhesion and survival	(49)

CD11c+ MICROGLIA IN HOMEOSTATIC CONDITIONS

In Development

Even before microglia were formally identified, the presence of fat-laden cells had been reported and suggested to be a part of the normal developing CNS (50–52), and to participate in either cell death processes (53) or myelin formation (54–56). Early after the initial description of microglial cells, neuroanatomists began to track and map microglia in the CNS. Del Rio-Hortega was the first to describe "fountains of microglia" in the developing brain, having amoeboid morphology and being preferentially located in the white matter (57). Already in 1925, Penfield reported that what he describes as "neuroglia of mesodermal origin" "were variously considered to be normal and having to do with myelination or to indicate an abnormal inflammatory process" (58).

In the mid- to late 1970s, with del Rio-Hortega's "fountains of microglia" in mind, these cells were investigated again using

light and electron microscopy. Most studies describe round, amoeboid, highly vacuolated cells with fat-containing granules, which are found in developing white matter, particularly along unmyelinated axonal tracts in the corpus callosum of rabbits (59), rats (60), mice (61), birds (62), fish (63), and humans (64), as opposed to more highly ramified cells present in the gray matter. In all these studies, amoeboid or ovoid-shaped microglia invade the white matter before disappearing when increasing numbers of ramified microglia colonize the gray matter (peaking around postanatal day (P) 5 and disappearing around P10 to P15 in rodents). Multiple studies support this finding and extrapolate their potential function, stating either that they have enhanced phagocytic abilities for the elimination of apoptotic material coming from normal developmental cell death or that they participate in myelination (59, 60, 65–68). This involvement in myelination was reinforced by a study by Pont-Lezica et al. showing that microglial alteration early in development leads to impaired corpus callosum fasciculation (11). Their phagocytic abilities along with their morphology provoked debates regarding their origin (68), their fate (66), and even their microglia status with some studies modifying the nomenclature by referring to them as "brain macrophages" rather than "amoeboid microglia" (67, 68).

With the new notion of microglial phenotypes emerging, these early amoeboid microglia were hypothesized to have higher "activation" levels before becoming "deactivated" in a controlled manner, as this was believed to be temporarily helpful to scavenge debris coming from developmental cellular death. To corroborate this hypothesis, Hristova et al. attempted the first phenotypic analysis of these cells, and reported expression of high levels of integrins alpha X (*Itgax*, CD11c), alpha 4 (*Itga4*), alpha 5 (*Itga5*), and beta 2 (*Itgb2*) in microglia from periventricular white matter in comparison to cortical microglia at P7 by staining quantification in IBA1+ cells (37). In addition, *in situ* hybridization clearly showed transient *Igf1* and colony-stimulating factor 1 (*Csf1*) mRNA expression within microglial cells in the corpus callosum and periventricular white matter until approximately two postnatal weeks (37). In this study, expression of *Igf1* and *Csf1* by microglia were hypothesized to play a protective role, preventing axonal damage for instance, which has since then been confirmed in a study by Ueno et al. (12).

This finding was reinforced by our own study showing that microglial cells expressing high levels of *Itgax* and *Igf1* are present in the white matter (cerebellum and corpus callosum) of developing mouse brains particularly between P3 and P5 where they make up almost 20% of all microglia and decrease in numbers already at P7 before being almost completely undetectable by P28 (35). Presence of *Igf1*-expressing microglia in these locations in P5 brains was further confirmed by *in situ* hybridization (69). We performed RNA-seq on these cells between P3 and P5 after FACS-sorting based on $CD45^{dim}$ CD11b+ CD11c+ gating comparing them to their CD11c− counterparts. We identified a robust neurodevelopmental gene signature for developmental CD11c+ microglia, including factors involved in astrocyte and neuronal differentiation, tissue remodeling, and myelinogenesis accompanied by downregulation of immune function-related genes. Of note, *Itgax*, *Itga4*, *Csf1*, and *Igf1*, which were highlighted in the Hristova study, were also part of this signature. Importantly, we demonstrated that *Igf1* expression by CD11c+ microglia during development is crucial for primary myelination. Indeed, selective deletion of *Igf1* specifically from CD11c+ cells led to myelination defects in P21 brains (35). Interestingly, all neonatal microglia expressed neuroectodermal genes including *Nestin*.

A concomitant study by Hagemeyer et al. similarly identified amoeboid microglia in the developing white matter of the corpus callosum and cerebellum particularly between P1 and P8 before being almost undetectable by P14 (70). Interestingly, they used a Mac-3 staining to identify these cells, reminiscent of a study by Valentino and Jones who reported Mac-3 expression in "fountain microglia" in a footnote (68). They identified a signature akin to the one we found (38 genes in common out of 61 upregulated genes including *Itgax*, *Csf1*, and *Igf1*) by comparing "fountain microglia" from corpus callosum at P7 with cortical microglia at the same age by whole-genome microarray (70). Of note, the study underscores that many of the most upregulated genes were related to a primed or activated microglial phenotype and they confirmed CD11c expression in the "fountain of microglia" cells with a reporter mouse. In addition, by depleting all microglia during the critical period of the first postnatal week, they showed that the number of oligodendrocyte progenitor cells was reduced and a long-lasting effect on myelination was induced into adulthood (70), in line with our own results.

Two recent studies used single-cell RNA-seq to elucidate microglial heterogeneity during development (24, 36). The Barres lab study used deep single-cell RNA-seq on microglial cells sorted based on CD11b+ gating and CD45 levels from six different brain regions at E14.5, P7, and P60 (24). They found a cluster of cells they named "proliferative region-associated microglia" (PAM), mainly found at P7 in the white matter, that have an amoeboid morphology and phagocytose newly formed oligodendrocytes (24). In addition, they reported enhanced expression of *Igf1* and *Itgax* in this cluster compared to any other at P7 or other time points. These cells were observed as early as E17.5 in the embryonic brain, their numbers peaking around P7 and were almost absent from P14 brains (24). All these features fit with CD11c+ microglia from our study and the historical "fountain of microglia" cells.

The Stevens lab used high-throughput RNA-seq on microglial cells from the whole brain sorted based on a $CD45^{dim}$ $CD11b^{hi}$ $CX3CR1^{hi}$ gating at E14.5, P4–5, P30, P100, and P540 and in injury contexts, prioritizing high numbers of cells over depth of sequencing (36). They identified a cluster of cells exclusive for the P4–5 time point, which have an amoeboid morphology, express phagocytosis-related genes, and are restricted to the corpus callosum and cerebellum, associating closely with axonal tracts, which they named "axon tract-associated microglia" (ATM) (36). Again, the features of this subset resembled closely the features of CD11c+ microglia and "fountain of microglia" cells described above. Interestingly, their study showed no evidence for a sex bias, the number of cells associated to this cluster being similar for neonatal female and male pups (36).

In addition, Anderson et al. (71) described gene signatures of retinal microglia in P7 mice, 60% of which were found to express CD11c. The microglial signature in the P7 retina fit the signature associated to developmental CD11c+ microglia as *Itgax*, *Lpl*, *Clec7a*, and *Igf1* were enriched in sorted CD11c^{hi} vs. CD11c^{low} cells at P7, whereas *P2ry12* and *Tmem119* were downregulated (71).

We therefore hypothesize that CD11c+ microglia, fountains of microglia, PAMs, and ATMs, although described in different studies by different methods under different names, actually represent the same population of cells. Comparison of the transcriptomic signature found in each of these studies leads to a core signature of 11 genes found in all four studies (*Gpnmb*, *Itgax*, *Spp1*, *Fam20c*, *Fabp5*, *Hpse*, *Igf1*, *Folr2*, *Csf1*, and *Anxa5*) and 28 additional genes found in at least three of these studies (*Atp6v0d2*, *Slpi*, *Cd28*, *Crip1*, *Lgals1*, *Anxa2*, *Vat1*, *Ifitm2*, *Gm1673*, *Plaur*, *S100a1*, *Colec12*, *Clec7a*, *Atf3*, *Atp1a3*, *Ephx1*, *Nceh1*, *Lpl*, *Pld3*, *Plin2*, *Aplp2*, *Ccl3*, *Bnip3*, *Ccl9*, *Gpx3*, *Slc16a3*, *Lag3*, and *Lilrb4*) (**Figure 1**). Interestingly, *Csf1*, one of the genes of the core signature, has been identified as one of the prominent genes characteristic of the pre-microglia homeostatic signature (23). These 39 genes constitute the "developmental signature" of the microglial population described in this section. Of note, homeostatic microglia markers, such as *Tmem119*, *P2ry12*, *Sall1*, *Tgfbr1*, *Fcrls*, and *Cx3cr1*, have been shown to be expressed by this subset, although in most reports at slightly lower levels than in adult microglia or other neonatal microglia (24, 35, 36, 70). Later in this review, we will refer to this population as "developmental CD11c+ microglia". Features of this population include peak numbers between P3 and P7, amoeboid morphology, phagocytic abilities, and location in white matter (**Figure 1**). In addition, studies mentioned in this section clearly reveal a critical functional role of developmental CD11c+ microglia in the myelination process. Their presence in high numbers in the white matter makes them strategically placed in both space and time to take on that role. The aforementioned data support their involvement in phagocytosis of newly formed oligodendrocytes, probably linked to the proper establishment of primary myelination (24, 35, 36, 70). Two of the studies show the long-term importance of these cells on oligodendrocytes and myelination later in life (35, 70).

Although the number of common genes in the developmental signature might appear low, we would argue that this is probably due to discrepancies in the transcriptomic techniques used (microarray, bulk RNA-seq, high-throughput single-cell RNA-seq, deep single-cell RNA-seq), as well as the isolation techniques used (FACS-sorting based on various gatings, presence or absence of perfusion, whole brain dissection, or region microdissection) (see **Table 2**) [discussed in (76)]. However, similarities in the localization, colonization kinetics, morphology, and functional role leave little room for doubt regarding the uniqueness of the population described.

In Adulthood

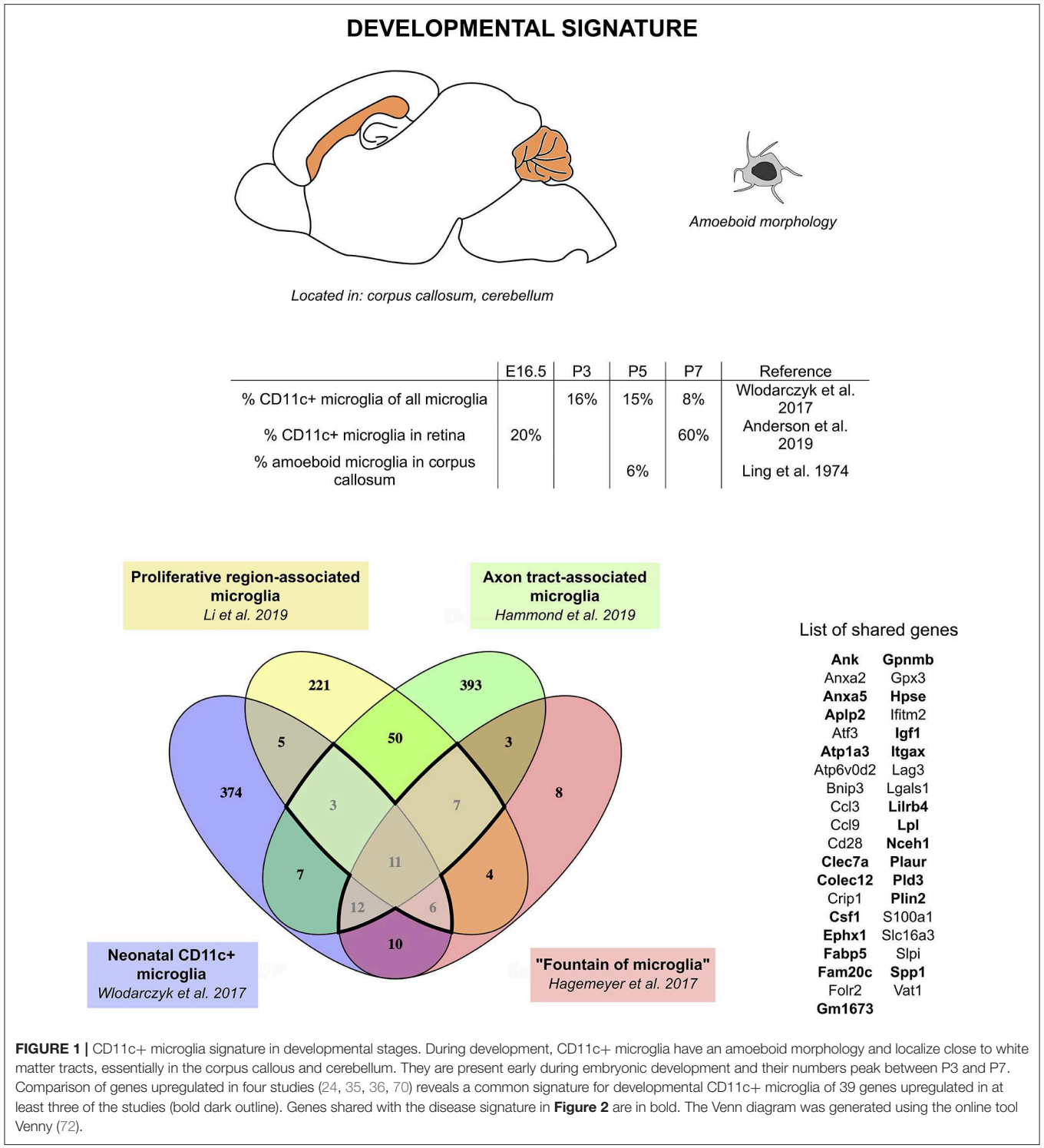
Recent studies have described the homeostatic adult brain as the state with lowest microglial heterogeneity (24). In addition, most high-throughput studies investigating adult microglia in steady state generally report very homogeneous populations in the homeostatic clusters, whether by mass cytometry (77) or single-cell RNA-seq (36), characterized by robust expression of classical microglial homeostatic markers.

However, in a CD11c-eYFP reporter mouse, YFP-expressing cells have been found throughout the brain and retina in adulthood. Although initially thought to be DCs (40), they have since then been shown to exhibit a phenotype resembling microglia (41, 78). Interestingly, a particular abundance of these cells is found in ventral areas of the brain, white matter tracts, and areas of adult neurogenesis (78). This is in line with a report that CLEC7A+ microglia are found in neurogenic niches in the adult mouse (24), showing that in the homeostatic adult brain, microglia with a phenotype similar to developmental CD11c+ microglia could remain in low number in selected areas. Consistent with this, a subset of microglia (also positive for TMEM119 and P2RY12) expressing higher levels of CD11c was found in the human subventricular zone and thalamus (79). In reporter mice, expression of CD11c has been shown to not always follow the expression of the YFP reporter and should therefore be taken cautiously (78). The existence of CD11c-expressing microglia has however been confirmed in the adult homeostatic brain (around 2% of total microglia) (33–35, 42, 80, 81). Similarly, a small population of cells from the choroid plexus of adult mice was shown to be transcriptionally distinct from other choroid plexus cells and border-associated brain macrophages. This population named "Kolmer's epiplexus cells" closely resembles microglial cells and was associated with enriched expression of *Spp1*, *Apoe*, and *Igf1* (82). Although *Itgax* was not among the significantly upregulated genes in this study, CD11c+ cells expressing low levels of CD45 have previously been described in the choroid plexus of adult mice (78).

In Aging

Change in microglial gene expression and phenotype in steady-state aging has been studied extensively. Although reports agree on the changes in morphology and general phenotype of microglia toward dystrophic microglia (deramification, cytorrhexis, and fragmentation) in aging [reviewed in (83)], genomic studies have given discrepant results, with some arguing for shift toward neuroprotection (84) and others highlighting a "primed phenotype" with higher immune activation (85). That said, having a second look at datasets from various studies brings to light common highly expressed genes in aged microglia compared to young microglia: *Spp1*, *Clec7a*, *Igf1*, *Lpl*, *Axl*, *Apoe*, *Lgals3*, *Itgax*, *Cst7*, etc. are indeed found across several studies (84–86), although not all and not always in the same range of upregulation (87). In a later study, Holtman et al. related the "primed" microglial signature they found from two aging models (one physiological aging model and one accelerated aging model) to the study by Hickman et al. and found a high correlation between the datasets (88).

High-throughput single-cell methods are a good way to decipher complex populations with mixed subsets. A mass-cytometry study revealed that a specific subset of microglia emerges during aging that overexpresses surface CD11c and CD14, CLEC7A, and CD68 as compared to other microglia



	E16.5	P3	P5	P7	Reference
% CD11c+ microglia of all microglia		16%	15%	8%	Wlodarczyk et al. 2017
% CD11c+ microglia in retina	20%			60%	Anderson et al. 2019
% amoeboid microglia in corpus callosum			6%		Ling et al. 1974

FIGURE 1 | CD11c+ microglia signature in developmental stages. During development, CD11c+ microglia have an amoeboid morphology and localize close to white matter tracts, essentially in the corpus callous and cerebellum. They are present early during embryonic development and their numbers peak between P3 and P7. Comparison of genes upregulated in four studies (24, 35, 36, 70) reveals a common signature for developmental CD11c+ microglia of 39 genes upregulated in at least three of the studies (bold dark outline). Genes shared with the disease signature in **Figure 2** are in bold. The Venn diagram was generated using the online tool Venny (72).

at the same age, although they downregulate CX3CR1 and MERTK (77, 89). CD11c expression of microglia in the white matter and caudal areas of the CNS of aged mice was also shown using immunohistochemistry (90). This study also reports expression of CLEC7A in white matter tracts of aged animals and reports numerous changes in white matter microglia associated with aging. Similarly, single-cell RNA-seq revealed that several populations of microglia that were present in younger age at very low numbers become increasingly prevalent with aging. One of these populations (referred to as OA2) is characterized by genes from the developmental signature and genes classically associated with neurodegeneration (*Spp1*, *Lpl*, *Lgals3*, *Lilrb4*,

TABLE 2 | Specification of studies used to establish the signatures.

	References	Condition	Tissue	Isolation technique	Transcriptomic technique
Development	(35)	P4–6	Whole brain	**FACS** $CD45^{dim}CD11b^{+}CD11c^{+}$ *compared to* $CD45^{dim}CD11b^{+}CD11c^{-}$	**RNA-seq** Illumina HiSeq 2500
	(70)	P7	Cortex *compared to* Corpus callosum	**FACS** $CD45^{dim}CD11b^{+}Gr1^{-}$	**Microarray** Affymetrix Mouse Gene 2.0 ST Arrays
	(24)	P7	Cortex Cerebellum Hippocampus Striatum Olfactory bulb Choroid plexus	**FACS** $CD45^{+}CD11b^{+}$	**Single cell RNA-seq** Smart-seq 2 Illumina NextSeq
	(36)	P4–5	Whole brain	**FACS** $CD45^{dim}CD11b^{+}CX3CR1^{+}$	**Single-cell RNA-seq** Chromium (10× genomics) Illumina NextSeq 500
Diseases	(73)	APP/PS1 18 months	Cortex	**MACS** $CD11c^{+}$ sorted fraction *compared to* $CD11c^{-}$ eluted $CD11b^{+}$ sorted	**Microarray** Agilent Technologies Mouse GE 4x44k V2 microarrays
	(74)	5XFAD 6 months	Whole brain	**FACS** $CD45^{+}$	**MARS-seq** Illumina NextSeq 500
	(75)	APP/PS1 9 months	Whole brain	**FACS** $FCRLS^{+}CLEC7A^{+}$ *compared to* $FCRLS^{+}CLEC7A^{-}$	**RNA-seq** Illumina NextSeq 500
	(35)	Symptomatic EAE	Whole brain	**FACS** $CD45^{dim}CD11b^{+}CD11c^{+}$ *compared to* $CD45^{dim}CD11b^{+}CD11c^{-}$	**RNA-seq** Illumina HiSeq 2500

Cst7, *Apoe*, *Fam20c*, *Anxa5*, *Plaur*, *Aplp2*, etc.) among others (36). By showing the existence of a mix of different microglial subsets in the context of aging, this study helps us understand the seemingly discrepant results obtained by bulk RNA-seq performed on whole brain microglia during aging.

EVIDENCE FOR CD11c+ MICROGLIA SIGNATURE IN REPOPULATION STUDIES

Under homeostatic conditions, microglia are long-lived, self-renewing cells. Although some studies suggest that microglia persist throughout the life of an individual (91), others show that their turnover rate is quite fast, at around 1% per day in the mouse (92, 93) and 28% per year in the human (94). Regardless, their relatively long lifespan has been proposed to be crucial in microglial priming and ultimately contributing to neurodegeneration (91). Similarly, microglia have been found to be detrimental in some disease contexts [reviewed in Wolf et al. (95)], leading researchers to entertain the idea of transient microglial depletion as a therapeutic strategy (96, 97). Indeed, the depleted microglial niche gets repopulated within a couple of weeks post-depletion (98, 99). It is not yet resolved whether this repopulation occurs from peripheral cells or from a local microglial progenitor, and whether this progenitor is Nestin-positive. Such depletion strategies have had either beneficial or detrimental outcomes, depending on the pathology and the depletion method [reviewed in Han et al. (100)]. More recently, studies have characterized repopulating microglia, to assess whether and how they differ from the original microglia and whether these differences could account for the positive outcomes of microglial depletion strategies. Although morphological differences have been reported (101), most studies focused on gene expression analysis (98, 101–104). Two of the early studies advocated for repopulating microglia being functionally similar to resident control microglia (98, 101). However, closer examination and more recent studies, including single-cell RNA-seq, suggest that these cells differ transcriptionally (98, 102–104). Interestingly, Zhan et al. compared the repopulating microglial signature to the neonatal microglial signature (104), putting forward the idea that newly formed microglia resemble developmental microglia, before adopting a more mature phenotype. When comparing their transcriptomic data to the CD11c+ neonatal microglia signature we describe above, we found nine overlapping genes (*Atp6v0d2*, *Clec7a*, *Spp1*, *Lgals1*, *Gm1673*, *Gpnmb*, *Atp1a3*, *Itgax*, and *Ank*). Similarly, seven genes (*Atp6v0d2*, *Spp1*, *Igf1*, *Gpx3*, *Gpnmb*, *Ccl3*, and *Lpl*) overlapped with the repopulating microglial

signature from Bruttger et al. (98), possibly indicating the presence of CD11c+-microglia-like cells in the repopulating clusters they described. This is reinforced by our study in which CD11c+ microglia could be found in repopulating microglia clusters after genetic microglial depletion (35). However, in contrast to Zhan et al. our analysis did not show neonatal-like, neurodevelopmental gene signature in repopulated microglia (35). The low extent of overlap between these studies and our newly defined neonatal CD11c+ microglia could be explained by heterogeneity of repopulating microglia, diluting the signal from CD11c+ microglia in bulk RNA-seq studies.

CD11c+ MICROGLIA IN DISEASE STATES

Microglia activation is a common feature in many neurological disorders including inflammatory, demyelinating, and degenerative diseases, as well as glioma and injury. Although microglia activation may have deleterious consequences, it has also been shown in many instances to exert protective and regenerative effects. It is now becoming clear that there is an emergence of CD11c+ microglia population in pathological conditions. In this section, we will discuss the importance and the role of this cell subset in several neurological diseases.

Alzheimer's Disease

For decades, it has been known that microglia localize around Aβ plaques, and engulf Aβ in AD, showing their importance in the disease. In recent years, interest in these cells has increased, largely due to a wave of transcriptomic and genome-wide association (GWAS) studies. In addition, a majority of AD risk genes are related to microglia, including triggering receptor induced on myeloid cells 2 (TREM2) [reviewed in McQuade and Blurton-Jones (105)]. Despite the enormous amount of data generated, no consensus has yet been reached on whether microglia are protective or detrimental in neurodegeneration. Some of the attempts to resolve this issue involved comparing transcriptomes of microglia sorted from healthy, aged, and diseased brains. The study by Holtman et al. cited above identified a microglial signature found not only in aging models but also in disease models including the APP/PS1 AD model and the SOD1 model for amyotrophic lateral sclerosis (ALS) (88). The common genes included *Itgax*, *Clec7a*, *Axl*, *Lgals3*, and *Apoe*, indicating the presence of a CD11c-expressing microglial population in these models. The gene module described in this study mostly contained genes related to phagocytosis and cell proliferation, with tissue protective elements (88). With a similar strategy, other studies demonstrated that microglia from aging brains and from amyloidosis (APP/PS1) and tauopathy (AAV-Tau P301L) shared a common gene signature including *Cst7*, *Itgax*, *Gpnmb*, *Clec7a*, *Lpl*, *Lgals3*, *Apoe*, and *Spp1* (86). Similar results were also obtained by Krasemann et al. in the APP/PS1 model. Such shared microglial characteristics led to the term "microglial neurodegenerative phenotype (MGnD) signature" (75). This is also in line with the presence of CD11c-expressing microglia in these models, with a phenotype similar to the one found in physiological aging.

The presence of CD11c+ microglia around Aβ plaques has been shown in several studies (30, 73, 74, 106, 107). A recent study by Kamphuis et al. extensively investigated the localization, proliferation status, and transcriptome of CD11c+ vs. CD11c− microglia in APP/PS1 mice (73). Importantly, this study also highlighted a steady increase in CD11c transcripts in brains of APP/PS1 and 3xTg-AD mice with aging as plaques appear, as well as in hippocampal samples from AD patients, although it declines in the later stages of the disease (73). The transcriptomic signature of CD11c+ microglia, when compared to their CD11c− counterparts, showed increased expression of *Gpnmb*, *Fabp5*, *Spp1*, *Igf1*, *Itgax*, *Gm1673*, *Cst7*, *Cox6a2*, *Apoe*, *Ch25h*, *Clec7a*, *Lilrb4*, *Csf1*, *Axl*, *Lpl*, *Sulf2*, *Egr2*, *Anxa5*, *Cd68*, *Timp2*, and *Ctsb* among others. Many of these genes are common with the developmental signature of CD11c+ microglia described above or with the signatures found in whole brain "primed" microglial signatures (73). These findings further support that the "primed" microglia phenotype described in many studies recapitulates the CD11c+ microglia signature diluted among CD11c− counterparts. The robustness of the signature is hardly surprising, considering that CD11c+ microglia make up for 23% of all Iba1+ cells in the aged APP/PS1 brain (73). Of note, strong upregulation of some CD11c+ microglia signature genes, including *Itgax*, *Clec7a*, and *Cst7*, was even detectable in whole tissue samples from cortex and hippocampus in AD models (73, 108).

High-throughput single-cell studies also contributed to our understanding of microglial populations in AD rodent models. The same study that identified CD11c and CD14 surface expression by mass cytometry on a microglia population emerging in aging also identified a similar population in APP/PS1 brains (77). Single-cell RNA-seq studies identified three microglial signatures in neurodegeneration models: the disease-associated microglia (DAM) signature (74), the late response microglia signature (109), and the activated response microglia (ARM) signature (80) that emerge in the 5xFAD, CK-p25, and APP^{NL-G-F} models for AD, respectively. All three studies described cell clusters showing nearly identical microglia populations, similar to the CD11c+ microglia signature observed in the Kamphuis study. Importantly, all of the DAM cells were CD11c+ (74) with highly overlapping gene signatures uncovered by bulk sequencing of sorted CD11c+ microglia (73). Microglia with characteristics from the ARM cluster are present in low numbers (ca. 2%) even in wild-type mice at young age, increasing as part of normal aging to reach up to about 12% of all microglia (80), consistent with observations discussed above of CD11c+ microglia in the steady state in adult and aging mice. ARM microglia are however most evident in APP^{NL-G-F} mice where they outnumber all other microglial clusters reaching 52% of all microglia at 21 months of age (80). This is in line with increases in CD11c+ microglia reported in other studies. Importantly, the signature observed in CD11c+/DAM/MGnD/ARM microglia is enriched for known AD risk genes (80). Of note, this transcriptomic signature is similar to that induced by retinal degeneration (110).

CD11c+ microglia have been demonstrated to be beneficial for and to correlate with increased Aβ uptake and induction of

IGF1-mediated neurogenesis in an animal model of AD (30). In addition, abundance of *Igf1*-expressing microglia around Aβ plaques was recently confirmed by *in situ* hybridization in an AD model (69). Functional analyses led to discrepant results suggesting either protective, immunosuppressive function as well as enhanced capacity for uptake and lysosomal degradation of Aβ (73), or pathogenicity via possible contribution to local arginine deprivation and subsequent neurodegeneration (111). Butovsky's group also proposed a detrimental role for these cells due to ameliorated Aβ deposition in 4-month-old TREM2-deficient mice that lack CD11c+ microglia (75). However, the role of TREM2 is not clear, since other data show either protective or detrimental roles for this protein depending on the age of the animals (75, 112–114). Nonetheless, all these studies demonstrate lack of microglial proliferation and clustering around plaques in TREM2-deficient animals, thus allowing for more dispersed Aβ localization in AD models (75, 112–116). This can be detrimental due to Aβ spreading that is not limited by microglia clusters, ultimately leading to severe axonal dystrophy (114). Moreover, it has been demonstrated that in TREM2-deficient animals older than 8 months, the Aβ burden is enhanced as compared to 4-month-old animals, suggesting that TREM2 signaling is necessary for limiting advanced stage pathology (117). Thus, CD11c+ microglia may actually be beneficial and protective in later stages of the disease as proposed by Keren-Shaul et al. (74). Human data further support this hypothesis since loss-of-function mutations in TREM2 have been identified as a strong risk factor for the development of AD and other neurodegenerative diseases [reviewed in McQuade and Blurton-Jones and Ulland and Colonna (105, 118)].

Collectively, CD11c+ microglia (also referred to as primed microglia, late response microglia, DAM, ARM, or MGnD) are a well-defined population of cells that show adaptation predominantly for phagocytic clearance of apoptotic/necrotic neurons and limiting Aβ spreading. Given that AD risk genes are enriched in this population (80), mutations in such genes may have an impact on the ability of CD11c+ microglia to cope with Aβ plaque burden, either promoting or limiting AD pathology.

Amyotrophic Lateral Sclerosis

ALS is a disease affecting motor neurons leading to their degeneration. Microglial contribution to the disease has been established since a robust microglial activation has been found in both patient and transgenic mouse tissue (119, 120). In addition, many risk factors for the disease have been shown to be expressed by microglia in the CNS, reinforcing the idea of an involvement of these cells in the disease (121). Microglial activation in the disease arises from accumulation of misfolded protein, and, similarly to observations made in other disease contexts, microglia have been reported to play a beneficial role in the pre-symptomatic phase of the disease before shifting to detrimental roles in the advanced disease state (122). However, microglial depletion in the context of ALS has not been found to increase survival (123), leading to the idea that both functions might be concomitant, constantly counteracting each other. Interestingly, a study from 2013 analyzed the transcriptome of microglia sorted from mice carrying an ALS-associated mutation and found a particular signature for these cells at the end stage of the disease compared to microglia from healthy brains (124). Once again, among the top regulated genes were genes related to Huntington's disease, AD, and Parkinson's disease (*Mapt*, *Psen2*, *Apoe*, etc.). The signature found in this study includes both factors reported to be beneficial in the context of ALS (*Igf1*, *Grn*, *Trem2*, *Tyrobp*, etc.), and factors known to be detrimental (*Mmp12*, *Optn*, *Cybb*, etc.), as well as some like *Spp1*, *Gpnmb*, and *Itgax* recurrently found in neurodegenerative diseases. Microglia were also found to upregulate surface CD11c. Microglia from SOD1 mice were also found to fit the abovementioned MGnD signature, in addition to expressing *Clec7a* levels increasingly during disease progression (75).

Stroke, Ischemia, and Injury

Neuron degeneration and nerve injury have been linked to microglia in various models for traumatic brain injury (TBI) (125), spinal cord injury (SCI) (126), nerve injury (93), and ischemic stroke (127). Much like in inflammation models, microglial contribution in all of these models is still rather unclear and they may play a double role considering their association with both beneficial and detrimental effects. Studying microglia in context of inflammation can get quite complicated due to massive infiltration of peripheral immune cells, notably monocytes and macrophages, occurring subsequently to TBI (128), SCI (129), and stroke (127, 130, 131). In a study comparing the transcriptomics of microglia and macrophages after ischemia in rats, it was reported that microglia played a detrimental role and macrophages played a beneficial role with regard to recovery, based on their expression of classical inflammation markers (132). Investigation of the genes enriched in microglia three days after middle cerebral artery occlusion compared to sham controls, however, revealed *Spp1*, *Gpnmb*, *Lgals3*, *Fabp5*, and *Axl* among others, fitting with the potential presence of CD11c+ microglia-like cells in this context, diluted among other microglia. Consistent with this, *Ccl2* mRNA was found to be increased in microglia and macrophages at this time point (132), an aspect that has been associated with the emergence of CD11c+ microglia (81). Another study, conducted in a model of phototrombic stroke on whole tissue, actually showed upregulation of *Gpnmb*, *Itgax*, and *Clec7a* in a cluster associated with early response (133), which the authors related to the DAM phenotype (74). In a study of facial nucleus axotomy, the authors also related the observed microglial phenotype (134) to the DAM phenotype, as well as to a phenotype found in the Ck-p25 model (109): 72 genes were regulated in common between all three studies representing almost 75% of all genes upregulated in the facial nucleus axotomy model. Interestingly, in an SCI transcriptomic study, a profile of microglia reminiscent of the CD11c+ phenotype was identified (with upregulation of *Gpnmb*, *Spp1*, *Lpl*, *Apoe*, *Igf1*, *Lgals3*, and *Itgax* among others) and persisted in a full transection model, whereas it contracted concomitantly to recovery in a hemisection model (135), indicative of the transitory nature of this subset. Conversely, in TBI, the microglial signature was further from the CD11c+ microglia signature,

although *Itgax* was among the upregulated genes 14 and 60 days post-injury, possibly indicating once again a dilution of the signature in all microglia (136). In addition, considering the difficulty associated with gating out macrophages from microglia in a context of extensive infiltration, macrophage contamination of the sorted samples cannot be excluded in these studies, potentially complicating interpretation of the observed transcriptomes.

Multiple Sclerosis

MS is an inflammatory, demyelinating disease of the CNS that can be modeled by EAE or toxin-induced demyelinating models. Recent advancement in our understanding of the disease points toward important roles for microglia in the pathomechanism. Although the evidence supporting their implication in initiation and facilitation of the disease is strong (95), there is a growing body of evidence for their protective functions including involvement in remyelination (137).

We have identified CD11c+ microglia during EAE accounting for around 10% of total microglia in whole CNS (33, 34). Of note, this subset is even more abundant in the spinal cord at the peak of the diseases reaching up to 60% of total microglia (Wlodarczyk, unpublished). The emergence of the CD11c+ microglia is a dynamic process starting at the onset, reaching a maximum at the peak and contracting in the chronic phase of EAE (77, 138). These cells are localized in the demyelinated spinal cord lesions (33). CD11c+ microglia from EAE again showed upregulation of similar genes as in neurodegenerative models including *Itgax*, *Gpnmb*, *Spp1*, etc. (35). A similar signature was confirmed by Krasemann et al. (75). In addition, deep analysis of genes that were upregulated in CD11c+ microglia population pointed to their involvement in immune responses (35).

A key aspect of neuroinflammation in EAE is the recruitment and reactivation of encephalitogenic T cells to express their effector functions. Many cell types are implicated in this process, including blood-derived DCs and monocytes/macrophages but also parenchymal microglia (139). In EAE, CD11c+ microglia express MHCI, MHCII, and costimulatory molecules CD80/CD86 (34, 140), which is in line with recent high-throughput mass-cytometry reports (77, 138). We have provided evidence that CD11c+ microglia are able to induce similar proliferative response of encephalitogenic CD4+ T cells as blood-derived professional antigen-presenting cells (32, 34). Interestingly, in contrast to CD11c+ blood-derived cells and CD11c− microglia, CD11c+ microglia completely lacked mRNA expression for IL-23 (34) that is known to induce GM-CSF-producing CD4+ T cells, critical for EAE pathology (141). This indicates that although CD11c+ microglia alone might contribute to T cell expansion, they are unlikely to induce pathogenic T cell responses. Importantly, a subsequent study showed that they were a major source of message for myelinogenic IGF1, suggesting that they might exert protective roles in EAE (33). This is supported by our recent study showing that stimulation of CSF1R with its ligands during symptomatic EAE significantly reduced demyelination and ameliorated disease progression most likely through induction of CD11c+ microglia (81). Moreover, decreasing CD11c+ microglia by blocking of TREM2 signaling (as discussed below) led to increased severity of EAE and exacerbated demyelinating lesions in the spinal cord (142), further supporting protective roles of CD11c+ microglia.

Microglia are known to contribute to remyelination by creating an environment supporting OPC recruitment and differentiation by phagocytosing myelin debris, secreting growth factors and modulating extracellular matrix [reviewed in Lloyd and Miron (137)]. Circumstantial evidence for remyelinating properties of CD11c+ microglia includes our first demonstration of the expansion of these cells in cuprizone-demyelinated corpus callosum (32). A microarray study by Olah et al. identified a pro-remyelinating microglial signature that includes several genes reminiscent of the CD11c+ microglia characteristics described above (*Itgax Igf1*, *Clec7a*, *Apoe*, *Spp1*) (143). Moreover, CD11c immunoreactive microglia were present in remyelinating corpus callosum (32). A similar microglial signature was later confirmed in both demyelination and remyelination phases (144). Conversely, microglia expressing the CD11c+ microglia signature including *Apoe*, *Axl*, *Igf1*, *Lyz2*, *Itgax*, and *Gpnmb* were identified by single-cell transcriptomics in both de- and remyelinated lesions (145). Recently, cuprizone-mediated demyelination was shown to be alleviated in mice lacking microglial SIRPα that have increased numbers of CD11c+ microglia, pointing to their protective role (89). In line with the induction of CD11c+ microglia (81), stimulation of CSF1R ameliorated cuprizone-induced demyelination (146).

Another line of evidence comes from the influence of TREM2 deficiency, which leads to absence of CD11c+ microglia in adult mice (74, 75), on remyelination after cuprizone demyelination. The data indicate that TREM2 deficiency had no impact on the initial demyelination, but affected subsequent remyelination when the cuprizone treatment was prolonged, most likely by impairing myelin removal as well as myelin regeneration, which further supports a protective role for CD11c+ microglia in this paradigm (144, 147). Additionally, it was reported that microglial necroptosis in circumstances of lysophosphatidylcholine demyelination leads to repopulation by pro-regenerative CD11c+ microglia, as blocking of this mechanism prevented remyelination (148). Of note, demyelination induced by mouse hepatitis virus also led to enrichment of CD11c+ microglial gene signature in the spinal cord (149).

Taken together, association of CD11c+ microglia to white matter (89) as well as their role in primary myelination strongly support their importance in induction and facilitation of remyelination. This opens the possibility for induction of innate repair programs in diseased CNS via promotion of the emergence of CD11c+ microglia.

Glioma

Very early studies identified microglial cells close to gliomas to resemble the amoeboid form described during development and to take on phagocytic functions (58). More recent studies have shown that parenchymal microglia are attracted to the tumor in glioma-affected brains, representing up to 30% of the tumor mass (150). Microglia associated to the tumor have been termed glioma-associated microglia/macrophages (GAM). These

cells initially exhibit beneficial anti-tumor abilities but have been found to be hijacked by the tumor to exert tumor-promoting functions [reviewed in Li and Graeber (151)]. A study from 2015 identified a signature for GAMs, and emphasized their high expression of SPP1 and GPNMB (152). They compared this signature to classical macrophage activation markers (M1/M2) and concluded a lack of overlap between the GAM signature and these classical phenotypes. Of note, the signature also includes genes such as *Itgax*, *Fabp5*, and *Clec7a* among others recurrently found in disease signatures (152).

Microglial Disease Signature

Considering the similarities observed in gene expression from the different studies aforementioned, we compared the transcriptomic signatures obtained in studies comparing specifically microglia sorted based on a typical marker for this specific subset of microglia or from single-cell RNA-seq (three of the AD studies and one EAE study, **Figure 2**). We found a core disease signature for microglia consisting of 89 genes shared between all four studies (**Figure 2**). *Itgax* being once again a part of this signature and with clarity in mind, we will refer to this signature as the "CD11c+ microglia disease signature" henceforth. Once again, the microglial nature of this subset is supported by expression, although slightly lower than in homeostatic microglia, of *Tmem119*, *Cx3cr1*, *P2ry12*, *Sall1*, and *Tgfbr1* among other homeostatic genes (35, 73–75).

CD11c+ MICROGLIA SIGNATURE

Over the years, advancements in technology have allowed the scientific community to investigate cells and cell populations in increasingly detailed ways, particularly at the molecular level. This investigation has been done using a multiplicity of different conditions and models, leading to increasing amounts of data generated. Although invaluable, this work has also led to redundancy in the microglial profiles that were identified (154).

Our investigation led us to define two particularly strong signatures for CD11c+ microglia in development (**Figure 1**) and in disease (**Figure 2**). Interestingly, Li et al. (24) as well as Anderson et al. (71) related the developmental microglia signature observed in their studies to the DAM microglial signature. These similarities prompted us to compare the signatures we identified from the literature.

Comparison of the developmental signature and the disease signature resulted in defining of a "core" signature common to CD11c+ microglia across all contexts, which consists of 22 genes: *Ank*, *Anxa5*, *Aplp2*, *Atp1a3*, *Clec7a*, *Colec12*, *Csf1*, *Ephx1*, *Fabp5*, *Fam20c*, *Gm1673*, *Gpnmb*, *Hpse*, *Igf1*, *Itgax*, *Lilrb4*, *Lpl*, *Nceh1*, *Plaur*, *Pld3*, *Plin2*, and *Spp1* (**Figure 3** and **Supplementary Table 1**). Interestingly, the protein network linked to these genes had significantly more links than what can be expected, indicating at least a partial biological connection between these genes (**Figure 3**). Further investigation of the physiological function of the proteins related to the genes present in the core signature revealed their involvement in lipid metabolism, cell migration and proliferation, and, to a lesser extent, immune function (**Supplementary Table 1**). As expected, all of these proteins had been associated with various brain diseases (**Supplementary Table 1**). Of note, many of these proteins assume similar function or have been found to interact directly or indirectly with each other (**Supplementary Table 1**). Further investigation of these genes and proteins in link with one another would most likely unveil interesting mechanisms underlying CD11c+ microglia function.

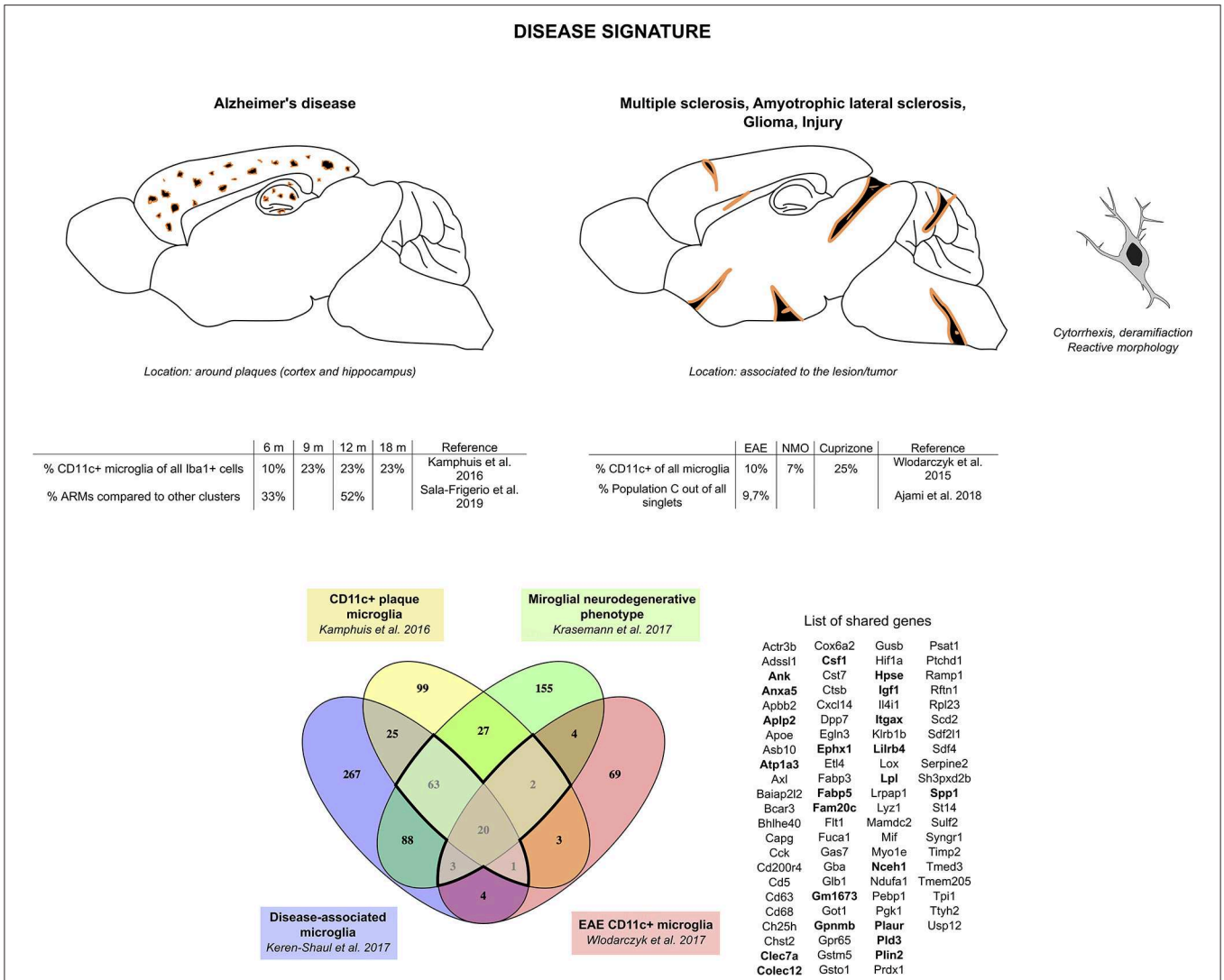
Although described previously as different microglial subsets, we argue that the robust core signature we have identified can be found for this subset across all these different stages. We suggest that the differences in this subset observed between conditions reflect methodological discrepancies (**Table 2**) or microenvironment-linked context-specific changes and the subset's own phenotypic plasticity in coping with these variations, rather than fundamental differences in cell lineage.

EMERGENCE OF CD11c+ MICROGLIA

The dynamics of CD11c+ microglia seem tightly spatio-temporally regulated. They first emerge during the first postnatal week, peaking at P5 and gradually decreasing as animals age, being barely detectable in the healthy adult CNS (33–35, 42, 80, 81) to increase again in aging or disease (33, 73, 81, 85, 89). Importantly, none of the studies that have investigated induction of inflammation by means of lipopolysaccharide, poly(I:C), or other immune challenges could recapitulate the robust CD11c+ signature found in steady state and disease and injury contexts (86, 88, 90, 124, 155, 156). Below, we present factors that participate in controlling the induction of this population (**Figure 4**).

Activation of the TREM2–APOE Pathway

One candidate that has been extensively studied with regard to CD11c+ microglia is the TREM2 pathway. TREM2-deficient animals were shown to downregulate the CD11c+ microglia signature in cuprizone-induced demyelination (144) and in an AD model (113). In addition, in the study from the Amit lab, TREM2 deficiency in an AD mouse model led to an arrest of microglia in an intermediate state between the homeostatic state and the CD11c+ microglia stage. Barely any microglia in these mice exhibited the CD11c+ microglial signature (74). This suggests that CD11c+ microglia induction is a two-step process, where the first step, to leave the homeostatic state, is TREM2-independent and the second step, to reach the complete CD11c+ microglia phenotype, is TREM2-dependent. These observations were confirmed by Krasemann et al. in another TREM2-deficient AD model (75). Similarly, APOE-deficient mice exhibit lower numbers of CD11c+ microglia in AD, ALS, and MS mouse models (75, 80). This is suggestive of a positive feedback loop, as this population itself strongly upregulates APOE (75). Surprisingly, the Barres lab showed that induction of CD11c+ microglia during postnatal development in contrast to adulthood is TREM2–APOE-independent (24). A similar TREM2 independence of CD11c+ microglia induction was shown in the developing retina (71).



	6 m	9 m	12 m	18 m	Reference
% CD11c+ microglia of all Iba1+ cells	10%	23%	23%	23%	Kamphuis et al. 2016
% ARMs compared to other clusters	33%		52%		Sala-Frigerio et al. 2019

	EAE	NMO	Cuprizone	Reference
% CD11c+ of all microglia	10%	7%	25%	Wlodarczyk et al. 2015
% Population C out of all singlets	9,7%			Ajami et al. 2018

FIGURE 2 | CD11c+ microglia signature in disease states. In diseased CNS, CD11c+ microglia adopt an amoeboid, reactive morphology. In AD, they are found surrounding Aβ plaques. Similarly, in MS and ALS models and in injury, they are found around and in the lesions. In glioma, they are found mixed with tumor cells. CD11c+ microglia numbers in diseased CNS vary considerably, ranging from 10 to 50% of all microglia. Comparison of genes upregulated in four studies (35, 73–75) reveals a common signature for CD11c+ disease microglia of 89 genes upregulated in at least three of the studies (bold dark outline). Genes shared with the developmental signature in **Figure 1** are in bold. Raw data for the Krasemann study were obtained using the Gene Expression Omnibus Database and the differential gene expression analysis was performed using the DEBrowser package in R (153). The Venn diagram was generated using the online tool Venny (72).

Cell Death

Krasemann et al. highlighted phagocytosis of apoptotic neurons and monocytes as a trigger for the induction of the CD11c+ microglia phenotype (75). Of note, induction of this phenotype was not observed upon microglia exposure to *Escherichia coli*, zymosan particles (75), or microparticles (Marczynska et al., unpublished), suggesting that induction of CD11c+ microglia is a tightly controlled reaction to local cell damage or apoptosis, rather than to phagocytosis itself. Interestingly, microglial necroptosis in demyelination models leads to brain repopulation with CD11c+ microglia from nestin+ resident microglia (148). Similarly, nestin+ microglia colonizing the brain after microglia ablation expressed surface CD11c (98). The gene expression in repopulating microglia highly overlapped with the CD11c+ microglia signature. We showed that genetic or toxin-induced ablation of neonatal CD11c+ cells led to their instant repopulation (35). Whether the observed concomitant decrease of CD11c− microglia (35) reflects induction of CD11c+ phenotype in CD11c− cells by phagocytosis of dying microglia has not been determined. Interestingly, a dramatic decrease in CD11c+ microglia was observed in the postnatal retina of mice deficient in *Bax*, a pro-apoptotic gene that is essential for developmental death of neurons (71). This emphasizes that apoptotic cells are a strong and common inducer of CD11c+ microglia regardless of age and condition. This is also in line with several studies where developmental cell death has been linked

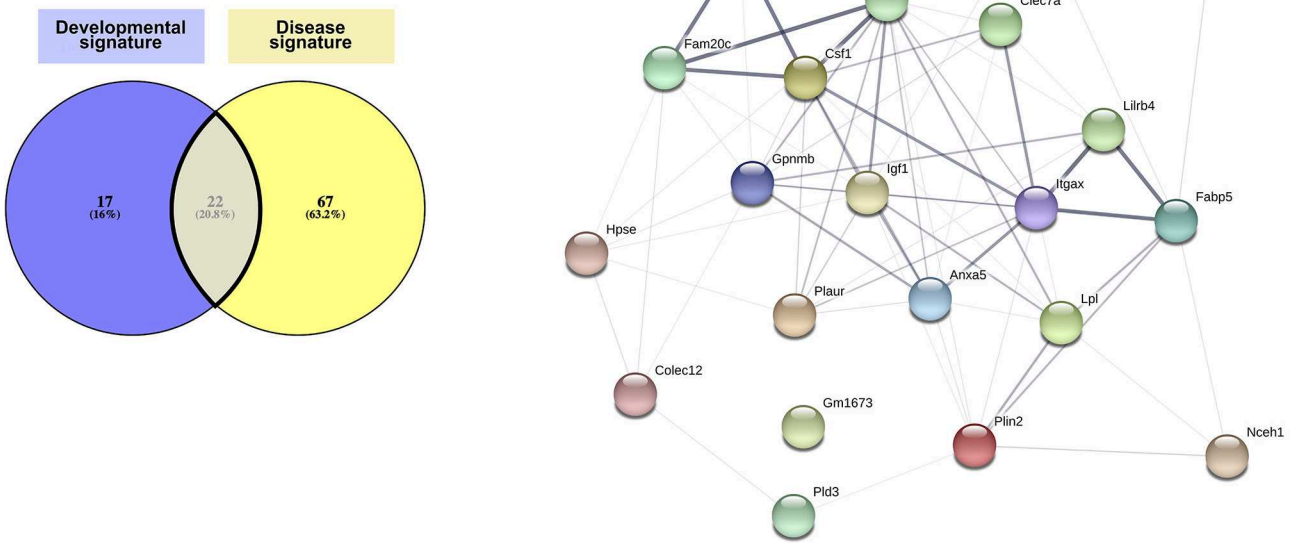


FIGURE 3 | Core CD11c+ microglia signature. Considering similarities between the transcriptomic signatures and functions in the CD11c+ microglia subset in development and in disease, we compared both signatures to obtain a core of genes upregulated in this subset across all conditions. We observe overlap of 20% of the genes between both signatures, corresponding to 22 shared genes. Upon interrogating the STRING database (Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, von Mering C. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019 Nov; 47:D607–613.), we observed that the network formed by the proteins corresponding to the genes in the core signature had significantly more interactions than expected from a similar set of random proteins, indicating that these proteins related to the genes in the core signature are at least partially biologically connected. The thickness of the edges linking the different genes is proportional to the strength of the evidence linking the two proteins. The Venn diagram was generated using the online tool Venny (72).

to microglial entry in the developing CNS (61). In addition, retinal CD11c+ microglia were resistant to depletion induced by either CSF1R deficiency or blocking, contrary to their CD11c− counterparts. In line with this, our own data showed that despite using several depletion regimens, CD11c+ microglia could not be depleted from postnatal brain as they were immediately repopulated (35).

Cytokines

We have shown that both populations of adult microglia (CD11c+ and CD11c−) express equal levels of CSF1R (33). Importantly, stimulation of this receptor by its ligands, interleukin (IL)-34 and CSF1, induced a significant increase in CD11c+ microglia numbers, with faster kinetics for IL-34 (81). Moreover, such stimulation induced CCL2 in the brain, and we showed that overexpression of CCL2 leads to a dramatic expansion of CD11c+ microglia in a CCR2-independent manner (81).

Butovsky et al., on the other hand, showed that another cytokine, IL-4, can induce CD11c+ expression on Aβ pretreated microglia (30, 157). Moreover, they demonstrated that GA vaccination leads to an increase of CD11c+ microglia surrounding Aβ plaques and suggested that this was induced by T-cell-derived IL-4 (30).

Inhibition of SIRPα/CD47 Signaling

Recently, the emergence of CD11c+ microglia in the adult brain has been shown to be homeostatically controlled by SIRPα/CD47 interaction. Genetic ablation of SIRPα in microglia or global lack of CD47 equally resulted in increased numbers of CD11c+ microglia, suggesting that microglial SIRPα suppresses CD11c expression in the same cells (89).

CONCLUSION

Here, we have demonstrated that the subpopulation of microglia described in many recent studies (and named PAM, ATM, fountain of microglia, DAM, ARM, MGnD, and late response microglia) indeed reflects the characteristics of CD11c+ microglia, originally identified over a decade ago. Thus, we believe that a unification of the nomenclature by referring to the microglial subset expressing the described signature, from development to old age, as CD11c+ microglia is a necessary step to progress our understanding of microglia biology. This subset emerges in development before contracting during adulthood but is triggered to re-emerge in aging as well as in the context of disease or tissue injury (**Figure 4**). The summary of the data that mentioned microglia showing the aforementioned signature strongly points to the importance

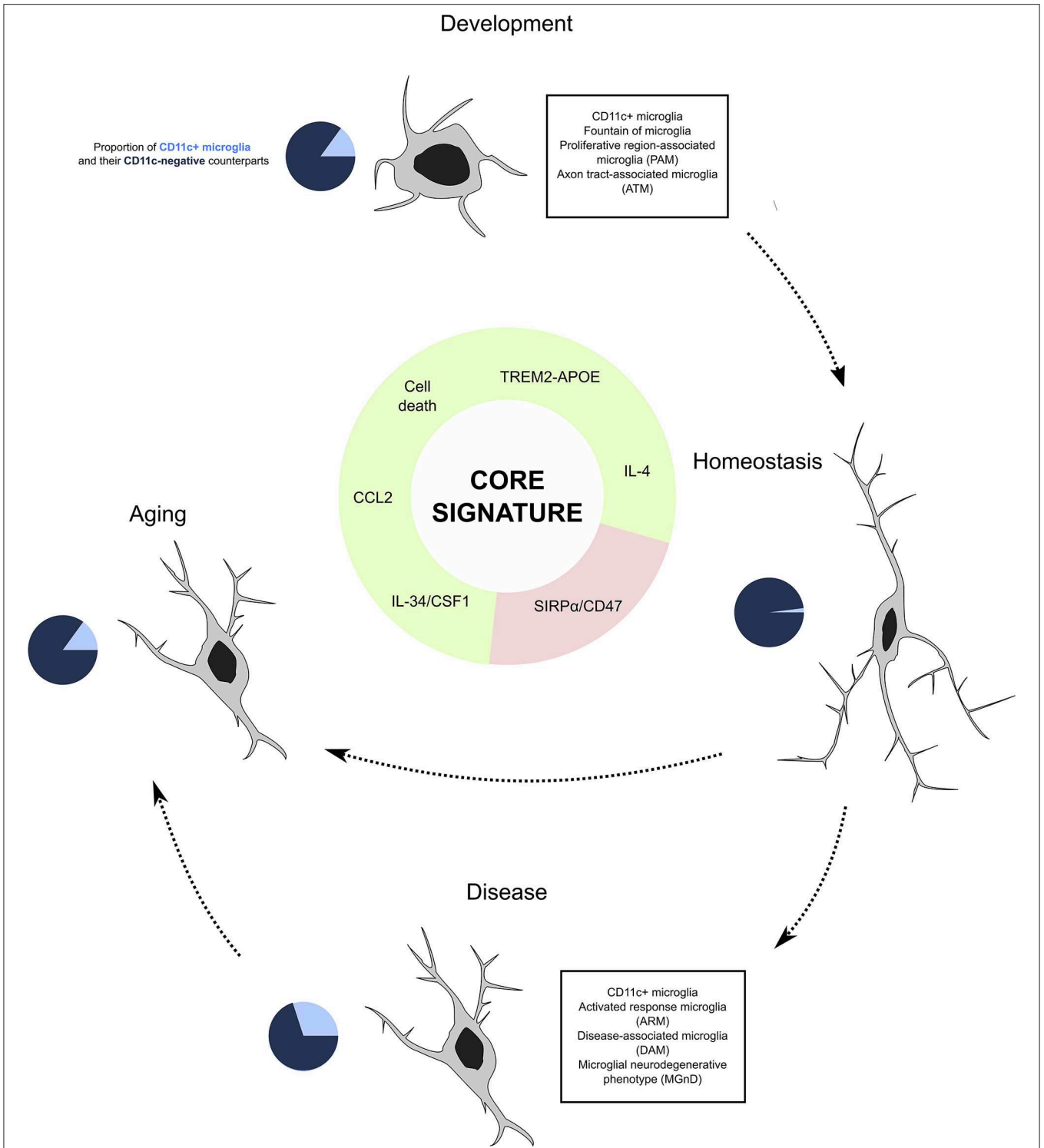


FIGURE 4 | CD11c+ microglia as a subset of microglia present through life and across conditions. Our investigation leads us to believe that CD11c+ microglia represent a subset of microglia characterized by a robust signature of 22 genes expressed by this subset at any age and in various disease states. Emergence of this subset is induced by various factors including signaling through the TREM2–APOE pathway, cell death, IL-4 signaling, and cytokine signaling through CSF1R inducing CCL2, and is inhibited by CD47/SIRPα signaling. In physiological conditions, CD11c+ microglia account for around 15% of all microglia, before contracting to 2% in adulthood and being re-induced by aging at levels similar to development. In disease states, their numbers oscillate between 10 and 50%. We argue that despite the numerous names given to this subset across conditions, it is unique and should be referred to as "CD11c+ microglia".

of CD11c+ microglia in primary myelination during CNS development as well as their protective, remyelinative, and regenerative capacities in CNS pathology. This opens new perspectives for therapeutic targeting of microglia in neurological conditions.

AUTHOR CONTRIBUTIONS

AW and AB-B designed the manuscript. AB-B analyzed the transcriptomic data and prepared the figures. AB-B, AW, and TO wrote and approved the 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/fimmu.2020.00430/full#supplementary-material

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REVIEW
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The Peripheral Immune System and Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease that is defined by loss of upper and lower motor neurons, associated with accumulation of protein aggregates in cells. There is also pathology in extra-motor areas of the brain, Possible causes of cell death include failure to deal with the aggregated proteins, glutamate toxicity and mitochondrial failure. ALS also involves abnormalities of metabolism and the immune system, including neuroinflammation in the brain and spinal cord. Strikingly, there are also abnormalities of the peripheral immune system, with alterations of T lymphocytes, monocytes, complement and cytokines in the peripheral blood of patients with ALS. The precise contribution of the peripheral immune system in ALS pathogenesis is an active area of research. Although some trials of immunomodulatory agents have been negative, there is strong preclinical evidence of benefit from immune modulation and further trials are currently underway. Here, we review the emerging evidence implicating peripheral immune alterations contributing to ALS, and their potential as future therapeutic targets for clinical intervention.

Keywords: amyotrophic lateral sclerosis (ALS), T lymphocytes, monocyte, cytokine, inflammation, immunity

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, defined by the presence of muscle weakness and the progressive death of upper and lower motor neurons (1). ALS leads to respiratory failure with the length of survival being predicted by respiratory muscle weakness (2). However, ALS is more than just a motor neurone disease. ALS also has extra-motor features, including cognitive and behavioral disturbance (3–5). ALS is markedly heterogeneous in clinical features, such as site of onset of weakness and rate of progression (6, 7), and is more common in men than in women (8).

ALS can be sporadic (SALS) or familial (FALS), although the distinction can be difficult to assign (9). Genetic susceptibility (10, 11) and environmental exposure (12) contribute to the pathogenesis of ALS, possibly through a multi-stage process (13, 14). Causative genes exist in patients with FALS, and mutations in these genes occur in some patients with SALS (15). Calculations suggest that 61% of the variance in risk of developing ALS is due to genetic factors (16), which means that ∼40% of the variance in risk is due to non-genetic factors, which could include environmental exposures. The pathological features of ALS include aggregation of insoluble protein within cells (17), but the type of protein aggregate varies among patients. It has been thought that the majority of patients have accumulation of tar DNA binding protein 43 (TDP-43), (as well as others), with a small group of patients having accumulations of superoxide dismutase 1 (SOD1) (18–20). However, recent evidence suggests that SOD1 may aggregate in the spinal cord in a majority of ALS patients

(21, 22). The genes that cause ALS usually encode for proteins or polypeptides that accumulate within cells or are involved in the metabolism of protein aggregates (19, 23). There is evidence that some of the aggregated proteins can transfer from cell to cell in a prion-like fashion (24, 25) which could explain the characteristic spread of weakness from the site of onset to other regions.

A number of possible pathways of disease have been described, including mitochondrial dysfunction, glutamate excitotoxicity (26, 27), problems with autophagy (28) and altered RNA metabolism (29). Furthermore, the death of motor neurons can be "non-cell autonomous," meaning that other types of cells such as astrocytes, microglia and possibly oligodendrocytes can drive motor neuron death (30, 31). There has been considerable research on the type of cell death that occurs in ALS. It has been previously thought that neuronal cell death in ALS is due to apoptosis (32–35) which is mediated through caspases. Evidence for apoptosis in ALS has been found with TUNEL staining of human tissues (36) and with measurements of bcl-2 (37). Others found increased p53 in ALS (38). In ALS there is also evidence of caspase activation (35). However, more recently there has been a suggestion that necroptosis, an inflammatory form of cell death which is caspase independent and involves RIP kinase activation, is a common form of cell death in neurodegenerative disease (39). Necroptosis is the mechanism of cell death from glutamate toxicity (40), which is one of the most important mechanisms proposed for the pathogenesis of ALS. There is evidence that necroptosis occurs in a cell culture model of ALS (41). Mutations in optineurin, a rare genetic cause of ALS, allow the activation of RIP kinases to promote necroptosis (42). More recently still, ferroptosis, an oxidative form of cell death (43), has been reported to occur in ALS (44).

The death of motor neurons, possibly stimulated by the pathways described above, and occurring through one of the types of cell death described above, is the cardinal feature of ALS. However, the pathology of ALS in the brain and spinal cord also involves more than death of motor neurons, with evidence of involvement of the immune system (45). There is neuroinflammation with microglial activation and a modest level of T lymphocyte infiltration (46–48). In ALS patients, microglial activation is visible with PET imaging suggestive of an ongoing neuroinflammatory process (49). Such inflammatory pathology could be a reaction to cellular damage. Once established, such inflammation could aggravate disease. However, it must be also noted that the immune system can also be protective, particularly after injury (50, 51). Thus, the role of the immune system in pathogenesis could be either harmful or helpful, and work is required to delineate the precise role of each immune pathway to ALS pathology.

There is also evidence of abnormality of the peripheral immune system in ALS, and this is the topic of the present review. As with inflammation in the CNS, peripheral immune activation could be a reaction to tissue damage, but once established, could exacerbate disease. This review will focus on describing the abnormalities of circulating blood cells, different immune system proteins, and their key inflammatory mediators, cytokines. These are summarized in **Tables 1**, **2**. To consider whether the immune abnormalities contribute to disease pathogenesis, we list some evidence that these abnormalities are correlated with human disease or are pathogenic in animal models of ALS. If immune abnormalities contribute to pathogenesis, then modification of the immune response could be beneficial to patients, so we also highlight the results of forthcoming and completed clinical trials of immune interventions in ALS.

ABNORMALITIES OF PERIPHERAL BLOOD CELLS

Total Leukocyte Count/Granulocytes

Several studies have provided evidence of immune activation in the peripheral blood in ALS. The total leukocyte count is elevated in patients with ALS, and correlates with progression of disease (52). The ratio of neutrophils to monocytes was also shown to be increased (53), as was the total number of granulocytes (54). A micro-array study further confirmed evidence for mild neutrophilia in ALS patients (55). In the $SOD1^{G93A}$ transgenic mouse ALS model, circulating neutrophils are increased (73), and neutrophils and mast cells are present along peripheral motor axons, with masitinib treatment leading to reduction of axonal damage (56). This suggests these cells are harmful and contribute to disease progression.

Lymphocytes

$CD4^+$ T Cells

Some studies demonstrate increased levels of $CD4^+$ helper T lymphocytes in patients with ALS (54, 57, 58), but others have found reduced numbers of these cells (59). It is possible that this variation is related to the variation in immune responsiveness of individuals. $CD4^+$ T cells in the CNS are thought to be neuroprotective in an animal model of ALS (60), and a lack of $CD4^+$ T cell mediated neuroprotection could be detrimental, in patients with reduced numbers. This protection is mediated through Treg cells that are discussed below.

$CD8^+$ T Cells

There are reports of reduced levels of $CD8^+$ cytotoxic T lymphocytes in ALS (57), reports of increased levels of $CD8^+$ cytotoxic T lymphocytes (54, 61), and reports of no alterations in these cells (59). Once again this could be related in part to individual variability. In the $SOD1^{G93A}$ mouse ALS model, cytotoxic lymphocytes cause death of motor neurons (62), so increased numbers could be detrimental.

NKT Cells

NK T cells recognize lipid antigens through CD1,and secrete an array of cytokines (95). A study in people with ALS found increased levels of natural killer T (NKT) cells (61). In the $SOD1^{G93A}$ mouse model, there are also increased NKT cells, especially in the liver (63); furthermore treatment that reduced the numbers of peripheral NKT cells led to prolongation of life-span, suggesting that these cells are harmful in ALS.

Th 17 Cells

The co-stimulatory pathway activated through CD40 ligand is upregulated in some human subjects with ALS (96), and there

TABLE 1 | Changes in peripheral blood cells in ALS.

Cell	Change in ALS	Evidence of disease association	Reference(s)
Total leukocytes	Increased	Level correlates with rapidity of progression	(52)
Granulocytes/neutrophils	Increased granulocytes, increased neutrophils, increased ratio of neutrophils to monocytes	Treatment with mastinib reduces axonal degeneration in animal model	(53–56)
$CD4^+$T cells	Conflicting reports, most suggest an increase	$CD4^+$ T cells are protective in animal model	(54, 57–60)
$CD8^+$ T cells	Conflicting reports	Cytotoxic cell cause death of motor neurones in animal model	(54, 57, 59, 61, 62)
NK T cells	Increased	Reduction in numbers led to prolonged survival in animal model	(61, 63)
Treg cells	Reduced and dysfunctional	Inverse correlation with rate of progression	(57, 61, 64–66)
$CD14^+$ monocytes	Variable reports of numbers, but evidence of activation, increased ratio of classical to non-classical monocytes	Monocyte activation correlates with disease progression	(52, 57, 58, 67–69)

TABLE 2 | Changes in peripheral blood proteins in ALS.

Protein	Changes in ALS	Evidence for role in pathogenesis	References
IgG	Increased	Passive transfer leads to motor neurone degeneration	(70–72)
Complement	Increased complement in ALS	Lack of C5a is protective in animal model	(59, 73–75)
Tumor necrosis factor	Increased	Mixed effects on motor neuron survival, depending on receptor	(76–82)
Interleukin 1β	Increased	Blocking IL-1 led to prolonged survival in animal model of ALS	(77, 79, 83)
Interleukin 33	Reduced	Treatment with IL33 reduced disease in animal model of ALS	(84, 85)
Interleukin 6	Increased	Genetic variation of IL-6 receptor influences the severity of ALS. However, IL6 deficiency has no effect of animal model	(79, 86–90)
Interleukin 17	Increased	Unknown- but usually pro-inflammatory	(91)
C reactive protein	Increased	Unlikely—this is evidence of inflammation	(89, 92–94)

is thought to be a particular activation of Th-17 T lymphocytes (97). Th17 lymphocytes are pro-inflammatory and thought to be harmful, but can exhibit plasticity and change to other less harmful functions (98).

Treg Cells

Much work has focused on regulatory T cells (Tregs) in ALS (99). There are reduced levels of Tregs in ALS patients (57, 61, 64), and these cells are also found to be dysfunctional (65). The level of Tregs correlates inversely with progression of disease (64). Another study also found that there was an inverse correlation between Treg numbers and the rate of disease progression (66). In a human trial, three patients were given autologous expanded Tregs (100), which showed a possible reduction in the rates of disease progression during infusion periods. A trial has been commenced to determine whether rapamycin, which increases levels of Tregs through the mToR pathway, can lead to increased levels of Tregs in ALS (101).

In $SOD1^{G93A}$ mice, there is evidence of dysfunction of Tregs and transfer of wild-type Tregs delays onset of disease (102). Another study in SOD1 transgenic mice showed that transfer of Tregs slowed disease progression (66). These studies are a promising area of research because of the suggestion that Tregs are able to control or reduce disease activity, but clearly requires larger, controlled and blinded human studies to validate their therapeutic potential.

NK Cells

NK cells are cells of the innate immune system, that mediate cytotoxicity. There is an increase in NK cells in patients with ALS compared to controls (52, 54). NK cells are found in the CNS of SOD1 G93A mutant mice where they are thought to be harmful. Thus, NK cells could possibly be pathogenic and a trial of anti-NK therapy has been proposed in ALS (http://grantome.com/grant/NIH/R21-NS102960-01A1).

Monocytes

Monocyte Classification

With measurements of expression of CD14 (the lipopolysaccharide receptor) and CD16 (the FcγIII receptor), monocytes can be separated into three groups; these are classical ($CD14^{++}$ $CD16^-$), intermediate ($CD14^{++}CD16^+$) and non-classical ($CD14^+CD16^{++}$) (103, 104). HLA DR is expressed in $CD16^+$ monocytes, while $CD14^+$ monocytes reduce HLADR expression when activated (105). Other markers can also be used to distinguish monocyte subsets (106).

Monocyte Numbers and Proportions

There is a report of a mild increase in $CD14^+$ monocyte numbers in ALS (52). However, another study reported reduced levels

of $CD14^+$ cells in the early stage of disease (57). There is also a report that there is no difference on the numbers of $CD14^+$ monocytes between patients and controls (58). In addition, it has been reported there is a reduction in $CD16^-$ monocytes in ALS (53). These variations could be explained by differences in methodology and the lack of clear demarcation between these monocyte populations in flow cytometry gating strategies. There are also reports of alterations in the proportions of monocytes in ALS, with an increase in the ratio of classical to non-classical monocytes (67, 107).

In addition to population shifts, there have been reports of alterations in monocyte activation in ALS. $CD14^+CD16^-$ classical monocytes in ALS show an inflammatory microRNA profile (68). Another study reported increased production of neurotoxic cytokines by monocytes from twins with ALS compared to the unaffected twin (108). Increased peripheral monocyte expression of inflammatory genes correlates with disease progression (69). Another study reported expression of activation markers on monocytes but reduced expression of HLA-DR (57). In another study, patients with ALS could be separated into groups, with one group showing increased HLA-DR expression on monocytes (54). Another study found that there was increased expression of HLA-DR on $CD14^+$ monocytes in ALS and this correlated with the rate of disease progression (58). Another study used exosomes to activate monocytes, and found that monocytes from ALS patients were less responsive than those from healthy individuals (109). ALS monocytes are less responsive to purinergic stimulation than those from controls (110).

As outlined above, the pathology of ALS is characterized by the accumulation of aggregates of proteins in neurons. There is now evidence of abnormal accumulation/location of these proteins in monocytes. For example, altered location of TDP43 in monocytes of patients with genetic mutations in TARDBP, the gene encoding TDP-43 has been demonstrated (111). There is also a report that C9orf72 is expressed in myeloid cells and that expression in monocytes increases after activation (112). Ablation of the mouse homologue of C9orf72 led to macrophage dysfunction and microglial activation (113).

There is evidence that peripheral monocytes enter the CNS in ALS (107), although this is controversial. In $SOD1^{G93A}$ mice, numbers of inflammatory monocytes correlated with disease progression (114). Activated macrophages are found around degenerating nerve (115) and at the neuro-muscular junction in mouse models of ALS (116). These experimental studies suggest that a shift toward activated monocytes in ALS could contribute to ALS progression through secretion of inflammatory and potentially neurotoxic mediators. Further research is needed to precisely define the role of the monocyte in ALS.

ABNORMALITIES OF IMMUNE PROTEINS IN PERIPHERAL BLOOD

Immunoglobulin Levels

Some of the first studies of the role of the immune system in ALS were concerned with the presence of antibodies in the blood of subject with ALS, particularly reports of antibodies to voltage gated calcium channels (117, 118). In addition, there have been studies of non-specific changes in antibodies, as a recent study has shown an increase in IgG levels in subjects with ALS compared to controls (70). In mice, an experimental study showed that prolonged intra-peritoneal injection of immunoglobulin from human subjects with ALS led to loss of spinal motor neurons and loss of muscle strength (71). An earlier study showed that passive transfer of purified immunoglobulin from ALS patients led to motor neuron degeneration and accumulation of calcium containing organelles (72).

Complement System

There is clear evidence of activation of innate immune complement system in human subjects with ALS, with raised C5a levels and increased expression of C5a on human leukocytes (74). A two dimensional gel electrophoresis was used to study serum proteins in ALS subjects and found that components of complement C3 were increased compared to controls (75) and another study using nephelometry showed increased levels of complement C3 in the blood of ALS patients (59). Animal studies also indicate a role for terminal complement activation in motor neuron degeneration. In SOD1 and TDP43 animal models of ALS there is evidence of complement activation (119, 120), and genetic deficiency or pharmacological inhibition of the C5a receptor, C5aR1, is protective in rodent $SOD1^{G93A}$ models (73, 121–123). A comprehensive review of the involvement of complement in ALS has recently been written (124).

Cytokines

Tumor Necrosis Factor (TNF)

There are increased levels of TNF and soluble TNF receptor in the blood of patients with ALS (76–78). A meta-analysis found that TNF levels were significantly increased in ALS (79). RNA-seq analysis has identified TNF as a contributor to inflammation in the spinal cord of ALS patients (125). It is unknown whether this inflammation is harmful or beneficial. It has been suggested that TNF is harmful and that reduction would be beneficial (80). On the other hand, TNF stimulates a survival pathway in motor neurons and could be beneficial (81, 82).

In SOD1 mutant mice, signaling through the TNF receptor 2 lead to motor neurone death (126), whereas signaling through TNF receptor 1 was harmful (127). The recent suggestions that TNF inhibitors could be a risk factor for ALS (128) could indicate that TNF is beneficial in some way.

Interleukin 1 (IL-1)

Interleukin 1 exists as a family of proteins (129). One study found that interleukin 1 β (IL-1β) was undetectable in ALS patients (130) but other studies have found increased levels (77). A meta-analysis found that IL-1β was significantly increased in ALS (79). Pathways involving IL-1 are thought to be involved in ALS pathogenesis as shown in SOD1 and TDP-43 animal models (83, 131, 132). A proteomic study of plasma from ALS patients showed activation of pathways associated with inflammation and activation of two networks centered on NFκ B and IL-1 (133). In animal studies, blocking IL-1 led to prolonged survival (83). In

humans, there has been a pilot study that showed that blocking IL-1 with Anakinra was safe in ALS, although there was no prolongation of survival (134).

Interleukin 33 (IL-33)

IL-33, a cytokine related to IL-1, has a role both in inflammation, and in metabolism (135, 136). IL-33 binds to receptor, ST2. Levels of IL-33 are reduced in ALS, and levels of soluble ST2 are increased in ALS (84). In a study in SOD1^{G93A} transgenic mice, IL-33 treatment ameliorated disease (85), suggesting this is a key downstream mediator of ALS progression.

Interleukin 6 (IL-6)

IL-6 is considered to be a pro-inflammatory cytokine, and is part of an acute phase response, however, it also has some documented anti-inflammatory effects. Plasma levels of IL-6 are increased in ALS (79, 86, 87), and this was supported by a meta-analysis (79). One study suggested that this was a response to hypoxia rather than to the disease itself (137) (see below). IL-6 has been suggested to have a role in endothelial damage in ALS (138). Genetic variation in the IL-6 receptor has been shown to modify the severity of ALS (88). Treatment with the IL-6 blocking antibody toclizumab reduced levels of IL-6 and other cytokines in cells from some ALS patients (89); this study did not look for effects on clinical signs. In SOD1 mutant mice, IL-6 deficiency did not affect the severity of disease (90).

Interleukin 17 (IL-17)

IL-17 is a pro-inflammatory cytokine that also responds to stress (139, 140). Increased levels of IL-17 are reported in the serum of subjects with ALS (91, 141), but to date IL-17 has not been explored clinically as a therapeutic target.

Interleukin 13 (IL-13)

IL-13 regulates T lymphocytes and has been implicated in autoimmune disease (142). IL-13 levels are elevated in the blood of patients with ALS (77). IL-13 producing T lymphocytes have been found in the blood of subjects with ALS and correlate with the rate of disease progression (91, 143).

Interleukin 18 (IL-18)

IL-18 is another member of the IL-1 family of cytokines and stimulates many lymphoid cells (144). Although levels of IL-18 are increased in patients with ALS (130), there is no information about relation of IL-18 to disease activity to date.

Chemokines

Chemokines are small proteins that are involved in chemotaxis and activation of granulocytes and lymphocytes. In the CNS, chemokines also have a role in signaling between cells (145). The expression of MCP-1 receptor (CCR2) is reduced on circulating monocytes in ALS (146). Another study showed significantly increased expression of CXCR3, CXCR4, CCL2, and CCL5 on T lymphocytes in ALS patients compared to healthy controls (147). There are higher levels of the chemokine MCP-1 in patients with a shorter diagnostic delay, which is a marker of more severe rapidly progressing disease (148).

Other Evidence of Systemic Inflammation

There is also evidence of increased levels of C reactive protein and erythrocyte sedimentation rate (ESR) in subjects with ALS compared to controls, and evidence that levels correlate with the levels of disability as measured by the ALS functional rating scale (89, 92–94). Levels of lipopolysaccharide are elevated in patients with ALS, (149), as have levels of nitric oxide, suggesting systemic inflammation (78).

Evidence of Hypoxia

In ALS, there is evidence of hypoxia in neurons, and this is thought to contribute to pathogenesis. This can be seen as increased levels of hypoxia inducible factor−1α (150). There is also thought to be dysregulation of the pathways that protect from hypoxia (151, 152). In the peripheral blood monocytes of ALS patients there is also dysregulation of hypoxia pathways (153). A gene expression study found evidence of hypoxia related genes in peripheral blood of ALS patients (55). In an animal model of ALS, hypoxia aggravates the loss of motor neurons (154). The significance of these findings is presently unclear, but this is further evidence of peripheral immune changes in ALS.

NF-κ B Pathways

Nuclear factor κB (NF-κ B) is a protein complex that regulates the transcription of DNA. Evidence that NF-κ B is important in ALS comes from studies showing genetic abnormalities in optineurin (155). Analysis of cell transfection showed that the nonsense and missense mutations of OPTN abolished the inhibition of activation of NF-κ B. The authors proposed that NF-κ B is the final common pathway in ALS pathogenesis, and that inhibitors of NF-κ B could be used to treat ALS. Further, in animal studies it has also been found that the NF-κ B p65 subunit is a binding partner for TDP-43 and that dysregulation of TDP-43 leads to activation of NF-κ B (156). NF-κ B is expressed in astrocytes (157), and in activated microglia in ALS spinal cord (158).

Other evidence of a possible role of NF-κ B in ALS comes from a role for hypoxia in ALS. (153, 159) (see above). NF-κ B is activated during acute hypoxia and acts to up-regulate inflammatory factors such as IL-6, cyclo-oxygenase (COX 2), TNF-α, and prostaglandin E-2 (PGE-2) (159). Reactive oxygen species lead to induction of NF-κ B. This is mainly in lymphoid cells but also in neurones. It has been suggested that NF-Ƙ B is a transcription factor controlled by hypoxia and may contribute to neurological disorders (160).

In neurodegenerative disease it is thought that NF-κ B can augment cell death (161). There is some evidence about the role of NF-κ B from animal models of ALS. In SOD1^{G93A} mutant mice, treatment with a PPAR inhibitor led to clinical improvement and reduced expression of iNOS and NF-κ B reactivity (162). Phenylbutyrate induced NF-κ B translocation to the nucleus in ALS mice, and this led to reduced motor neuron death (163). Intrathecal injection of an adenovirus containing insulin like growth factor led to slowing of disease through inhibition of NF-κ B in an animal model of ALS (164). However, inhibition of NF-κ B in astrocytes did not reduce disease in ALS mice (165).

The above-mentioned studies focus predominantly on the role of NF-κ B within the CNS in ALS. Little is known about NF-κ B in the peripheral immune system in ALS, but given the central role of NF-κ B in the biology of the immune system (166), this warrants further study.

Immunometabolic Changes

There is considerable interaction between the immune system and metabolic pathways, which is a rapidly growing research field being known as "immunometabolism" (167, 168). For survival, metabolism and the immune system need to be linked, because there needs to be a mechanism for balancing the energy needed for basal and defensive processes (169). In ALS, there is evidence of alterations in metabolism (170). There are reports of alteration in the levels of metabolic proteins such as adipokines. This includes IL-6 but also other proteins such as leptin and adiponectin (86, 171). A proteomic study found dysregulation of pathways involved in lipid metabolism (133). In particular, there was dysregulation of the Liver X receptor/Retinoid X receptor (LXR/RXR) and the Farnesoid X receptor/Retinoid X receptor (FXR/RXR) pathways that are at the intersection of immunology and metabolism.

EVIDENCE FROM COMPLETED CLINICAL TRIALS

It is attractive to consider that modulation of the immune response will be a useful therapy in ALS. If neuroinflammation enhances disease activity, then control of neuroinflammation should be helpful (172), possibly by enhancing the protective immunity (173).

Overall, clinical trials of new disease-modifying therapies in ALS have been disappointing (174). Several of these trials have used medications that act on the peripheral immune system. Total body irradiation and stem cell therapy were of no benefit in ALS (175). Earlier attempts at immune therapy included treatment with intravenous immunoglobulin, (176), with cyclophosphamide, (177) and with azathioprine and prednisone which were also of no benefit (178). Glatiramer acetate, a synthetic polypeptide with immune effects that is used in multiple sclerosis, further demonstrated no benefit in ALS (179).

Minocycline, an anti-inflammatory agent, also failed in a trial in ALS, and in fact, patients on this treatment had a worse outcome (180). Celecoxib, another anti-inflammatory agent, also failed its clinical end-point (181). Sodium chlorite (NP001) which was proposed to deactivate macrophages, was also more recently shown to be unsuccessful (182, 183).

Masitinib, a tyrosine kinase inhibitor that targets mast cells, microglia, and macrophages despite showing positive results in SOD1 transgenic mice (184), failed in its phase II study in humans (185). Finally, a trial of granulocyte colony stimulating factor led to a decrease in levels of MCP-1 and IL-17 in subjects with ALS (186).

IS THERE AN INFLAMMATORY SUBGROUP?

One of the challenging features of ALS is its heterogeneity- of clinical features, of rate of progression and also in the underlying pathological aggregation of proteins. This heterogeneity could indicate that the pathogenesis of disease varies among patients, and there could be sub- groups of patients in whom immune processes are more or less important.

A study of gene expression indicated that patients can be grouped into patients with higher expression of IL-6R and myeloid lineage-specific genes, and patients with higher expression of IL-23A and lymphoid-specific genes (55). The results from a clinical trial of Toclizumab also led the authors to note that Toclizumab reduced IL-6 and other cytokines in cells from some ALS patients (i.e., an "inflammatory group") but not others (89).

INTERACTION AMONG THE NERVOUS SYSTEM, THE IMMUNE SYSTEM AND THE GUT MICROBIOTA

The gut microbiota has been increasingly recognized as playing an important role in human health, and has been implicated in neurodegenerative disease including ALS, as we have recently reviewed (187). One of the many functions of the gut microbiota is to regulate the immune system. It is therefore possible that some of the immune abnormalities in ALS are linked to the gut microbiota. However, this field is complex and analysis requires large numbers of subjects so more remains to be discovered regarding this possible interaction.

IMMUNOGENETICS OF ALS

If immune genes played a role in the susceptibility to ALS or modified the course of ALS, this would be evidence of involvement of the immune system in disease. In autoimmune diseases, there is an association of disease with HLA loci (188). This is not the case in ALS, except for a possible association with HLA class I antigens (189, 190).

The association with HLA class I antigens could be due to linkage with the haemochromatosis locus (HFE), which is found in the HLA region. Some years ago, an association with the H63D polymorphism was reported (191). More recently, a meta-analysis has discounted this association but instead suggested an association with the C282Y polymorphism (192).

There are numerous polymorphisms that affect the immune system. These have been linked to autoimmune diseases such as multiple sclerosis and type I diabetes (193) but not to ALS (194). However, it would seem likely that genetic variation in immune genes could influence the immune abnormalities described above, For example, polymorphisms in cytokine genes can influence the levels of cytokines such as IL-6, (195) and TNFα (196) and the IL33/ST2 pathway (197).

The field of immunogenetics of ALS would appear to be a fruitful topic for further exploration, and possibly could explain

why some patients have a stronger immune response than others, and why some patients show an "inflammatory" phenotype. Immunogenetics, and variation in the immune response to disease could therefore contribute to the known heterogeneity of ALS.

CONCLUSION

There is clear evidence of immune activation in some patients with ALS and in animal models of disease. It is possible that there is a subgroup of patients in whom inflammatory pathways are important in pathogenesis. In some cases, the immune abnormalities are correlated with disease severity, but it is not clear whether this is cause or effect. The immune system has both harmful and beneficial effects and there is a need to focus research efforts on enhancing the beneficial effects of protective immunity. Clinical trials so far have been disappointing, but there is still scope for further attempts at immune intervention to ameliorate this disease.

AUTHOR CONTRIBUTIONS

PM conceived the idea and wrote the first draft. JL, TW, and RH read and revised the manuscript.

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REVIEW
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B and T Cells Driving Multiple Sclerosis: Identity, Mechanisms and Potential Triggers

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Historically, multiple sclerosis (MS) has been viewed as being primarily driven by T cells. However, the effective use of anti-CD20 treatment now also reveals an important role for B cells in MS patients. The results from this treatment put forward T-cell activation rather than antibody production by B cells as a driving force behind MS. The main question of how their interaction provokes both B and T cells to infiltrate the CNS and cause local pathology remains to be answered. In this review, we highlight key pathogenic events involving B and T cells that most likely contribute to the pathogenesis of MS. These include (1) peripheral escape of B cells from T cell-mediated control, (2) interaction of pathogenic B and T cells in secondary lymph nodes, and (3) reactivation of B and T cells accumulating in the CNS. We will focus on the functional programs of CNS-infiltrating lymphocyte subsets in MS patients and discuss how these are defined by mechanisms such as antigen presentation, co-stimulation and cytokine production in the periphery. Furthermore, the potential impact of genetic variants and viral triggers on candidate subsets will be debated in the context of MS.

Keywords: Th1/Th17, T-bet$^+$ B cells, CD8$^+$ T cells, Epstein-Barr virus, genetic risk, transmigration, germinal center, IFN-γ

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INTRODUCTION

In multiple sclerosis (MS) patients, pathogenic lymphocytes are triggered in the periphery to infiltrate the central nervous system (CNS) and cause local inflammation and demyelination. Anti-CD20 therapy has recently been approved as a novel treatment modality for MS (1–3). Although this underscores the fact that B cells play a key role in MS, the exact triggers, subsets and effector mechanisms contributing to the disease course are incompletely understood. The impact of this therapy on the antigen-presenting rather than the antibody-producing function of B cells in MS indicates that their interaction with T cells is an important driver of the pathogenesis (1, 4). Alterations in cytokine production, co-stimulation and antigen presentation most likely contribute to the development of pathogenic B and T cells that are prone to enter the CNS (4, 5). Such mechanisms might be influenced by the interplay between genetic and environmental risk factors (6). The major *HLA-DRB1*1501* locus accounts for 30% of the overall risk (6) and has been shown to promote B cell-mediated induction of brain-infiltrating T helper (Th) cells in MS patients (4). Besides for *HLA-DRB1*1501*, other genetic risk variants that have been identified in the past decades also appear to potentiate B and Th cell activation, a feature that is shared amongst several

autoimmune disorders (7). Furthermore, infectious triggers such as the Epstein-Barr virus (EBV) alter their function and reactivity in MS (5, 6, 8, 9). The current view is that transmigration of lymphocyte subsets into the CNS signifies relapsing disease, while compartmentalized CNS inflammation, as seen during disease progression, seems to be driven by tissue-resident populations (10, 11). Since there is a clear association of relapse occurrence and radiological disease activity early in MS with the severity of disability progression later in MS (12), it is crucial to understand what motivates these cells to invade the CNS and why these cells instigate local pathology in MS patients.

In this review, we will discuss which and how brain-infiltrating lymphocyte subsets can contribute to MS pathogenesis. These pathogenic events are characterized by: (1) peripheral escape of pathogenic B cells from T cell-mediated control, (2) mutual activation of pathogenic B and T cells within peripheral germinal centers, and (3) re-activation of infiltrating B and T cells within the CNS. We will use current knowledge to consider the extent to which genetic and viral triggers may drive these pathogenic events in MS.

IMPAIRED T CELL-MEDIATED CONTROL OF PATHOGENIC B CELLS IN MS

B and T cells closely interact in secondary lymphoid organs to generate an optimal immune response against invading pathogens. Within follicles, B cells recognize antigens via the highly specific B-cell receptor (BCR), resulting in internalization, processing and presentation to T cells. This mechanism is unique and tightly coordinated involving five consecutive and interdependent steps: (1) B-cell receptor signaling, (2) actin remodeling, (3) endosomal formation and transport, (4) HLA class II synthesis and trafficking to specialized late endosomes (i.e., MIICs), and (5) antigen processing and loading onto HLA class II molecules for presentation to $CD4^+$ Th cells (13, 14). Through their interaction with Th cells, germinal center (GC)-dependent and -independent memory B cells are formed, a process that is governed by the strength of the HLA/peptide signal (15). GC B cells respond to interleukin (IL)-21-producing follicular Th (Tfh) cells to develop into class-switched (IgG^+) subsets or antibody-producing plasmablasts/plasma cells (15, 16). Memory B cells, in return, specifically trigger Th effector subsets that help $CD8^+$ cytotoxic T cells (CTLs) to kill the infected cell (17). In MS, this crosstalk between B and T cells is likely disturbed, eventually causing pathogenic instead of protective immunity. This may already start during selection of naive autoreactive B cells in the periphery.

Normally, after removal of the majority of B-cell clones expressing polyreactive antibodies in the bone marrow (central tolerance), surviving autoreactive B cells are kept in check by peripheral tolerance checkpoints (18). In contrast to most other autoimmune diseases, only peripheral and not central B-cell tolerance checkpoints are defective in MS, which coincides with increased frequencies of naive polyreactive populations in the blood (18–21). Although the exact cause is currently unknown, the escape of pathogenic B cells from peripheral control may be related to (1) chronic T-cell stimulation and (2) T cell-intrinsic defects (see **Figure 1**).

Epstein-Barr virus is one of the most thoroughly investigated pathogens regarding T-cell responses in MS. Many theories have been proposed how EBV can influence MS pathogenesis (9). One hypothesis is that, due to the chronic nature of this infection, continuous antigen presentation by B cells leads to functionally impaired, so-called "exhausted" T cells (8, 22). This, together with the impact of HLA and other risk alleles (23), may result in inappropriate T cell-mediated control of EBV-infected (pathogenic) B cells. Consistent with this, peripheral $CD8^+$ CTLs show decreased responses to EBV and not to cytomegalovirus antigens during the MS course (8). EBV antigens can also induce IL-10-producing $CD4^+$ T regulatory cells (Tregs) capable of suppressing effector T-cell responses to recall antigens (24), as seen for other persistent viral infections such as lymphocytic choriomeningitis virus (25, 26). However, forkhead box P3 ($FOXP3^+$) Tregs have also been described to control infections (27), suggesting that additional T cell-intrinsic defects are involved. For example, Treg populations that are enriched in MS patients produce increased levels of interferon gamma (IFN-γ), express reduced levels of FOXP3 and have defective suppressive activity *in vitro* (28). This is not only accompanied with less suppression of effector T cells (29, 30), but possibly also with impaired removal of pathogenic B cells, as described for other autoimmune diseases (18, 31, 32). The direct impact of Tregs on B cells in MS patients is still unknown. Treg function may be altered by variation in *IL2RA* and *IL7RA*, two known MS risk loci (33, 34). FOXP3 correlates with IL-2 receptor (IL-2R) as well as IL-7 receptor (IL-7R) expression in Tregs (35). It can thus be expected that *IL2RA* and *IL7RA* (33, 34), but also *BACH2* (36) variants impair Treg development in MS. This may even influence FOXP3- and IL-2R-expressing $CD8^+$ T cells, which can suppress pro-inflammatory $CD4^+$ Th cells (37) and are reduced in the blood during MS relapses (38–40).

THE GERMINAL CENTER AS A POWERHOUSE OF PATHOGENIC B- AND TH-CELL INTERACTION IN MS

Th Cells as Inducers of Pathogenic Memory B Cells

After their escape from peripheral tolerance checkpoints, naive B cells likely interact with Th cells in GCs to eventually develop into memory populations potentially capable of infiltrating the MS brain (**Figure 1**). Little is known about how peripheral effector Th cells mediate the development of such pathogenic B cells in MS patients. In GCs of autoimmune mice, autoreactive B cells are triggered by Tfh cells producing high levels of IFN-γ (16). IFN-γ induces the expression of the T-box transcription factor T-bet, which upregulates CXC chemokine receptor 3 (CXCR3), elicits IgG class switching and enhanced antiviral responsiveness of murine B cells (41–43). Recently, we found that B cells from MS patients preferentially develop into $CXCR3^+$

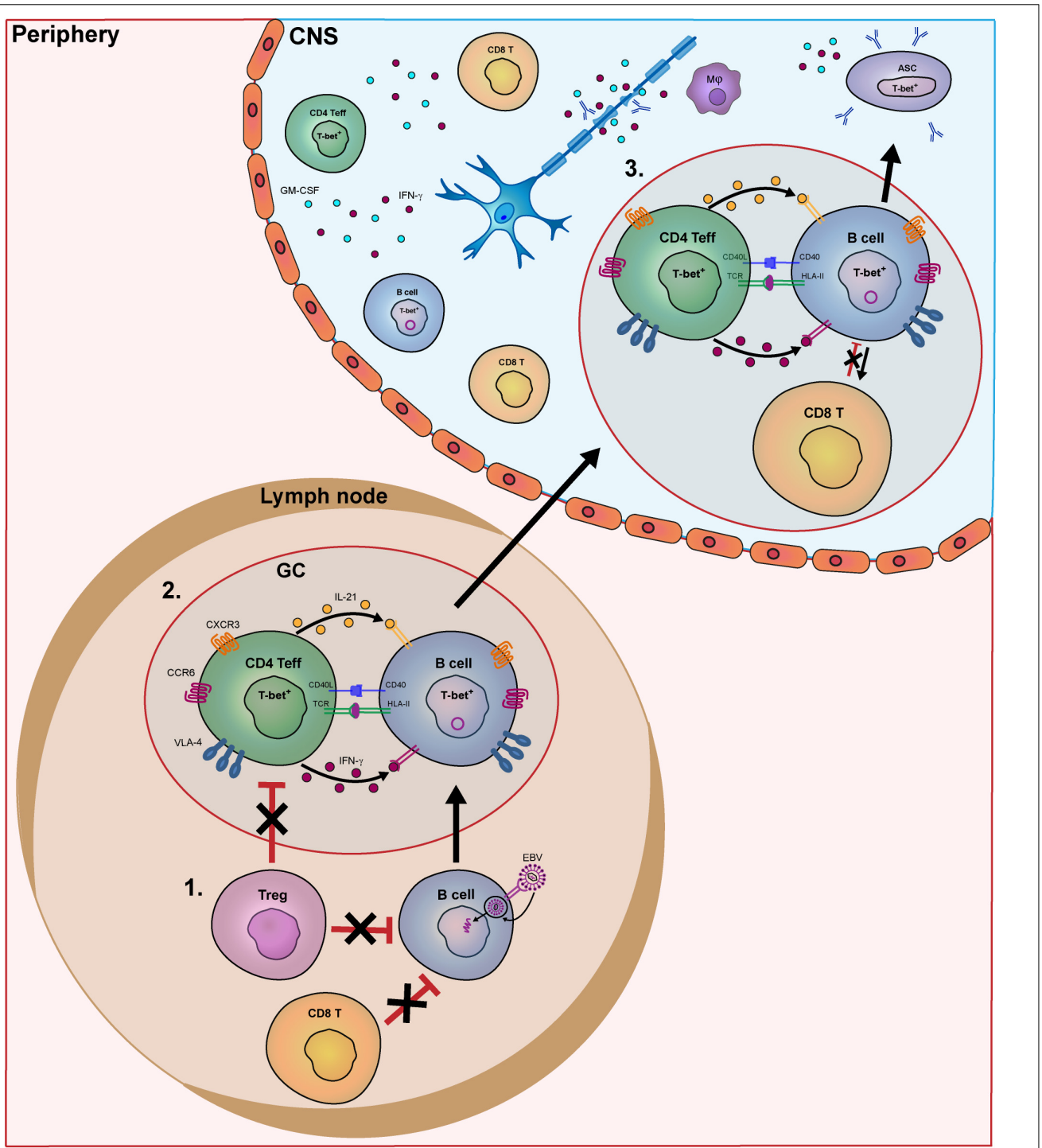

FIGURE 1 | Model of the key pathogenic events involving human B- and T-cell subsets driving MS disease activity. In MS patients, B- and T-cells interact in the periphery and central nervous system (CNS) to contribute to disease pathogenesis. In this model, we put forward three important meeting points of pathogenic B and T cells that drive the disease course of MS. In secondary lymphoid organs, B-cell tolerance defects in MS patients allow EBV-infected B cells to escape from suppression by $CD8^+$ and T regulatory (Treg) cells **(1)**. Subsequently, these activated B cells enter germinal centers (GCs) and interact with follicular Th cells to further differentiate into pathogenic memory B cells. Under the influence of IFN-γ and IL-21, B cells develop into T-bet-expressing memory cells, which in turn activate Th effector cells such as Th17.1 **(2)**. These subsets are prone for infiltrating the CNS of MS patients by distinct expression of chemokine receptors (CXCR3, CCR6), adhesion molecules (VLA-4) as well as pro-inflammatory cytokines. **(3)** Within the CNS, IFN-γ-, and GM-CSF-producing T cells and T-bet^+ memory B cells probably come into contact in follicle-like structures, resulting in clonal expansion inflammation and demyelination. T-bet^+ memory B cells further differentiate into plasmablasts/plasma cells to secrete high numbers of potentially harmful antibodies (oligoclonal bands).

populations that transmigrate into the CNS (44). The IFN-γ receptor (IFNGR) and downstream molecule signal transducer and activator of transcription (STAT)1 in B cells are major determinants of autoimmune GC formation in mice (45, 46). After ligation of the IFNGR, STAT1 is phosphorylated, dimerizes and translocates into the nucleus to induce genes involved in GC responses, such as T-bet and B-cell lymphoma 6 (BCL-6) (16, 47). Although IFN-γ-stimulated B cells of MS patients show enhanced pro-inflammatory capacity (44, 48), it is unclear whether alterations in the IFN-γ signaling pathway contribute to the development of T-bet$^+$ B cells infiltrating the CNS. Interestingly, a missense SNP in *IFNGR2* has been found in MS, which may alter their development (49, 50). Another target gene of the IFN-γ pathway is *IFI30*, which encodes for the IFN-γ-inducible lysosomal thiol reductase (GILT) and is considered one of the causal risk variants in MS (7). GILT is a critical regulator of antigen processing for presentation by HLA class II molecules (51–53). Together, these findings point to T-bet-expressing B cells as potent antigen-presenting cells that are highly susceptible to triggering by IFN-γ-producing Th effector subsets in MS (44, 54) (**Figure 2**).

Epstein-Barr virus may be an additional player in the formation of T-bet-expressing B cells. In mice, persistent viral infections sustain the development of these types of B cells, in which T-bet enhances their ability to recognize viral and self-antigens (41, 55). EBV is hypothesized to persist latently in pathogenic B cells and mimic T-cell help for further differentiation in GCs (5, 22, 56, 57). During acute infection, EBV uses a series of latency programs that drive B cells toward a GC response in an antigen-independent manner. Latent membrane protein (LMP)2A and LMP1 resemble signals coming from the BCR and CD40 receptor (56, 57). In addition to their regulation of GC responses independently of T-cell help (58), recent evidence implicates that LMP2A and LMP1 can synergize with BCR and CD40 signaling as well (59). Interestingly, downstream molecules of the BCR (e.g., Syk, CBL-B) and CD40 receptor (e.g., TRAF3) are genetic risk factors for MS (23, 60), therefore potentially cooperating with these latent proteins to enhance pathogenic B-cell development (**Figure 2**). This is supported by the binding of LMP2A to Syk in B cells and their escape from deletion in GCs of transgenic mice (61). Alternatively, pathogenic B cells can be induced via pathogen-associated TLR9, which binds to unmethylated CpG DNA and further integrate with BCR, CD40, and cytokine signals (62–65). Moreover, pathogenic B-cell responses in systemic autoimmune diseases such as systemic lupus erythematosus are enhanced after IFN-γ and virus-mediated induction of the T-bet (45, 55, 64, 65). In MS patients, TLR9 ligation is also a major trigger of pro-inflammatory B cells (48) and crucial for the differentiation of T-bet-expressing IgG1$^+$ B cells during IFN-γ- and CD40-dependent GC-like cultures *in vitro*. Thus, under influence of specific genetic factors, EBV might join forces with IFN-γ-producing Th cells to stimulate pathogenic (T-bet$^+$) GC B cells both in a direct (via infection and persistence in pathogenic subsets) and indirect (via TLR7/9) fashion in MS (**Figure 2**).

B Cells as Inducers of Pathogenic Memory Th Cells

Synchronously, within peripheral GCs, T-bet-expressing memory B cells are ideal candidates to trigger IFN-γ-producing, CNS-infiltrating Th cells in MS (**Figure 1**). In both mice and humans, T-bet promotes the antigen-presenting cell function of B cells. This may be related to the impact of EBV infection on B cell-intrinsic processing and presentation of antigens such as myelin oligodendrocyte glycoprotein (MOG) (5). The potent antigen-presenting cell function of B cells in MS patients is further reflected by the effective use of anti-CD20 therapy. This therapy does not affect antibody serum levels, but significantly reduces pro-inflammatory Th-cell responses in MS, both *ex vivo* and *in vivo* (1). CD20 was found to be enriched on IFN-γ-inducible T-bet-expressing IgG$^+$ B cells in MS blood (44), pointing to this pathogenic subset as an important therapeutic target. Furthermore, genetic changes in HLA class II molecules, as well as costimulatory molecules [e.g., CD80 (66, 67) and CD86 (68)], may additionally enhance Th cell activation by such memory B cells (**Figure 2**). HLA class II expression on murine B cells was reported to be indispensable for EAE disease onset (69, 70). The *in silico* evidence that autoimmunity-associated HLA class II molecules have an altered peptide-binding groove (71, 72), together with the potential role of several minor risk variants in the HLA class II pathway [e.g., *CIITA, CLEC16A, IFI30* (**Figure 2**)], insinuates that antigens are differently processed and presented by B cells (4, 5). This is supported by the increased ability of memory B cells to trigger CNS-infiltrating Th cells in MS patients carrying *HLA-DRB1*1501* (4). These CNS-infiltrating T cells induced by B cells showed features of both Th1 and Th17, therefore representing highly pathogenic subsets. Such subsets are characterized by master transcription factors T-bet and RORγt (73, 74), of which the latter is involved in the co-expression of IL-17 and GM-CSF in mice but not in humans (75, 76). GM-CSF is an emerging pro-inflammatory cytokine produced by Th cells in MS (33, 75, 77). Our group recently revealed that a Th subset producing high levels of IFN-γ and GM-CSF, but low levels of IL-17, termed Th17.1, plays a key role in driving early disease activity in MS patients (78). Proportions of Th17.1 cells were reduced in the blood and highly enriched in the CSF of rapid-onset MS patients. In addition, Th17.1 cells and not classical Th1 and Th17 cells accumulated in the blood of MS patients who clinically responded to natalizumab (anti-VLA-4 mAb). The increased pathogenicity of Th17.1 is further exemplified by their high levels of multidrug resistance, anti-apoptotic and cytotoxicity-associated genes *ABCB1* (MDR1), *FCMR* (TOSO) and *GZMB* (granzyme B), respectively (78–81). Th17.1 cells also show pronounced expression of the IL-23 receptor (IL-23R) (78), which is essential for maintaining the pathogenicity of Th17 cells during CNS autoimmunity (82). IL-23 signals through the IL-23R and IL-12 receptor beta chain (IL-12Rβ1), resulting in JAK2-mediated STAT3 and TYK2-mediated STAT4 phosphorylation, and thereby inducing RORγt and T-bet, respectively (83). *IL-12RB1*, *TYK2*, *STAT3,* and *STAT4* are known genetic risk variants and thus may directly induce Th effector cells in MS (**Figure 2**). In addition to its potential effect on Tregs

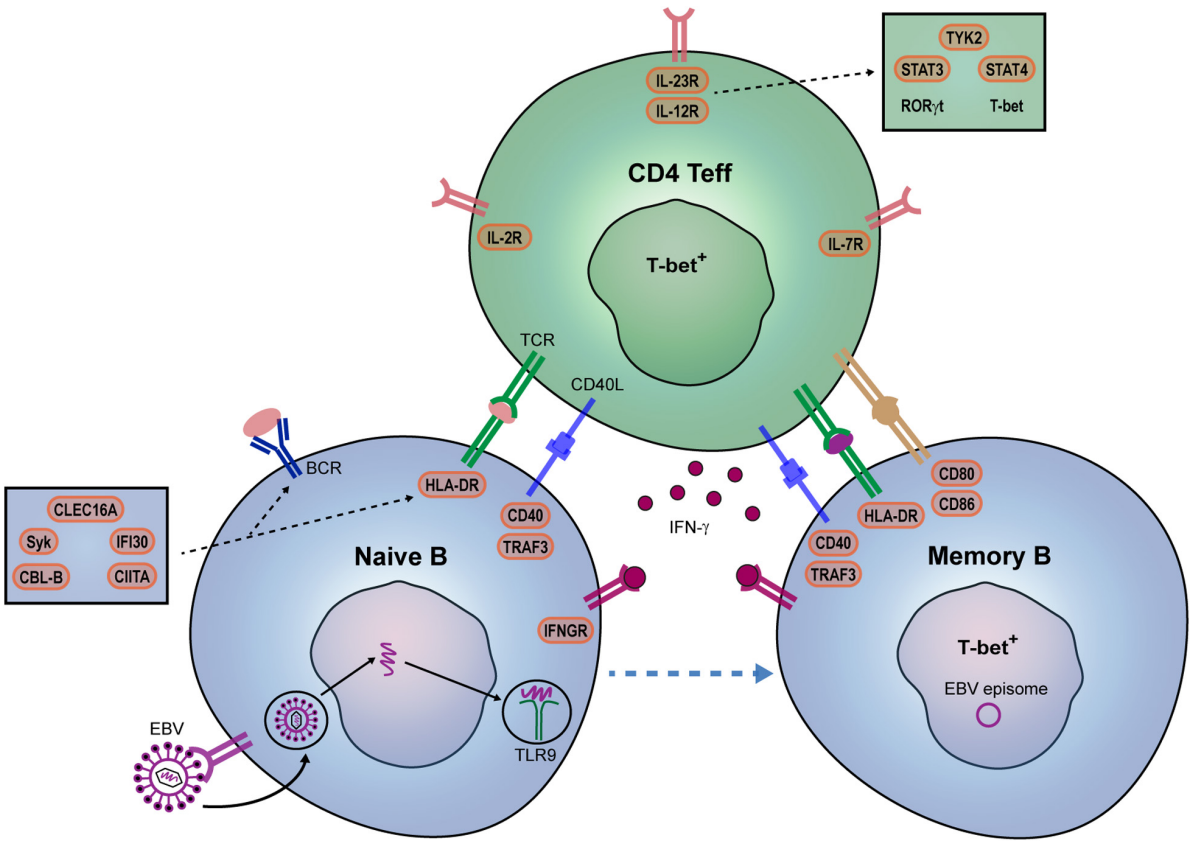

FIGURE 2 | Potential contribution of EBV and genetic risk factors to pathogenic B- and Th-cell development in MS patients. IFN-γ is a key player in autoreactive B- and Th-cell interaction and autoimmune germinal center (GC) formation in mice. In MS, we propose that EBV infection together with specific genetic risk variants promote the IFN-γ-mediated interplay between B and T cells within GCs. EBV directly infects naive B cells and mimic GC responses. EBV DNA can also bind to TLR7/9, and together with IFN-γ, induces T-bet$^+$ memory B cells. Their interplay may be additionally stimulated by both B cell-intrinsic (IFN-γ sensitivity: *IFNGR2*; B cell receptor-antigen uptake: *CBLB*, *SYK*, *CLEC16A*; HLA class II pathway: *CLEC16A*, *CIITA*, *IFI30*; co-stimulation: *CD80, CD86*) and Th cell-intrinsic (surface receptors: *IL2RA*, *IL7RA*, *IL12RB1*; downstream molecules: *TYK2*, *STAT3*, *STAT4*) genetic risk variants. IL12R/IL-23R complexes trigger JAK2/STAT3-dependent RORγt and TYK2/STAT4-dependent T-bet expression in Th effector cells.

(see above), MS-associated risk variant *IL-2RA* enhances GM-CSF production by human Th effector cells (33). To confirm the influence of these and other risk loci (84) on the induction of pathogenic Th cells such as Th17.1 in MS, functional studies need to be performed in the near future.

The increased pathogenicity of Th effector cells may additionally be skewed by IL-6-producing B cells (85, 86), which have been shown to trigger autoimmune GC formation and EAE in mice (87, 88). Blocking of IL-6 prevents the development of myelin-specific Th1 and Th17 cells in EAE (89). The IL-6-mediated resistance of pathogenic Th cells to Treg mediated suppression in MS (90, 91) further links to the abundant expression of anti-apoptotic gene *FCMR* in Th17.1 (78, 92). Intriguingly, B cell-derived GM-CSF can be an additional cytokine driving pathogenic Th cells in MS patients by inducing pro-inflammatory myeloid cells (93). Although the causal MS autoantigen is still unknown, previous work implies that B cell-mediated presentation of EBV antigens at least contributes to pathogenic Th-cell induction (5, 94). As mentioned above, antiviral CD8$^+$ CTLs can become exhausted during persistent viral infections. Normally, this mechanism is compensated by the presence of cytotoxic CD4$^+$ Th cells, which keep these types of infections under control (95). Such Th populations have been associated with MS progression (96) and are also formed after EBV infection, producing high levels of IFN-γ, IL-2, granzyme B, and perforin (97, 98). Similarly, EBV- and myelin-reactive Th cells from MS patients produce high levels of IFN-γ and IL-2 (6) and strongly respond to memory B cells presenting myelin peptides (99). These studies indicate that the involvement of EBV-infected B cells, especially those expressing T-bet (see section "Th Cells as Inducers of Pathogenic Memory B Cells"), in activating Th effector cells with cytotoxic potential (78, 100, 101) deserves further attention in MS.

REACTIVATION OF CNS-INFILTRATING B AND T CELLS IN MS

Mechanisms of Infiltration

Under normal physiological conditions, the CNS has been considered an immune privileged environment and consists of a limited number of lymphocytes that cross the blood brain barrier

(BBB) (102). However, the revelation of meningeal lymphatic structures emphasized the cross-talk between CNS and peripheral lymphocytes in secondary lymphoid organs (103). The choroid plexus has been identified as the main entry of memory cells into the CNS, which is in the case of T cells mostly mediated by CCR6 (104, 105). The normal human CSF, as is acquired from the arachnoid space by lumbar spinal taps, contains more $CD4^+$ Th cells compared to $CD8^+$ T cells with central memory characteristics (106–108). The arachnoid space is a continuum with the perivascular space surrounding penetrating arterial and venous structures into the parenchyma (109). Within the brain parenchyma, more $CD8^+$ T cells than $CD4^+$ Th cells are found, however, their numbers remain low and can be found virtually restricted to the perivascular space (11, 110). These T cells display a phenotype mostly associated with non-circulating tissue resident memory T cells. The perivenular perivascular space has been argued to be the common drainage site of antigens mobilized with the glymphatics flow (111). The exact relationship between memory T cells in the subarachnoid and perivascular space has been poorly identified in terms of replenishment and clonal association.

The BBB is dysfunctional during the early phase of MS, resulting in or is due to local recruitment of pathogenic T and B cells (112). Differential expression of pro-inflammatory cytokines, chemokine receptors and integrins by infiltrating lymphocytes have been argued to mediate disruption of the BBB in MS (104, 113). Myelin-reactive $CCR6^+$ and not $CCR6^-$ memory Th cells from MS patients not only produce high levels of IL-17, but also IFN-γ and GM-CSF (80). Previous studies mainly focused on the migration of IL-17-producing $CCR6^+$ Th cells through the choroid plexus in EAE and *in vitro* human brain endothelial cell layers in MS brain tissues (104, 114). In our recent study, we subdivided these $CCR6^+$ memory Th cells into distinct Th17 subsets and found that especially IFN-γ producing Th17.1 ($CCR6^+CXCR3^+CCR4^-$) cells were capable of infiltrating the CNS, both in *ex vivo* autopsied brain tissues and in *in vitro* transmigration assays (78). The fact that Th17.1 cells have cytotoxic potential and strongly co-express IFN-γ with GM-CSF (78) suggests that these cells are involved in disrupting the permeability of the BBB in MS (115, 116). The impact of CXCR3 on their transmigration capacity is likely the result of binding to the chemokine ligand CXCL10, which is produced by brain endothelial cells and is abundant in the CSF of MS patients (117, 118). Similar observations were made for CXCR3 $(T\text{-bet})^+$ B cells (44). CCR6 is also highly expressed on memory B-cell precursors within the Th cell-containing light zone of GCs (119), and on IFN-γ-producing $CD8^+$ T cells infiltrating the MS brain (120). This implies that both populations are susceptible to enter the CNS of MS patients. In addition to chemokine receptors and pro-inflammatory cytokines, adhesion molecules such as activated leukocyte cell adhesion molecule (ALCAM) enhance transmigration of pathogenic B and T cell subsets (115, 121, 122). Furthermore, CXCR3 is co-expressed with integrin α4β1 (VLA-4), which allows both B- and T-cell populations to bind to vascular cell adhesion protein 1 (VCAM-1) on brain endothelial cells (123). This is supported by the reducing effects of VLA-4 inhibition on B- and Th17-cell infiltration into the CNS and disease susceptibility in EAE (124). Natalizumab, a monoclonal antibody against VLA-4, is used as an effective second-line treatment for MS (125). Discontinuation of this treatment often results in severe MS rebound effects (126). Hence, the peripheral entrapment of populations like Th17.1 and $T\text{-bet}^+$ B cells in natalizumab-treated patients (44, 78) probably underlies the massive influx of blood cells causing these effects. The same is true for EBV-reactivated B cells, which are enriched in lesions from MS patients after natalizumab withdrawal (127). A previous gene network approach using several GWAS datasets further highlights the relevance of adhesion molecules on the BBB endothelium for the crossing of T and B cells (128), especially those affected by IFN-γ (115).

Local Organization and Impact

Both B and T cells accumulate in active white matter lesions of the MS brain (10, 129). In diagnostic biopsy studies, T cell-dominated inflammation is a characteristic of all lesion-types observed (130). Also in post-mortem MS lesions, white matter MS lesions with active demyelination associate with an increase in T cell numbers (10, 129). Although $CD4^+$ Th cells are in general outnumbered by $CD8^+$ CTLs in brain lesions as investigated in autopsy studies (10), their role as triggers of local pathology should not be overlooked in MS. This is consistent with the enrichment of $CD4^+$ Th cells in white matter lesions with active demyelination (10). An abundant number of $CD4^+$ Th cells were also visible in pre-active lesion sites, suggesting an involvement of these cells in the early stages of lesion formation (131). Additionally, it was demonstrated that in contrast to $CD8^+$ CTLs, brain-associated $CD4^+$ Th-cell clonotypes are reduced in MS blood, indicating specific recruitment (as described above) or, alternatively, clonal expansion in the CNS (132). Furthermore, dominant Th-cell clones were undetectable following reconstitution after autologous hematopoietic stem cell transplantation in MS patients, which was not seen for $CD8^+$ T cells (133). Interestingly, T-cell clones are shared between CNS compartments within a patient, including CSF and anatomically separated brain lesions (132, 134–137). This suggests that brain-infiltrating T cells bear similar reactivity against local (auto)antigens.

In subsets of MS autopsy cases with acute and relapsing remitting MS, B cells can also be found predominantly in the perivascular space in association with active white matter lesions (10). The role of these perivascular B cells, including $T\text{-bet}^+$ B cells (44), could be to re-activate (infiltrating) pro-inflammatory $CD4^+$ and $CD8^+$ T cells to cause MS pathology (**Figure 1**). Identical B-cell clones have been found in different CNS compartments of MS patients, including the meninges (138, 139). Within the meninges, B- and T cell-rich follicle-like structures have been found that localize next to cortical lesions, presumably mediating progressive loss of neurological function in MS (140, 141). Interestingly, MS brain-infiltrating lymphocytes express and respond to IL-21 (142), the cytokine that drives follicular T- and B-cell responses. Additionally, IFN-γ triggering of B cells promotes ectopic follicle formation in autoimmune mice (16, 45), suggesting that the structures observed in the MS CNS are induced by B cells interacting with IFN-γ-producing

T cells. However, the role of IL-17 in this process should not be ruled out, as shown in EAE (143).

Besides mediating migration and organization of pathogenic lymphocytes in the MS brain, cytokines are likely relevant effector molecules. IFN-γ production by Th cells also associates with the presence of demyelinating lesions in the CNS (144–146). IFN-γ, and possibly also GM-CSF, can activate microglia or infiltrated macrophages to cause damage to oligodendrocytes (93, 147, 148). As for B cells, increased production of TNF-α, IL-6, and GM-CSF has been found (48, 87) and we have recently shown that during Tfh-like cultures, IFN-γ drives IgG-producing plasmablasts in MS (44). One could speculate that after their re-activation by IFN-γ-producing Th cells within the meningeal follicles, T-bet$^+$ memory B cells rapidly develop into antibody-producing plasmablasts/plasma cells (**Figure 1**). IFN-γ-induced GC formation promotes the generation of autoantibodies in lupus mice (16, 45). The targeting of B cells and not plasmablasts/plasma cells by clinically effective anti-CD20 therapies in MS, as well as the abundance of oligoclonal bands in MS CSF, at least support the local differentiation of B cells into antibody-secreting cells (48, 149). We argue that IgG secreted by local T-bet-expressing plasmablasts/plasma cells are highly reactive in the MS brain (43, 44, 55), although the (auto)antigen specificity and pathogenicity of such antibodies remain unclear in MS, as well as their contribution as effector molecules to MS pathology.

Several antigenic targets have been proposed to contribute to MS pathology. Next to myelin, which is one of the most intensively studied antigens (150), also EBV antigens are considered as major candidates. EBNA-1 specific IgG antibodies are predictive for early disease activity (151) and are present in CSF from MS patients (152, 153). Some studies imply that reactivated B cells in ectopic meningeal follicles (154, 155) cross-present EBV peptides to activate myelin- and EBNA-1 specific Th cells (6, 156, 157). Whether EBV is detected in the brain or solely recognized in the periphery and how this contributes to local pathology is still a matter of intense debate in the field (127, 158–162). In addition to myelin (150) and EBV (6), other antigenic targets of locally produced IgG and infiltrating T cells have been suggested, such as sperm-associated antigen 16 [SPAG16 (163)], neurofilament light, RAS guanyl-releasing protein 2 [RASGRP2 (4)], αB-crystallin and GDP-l-fucose synthase (135).

CONCLUDING REMARKS

In this review, we have discussed potential triggers and mechanisms through which interacting B and T cells drive the pathogenesis of MS. In our presented model, peripheral B cells escape from tolerance checkpoints as the result of impaired control by chronically exhausted or genetically altered regulatory T cells. Subsequently, B cells interact with IFN-γ-producing effector Th cells in germinal centers of lymphoid organs to create a feedforward loop, after which highly pathogenic subsets break through blood-CNS barriers and, together with infiltrating CD8$^+$ CTLs are locally reactivated to cause MS pathology. Although definite proof is still lacking, these pathogenic events are likely mediated by an interplay between persistent infections such as EBV and genetic risk variants. Together, these factors may alter the selection, differentiation and pathogenic features of B- and T-cell subsets. In our view, more in-depth insights into how infections and genetic burden define the CNS-infiltrating potential and antigen specificity of such subsets should be the next step to take in the near future. The development of small molecule therapeutics against subsets driving the disease course would be an effective way of generating clinically relevant benefits without harmful effects in MS patients.

AUTHOR CONTRIBUTIONS

JL, LR, and ML designed and wrote the manuscript. ML and JS revised the manuscript.

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REVIEW
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Traumatic Spinal Cord Injury and the Gut Microbiota: Current Insights and Future Challenges

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Individuals with traumatic spinal cord injury (SCI) suffer from numerous peripheral complications in addition to the long-term paralysis that results from disrupted neural signaling pathways. Those living with SCI have consistently reported gastrointestinal dysfunction as a significant issue for overall quality of life, but most research has focused bowel management rather than how altered or impaired gut function impacts on the overall health and well-being of the affected individual. The gut-brain axis has now been quite extensively investigated in other neurological conditions but the gastrointestinal compartment, and more specifically the gut microbiota, have only recently garnered attention in the context of SCI because of their vast immunomodulatory capacity and putative links to infection susceptibility. Most studies to date investigating the gut microbiota following SCI have employed 16S rRNA genomic sequencing to identify bacterial taxa that may be pertinent to neurological outcome and common sequalae associated with SCI. This review provides a concise overview of the relevant data that has been generated to date, discussing current understanding of how the microbial content of the gut after SCI appears linked to both functional and immunological outcomes, whilst also emphasizing the highly complex nature of microbiome research and the need for careful evaluation of correlative findings. How the gut microbiota may be involved in the increased infection susceptibility that is often observed in this condition is also discussed, as are the challenges ahead to strategically probe the functional significance of changes in the gut microbiota following SCI in order to take advantage of these therapeutically.

Keywords: spinal cord injury, neurotrauma, gut dysbiosis, gastrointestinal dysfunction, inflammation, infection

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INTRODUCTION

It is well-recognized that traumatic spinal cord injury (SCI) leads to permanent sensorimotor impairments, but perhaps less appreciated is the fact that individuals with SCI also suffer from debilitating multi-system physiological dysfunction (1, 2). For example, autonomic dysreflexia, SCI-induced immune depression syndrome (SCI-IDS) and bladder/bowel dysfunction can all develop after SCI due to a disruption of autonomic pathways between the brain and the spinal cord (3–6). In the context of inflammation and immune function after SCI, the majority of neurotrauma research has focused on defining the activity of specific immune effectors, placing particular emphasis on how these impact on lesion site development. What is much less understood, however, is why the immune response to SCI is aberrant and involves co-existing pro-inflammatory and

immunosuppressive elements, both of which are notably not contained to the lesion itself. The overall picture of an obviously multi-faceted and paradoxically-acting immune system therefore remains blurred, including much of its relationship to the secondary sequalae of SCI. Whilst overall mortality has reduced considerably, current treatments do little to combat the more chronic consequences of SCI, including serious visceral comorbidities, and there has been no discernible progress in improving life expectancy and overall quality of life for affected individuals (7–9).

With the increasing recognition of an impaired and/or aberrantly acting immune system, more recent work in the field has shifted its focus toward investigating the possibility of extraneous stimuli or signals that may be influencing the immunological changes that occur after SCI. One logical candidate here may be the gut. The vast microbial communities that reside in the gut (and indeed in other niches in the body) coordinate critical functions for host survival and they have many complex interrelationships with other organs in the body, to the extent that the microbiota is now regarded an organ in its own right. The gut's microbial ecology and intrinsic immune compartment are known to exert considerable influence over basal immunological activity, any perturbations to homeostatic conditions in the gut can therefore have a robust impact on immune function (10–13). How SCI affects this aspect of the gut is only just beginning to be understood. Surveys amongst SCI patients typically reveal gut dysfunction and neurogenic bowel conditions that culminate in reduced intestinal motility, impaired defecation, abdominal pain and associated infection risk as major issues that undermine their overall quality of life, arguably more so than physical paralysis (1, 2, 14, 15). A better fundamental understanding of how the gut contributes to the pathophysiological changes and chronic consequences of SCI is therefore of paramount importance.

THE GUT MICROBIOTA

The pivotal influence and contribution of the gut microbiota to overall health is due in part to the presence of 10^{14} microbes with a taxonomic diversity encompassing bacterial, archaeal and eukaryotic species (10, 12). The microbial inhabitants of the gut are diverse between individuals and while a core microbiota of defined microorganisms does not exist, high-throughput metagenomic sequencing has revealed the reliable presence of 12 bacterial phyla, chiefly *Firmicutes* and *Bacteroidetes*, archaeal phyla, as well as rich fungal communities (10, 16–18). Together, they share a high degree of functional redundancy through a genomic content ∼150 times larger than the human genome (19, 20). Diet changes, host behavior and antibiotic treatment throughout life significantly impact on both the gut microbiota and disease susceptibility. At an extreme, work with gnotobiotic mice allude to a compromised ability of the body to effectively manage immunity; this seminal discovery already has wider implications for the use of laboratory animals that have fundamentally misrepresented (or even absent) gut microbiota, in particular when considering the translational value of these experiments (21).

The immune-modulatory capacity of the gut microbiota spans between the production of stimulatory metabolites, and the priming of immune cells that are critical for maintaining the health of the host; however, these influences can become detrimental if the microbial balance is lost. To avoid inappropriate immune activation by "non-self" material, the microbiota is largely kept separate from surveilling host immune cells via physical, biochemical and immunological means (10, 22). There are however certain commensal bacteria that actively interact with immune effectors such as *Bacteroides fragilis*, a member of the *Bacteroidetes* phylum, which directly stimulate regulatory $CD4^+$ T cells to enable its own colonization into the epithelium and simultaneously induce beneficial immunosuppression (23, 24). The secretion of immunoglobulin A stimulated by segmented filamentous bacteria (SFB) goes toward limiting the exposure of the epithelium to other pathogenic microbes (10, 25, 26). On the other hand, the pathobiont (i.e., a commensal microbe with the potential to become a pathogenic) *Escherichia coli* similarly adheres to the epithelium, but can trigger the recruitment of Th17 rather than regulatory $CD4^+$ T cells, which effectively enhances inflammation in the gut (18, 27). In considering the immunomodulatory potential of metabolites, microbial short-chain fatty acids (SCFAs) derived via anaerobic fermentation have the capacity to exert widespread influence over host cellular function, including epigenetic regulation, stem cell proliferation and gut barrier modulation and, importantly, they also act as potent anti-inflammatory mediators (18, 28). Microbe-directed immune cell manipulation is thought to be necessary for homeostatic immune control, though a push toward pro-inflammatory conditions in disease contexts such as inflammatory bowel disease implicate the pathological potential of these microorganisms if the delicate balance they maintain with the host becomes disturbed.

INTERACTIONS BETWEEN THE GUT MICROBIOTA AND THE CENTRAL NERVOUS SYSTEM

The activity and influence of the gut microbiota is not contained to local immune-gut interactions but extends via critical communication axes to distant organs including the brain. This relationship was made apparent in the association of gastrointestinal disorders with psychiatric conditions, as well as in multiple cases where antibiotic treatment modulated disease outcomes in the central nervous system (CNS) in sterile contexts (18, 29–31). Specific routes of direct communication include afferent fibers from the enteric nervous system (ENS; the intrinsic neural network of the gut), autonomic signaling and humoral pathways such as the hypothalamic-pituitary-adrenal axis and enteroendocrine/mucosal immune system communications (32). Observations that diet-induced changes in the intestinal microbiota were accompanied by increased

exploratory behavior in mice suggest that there may be additional pathways for communication with the brain that are independent of the aforementioned routes and instead rely on microbial-derived factors interacting with the CNS (29).

Effects of the microbiota-gut-brain axis have also been implicated in CNS injury and diseases. Investigations of blood-brain-barrier (BBB) development revealed that germ-free mice have increased BBB permeability, which renders the CNS vulnerable; this phenotype was only rescued with the introduction of normal microflora (33). In autism spectrum disorders, a reduced integrity of the BBB, in addition to abnormal neural development and altered gene expression, has also been linked to the gut microbiota (34). The use of germ-free mice has increased appreciation of how the microbiota shapes neuroinflammation, including in the context of experimental autoimmune encephalomyelitis (EAE—the animal model of multiple sclerosis), with both pro- and anti-inflammatory effects (29, 35). Here, germ-free and antibiotic-treated mice show reduced EAE severity compared to normal mice (36), whilst the introduction of segmented filamentous bacteria (SFB) into the gut of these germ-free mice was sufficient to instigate EAE, reinstating the ability of these mice induce Th17 cells (35, 37). Colonization of the gut with *B. fragilis* provided greater protection, however, from EAE symptoms through increased regulatory T cell activity (36, 38). The critical involvement of the microbiota in CNS disease appears recapitulated in humans, as alterations in the microbiome of multiple sclerosis patients correlated with specific gene expression patterns that direct host immune activity (39).

In the context of traumatic CNS injuries, pivotal work by Houlden et al. (30) showed that the gut microbiota undergoes significant change (i.e., gut dysbiosis) after experimental stroke and traumatic brain injury (TBI). A disturbance of the microbiota-gut-brain axis is thought to underpin the symptoms of abdominal pain, intestinal immobility and gastric ulcer formation that occur in these patients following injury (31). After an insult to the brain, a significant loss of cholinergic neurons in the gut submucosa, accompanied by an upregulation of noradrenaline from sympathetic terminals in the gastrointestinal tract, dysregulate the gut microbial content (30, 40). Changes in the gut microbiota that favor pathogenic bacteria (gram-negative species of *Bacteroidetes* and *Proteobacteria*) over beneficial species (from the *Firmicutes* phylum) can be observed as early as 2 h post-injury and persist for a week; interestingly, these alterations in microbial abundance are predictive of the lesion volume and associated behavior deficits in an almost dose-dependent manner (41). To counter gut dysbiosis after TBI, antibiotic treatment targeting pathobionts as well as probiotic interventions that support anti-inflammatory activity have been successful in decreasing pathology in the gut, thereby conferring neuroprotection. Whilst these findings emphasize the role of the microbiota-gut-brain axis in CNS injury, the exact mechanisms behind these observations and the associated clinical implications have not been addressed.

CURRENT RESEARCH INTO SCI AND THE GUT MICROBIOTA

The gut microbiota has also been rapidly gaining interest for potential "disease-modifying" effects in SCI (42). Whilst this is unsurprising given the obvious parallels between TBI and SCI, it is important to consider the direct innervation of the gut from the spinal cord, and how this may be differentially affected between these conditions as well as within SCI itself based on the neurological level of the lesion. Sympathetic nerve fibers providing autonomic input into the ENS originate from the thoracic region of the spinal cord, whilst visceral sensory afferents carrying feedback from the gut synapse with spinal cord neurons that eventually transit to the brain (43). Afferent vagal fibers also report to the brain, specifically informing it of the conditions of the intestinal environment (18). Interruption or loss of control over these various pathways and feedback loops push the intrinsic ENS circuits away from homeostasis, and this autonomic imbalance in part explains why SCI patients also suffer from severe gut immobility, fecal retention and increased risk of infections, all of which culminate in a considerably reduced quality of life (1). The impact of SCI on the gut microbiota and the subsequent consequences on inflammation and immune function are only now beginning to be systematically interrogated (see **Table 1** for a summary overview).

The first report of changes in the gut microbiome of SCI patients identified a specific reduction of beneficial butyrate-producing microbes of the *Firmicutes* phylum at 12 months or more post-injury compared to healthy controls (44). Although this work was primarily descriptive via the use of 16S ribosomal RNA (rRNA) genomic sequencing, it was suggested that this microbiome profile may be pointing toward a reduced immunomodulatory metabolite content of the gut. Later pre-clinical work in a thoracic level 9 (T9) contusion SCI mouse model by Kigerl et al. (54) reported that SCI increases gut permeability 1 week after injury, and it was postulated that, similar to stroke (40), this may allow for bacterial translocation to distant organs (45). This landmark study also sequenced 16S rRNA, which was extracted from fecal samples of mice with a moderate-severe T9 contusive SCI up to 28 days post-injury. Their results showed that the bacterial orders *Bacteroidales* decreased while *Clostridiales* significantly increased over time post-SCI. Although there is no doubt that profound changes to the gut microbiota did occur in these SCI mice, a consideration around this study is that the experimental design did not include fecal samples from sham-operated controls beyond the sub-acute phase (>7 days post-SCI). The 16S rRNA sequencing results for these mice were further presented as pooled data rather than being split between the acute (0–3 days) and sub-acute (5–7 days) phases post-surgery. A more recent study by Schmidt et al. (45) showed acute effects of surgery (i.e., laminectomy) and/or anesthesia on the gut microbiome, albeit in rats, and others have reported rapid and profound shifts in the gut microbiome profile of poly-traumatized human patients with no documented history of neurological injury (46). Going forward, the impact of trauma itself, SCI severity, lesion level and possible

TABLE 1 | A summary of gut microbial changes after SCI in pre-clinical and human investigations.

	Study details	PCR gene primers	Microbial changes (vs. control) Phyla + lower taxonomic ranks	Intervention/ Treatment	References
Pre-clinical studies	**Animal**: Female C57BL/6 mice **SCI:** 75 kdyn T9 contusion (cont.) SCI **Controls:** T9 Laminectomy + naïve **Timepoints:** ≤28 days hard enter Separately housed, no antibiotics. Food intake equilibrated across all animals.	16s rRNA V4–V5 515F 806R	↑ *Firmicutes* ↑ (o) *Clostridiales* ↓ *Bacteroidetes* ↓ (o) *Bacteroidales*	**VSL #3 probiotic** ↓ Gut dysbiosis ↑ Functional recovery	(54)
	Animal: Adult female Fischer rats **SCI:** moderate-severe T9 cont. SCI (weight drop: 10 g rod from 25.0 mm) **Controls:** T9 Laminectomy **Timepoints:** 8 weeks (wks) Co-housed in injured + non-injured pairs, 7-day gentamicin treatment. *Ad libitum* access to food and water.	16s rRNA V4 Unknown primers	= α diversity *Actinobacteria* ↑ (f) *Bifidobacteriaceae* ↑ (s) *B. choerinum* *Firmicutes* ↑ (f) *Clostridiaceae* ↑ (s) *C. disporcum* ↓ (s) *C. saccharogumia* (f) *Lactobacillaceae* ↑ (s) *L. intestinalis*	–	(50)
	Animal: Adult female C57BL/6 mice **SCI:** 50 kdyn T9 cont. SCI **Controls:** T9 Laminectomy **Timepoints:** ≤6 weeks Co-housed in exp. group, no antibiotics	16s rRNA V3–V5 (V4) Unknown primers	↑ Increased bacterial load ↓ *Firmicutes* ↑ *Bacteroidetes* ↑ *Proteobacteria*	**PDE4B$^{-/-}$ KO mice** ↓ Gut dysbiosis ↓ Neuroinflammation ↑ Functional recovery	(53)
	Animal: Adult female C57BL/6 mice **SCI:** 70 kdyn T10 cont. SCI **Controls:** T10 Laminectomy **Timepoints:** 28 days No antibiotics. *Ad libitum* access to food and water.	16s rRNA V3–V4 338F 806R	↑ α diversity *Firmicutes* ↓ (o) *Lactobacillales* ↓ (g) *Lactobacillus* ↑ (o) *Clostridiales* ↑ (f) *Lachnospiraceae* *Actinobacteria* ↓ (o) *Bifidobacterialis*	**Melatonin** ↓ Gut dysbiosis ↓ Leaky gut ↑ Functional recovery	(55)
	Animal: Adult female Lewis rats **SCI:** 125 kdyn unilateral C5 cont. SCI **Controls:** C5 Laminectomy and naïve **Timepoints:** preinjury, 3 days, 4 weeks Co-housed in exp. group; no antibiotics. *Ad libitum* access to food and water.	16s rRNA V4 Unknown primers	↑ α diversity in all groups at 3 dpi Significantly different OTUs (g/s level): 155 = SCI vs. healthy 40 = SCI vs. sham *Analysis of phylogenetic differences in supplementary data*	**Fecal Transplant** ↓ Gut dysbiosis ↓ Anxiety-like behavior	(45)
Human studies	**SCI:** AIS grade A Cont. SCI **Control:** Healthy individuals **Further comparisons:** Upper motor neuron (UMN) + lower motor neuron (LMN) bowel syndrome **Timepoints:** ≥1 year post-injury 1–3 weeks standard diet, 3 weeks no antibiotics	16s rRNA V4 515F 806R	*Firmicutes* ↓ (g) *Pseudobutyrivibrio* ↓ (g) *Dialister* (UMN) ↓ (g) *Megamonas* ↓ (g) *Marvinbryantia* (UMN vs. LMN) ↓ (g) *Roseburia* (LMN)	–	(44)
	SCI: AIS grade A SCI **Control:** Healthy males **Further comparisons:** Quadriplegia (quad) vs. paraplegia (para) **Timepoints:** ≥ 6 months post-injury 2 weeks standard diet, 1 month no antibiotics	16s rRNA V3–V4 338F 806R	↓ α diversity ↓ *Firmicutes* (Quad vs. Healthy and Para) ↓ (g) *Dialister* ↓ (g) *Megamonas* ↓ (g) *Eubacterium* ↓ (g) *Subdoligranium* ↓ (g) *Faecalibacteria* (Quad) ↑ (g) *Blautia* ↑ (g) *Lachnoclostridium* ↑ (g) *Phascolarctobacterium* (Para) *Bacteroidetes* ↓ (g) *Prevotella* ↑ (g) *Bacteroides* ↑ (g) *Parabacteroides* (Para) ↑ *Proteobacteria* ↑ (g) *Escheria/Shigella* ↑ *Verrucomicrobia*	–	(14, 52)* **Same quadriplegic patient cohort*

interspecies differences therefore all require careful investigation as to how they impact on the gut microbiota and, if so, for how long these changes persist or perhaps even diverge with time (46–49). This becomes particularly important when exploring correlations between select changes in the gut microbiota and the neurological outcome.

A separate study by O'Connor et al. also examined differences in microbial content of the gut following T9 contusion SCI in rats (50). They detected significant modifications in the gut microbiome after SCI during the intermediate/chronic phase of SCI (8 weeks post-injury) as compared to the sham-operated control group. Somewhat counterintuitively perhaps is that this study found a greater prevalence of *Lactobacillus intestinalis,* a lactic acid-producing probiotic bacterial species generally considered to be beneficial. By the same token, the microbiota of SCI animals also showed unexpected post-SCI rises in certain *Clostridaiceae* and *Bifidobacterium* species that are primarily thought to be beneficial. It may be that the activity of these commensal bacteria becomes pathogenic (and/or of lesser influence) within an inflammatory environment (51). Certainly, pro-inflammatory cytokines such as IL-1β, IL-12 and MIP-2 were significantly elevated in intestinal tissue 4 weeks after SCI, the extent of which was also correlated with a reduction of beneficial butyrate-producing bacteria in the gut, which falls in line with previous human SCI work (44). It is important to note, however, that all animals in this study received a 7-day course of gentamicin treatment following surgery. How this and also the use of general anesthesia in experimental studies impacts on the gut microbiota, including the shaping of any SCI-associated changes therein, remains unclear. Whilst the study by Kigerl et al. (54) therefore may provide more specific insights how traumatic SCI itself impacts on the gut microbiota, the findings of O'Connor et al. (50) are still of significant translational value given that most human patients undergo surgery and typically receive prophylactic antibiotic as well as probiotic treatment after their injury.

Several more recent reports have attempted to better define the consequences of SCI-induced changes in the gut microbiota, linking these directly to specific bacterial types that could be directly therapeutically targeted. For instance, in a Chinese cohort of male SCI patients, Zhang et al. (14) reported that the overall diversity of the gut microbiota was significantly reduced 6 months after SCI compared to healthy controls. Amongst a spectrum of changes in bacterial phyla and genera and an overall decrease in microbial diversity after SCI, these authors found that *Bacteroides*, a genus of the *Bacteriodales* order, increased with SCI (14); they also observed an increase in the abundance of bacteria from the *Proteobacteria* and *Verrucomicrobia* phylum. These changes were directly compared with aspects of neurogenic bowel dysfunction as well as the extent of physical paralysis, which again revealed more specific microbial alterations. A more recent follow-up investigation by this group correlated these established changes in the microbial profile to the serum lipid profiles of this patient cohort (52). Another investigation in mice by Myers et al. also characterized SCI-induced gut dysbiosis, noting a significant increase of the *Proteobacteria* phylum at 6 weeks after injury compared to an uninjured control group, which is in agreement with human SCI findings and perhaps suggests a bias toward gram-negative endotoxin-containing bacteria as drivers gut pathogenesis in this condition (53). The findings of this study also pointed toward a reduction in *Firmicutes*, along with an increase in *Bacteroidetes* phyla. Genetic ablation of the phosphodiesterase PDE4B prevented these changes in bacterial phyla, which coincided with improved functional recovery via inflammatory modulation. An investigation by Jing et al. (55) measured an overall increase in bacterial diversity in SCI mice [which goes against some human SCI microbiome analysis (14)], in particular a relative increase in the abundance of *Clostridiales*, as was found in previous work (54), and a decrease in *Lactobacillales* and *Bifidobacteriales*. Daily melatonin treatment post-injury appeared to reverse some of these changes, and this was correlated with a more favorable cytokine profile and improvements in gut barrier integrity and functional recovery (55). Lastly, the study by Schmidt et al. that was alluded to earlier documented transient changes in the gut microbiota of rats with a unilateral mild cervical contusion SCI, which occurred as early as 3 dpi before resolving by 4 weeks, and correlated these changes with anxiety-like behaviors (45). Treatment of these rats with fecal transplants from naïve animals resulted in a normalization of the gut microbiota based on 16S rRNA sequencing results, and also prevented the onset or development of anxiety-like behaviors. No improvements in lesion pathology and locomotor recovery were observed in association with this intervention (45). Interestingly, the study by Kigerl et al. (54) showed that the extent of neuroinflammation at the site of SCI could, at least partly, be ameliorated with the therapeutic use of the probiotic VSL #3, which contains lactic acid-producing bacteria from the *Lactobacillus* and *Bifidobacterium* genera. Whilst the root causes that drive SCI-induced gut dysbiosis remain unknown, and also how VSL #3 provides neuroprotection at the lesion site, this finding clearly holds promise for clinical translation. It also emphasizes the point that either preventing gut dysbiosis or, alternatively, restoring the composition of the gut microbiota to a pre-injury state may not necessarily lead to beneficial outcomes, but rather that introduction and/or boosting of beneficial microbial communities may be required to skew the inflammatory response toward one that improves the neurological outcome.

Integrating all these specific microbial alterations at various taxonomic ranks and the functional significances of these will now form the next challenge, especially given the vast amount of data that is typically acquired from sequencing studies. On the whole, most investigations have reported a decrease of bacterial taxa in the *Firmicutes* phylum that occasionally coincides with an increase of the *Bacteroidetes* phylum. In combination, this may be indicative of a SCI-associated adjustment in the "*Firmicutes*:*Bacteroidetes*-ratio." It should be noted that the studies of Kigerl et al. (54) and O'Connor et al. (50) seemingly reported opposing results here, but these are likely attributable to experimental deviations. Specifically, Kigerl et al. (54) housed their mice individually to avoid coprophagia whilst animals were co-housed in the study by O'Connor et al. (50). As mentioned earlier, O'Connor et al. (50) also prophylactically gave their SCI rats 7 days of gentamicin treatment whereas the study

by Kigerl et al. (54) avoided the use of antibiotics altogether. More broadly speaking, recent pivotal work by The Human Microbiome Project showed that the *Firmicutes*:*Bacteroidetes*-ratio may be more reflective of an organism's "microbial equilibrium" and therefore not necessarily as suitable a measure of dysbiosis between individuals as previously thought (56). The *Proteobacteria* phylum also appears to increase in abundance in both a pre-clinical and SCI patient setting. Given that certain *Proteobacteria* genera have been implicated in driving peripheral inflammation (57), future studies should therefore derive a clearer putative mechanism for this phylum in the context of SCI-associated pathology.

Taken together, the above-listed exploratory studies have been instrumental in substantiating the association between the gut microbiome and SCI-associated pathology, although the findings remain correlative for the most part and the drivers of dysbiosis are still currently unknown. All of these investigations employed 16S rRNA gene sequencing to map out bacterial diversity of the gut via the generation of big genomic datasets. It is important to recognize that, when used in isolation, this technique has some major caveats: (1) archaeal and fungal communities are omitted from these analyses and, perhaps more importantly, (2) the identification of specific organisms may not necessarily be conducive to defining the causes of dysbiosis, altered gut function and its wider peripheral consequences. This may run the risk of potentially convoluting our understanding of how certain microorganisms drive and/or link to pathophysiological changes. The reliance that this type of analysis places on designating operational taxonomic units of interest overlooks the global metabolic/physiological potential of the gut microbiota as a whole, which ultimately may provide a more informative and complete perspective on gastrointestinal activity after SCI. It should also be noted that examining both the murine and human microbiomes at a genus/species level may not be appropriate at times, and interrogating the broader functional perspective of the microbiota instead, perhaps via the use of enterotypes, may be a more applicable and translatable approach in this field (58).

ENTEROTYPES AS A WAY FORWARD TO INTERPRET THE SIGNIFICANCE OF CHANGES IN MICROBIOTA BETWEEN CONDITIONS AND SPECIES?

A multitude of techniques, experimental design options and analysis strategies have been recommended to better resolve the functional profile of the gut microbiota in SCI and the putative implications thereof [reviewed in Kigerl et al. (59)]. One additional approach may, however, be to consider the entire microbiota of an organism as a whole via the use of global classifications, also known as enterotypes (58). This stratification strategy aids in removing bias that researchers often place on changes in specific microbial genera/species, which may be overstating the functional relevance of these (60, 61). The human gut microbiome was the first to be stratified into three enterotypes based on bacterial compositional clustering around a central/driver taxon, with profiles aligned around particular functional characteristics, such as the synthesis of different vitamins and various metabolic activities (60). Enterotypes have been claimed to represent the majority of inter-individual diversity in humans as opposed to a continuum of microbial differences and, notably, certain abundant physiological functions were associated with relatively rarer bacteria genera (60). The delineation of specific enterotypes continues to receive much scrutiny, however, with some recent work proposing a gradient of microbial variation, and others being concerned by the inherent risk of oversimplification with a stratification model (61–63). It is nonetheless evident that analyzing global microbial patterns is likely to prove quite informative given the overlap in enterotypes observed in humans and model organisms (58, 64). Hildebrand et al. (64) indeed revealed the presence of such enterotypes in various strains of laboratory mice, with a low-richness cluster that was dominated by *Bacteriodetes* (similar to the human equivalent "enterotype 1") and a high-richness cluster was populated with *Ruminococcaceae* (similar to the human equivalent "enterotype 3"). The microbial richness of these murine clusters was also found to be associated with varying levels of the calprotectin protein, a marker of intestinal inflammation, suggesting that certain mice may more readily induce inflammation depending on their gut enterotype (64). The presence of similar enterotypes in humans and laboratory mouse strains therefore demands a greater awareness to be given to enterotypes during experimental design in pre-clinical research, particularly from a translational perspective, as they may not necessarily be reflected in genera/species-specific compositional differences (58). To date, trauma-related perturbations of enterotypes have not been studied in humans or mice. Future work may therefore benefit from profiling potential enterotype-like clustering in animals prior to and after injury, in order to examine the possible impact of identified gut patterns or changes therein, and to also ascertain the impact of an "injury-state" enterotype on whole organism physiology (61).

THE ROLE OF THE GUT MICROBIOTA IN INFECTION SUSCEPTIBILITY

SCI patients are highly susceptible to life-threatening infections, and this is often attributed to the peripheral immune depression that many patients experience following their injury, a phenomenon known as SCI-IDS. SCI-IDS manifests as a reduction of circulating leukocytes, acute lymphoid organ atrophy and, in animal models, an increase in bacterial colony-forming units (CFUs) that can be cultured from e.g., the lungs (65, 66). Seminal work by Prüss et al. (65) showed that an adrenal gland removal/transplantation paradigm (which restores only cortical function of the adrenal glands), could achieve a "homeostatic" re-balancing of noradrenaline and glucocorticoid levels. This was found to alleviate the consequences of SCI-IDS after high-level (T1) SCI, with reductions noted in the number of CFUs that could be cultured from the lungs. This study did not establish, however, the critical mechanisms that allow for this "spontaneous pneumonia" to develop, leaving the question of how an imbalance in the aforementioned stress factors leads to

an increased presence and/or growth of microbes in the lungs as of yet unanswered. Kigerl et al. (54) suggested that the gut may perhaps "leak" infectious microbes after SCI, and that this may lead abnormal bacterial presence in extraneous tissues such as the lungs; however, the capacity of the gut microbiota to instigate infection through translocation is yet to be proven.

Support for a role of the gut microbiota as a source of disseminating bacteria comes from prior investigations into infection susceptibility following ischemic stroke (40). Here, Stanley et al. (40) demonstrated that airway infections after stroke are only observed in specific-pathogen-free mice (i.e., mice with microbiota but devoid of known pathogens), and not germ-free mice (i.e., mice with no microbiota). Sequencing of the lung microbiome after injury and bacterial tracking experiments led to the conclusion that the microbial source of infection was derived from the gut content. These findings are consistent with observations in other conditions where a translocation of bacteria to the lungs has been described, including sepsis and acute respiratory distress syndrome (67). In their exploration of potential mechanisms driving gut dysregulation after stroke, Stanley et al. (40) showed that the intestinal barrier was "leaky" as a result of increased gut permeability and altered epithelial tight junction distribution. These researchers also suggested that disrupted sympathetic innervation of the gut triggers the movement of commensal bacterial from the gut, which they verified via a reduced post-stroke infection incidence with adrenergic receptor inhibition (40). Taken together, this investigation offered a highly novel concept for the occurrence of airway infections after an acquired CNS insult, which may rationalize the theoretically-coupled incidences of SCI-induced gut dysfunction and dysbiosis with the heightened infection susceptibility in this patient population.

It is interesting to note, however, that although Stanley et al. (40) provided evidence in support of the premise that a disrupted neuronal circuitry instigated gut permeability and dysregulation after experimental stroke, blocking adrenergic signaling only resulted in a decreased bacterial load as opposed to a complete elimination of microbes from the lungs, suggesting

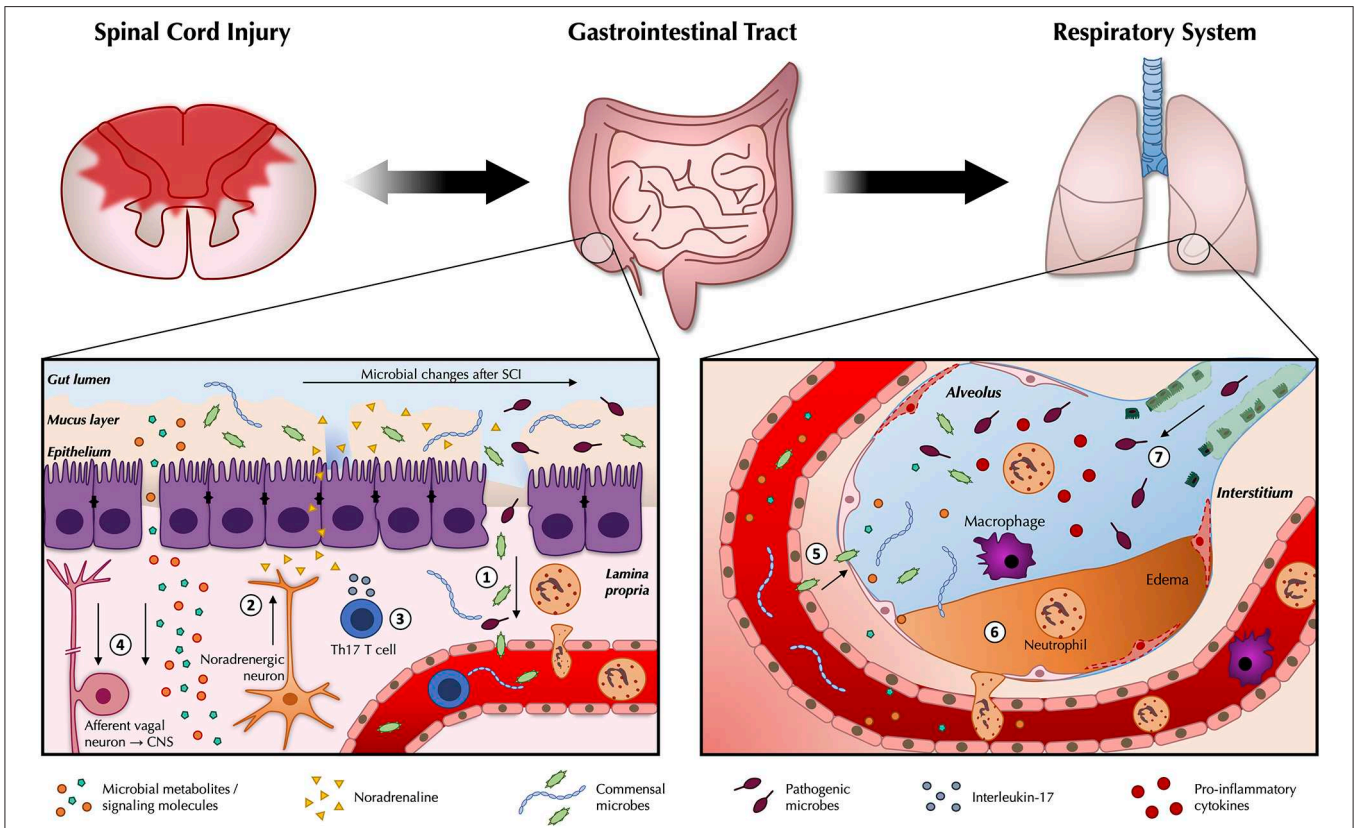

FIGURE 1 | Pathogenic changes in the gut microbiota after traumatic spinal cord injury. As part of the wider systemic response to SCI, inflammatory changes in the gut are likely to contribute to reduced intestinal function and barrier integrity (1). Leakiness of the gut epithelium can dysregulate the microbial community in the gut lumen and allow for bacterial translocation. Release of noradrenaline from post-ganglionic sympathetic terminals is thought to further contribute to gut dysbiosis (2). A greater abundance and/or expansion of specific pathobionts after SCI can induce a Th17 response, which propagates further inflammation (3). Afferent sensory feedback signals report perturbations in the intestinal environment to the brain via the vagus nerve, thereby completing the bidirectional loop between the gut and the central nervous system, while gut microbes release metabolites and signaling molecules such as short-chain fatty acids (SCFAs) that shape peripheral immunomodulatory processes (4). These changes may modulate the immune response to e.g., airway infections. Commensal microbes from the gut may also be able to take up inappropriate residence in the lungs (5). Local inflammatory changes in the lungs in response to SCI may compromise the respiratory epithelium (6), and interfere with local defense mechanisms against extraneous pathogens (7).

the putative existence of other/concurrent mechanisms that add to infection susceptibility. Intestinal damage resulting in the "leaky gut" phenotype may additionally be explained by excessive inflammatory processes that already exist in the context of the original disease/injury occurring in the host. For instance, in graft-vs.-host disease, neutrophils are recruited to the intestinal wall and are responsible for tissue damage via the induction of reactive oxidative species. Their destructive activity appeared dependent on the presence of translocating microbes into the peri-intestinal tissue, as neutrophils were not recruited in germ-free mice (68). Thus, displaced microbes may act as a chemotactic stimulus for inflammatory immune cells, whereby their movement could dictate the site of inflammation. When considering SCI, it is well-established that neutrophils do not just accumulate at the lesion site itself (69), but also in peripheral tissues. Here, systemically circulating neutrophils can cause widespread tissue damage in other organs such as the liver, spleen, lung and kidneys, a phenomenon more generally known as Systemic Inflammatory Response Syndrome (SIRS) (70, 71). It has already been noted that patients with severe SIRS also endure gut dysbiosis (72), so it is tempting to speculate that the early mobilization and priming of neutrophils by a CNS insult like SCI may also deleteriously affect the gut. Alternatively, the immunomodulatory activity of commensal gut microbes and/or the changes therein, as described earlier, may also add to altered systemic immune function. These possibilities are not mutually exclusive.

Perturbations to normal crosstalk between the gut microbiota and the immune system may disrupt this delicate homeostatic balance and provoke the unwanted residence and/or growth of extraneous pathogens in the airways of the host (11). For example, protection against pneumonia instigated by *S. aureus* is conferred in part by the activity of SFB in the gut which again promote pulmonary Th17 immunity (73). The gut microbiota normally positively regulates host defense against pneumococcal pneumonia by limiting bacterial dissemination, controlling inflammation, and by enhancing the phagocytic function of resident alveolar macrophages; these protective influences of the gut microbiota are dysregulated in germ-free mice (11, 74). These results are also corroborated by experiments in $Rag^{-/-}$ mice (which are deficient in T and B cells), as gut SFB can still instruct innate immune effectors here to resolve infections via the gut-lung axis (75). Lastly, unregulated secretion of anti-inflammatory SCFAs may also negatively interfere with host immunity and play into the pathophysiology of respiratory diseases, as has been documented in a cohort of tuberculosis-suffering patients (76). Studies into the intestinal microbiota of human patients already indicate changes in beneficial butyrate-producing microbes after SCI, warranting further investigations as to how this may play into impaired host immunity (44). Given the high prevalence of airway infections after SCI, a better understanding of how disruption in critical feedback circuits with the gut microbiota can work together with SIRS as a possible propagator of tissue damage may further rationalize the degree of vulnerability patients have toward extraneous sources of infection (i.e., of nosocomial origin) after injury (77, 78) (see **Figure 1**).

CONCLUSION

It is clear that individuals with SCI suffer from severe gastrointestinal dysfunction, the extent of which significantly impacts on their overall quality of life. As the importance of the gut microbiome for overall health and well-being is increasingly recognized, the significance of investigating the impact of SCI thereon is without question. Recent investigations all corroborate evidence that SCI undeniably changes the gut microbiota, and future studies can now aim to more specifically address how altered signaling via the CNS-gut axis may influence outcomes. Moving forward, future studies should aim to engage advanced metagenomic techniques so that the overall immunological and functional influence of the gut microbiota can be evaluated more thoroughly. As gut dysfunction may play a role in the increased infection susceptibility of this patient population, the net influence of changes in the gut microbiota over host immune function after SCI need to be better understood. It will be imperative, however, that all aspects of the gut microbiota are considered here to generate wholistic perspective of immunological dysfunction and microbial alterations after SCI, in order for these to be successfully translated into effective intervention strategies for SCI patients.

AUTHOR CONTRIBUTIONS

TJ drafted the manuscript. MR provided critical feedback. Both authors contributed to editing and approved of the final manuscript.

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REVIEW
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Visualizing the Central Nervous System: Imaging Tools for Multiple Sclerosis and Neuromyelitis Optica Spectrum Disorders

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Multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) are autoimmune central nervous system conditions with increasing incidence and prevalence. While MS is the most frequent inflammatory CNS disorder in young adults, NMOSD is a rare disease, that is pathogenetically distinct from MS, and accounts for approximately 1% of demyelinating disorders, with the relative proportion within the demyelinating CNS diseases varying widely among different races and regions. Most immunomodulatory drugs used in MS are inefficacious or even harmful in NMOSD, emphasizing the need for a timely and accurate diagnosis and distinction from MS. Despite distinct immunopathology and differences in disease course and severity there might be considerable overlap in clinical and imaging findings, posing a diagnostic challenge for managing neurologists. Differential diagnosis is facilitated by positive serology for AQP4-antibodies (AQP4-ab) in NMOSD, but might be difficult in seronegative cases. Imaging of the brain, optic nerve, retina and spinal cord is of paramount importance when managing patients with autoimmune CNS conditions. Once a diagnosis has been established, imaging techniques are often deployed at regular intervals over the disease course as surrogate measures for disease activity and progression and to surveil treatment effects. While the application of some imaging modalities for monitoring of disease course was established decades ago in MS, the situation is unclear in NMOSD where work on longitudinal imaging findings and their association with clinical disability is scant. Moreover, as long-term disability is mostly attack-related in NMOSD and does not stem from insidious progression as in MS, regular follow-up imaging might not be useful in the absence of clinical events. However, with accumulating evidence for covert tissue alteration in NMOSD and with the advent of approved immunotherapies the role of imaging in the management of NMOSD may be reconsidered. By contrast, MS management still faces the challenge of implementing imaging techniques that are

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capable of monitoring progressive tissue loss in clinical trials and cohort studies into treatment algorithms for individual patients. This article reviews the current status of imaging research in MS and NMOSD with an emphasis on emerging modalities that have the potential to be implemented in clinical practice.

Keywords: multiple sclerosis, neuromyelitis optica spectrum disorders (NMOSD), magnetic resonance imaging, optical coherence tomography, neuroimaging

INTRODUCTION

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) are inflammatory, autoimmune central nervous system conditions that have shown increasing incidence and prevalence over the past decades (1–5). While MS is the most frequent inflammatory CNS disorder in young adults, NMOSD is a rare disease. Relative frequency within the demyelinating CNS diseases varies widely among different ethnicities and regions, accounting for ∼1% of demyelinating disorders (6, 7). Based on results from population-based studies, NMOSD prevalence broadly ranges from 0.52 to 7.7 per 100,000 (7). Although NMOSD frequency in Asian and White/Caucasian ethnicities seems to be comparably similar (4, 8), Blacks seem to have highest NMOSD prevalence of up to 13/100,000 as inferred from mixed Northern American populations (9, 10).

For a long time, NMOSD had been seen as a rare variant of MS; however, the seminal discovery of a highly specific serum IgG autoantibody to the astrocyte water channel aquaporin-4 (AQP4) in up to 80% of NMOSD patients and subsequent research into the role of these antibodies in disease pathogenesis and lesion formation has made clear that this is a condition distinct from MS (11–17). Clinical experience has then shown that most immunomodulatory drugs used in MS are inefficacious or even harmful in NMOSD, emphasizing the need for a timely and accurate diagnosis and distinction from MS (18–21). Despite distinct immunopathology and differences in disease course and severity, there might be considerable overlap in clinical and imaging findings, posing a diagnostic challenge for managing neurologists. Differential diagnosis is facilitated in case of a positive serology for AQP4-abs obtained with a highly specific cell-based assay but might be difficult in seronegative cases or when less specific assays for AQP4-abs are used (22, 23).

Imaging of the brain, optic nerve, retina, and spinal cord is a procedure of paramount importance when managing patients with inflammatory CNS conditions at first presentation to enable diagnosis and differential diagnosis (24–28). Once a diagnosis has been established, imaging techniques are often deployed at regular intervals over the disease course as surrogate measures for disease activity and progression and to surveil treatment effects (29, 30). Although the application of some imaging modalities for monitoring of disease course was established decades ago in MS, the situation is less clear in NMOSD in which work on longitudinal imaging findings and their association with clinical disability is scant (26). Moreover, as long-term disability is mostly attack-related in NMOSD and does not stem from insidious progression as in MS, regular follow-up imaging might not be useful in the absence of clinical events. However, with accumulating evidence for covert tissue alteration in NMOSD and with the advent of approved immunotherapies, the role of imaging in the management of NMOSD might have to be reconsidered in the near future (31–37). In addition, imaging markers indicating impending relapses are an unmet need in NMOSD. On the contrary, MS management still faces the challenge of implementing imaging techniques that are capable of monitoring progressive tissue loss (for example brain or spinal cord atrophy) in clinical trials and cohort studies into treatment algorithms for individual patients (38–40).

This article reviews the current status of imaging research in MS and NMOSD with an emphasis on emerging modalities that have the potential to be implemented in clinical practice for diagnosis, differential diagnosis, and monitoring of disease course and immunotherapies.

MULTIPLE SCLEROSIS

As in previous versions of the MS diagnostic criteria, conventional MRI of the brain and spinal cord (T2/Flair/T1 post gadolinium sequences) is a cornerstone for an MS diagnosis within the 2017 revision of the McDonald criteria (41, 42), taking potential "red flags" and "MS mimics" into consideration that may point to an alternative diagnosis (24, 25). However, sensitivity of the 2017 criteria might have improved, and time to diagnosis appears to be shorter at the expense of specificity (43–45). Thus, frequent misdiagnosis of MS based upon misinterpretation of imaging findings on conventional MRI in conjunction with atypical clinical presentations even by MS experts has remained an alarming issue (46–49).

Recently the so-called "central vein sign" (CVS) was proposed as a potential new biomarker for a more specific MS diagnosis, emerging from observations, mostly at ultra-high field MRI studies, that MS lesions are frequently characterized by a small intralesional vein in contrast to relevant imaging differential diagnoses, such as NMOSD, small vessel disease, inflammatory CNS vasculopathies, Susac syndrome, and others (50–56).

CVS is now reliably assessable at 3T, for example, using T2*/FLAIR and co-registered SWI images, and might, therefore, become a clinically applicable imaging feature to discriminate MS from classical mimics at a high specificity (56–58) (**Figure 1**). In one study, a threshold of 50% perivenular lesions discriminated MS from inflammatory vasculopathies, such as Behcet disease, primary angiitis of the CNS, antiphospholipid syndrome, Sjögren syndrome, and systemic lupus erythematosus (SLE), with 100% accuracy (56), and another multicenter study conducted by

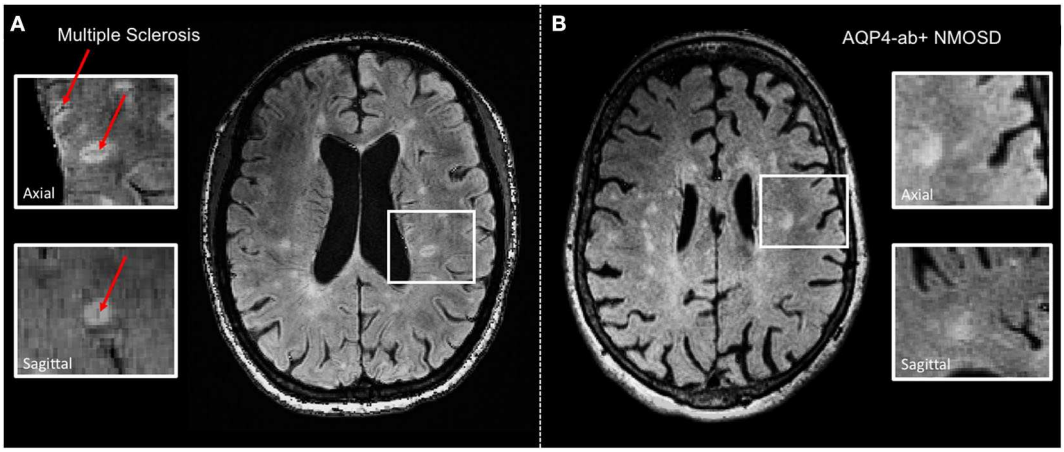

FIGURE 1 | Representative axial 3 T FLAIR-SWI images from individuals with **(A)** relapsing–remitting multiple sclerosis (RRMS; 28-year-old woman) and **(B)** AQP4-antibody-positive neuromyelitis optica spectrum disorder (AQP4+-NMOSD; 76-year-old woman). The central vein sign *(red arrows)* is present in the majority of MS lesions but not in white matter lesions in NMOSD. White boxes show magnified views of lesions in axial and sagittal plane. T, Tesla; FLAIR, fluid-attenuated inversion recovery; SWI, susceptibility-weighted imaging; RRMS, relapsing-remitting multiple sclerosis; AQP4-ab+, AQP4-antibody positive; NMOSD, neuromyelitis optica spectrum disorder.

the MAGNIMS consortium reported a specificity of 83% for a 35% CVS proportion threshold for discriminating MS from mimics such as NMOSD, SLE, migraine, cluster headache, diabetes, and other types of small vessel disease (58). Perhaps less onerous in the clinical situation is the three-lesion CVS criterion, which yielded a specificity of 89% for discriminating MS from other conditions. In this study, sensitivity was better with an optimized T2*-weighted sequence. These findings require replication in prospective studies enrolling patients with various ethnic backgrounds and from different regions of the world and will hopefully lead to a novel imaging biomarker with high specificity for MS that might find its way into a future revision of the McDonald criteria.

Although MRI T2 hyperintense lesions represent one of the major diagnostic hallmarks of MS, macroscopic MRI-visible lesions are commonly termed as "tip of the iceberg" because many more lesions are detected by histopathology at a microscopic level (59). Particularly, cortical lesions are widely elusive to conventional MRI at 3 Tesla although introduction of ultra-high field 7 T MRI more than doubles detection of cortical MS lesions (60) (**Figure 2**). Of note, post mortem studies showed that sensitivity to detect cortical lesions at 7 T is strongly influenced by their histopathological subtype, ranging from 11 to 100% (61). Hence, cortical pathology still remains more extensive than even 7 T MRI can reveal.

Cortical lesions are considered a distinctive feature of MS and are rarely present or even totally absent in other conditions mimicking multiple sclerosis, such as migraine or NMOSD (60). Intriguingly, presence and number of cortical pathology appears to correlate with clinical outcomes, most notably cognitive impairment in MS (62). However, clinical significance of cortical lesions is controversially discussed throughout the literature, and further 7 T MR studies, including investigations with improved visualization at magnetization-prepared 2 rapid acquisition gradient echoes (MP2RAGE), are highly warranted to clarify potential diagnostic and prognostic value of MS cortical pathology (63).

Brain and spinal cord volumetric imaging is another MR-based measure that might have the potential to be used in clinical practice to monitor disease progression and treatment response. Both neuropathology and imaging studies have shown that atrophy of the entire brain, including cortical and deep gray matter (DGM) as well as the spinal cord, are typical hallmarks of MS from earliest disease stages (64–68) and that, particularly, cerebral gray matter volume loss (above all, the deep gray matter) and spinal cord atrophy correlate with clinical disability and cognitive impairment and are predictive of further disease progression in longitudinal studies (69–78). In clinically stable and untreated MS patients, annual brain volume loss ranges from ∼0.5 to 1.0% in comparison to 0.1–0.3% for healthy subjects (73, 79). In a recent large European multicenter study comprising more than 1,200 patients with MS and more than 200 healthy subjects, volumes of deep and cortical gray and white matter were obtained, and participants followed over an average of 2.41 years (69). Deep gray matter showed the fastest annual atrophy rates, which ranged from −1.34 to −1.66% in various MS forms and was −0.88% in CIS and −0.94% in HC. Of all regional volumes quantified at baseline, only deep gray matter volume predicted time to EDSS progression, which underscores the relevance of DGM loss for disability accumulation. A 7.5-year longitudinal study (range 1–12 years), 206 MS patients and 35 healthy controls reported a cutoff of −0.4% annualized brain volume change to have a sensitivity of 65% and a specificity of 80% for discriminating physiological from pathological brain volume loss (80). The clinical relevance of this cutoff remains to be demonstrated. Various immunotherapies have been shown to decelerate brain volume loss; however, it is currently unclear how this observation would inform treatment decisions in

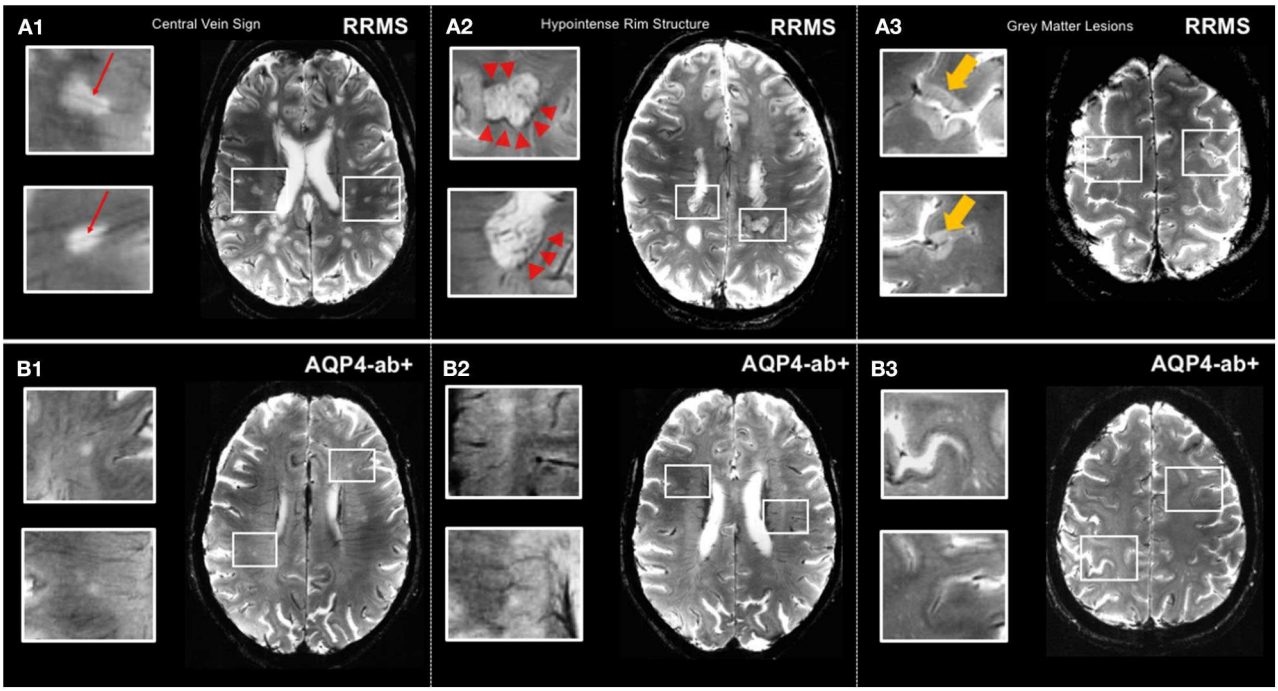

FIGURE 2 | MS-specific 7 T MR imaging markers displayed by T2*-weighted sequence. **(A1)** Lesions in relapsing-remitting MS commonly exhibit a central vein *(red arrows)*. **(A2)** Hypointense rim structures *(red arrow-heads)* are prevalent in a subset of MS lesions. **(A3)** 7 T MRI allows for the delineation of gray matter lesions in great detail. **(B1,B2)** Central vein sign and hypointense rim structures are absent in lesions of AQP4+-NMOSD patients. **(B3)** Gray matter lesions are commonly absent in AQP4+-NMOSD. MS, multiple sclerosis; T, Tesla; FLAIR, fluid-attenuated inversion recovery; SWI, susceptibility-weighted imaging; RRMS, relapsing-remitting multiple sclerosis; AQP4-ab+, AQP4-antibody positive; NMOSD, neuromyelitis optica spectrum disorder. LGN, lateral geniculate nucleus; V1, primary visual cortex.

individual patients (81–85). Brain volumetric measurements for use in individual patients are still hampered by numerous technical challenges, such as inter-session variability, influence of physiological factors (for example, hydration status), normal aging and comorbidities on brain volumes, time of day of MR scan, effect of lesion filling on post-acquisition quantitation procedures, and systematic differences pertaining to scanners and sequences parameters (38, 86). Therefore, despite sufficient accuracy of brain volume measurements in observational and interventional cohort studies, the technology is not yet apt to reliably investigate changes in individual patients within periods of less than a few years and therefore—also in light of the various physiological sources of error—atrophy measurements are currently not usable to monitor therapy in MS (30, 73, 87). Besides technical advances to reduce measurement variability, a better understanding into the neuropathological correlates and drivers of deep and cortical gray matter atrophy and whole brain volume loss is urgently required (38). The same applies to spinal cord atrophy, which is relatively easy to measure at the cervical level (mean upper cervical cord area or MUCCA) even on brain scans that cover the superior part of the spinal cord down to the C2/C3 level (88). However, physiological fluctuations and change over time of this measure in healthy subjects are unknown, and although some studies have reported spinal cord atrophy rates of between <0.5% and more than 2% per year, with progressive and clinically deteriorating patients exhibiting faster atrophy rates, it is not established how MUCCA could be used to monitor individual patients (88–93). However, a recent study suggests that conventional measures of spinal cord involvement, such as focal lesions and emergence of new lesions, can be used to estimate risk of secondary progressive MS and EDSS at 15 years in patients with clinically isolated syndrome (94).

Other advanced MRI techniques have been recently applied to investigate pathogenetic processes associated with neurodegeneration and disease progression. Amid other emerging quantitative MRI approaches, diffusion tensor imaging (DTI), which relies on the detection of changes in the random translational motion of water molecules and thereby estimates the level of tissue degradation in the normal-appearing white matter, provides promising imaging markers to detect neuronal damage (83). Post mortem investigations showed fractional anisotropy (FA) decrease to be to associated with axonal loss and myelin density, thereby suggesting DTI FA to be a useful indicator of both neurodegeneration and demyelination in MS (95) (**Figure 3**). However, future histopathological and clinical studies on quantitative MR markers are highly warranted to validate the capacity of modern MRI in detecting and monitoring neurodegenerative MS pathology that remains elusive to conventional structural MRI.

In clinical management, the use of MRI to monitor treatment response still relies on conventional parameters, such as new or enlarging T2 lesions and gadolinium-enhancing lesions

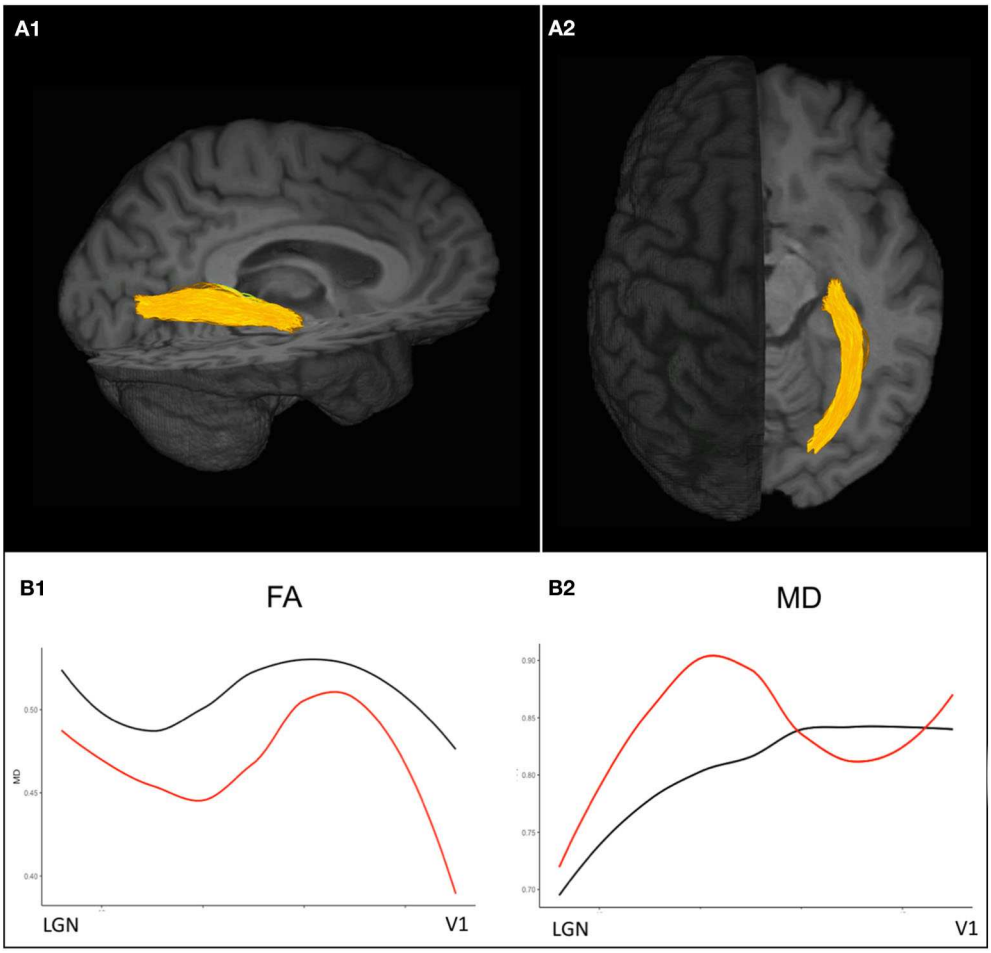

FIGURE 3 | Diffusion-weighted imaging based probabilistic tractography allows for the delineation of the optic radiations displayed in **(A1)** sagittal and **(A2)** axial view. **(B1)** Diffusion tensor imaging (DTI) values along the optic radiation of an exemplary ON patient 3 years after attack *(red)* show decreased FA values compared to a healthy control *(black)* indicating trans-synaptic neurodegeneration after ON. **(B2)** MD values are pathologically increased in an exemplary ON patient almost throughout the entire course of the optic radiations compared to the exemplary healthy control. ON, optic neuritis; FA, fractional anisotropy; MD, mean diffusivity.

in conjunction with clinical measures (relapses, disability progression), measures summarized under the term "NEDA" (no evidence of disease activity) (30). However, NEDA seems to be a questionable treatment goal given that, in real-world observational studies, <10% of patients retain a NEDA status after more than 5 years (96), and even with highly effective immunotherapies, NEDA rates hardly exceed 50% (97). Moreover, the clinical relevance of this composite score has been called into question, for example, by data from a large prospective observational study with more than 500 MS patients from California showing that meeting the NEDA status at 2 years was not predictive of long-term stability (98). In addition, the NEDA concept has been heavily criticized because of ignoring other relevant and disabling symptoms of the disease, such as fatigue, cognitive problems, sleep disorders, depression, etc. (71, 99–109). Moreover, recent safety concerns as to the deposition of gadolinium-based contrast agents (predominantly linear compounds) in the dentate nucleus and other brain regions provide arguments against their frequent use in monitoring radiographic disease activity in otherwise stable patients (110–113). For detection of new brain lesions, a T2/FLAIR sequence is sufficient as long as rigorous standardization of image acquisition to ensure maximum comparability is guaranteed (29). To overcome the shortcomings and downsides of the current NEDA concept, a new term ("minimal evidence of disease activity" or MEDA) has been proposed as well as a more sophisticated approach to monitor MS therapy taking also patient-reported outcomes into consideration ("multiple sclerosis decision model" or MDSM) (114, 115). However, both concepts lack prospective validation, so their use in clinical management cannot be unambiguously recommended. The same applies to the upgraded NEDA concept that includes brain atrophy into the composite measure (NEDA-4) (116, 117).

Numerous non-conventional and advanced imaging modalities are currently under investigation that may help improve visualization and quantification of (covert) tissue

damage in the gray and white matter of the brain and the spinal cord and could be used as an imaging surrogate of remyelination and repair; among them are magnetization transfer imaging, diffusion tensor imaging, myelin water imaging, susceptibility weighted imaging, magnetic resonance spectroscopy, sodium imaging, PET imaging, ultra-high field imaging at 7 Tesla, functional imaging with resting state fMRI, T1/T2-weighted ratio calculable from conventional T1- and T2-weighted images, machine-learning based imaging, magnetic resonance elastography, and several others, none of which will probably be used in clinical practice in the near future (27, 52, 83, 88, 118–135). Nonetheless, these endeavors are important to deepen our understanding of mechanisms of tissue damage in MS and to devise better imaging endpoints for clinical trials and routine care than those currently in use.

A recent emerging imaging tool in neuroinflammation is retinal optical coherence tomography (OCT), a technique that takes advantage of the retinal backscatter and reflection of low coherent light and enables the reconstruction of structural images of the various retinal layers with a resolution of a few microns and a very time-efficient image acquisition of only a few minutes in a cooperative patient (136).

OCT has been used for more than a decade in clinical neuroimmunology, mostly in cohort studies and occasionally as an endpoint in acute optic neuritis trials (137–139), and it is at the verge of entering clinical management of patients with MS and related disorders. Most widely used retinal measures of neuroaxonal damage are the (peripapillary) retinal nerve fiber layer (pRNFL) and the ganglion cell layer (GCL) that is often reported together with the inner plexiform layer (IPL) due to inaccuracy of segmentation and then displayed as ganglion cell/inner plexiform layer (GCIPL) (39, 136). In MS, OCT has been shown to be reliably applicable in a multicenter setting (140). Certain standards for quality control of OCT scans and reporting of data have been proposed, and confounders, for example, the influence of retinal vessels on neuroaxonal measures, have to be taken into consideration (141–143). Thinning of the RNFL or the GCL/GCIPL are consistently reduced according to a high number of studies both in MS eyes with a history of prior optic neuritis as well as to a lesser extent in MS eyes without prior ON (144–146). Retinal thinning in MS is detectable from the earliest disease stages (147, 148) and is associated with altered visual function, visual quality of life, VEP latencies, overall disability, cognitive performance, inflammatory brain lesions, and both spinal cord and brain atrophy, and has been shown to reflect clinical and radiographic disease activity in longitudinal studies (149–162). A recent meta-analysis comprising more than 1,000 eyes calculated an average pRNFL loss of 20 μm in eyes with prior ON and of 7 μm in eyes without history of ON (NON), and average GCIPL thinning was 16 μm in ON eyes and 6 μm in NON eyes (144). Annual rates of RNFL thinning in longitudinal studies range from ∼0.2 to 2.0 μm per year and depend on disease stage and treatment status. In general, patients with progressive MS tend to show more severe retinal thinning than RRMS patients (145). A retrospective, non-randomized "real-world" study suggested that MS immunotherapies may differentially affect the rate of annual ganglion cell loss with faster thinning in patients treated with interferon beta or glatiramer acetate and slower thinning in patients on natalizumab (163). In a longitudinal monocenter study in 72 patients with MS from Italy, NEDA status was associated with relatively preserved RNFL over 2 years; patients with NEDA (32% of the cohort) had an average RNFL loss of −0.93 μm as compared to −2.83 μm in the evidence of disease activity (EDA) group (164). Patients with stable EDSS over the course of the study had on average a RNFL loss of −1.33 μm as in contrast to −3.23 μm in patients with an EDSS worsening of ≥0.5 points. A cutoff of −1.25 μm RNFL loss was able to classify the NEDA status with a sensitivity of 80% and a specificity of 81.4%. A large retrospective multicenter study conducted by the International Multiple Sclerosis Visual System Consortium (www.imsvisual.org) in 879 patients with various stages of MS suggests that pRNFL may be used to predict disability worsening (165). Patients with a pRNFL below 92/93 μm (different OCT machines used) had a 60% increased risk of disability progression after 1 year, and those with a pRNFL <87/88 μm had a 4-fold increased risk of progression on the EDSS after 4–5 years.

Another retrospective study in 305 MS patients in different stages of the disease and with a median interval of 7.9 years from the acquisition of an OCT scan (using the older time domain technology to measure the pRNFL) (166) to the last EDSS assessment evaluated the relationship between both parameters (167). Each 1 μm decrease in the baseline pRNFL was associated with an increase in EDSS of 0.024 points, suggesting that a pRNFL measurement may help to prognosticate disability within 6–9 years later. Similar results were obtained when adjusting for the presence of previous optic neuritis episodes.

Also in a clinically isolated syndrome (CIS) scenario OCT may be helpful to assess the risk of further disease activity. A bicenter study from Germany grouped 89 patients with a CIS as a qualifying event into three groups according to their baseline GCIPL values in NON eyes (168). Patients in the lowest tertile (ranging from 58.7 to 69.2 μm) had a hazard ratio of 3.43 for not meeting NEDA status within the follow-up period (max 2.5 years) as compared to patients in the highest GCIPL tertile (ranging from 74.2 to 84.8 μm). In contrast, other established predictors of further disease activity in CIS patients, such as MRI T2 lesion load, sex, or ON as a qualifying symptom, were not predictive of a subsequent NEDA status. For the most recent revision of the McDonald criteria it was controversially discussed whether affection of the visual system should be used to demonstrate dissemination in space or time. However, "the panel felt the data … were insufficient to support incorporation into the McDonald criteria" but "studies to validate MRI, visual evoked potentials, or optical coherence tomography in fulfilling DIS or DIT in support of a multiple sclerosis diagnosis were identified as a high priority." (41). A first step toward this direction has been undertaken by the IMSVISUAL Consortium that recently pooled data from more than 1,500 patients with MS to determine the optimal intereye differences in RNFL and GCIPL thicknesses for identifying unilateral optic nerve lesions defined as history of acute unilateral optic neuritis (169). Using receiver-operating characteristic curve analysis, an intereye difference of 5 μm for RNFL and of 4 μm for GCIPL was demonstrated as an optimal

threshold for identifying unilateral optic nerve lesions. Eighteen percent of patients in the entire cohort had intereye differences of >5 μm for RNFL and 12% of >4 μm for GCIPL without history of acute ON. In line with another recent study (170), these findings suggest that these measures may complement MRI to demonstrate dissemination in space and time.

NEUROMYELITIS OPTICA SPECTRUM DISORDERS (NMOSD)

In 2015, new diagnostic criteria for NMOSD with and without (or with unknown) AQP4 antibodies have been proposed against the background of a broadening clinical spectrum that was recognized with the increasing number of patients tested for AQP4 antibodies (171). Imaging features regarded as characteristic yet not pathognomonic for NMOSD are a core element of the 2015 IPND criteria, in particular in seronegative patients or in subjects with unknown AQP4 ab status. The main goal of listing these imaging findings is to help clinicians discriminate NMOSD from other conditions, namely MS, and thus reduce the chance of misdiagnosis. Imaging abnormalities in NMOSD are described according to the anatomical location in the brain, optic nerve, and spinal cord. The establishment of the 2015 IPND criteria have led to a rise in the number of diagnosed NMOSD cases by up to 76%, and fortunately, diagnostic delay was considerably decreased from 53 months by the 2006 criteria to 11 months by the 2015 criteria (172, 173).

Over the past 15 years, an impressive number of imaging studies have made clear that—in contrast to earlier views—most NMOSD patients exhibit some kind of brain lesions. Lesions are not always located in areas of high AQP4 expression, and a considerable proportion (42%) may even meet Barkhof criteria for multiple sclerosis (24, 26, 174, 175). Most studies in NMOSD have used conventional MR sequences; non-conventional and advanced imaging studies are scant and have mostly yielded inconsistent results (176).

According to newer studies, the majority of NMOSD patients show some kind of brain lesions although findings considered highly suggestive and suspicious of an NMOSD diagnosis are less prevalent. Between 43 and 70% of NMOSD patients have brain lesions at onset, and up to 85% of patients meeting the 2006 Wingerchuk criteria for NMO and up to 89% of seropositive patients were reported to have brain abnormalities (12, 26, 177–180). Brain lesions considered highly suggestive of NMOSD are diencephalic lesions surrounding the third ventricle and cerebral aqueduct, which are often asymptomatic but may occasionally present with inappropriate antidiuretic hormone secretion, narcolepsy, hypothermia, hypotension, or hyperprolactinemia. Another very characteristic predilection site is the dorsal brainstem: Lesions adjacent to the fourth ventricle, including the area postrema and the nucleus tractus solitarii, are highly specific for NMOSD, reported in 7–46% of patients with NMO (26, 181). The typical clinical manifestation is with intractable hiccups, nausea, and vomiting (171). Lesions in the corpus callosum (CC) have been described in 12–40% of patients with NMOSD. Although the location in the CC is not a unique finding that differentiates NMOSD from MS, NMOSD callosal lesions are in contrast to MS located immediately next to the ventricles and follow the ependymal lining (26). CC lesions may extend into the cerebral hemisphere, forming an extensive and confluent white matter lesion. Acute CC lesions are often edematous and heterogeneous with a "marbled pattern" (182). Hemispheric white matter lesions may appear extensive and confluent, are often tumefactive (>3 cm in longest diameter), or have a long spindle-like or radial shape following white matter tracts; they usually have no mass effect. They may occasionally mimic posterior reversible encephalopathy syndrome (PRES) or Baló-like lesions or may resemble acute disseminated encephalomyelitis (ADEM) or CNS malignancies and were reported to be more frequent in AQP4 ab seropositive than seronegative patients (26). Hemispheric white matter lesions may disappear but may also remain as cyst-like or cavitary changes. Also corticospinal tracts may be involved in NMOSD with either unilateral or bilateral involvement and were reported in up to 44% of patients. These lesions may extend from the deep white matter in the cerebral hemisphere through the posterior limb of the internal capsule to the cerebral peduncles of the midbrain or pons. They are often contiguous, longitudinally extensive, and may follow pyramidal tracts. The reason for involvement in NMOSD is unclear as corticospinal tracts are not areas of high AQP4 expression. The probably most frequent type of brain lesions in NMOSD reported in up to 84% of patients are "non-specific" lesions: punctate or small (<3 mm) dots or patches of hyperintensities on T2-weighted or FLAIR sequences in the subcortical or deep white matter that are usually asymptomatic and tend to increase with age, presumably owing to age-related vascular comorbidities. These lesions may nonetheless pose diagnostic challenges vs. MS and other conditions. Few studies have looked into gadolinium-enhancing brain lesions in NMOSD; up to 36% of patients have shown enhancing lesions that are often poorly marginated, subtle, or show a patchy pattern. One study from Japan suggested "cloud-like enhancement" to be a characteristic enhancement pattern in NMOSD (178). Nodular enhancement or meningeal enhancement have also been described, and linear enhancement of the ependymal surface of the lateral ventricles ("pencil-thin lesion") was proposed as another imaging feature characteristic of NMOSD (183, 184).

In contrast to MS, cortical lesions are usually absent in NMOSD, which is supported by 3 T double inversion recovery and ultra-high field MR studies that investigated the cortex in NMO as well as by several histopathologic studies (54, 185, 186) (**Figure 2**). Additionally, a lower proportion of NMOSD lesions show the CVS or display hypointense rims compared to lesions in MS (54, 57) (**Figure 2**). The challenging overlap of brain lesion occurrence and numbers between NMOSD and MS have prompted the use of algorithmic approaches to improve differential diagnosis. For example, one study from the UK in 26 AQP4 ab seropositive NMOSD (63% of whom had brain T2 lesions and 16% met Barkhof criteria) and 50 RRMS patients replicated a few key features in both conditions that appeared to be discriminative, among them a smaller lesion size and fewer numbers in NMOSD as compared to MS, and MS exhibited a greater coherence of lesion location (most likely to occur adjacent

to the posterior of the body of the lateral ventricle in the parietal white matter) (187). In contrast, the lesional region with the greatest likelihood to be within the NMOSD group and not the MS group was adjacent to the fourth ventricle in the pons. Both groups had callosal lesions, but NMOSD patients showed no U fiber lesions and no Dawson's fingers. A combination of morphologic and locational criteria (at least one lesion adjacent to the body of the lateral ventricle and in the inferior temporal lobe or the presence of a subcortical U fiber lesion or a Dawson's finger–type lesion) could distinguish patients with MS from those with NMOSD with 92% sensitivity, 96% specificity, 98% positive predictive value, and 86% negative predictive value (187).

Of note, previous research shows that non-lesional tissue damage as measured by non-conventional imaging, such as DTI, may not occur in NMOSD except in the connecting tracts upstream and downstream of lesions (26). Although these findings lend support to the notion that NMOSD, in contrast to MS, may be a lesion-dependent disease that produces relapses without more generalized neurodegenerative pathology, the presence of potential subclinical tissue alterations in NMOSD affecting the afferent visual system has been controversially discussed. Recent DTI investigations in NMOSD patients without a clinical history of visual pathway affection showed structural retinal damage and pathological optic radiation DTI FA decrease outside attack-related lesions, suggesting a presumptive AQP4-ab–related astrocytopathy (188). These findings are in accordance with histopathological studies reporting on astrocytic end feet changes within LETM lesions and spinal cord atrophy in AQP4-ab–positive patients without previous myelitis attacks (189). Yet the question as to whether neurodegenerative non-lesion-related pathology exists in NMOSD is still under debate and needs to be further elucidated by future combined *in vivo* and *ex vivo* MRI investigations.

Longitudinally extensive myelitis lesions (LETM) spanning three or more contiguous vertebral segments have long been regarded as an imaging feature highly suggestive of NMOSD (**Figure 4**). Sensitivity and specificity for this criterion were 98 and 83%, respectively, in the patient cohort underlying the 2006 Wingerchuk criteria (177). Long cord lesions occur more frequently in the cervical cord from which they may extend into the brainstem and the upper thoracic spinal cord than in lower cord regions. Moreover, NMOSD spinal cord lesions occupy more than half of the cord area and show preferential involvement in the spinal central gray matter during the acute and chronic stages of spinal cord inflammation. By contrast, the majority of MS spinal cord lesions are localized in the lateral and posterior white matter regions of the cord (190, 191). In the acute stage, spinal cord lesions often appear hypointense on T1 weighted scans (in contrast to MS); the inflamed cord is often swollen and may show patchy contrast enhancement. In the chronic stage, extensive cord atrophy with or without T2 signal changes may develop in NMOSD (**Figure 4**). It is important to bear in mind that the timing of the spinal MRI in relation to the onset of clinical symptoms may be crucial for the detection of longitudinally extensive cord lesions (192) and that ∼15–20% of myelitis attacks in

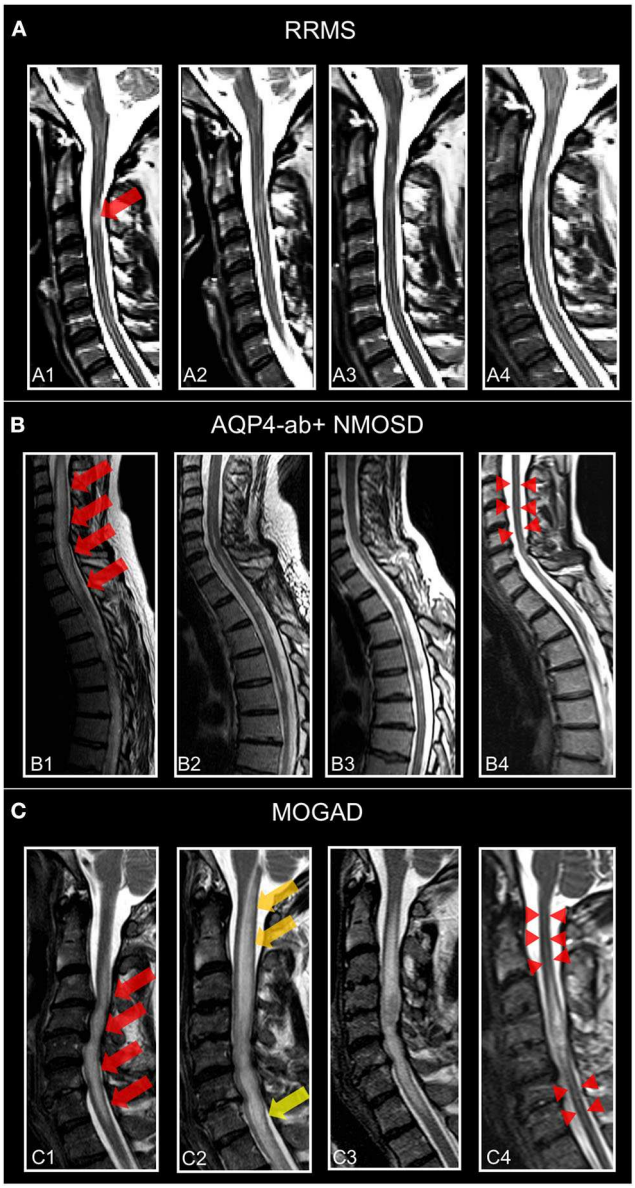

FIGURE 4 | Representative T2-weighted spinal cord images from individuals. **(A)** Patient with relapsing–remitting multiple sclerosis (30-year-old woman) and MS-related myelitis and spinal cord imaging at **(A1)** 1 months, **(A2)** 2 months, **(A3)** 24 months, and **(A4)** 72 months after attack. Short extent (<3 segments) spinal cord lesion *(red arrow)* at C3 with typical morphology of MS-related myelitis. **(B)** Patient with AQP4-antibody-positive neuromyelitis optica spectrum disorder (36-year-old woman) and NMOSD-related LETM and spinal cord imaging at **(B1)** 2 months, **(B2)** 5 months, **(B3)** 12 months, and **(B4)** 60 months after attack. Spinal cord lesion *(red arrows)* with longitudinal morphology (C2-Th1; >3 segments) and subsequent atrophy *(red arrow-heads)* typical of NMOSD-related LETM. **(C)** Patient with MOG antibody associated disease (41-year-old woman) and MOGAD-related LETM and spinal cord imaging at **(C1)** 7 months, **(C2)** 8 months, **(C3)** 24 months, and **(C4)** 48 months after attack. Initial LETM (C3-C7; *red arrows)* with remarkable increase in length after relapse at month 8 **(C2)** *(yellow arrows)* and subsequent atrophy *(red arrow-heads)*. RRMS, relapsing-remitting multiple sclerosis; AQP4-ab+, AQP4-antibody positive; NMOSD, neuromyelitis optica spectrum disorder; LETM, longitudinally-extensive transverse myelitis; MOGAD, myelin-oligodendrocyte-glycoprotein associated disease.

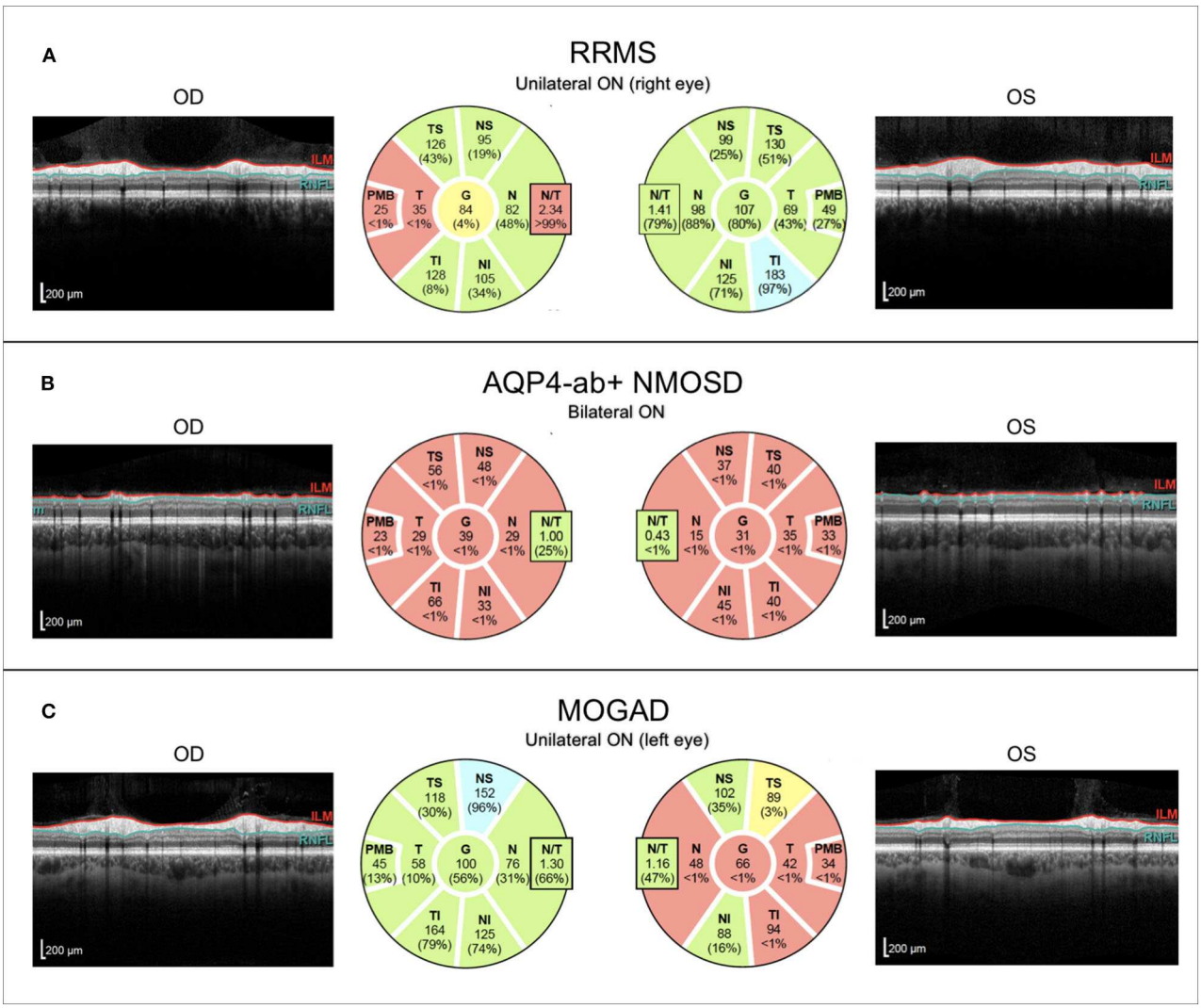

FIGURE 5 | Representative OCT images from individuals with **(A)** relapsing–remitting multiple sclerosis with unilateral right-sided ON (RRMS; 41-year-old woman), **(B)** AQP4-antibody-positive neuromyelitis optica spectrum disorder with recurrent bilateral ON episodes (AQP4+-NMOSD; 25-year-old woman), and **(C)** MOG antibody associated disease with left-sided unilateral ON (MOGAD; 46-year-old man). OCT, optical coherence tomography; RRMS, relapsing-remitting multiple sclerosis; ON, optic neuritis; OD, right eye; OS, left eye; ILM, inner limiting membrane; RNFL, retinal nerve fiber layer; AQP4-ab+, AQP4-antibody positive; NMOSD, neuromyelitis optica spectrum disorder; MOGAD, myelin-oligodendrocyte-glycoprotein associated disease.

NMOSD may show short transverse myelitis lesions, spanning 2.5 vertebral segments or less (193, 194). This means that a spinal cord lesion shorter than three vertebral segments does not rule out an NMOSD diagnosis. Interestingly, a recent study on 91 Chinese NMOSD patients compared patients with LETM and patients with short transverse myelitis and showed that the latter suffered less motor and bowel or bladder disability and had minor EDSS at clinical onset but exhibited shorter time to relapse (195). Moreover, although extensive spinal cord lesions are highly suggestive of NMOSD, numerous other conditions have to be taken into consideration, such as sarcoidosis, spondylotic myelopathy, autoimmune GFAP astrocytopathy, neoplasms, lymphoma, spinal cord infarction, and many others (196–201). Recently, "bright spotty lesions" on T2 weighted sequences were reported to be a discriminative feature of NMOSD myelitis with specificity values up to 100% (202, 203).

Recently, a study in 48 NMOSD (all AQP4 ab positive), 22 MS patients, and 24 patients with other causes of LETM from the United States assessed spinal cord imaging features that may help discriminate NMOSD from MS. Four findings were found to be most distinctive of NMOSD vs. other etiologies: bright spotty lesions, T1 dark lesions, centrally located lesions, and lesions involving more than 50% of the cord area on axial sequences (190).

Another study in 116 NMOSD patients (98 AQP4 ab positive) found a high proportion of patients (49%) without typical NMOSD brain and spinal cord lesions and 37% meeting the

2010 McDonald criteria. Nonetheless, a combination of easily applicable criteria for brain and spinal cord images enabled distinction from matched MS patients with good sensitivity and specificity regardless of serostatus (204).

Although the optic nerve is frequently involved in NMOSD, few studies with orbital MRI have been conducted. AQP4 ab–positive NMOSD tends to show more often posterior involvement of the optic nerve(s) including the chiasm and a more frequent intracranial and bilateral affection of the optic nerve as compared to MS (205). In AQP4 ab–positive patients with ON, lesion length on orbital MRI in the acute phase was a strong predictor of visual outcome (206). Another study reported a longitudinally extensive optic nerve lesion exceeding 17.6 mm to have a sensitivity of 81% and a specificity of 77% for NMOSD vs. RRMS (207).

Advanced imaging with volumetric analyses, DTI, spectroscopy, and others have increasingly been performed over the past 10 years (176), albeit with many inconsistent results, presumably owing to small sample sizes, ethnic differences of the cohorts investigated, heterogeneity of the samples with regard to AQP4 ab serostatus, and others. It is, for example, still a matter of debate as to whether progressive brain volume loss occurs in NMOSD over time as is the case in MS and how different compartments, such as white matter, cortex, or deep gray matter, are differentially affected (208–218) and how this might be associated with often-overlooked and insufficiently treated symptoms, such as cognitive impairment and pain (219–222). Presence of occult white matter damage as measured, for example, by DTI, MTR, or T1 relaxation time, has also remained contentious, presumably again owing to differences in inclusion criteria and different approaches to the correction for multiple comparisons problem (223–228). Few functional imaging studies with resting state fMRI (rs-fMRI) in NMOSD suggest that visual impairment due to severe optic neuritis causes brain network connectivity changes, in particular in visual networks (229–232). The vast majority of MR spectroscopy studies of the brain has found no clear indication for covert white matter damage (233–237), and low myoinositol/creatine values in the lesional cervical cord of NMOSD patients suggest astrocytic damage (238).

Spinal cord atrophy and reductions of MUCCA are a consistent feature of AQP4 ab–positive NMOSD even in the absence of myelitis attacks/spinal cord lesions (209, 239, 240) (**Figure 4**). In one study in 27 NMOSD patients with a history of myelitis and six NMOSD without history of myelitis and without spinal cord lesions (all participants AQP4 ab positive), MUCCA was reduced in both groups vs. healthy controls and correlated with clinical disability (241). The clinical relevance of MUCCA to monitor disease activity and covert progression requires further studies.

With the introduction of retinal OCT into clinical neuroimmunology, an increasing number of studies measuring retinal damage in NMOSD have been conducted over the past 10 years (242). Most studies have consistently shown that thinning of the RNFL and the GCIPL after an ON attack is on average more severe in AQP4 ab–positive NMOSD as compared to MS, a finding that aligns with the clinical experience of more severe vision loss in NMOSD (243–246). Impairment of visual quality of life caused by ON in NMOSD correlates with the extent of retinal damage measured by OCT, which underscores the potential clinical relevance of this technique (247) (**Figure 5**). Furthermore, this finding supports the strong recommendation for clinicians to treat ON as a neuroimmunological emergency as quickly and consequentially as possible because retinal ganglion cell loss starts early after clinical onset of symptoms, so timely administration of steroids or plasma exchange might help preserve retinal tissue and improve visual outcome (248–252). In NMOSD, ∼25% of patients show so-called microcystic macular edema (MME) in the inner nuclear layer (INL) following ON, a frequency that is higher than in MS (5–10%) (242, 253–255). MME is not specific to NMOSD as it was described in a wide range of optic neuropathies. MME may be dynamic over time and seems to be associated with a less favorable visual outcome although neither its clinical relevance nor its pathophysiological underpinnings are entirely clear. Presumed mechanisms causing MME are vascular damage with extracellular fluid accumulation, Mueller cell pathology, and vitreous traction (242, 256, 257). A contentious issue in vision research in NMOSD is the occurrence of subclinical and progressive retinal thinning in NMOSD. In line with the clinical experience that disability is almost exclusively attack-related in NMOSD, some studies did not find progressive retinal thinning independent of ON (258). However, recent work has suggested that there is attack-independent ganglion cell loss in NMOSD—a finding whose clinical relevance needs to be further investigated (259). In addition, foveal changes have been detected in NMOSD patients without clinical evidence of optic neuritis of affection of the visual system, which suggests that AQP4 ab may directly target astrocytic Mueller cells in the retina, thus causing a primary retinal astrocytopathy (188, 260, 261). This finding is backed by animal work and human neuropathology data, both providing evidence for complement-independent AQP4 loss in Mueller cells and a retinal astrocytopathy (262, 263). Mathematical models to investigate the foveal shape will help investigate whether fovea changes may be used as a differential diagnostic feature for NMOSD and how these change over time in conjunction with functional visual outcomes (264).

In the past few years, a plethora of publications has reported on serum antibodies to myelin oligodendrocyte glycoprotein (MOG) in a subset of adult patients with an NMOSD phenotype (with optic neuritis being the most frequent clinical manifestation) and beyond (involvement of cranial and peripheral nerves and encephalopathy with seizures have been reported) using highly specific immunoassays (265–275). The current discussion evolves toward recognizing this condition as a disease entity distinct from AQP4 ab positive NMOSD and MS for which the acronyms "MOGAD" (MOG antibody associated disease) or "MOG-EM" (MOG antibody associated encephalomyelitis) were proposed (15, 276–279).

Neuroimaging studies with MRI in MOGAD are scant, and the few reports suggest that there is a broad overlap with AQP4 ab–positive NMOSD as to the presentation on conventional

brain and spinal cord MRIs (279–284) although MOG patients were reported to show a more frequent involvement of the conus/lumbar spinal cord (285). From a clinical standpoint, it is important to bear in mind that up to 27% of patients with MOGAD may meet Barkhof criteria for MS (24). As in AQP4 ab–positive NMOSD, an algorithmic approach combining several criteria assessable on conventional brain MRIs (lesion adjacent to the body of a lateral ventricle and inferior temporal lobe lesion, U fiber lesion and Dawson's fingers) was able to discriminate between RRMS and MOGAD with good sensitivity and specificity but failed to distinguish MOGAD from AQP4 ab–positive NMOSD (286). These findings were replicated in Korean and Chinese populations in whom a distinction of MS from AQP4 ab–positive NMOSD and MOGAD was achievable with good sensitivity and specificity (287, 288); data on a distinction between MOGAD and AQP4 ab–positive NMOSD were not provided. Using principal component analysis on conventional brain images, another study was also not successful in accurately discriminating MOGAD from AQP ab–positive NMOSD (289).

In contrast, orbital MRI seems to exhibit distinctive features. A combined brain and optic nerve MRI study from Australia in 11 AQP4 ab–positive, 19 MOGAD, and 13 MS patients with a first ON in the investigated eye found more frequent optic nerve swelling in MOGAD and more frequent bilateral optic tract and chiasmal involvement in AQP4 ab–positive NMOSD (205). A predominant affection of the anterior structures of the optic nerve and bilateral involvement were also reported in MOGAD patients from the United States (281).

Retinal OCT findings in MOGAD have been inconclusive. Although some studies suggest that ON in MOGAD causes less severe retinal damage in comparison to AQP4 ab–positive NMOSD (206, 290, 291), others have found comparable thinning of the RNFL and the GCIPL in MOGAD and AQP4 ab–positive NMOSD, probably resulting from the higher ON attack rate in MOGAD (292) (**Figure 5**). These studies are consistent in suggesting that a single ON episode in MOGAD probably is more benign regarding its effect on the retina than a single ON attack in AQP4 ab–positive NMOSD. This is in line with several other studies reporting a generally favorable outcome from ON in MOGAD; however, exceptions to this rule with poor outcome have also been published (293–296). Interestingly, MOGAD patients seem to have better visual outcomes after ON than AQP4 ab–positive NMOSD despite similar severity of macular GCIPL thinning (297). The issue of subclinical retinal involvement in MOGAD in the absence of ON has not been well-explored. One cross-sectional study found pRNFL thinning in MOGAD NON eyes and an MME prevalence of 26% (298), and one longitudinal study with 38 eyes (18 without ON history, 20 with ON) from 24 MOGAD patients detected a higher rate of annual RNFL thinning than in healthy subjects (299). However, this was not accompanied by progressive GCIPL thinning and the reduction of RNFL over time was driven by a subgroup of patients with thicker RNFL at baseline so the question as to whether progressive retinal thinning occurs in MOGAD requires further investigation.

FUTURE DIRECTIONS

Although previous research in advanced neuroimaging led to a tremendous amount of new methods, parameters, and insights into MS and NMOSD diagnostic approaches and pathophysiological processes, further efforts are highly required to make these advances applicable to the clinical setting. Main short- to mid-term aims are (1) standardization of MRI and OCT parameters related to image acquisition and post-processing, (2) transfer and integration of non-conventional techniques into clinically usable procedures, and (3) validation by comparing these readily accessible techniques with current standards within the framework of large patient cohort studies and real-world research. The ultimate goal is to provide the most accurate and most cost- and time-effective markers for clinical diagnostics, therapeutic monitoring, and prognostic forecasting in individual MS and NMOSD patients.

Among the multitude of potential candidates, a selection of promising markers to be introduced into the clinical setting in the near future are the central vein sign at 3 Tesla MRI that has proven to substantially increase specificity of current McDonald 2017 diagnostic criteria in the detection of MS (58), global cerebral and specific regional cortical and deep-gray matter atrophy for monitoring and predicting disease progression and cognitive dysfunction in MS (73), OCT retinal ganglion cell layer thickness as a prognostic marker for future disease activity in patients with clinically isolated syndrome (168), and spinal cord atrophy markers for diagnostic discrimination between AQP4 ab-positive NMOSD and MOGAD and to monitor disease activity in these entities (239). However, strong efforts in terms of observational studies and testing of these markers in clinical trials are necessary to foster their establishment in clinical research and routine.

Because availability of ultra-high field (7 T) scanners has gradually increased during recent years, a noticeable shift of neuroimaging research to higher field strengths will take place in the future. By use of its higher spatial resolution and benefits to imaging contrasts inherent to higher field strengths, 7 T MRI may be used to advance quantitative neuroimaging that may have reached its technical limits at 3 T (131, 300). Moreover, readily accessible 7 T MRI markers, i.e., central vein sign, lesional hypointense rim structures, and gray matter lesion detection might aid to establish accurate diagnoses of MS, especially in patients with conflicting neuroinflammatory disease presentation, when introduced into clinical work-up (52). However, thorough research efforts are necessary to prove potential benefits of ultra-high field MRI compared to conventional MRI in the clinical setting.

Another steadily expanding field of research in MS and NMOSD that will be of interest in the long-term future of neuroimaging, is the emerging application of MRI functional and structural connectome analyses. These techniques provide novel measures by assessing the integrity and functionality of the entire CNS system rather than evaluating separate regional or qualitative alterations in isolation (301). Pathological

changes in the functional network integrity in terms of network disruption or even "network collapse" show close correlations to higher order dysfunctions, i.e., predominantly cognitive deficits, in patients with MS (302). Analogously, ON status of CIS and NMOSD patients is associated with decreased connectivity in visual network density as revealed by recent application of graph theory–based tools to analyze functional imaging data (303). These findings are complemented by similar evidence in structural connectome disruption with associations to disease burden in CIS and MS (304). In addition, recent graph theory–based investigations that showed associations between decreased nucleus accumbens and caudate nucleus volumes with higher combined attack type count and longer disease duration in NMOSD lend support to the notion that multimodal network analyses including OCT and MRI parameters may help to identify subsets of promising useful imaging markers (305). However, because validity and potential clinical usefulness of these methods are still unclear, future studies will be undertaken to assess the true capacity of modern neuroimaging connectomics and graph theory–based methods to explain pathological mechanisms and to aid in monitoring and predicting specific disease activity in MS and NMOSD patients.

CONCLUSIONS

Imaging research in autoimmune inflammatory CNS disease has made impressive progress over the past 20 years. Yet, although we are able to deploy structural and functional imaging techniques even in patients at almost subcellular resolution that have significantly contributed to our understanding of mechanisms of tissue damage in these conditions, most of these technologies still await a validated implementation in clinical practice. This, however, is an indispensable prerequisite to make use of these advances to inform treatment decisions and monitor disease activity in individual patients. Because this has remained an unmet need from our patients' perspective, this task will hopefully be tackled despite further thrilling developments in the field of neuroimaging in autoimmune neuroinflammation, for example, OCT angiography, improved post-processing and segmentation techniques, and the use of deep learning and artificial intelligence algorithms (264, 306, 307). Similar endeavors are underway in magnetic resonance imaging that will likely revolutionize our approaches to visualize the brain (128).

AUTHOR CONTRIBUTIONS

FP designed the concept of the manuscript, drafted and wrote the work, and critically revised the manuscript. JK assembled, prepared and interpreted imaging data and critically revised, and added to the manuscript regarding important intellectual content.

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MINI REVIEW
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The NMDA Receptor Antibody Paradox: A Possible Approach to Developing Immunotherapies Targeting the NMDA Receptor

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N-methyl-D-aspartate receptors (NMDAR) play a key role in brain development and function, including contributing to the pathogenesis of many neurological disorders. Immunization against the GluN1 subunit of the NMDAR and the production of GluN1 antibodies is associated with neuroprotective and seizure-protective effects in rodent models of stroke and epilepsy, respectively. Whilst these data suggest the potential for the development of GluN1 antibody therapy, paradoxically GluN1 autoantibodies in humans are associated with the pathogenesis of the autoimmune disease anti-NMDA receptor encephalitis. This review discusses possible reasons for the differential effects of GluN1 antibodies on NMDAR physiology that could contribute to these phenotypes.

Keywords: GluN1, immunotherapy, NMDA receptor, neuroprotection, stroke, epilepsy

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INTRODUCTION

Antibody-based immunotherapies form a key component of the pharmacological arsenal for treatment of cancer (1), and inflammatory diseases (2), with profound clinical success achieved for these conditions. Monoclonal antibody therapies have several desirable attributes over traditional small molecule drugs including long half-lives and high specificity for the target molecular disease driver leading to reduced off-target toxicity and a lower adverse effect profile. The pipeline of immunotherapies for central nervous system disorders is not as extensive and has largely been dominated by active or passive immunization approaches for Alzheimer's disease and Parkinson's disease that aim to modify disease progression by targeting proteins implicated in disease pathogenesis (3). Different strategies have been employed including using antibodies to neutralize the actions of putative neurotoxic protein species or to promote clearance of the offending disease protein. Clinical trials have shown some promise (4), but much work is still required to improve the therapeutic efficacy of these approaches.

The potential of antibodies to modulate the function of other molecular targets in the central nervous system (CNS) for therapeutic benefit has not been extensively investigated. In this review, I will provide an overview of our studies and those of others exploring the possibility of an immunoprotective approach for neurological diseases including stroke and epilepsy involving antibody-mediated targeting of the N-methyl-D-aspartate (NMDAR) subclass of glutamate receptor.

THE NMDA RECEPTOR

The NMDAR plays a pivotal role in brain development, neuronal survival, and synaptic plasticity associated with learning and memory. The receptor is a hetero-tetramer composed of two obligatory GluN1 subunits of which there are eight distinct splice variants, and two variable subunits from the GluN2 (GluN2A-2D) or GluN3 (GluN3A-3B) subunit families. The combination of GluN1 with different GluN2/3 family members provides for the creation of diverse NMDAR subtypes varying in their regional distribution and functional properties. The majority of native NMDAR are triheteromeric, with GluN1/GluN2A/GluN2B receptors being the most common subtype in forebrain excitatory neurons (5).

The subunits are transmembrane-spanning and arranged to form an ion channel pore that is gated in a ligand- and voltage-dependent manner. The extracellular regions of the receptor resembling two clamshell structures with binding sites for glutamate on the GluN2 subunit and sites for glycine binding on the GluN1 subunit. The interaction between the distal amino terminal domain (ATD) of the receptor and other proteins regulate subtype-specific receptor assembly and receptor trafficking and sites for allosteric modulation of NMDAR function are also found in the ATD. The cytoplasmic C-terminus domain engages in interactions with scaffold proteins and intracellular messenger systems in the postsynaptic density.

The importance of NMDAR in the maintenance of physiological brain function is underpinned by observations that NMDAR-mediated hypofunction caused by either receptor loss, or altered distribution at synapses, is implicated in neurodevelopmental (autism spectrum disorders) (6) and neuropsychiatric disorders (schizophrenia) (7). Moreover, excessive glutamate release that leads to NMDAR overactivation contributes to neurodegeneration in acute or chronic neurodegenerative diseases including Alzheimer's disease (8, 9). The centrality of the NMDAR in the pathophysiology of a broad range of conditions makes these receptors an attractive drug target but human trials of NMDAR antagonists of different compound classes and at different sites of receptor action have been disappointing and are associated with a narrow therapeutic index and an unacceptable adverse effect profile (10). Greater insight into NMDAR function, and the discovery that synaptic and extrasynaptic NMDAR may be differentially linked to cell survival vs. cell death pathways, respectively has contributed to ongoing efforts to develop subunit-selective NMDAR antagonists. Weaker GluN2B-selective blockers that may preferentially target extrasynaptic NMDAR have a much-improved side-effect profile in humans than early generation broad spectrum antagonists (11). Other approaches to amplify the NMDAR-mediated cell survival signaling warrant investigation.

The NMDA Receptor as an Immunotherapeutic Target

We previously described an immunotherapeutic approach for stroke and epilepsy involving targeted vaccination against the GluN1 subunit of the NMDAR (12). Rats genetically immunized to express a full-length GluN1 subunit protein developed high-titer serum GluN1 autoantibodies and were more protected in rat models of temporal lobe epilepsy and stroke. Systemic injection of the neurotoxin kainate has been used extensively to induce seizure activity and a pattern of selective neuronal cell loss in the hippocampus that recapitulates the neuropathological features observed in human temporal lobe epilepsy (13). We found that following a challenge with kainate, fewer GluN1-vaccinated rats (22 vs. 68% control-vaccinated rats) developed seizures and of the two animals that experienced 45 min of prolonged status epilepticus, only one showed evidence of neuronal cell death in the hippocampus. Moreover, in a middle cerebral artery occlusion model of ischaemic stroke, infarct lesion sizes for the GluN1-vaccinated animals were significantly smaller compared to the control-vaccinated animals following infusion of endothelin-1 (12). We did not detect any evidence of cell-mediated immune responses suggesting the protective phenotype is likely to be GluN1 antibody-mediated. Moreover, GluN1 IgG was detected at low levels in the cerebrospinal fluid (CSF) of GluN1-vaccinated rats under basal conditions prior to any insult and GluN1 antibodies are bound to antigen suggesting low-level passage across an intact blood brain barrier (BBB) (12). It has been estimated that 0.1% of systemic IgG are able to traffic through the BBB into the brain parenchyma (14). In individual animals, we found that GluN1 antibodies reacted preferentially with a few specific extracellular epitopes rather than a broad range of epitopes. To identify regions of importance, we immunized rats with recombinant GluN1 peptides that contribute to various functional domains of the NMDAR (15). Differential effects on seizure expression and injury between the different GluN1 peptide treatments were observed. These results also confirmed the protective phenotype is not a unique feature of the immunization approach used. Almost no hippocampal cell death was observed in rats immunized with a peptide consisting of amino acids 654–800 of GluN1 (GluN1[654–800]) despite extensive kainate-induced seizures sufficient in duration and intensity to induce neuronal cell death. In contrast, rats immunized with a GluN1 peptide covering amino acids 21–375 (GluN1 21–375) was associated with reduced seizure severity as assessed by a 5-point seizure rating scale following kainate challenge but hippocampal cell death was clearly evident in these rats. Expression of heat shock protein 70 (HSP70) and brain-derived neurotrophic factor (BDNF) protein were elevated by ∼1.5-fold in the brains of the GluN1[654–800]-vaccinated animals that were protected against neuronal cell death compared to the control animals (naïve and Homer 1a immunized) suggesting that GluN1 antibody-mediated effects at NMDAR leads to downstream upregulation of signaling pathways linked to cell survival. These results indicate that GluN1 antibodies to specific functional domains of the NMDAR are able to induce a state of tolerance to insult akin to preconditioning whereby short-term exposure to NMDAR antagonists (16, 17) or NMDAR activation (18) can induce a state of resistance to subsequent insult.

Our studies suggest that a GluN1 immunotherapy could have broad utility for a range of neurodegenerative disorders but further mechanistic characterization is required to assess the

feasibility and safety of such an approach. This is of critical importance as within the last decade, NMDAR autoantibodies targeting the GluN1 subunit have been linked to the pathogenesis of the autoimmune disease NMDAR encephalitis.

Autoimmune Diseases Associated With NMDAR Antibodies

Anti-NMDAR encephalitis is a devastating autoimmune condition characterized by the onset of psychiatric manifestations including psychosis, rapid memory loss and seizures and the presence of high-titer CSF autoantibodies of the IgG class against the GluN1 subunit (19–21). The condition is more prevalent in women, and associated with the ectopic expression of NMDAR proteins in ovarian teratoma although there are also affected individuals who do not have detectable tumors (21, 22). The clinical features in patients and animal models resemble those caused by genetic or pharmacological attenuation of NMDAR function. Indeed, evidence from studies examining the effect of patient antibodies in cell and animal models have led to the hypothesis that the clinical syndrome is as a result of NMDAR hypofunction at a network level. Patient GluN1 autoantibodies cross-link NMDAR expressed on cultured neurons that triggers their loss at the synapse by internalization at extrasynaptic sites. Similarly, cerebroventricular infusion of patient NMDAR antibodies into rodent brain decreases NMDAR expression levels leading to impaired synaptic plasticity that is associated with memory deficits, anhedonia, depression-like behavior, and a low seizure threshold (23–26). Depleted NMDAR expression is consistent with observations in post-mortem brain from humans with anti-NMDAR encephalitis (23, 24, 27). The effects of patient antibodies are specific to NMDAR as no effect on expression of AMPA receptors or other synaptic proteins are found (27, 28).

Whilst the role of GluN1 autoantibodies in disease pathogenesis has been the key focus, more recently a mouse model of NMDAR encephalitis involving active immunization with intact native-like NMDAR GluN1/GluN2B tetramers embedded in a liposome scaffold has been described that recapitulates a broader range of features reminiscent of that found in the human disease (29). Immunized mice developed overt neurological signs include marked hyperactivity and stereotypic motor features including tight circling, seizures, and a hunched posture, or lethargy as early as 4 weeks, with nearly all animals showing abnormal behaviors by 6 weeks. This was associated with infiltration of peripheral immune cells and neuroinflammation by 6 weeks as supported by increased immunoreactivity to markers of plasma cells, CD4-positive T cells, and CD20-positive B cells, activated microglia, and astrocytes gliosis. Neuronal loss was rare. Serum autoantibodies that target epitopes on GluN1 was predominant but reactivity to GluN2 subunits as well as a peptide that lacked the amino-terminal domain of GluN1 was also observed by Western blot in the mice tested suggesting a polyclonal response by the time fulminant symptoms were present at 6 weeks after immunization. Chronic exposure of cultured hippocampal neurons to serum autoantibodies reduced NMDAR protein expression and associated NMDAR-mediated currents without an effect on synapse numbers (29). Studies of NMDAR encephalitis in humans has focused on the role of the autoantibodies, but this study suggests that mature T cells are also involved in causing a more complex disease pathogenesis leading to broader repertoire of symptoms by promoting neuroinflammation and potentiating B cell- and plasma cell–mediated antibody responses. The use of conformationally stable NMDAR holoproteins may be a critical component in initiating a more complex pattern of immunogenicity.

The NMDAR Autoantibody Paradox

The pathogenic effects induced by patient antibodies contrast sharply to the protective benefit achieved in our studies in rodent models. Single amino acid substitutions at key residues within the extracellular regions of the GluN1 subunit can significantly affect channel permeability (30), so it is entirely plausible that site-specific targeting by GluN1 antibodies to different extracellular regions on the NMDAR could have differential effects on receptor function or distribution. Our observations showing distinct differences between effects on seizure expression and neuroprotective effects following immunization with different GluN1 peptide fragment provide support for this hypothesis (15). Using a library of peptides that span the entire 938 amino acids of the native GluN1 subunit as a screening platform, we found that GluN1 IgG antibodies from individual rats genetically vaccinated with GluN1 cDNA react most commonly with peptides that correspond to domains that form part of the extracellular vestibule of the NMDA receptor channel, including regions important for glycine binding (12). Similarly, we found neuroprotection was associated with GluN1 antibodies targeting the GluN1 [654–800] region that contributes to the S2 loop of the glycine binding domain (15). We developed a recombinant protein consisting of the extracellular pre-TM1 region that includes the amino-terminal domain (ATD) linked to the extracellular loop between TM3-4 domains of GluN1 and immunized groups of rats with this recombinant protein (recGluN1). We found that the humoral response following immunization with this protein generated GluN1 antibodies that preferentially reacted with peptides that correspond to domains important for glycine binding when we screened an IgG fraction purified from pooled rat serum against our GluN1 peptide library. Structural modeling predicts that the binding of GluN1 antibody to this target region would promote closure of the NMDAR ion channel (31).

In contrast, NMDAR patient autoantibodies recognize conformational epitopes at the GluN-ATD (28). Screening of patient autoantibodies against a series of GluN1 protein deletion mutants showed amino acid residues N368/G369 at the GluN1-ATD were crucial for the creation of reactivity of patient antibodies. Moreover, patient antibodies did not immunostain a GluN1 protein lacking the ATD, suggesting that these antibodies do not target regions important in glycine binding (28). The GluN1-ATD is a major locus for interactions between the NMDAR and various synaptic proteins that regulate the trafficking, surface distribution, and function of NMDAR (32, 33). Any biologic agent or drug compound capable of

modifying these interactions could have significant effects on NMDAR signaling. Mechanistically, NMDAR encephalitis patient antibodies block the ability of Ephrin B receptors to regulate synaptic NMDAR numbers (33), leading to their depletion and a state of NMDAR hypofunction (20, 27, 34).

Conversely, neuroprotection in mouse models of stroke and experimental autoimmune encephalitis can be produced using GluN1 antibodies that target the interaction site of the serine protease tissue plasminogen activator (tPA) at the ATD (35–37). GluN1 antibodies directed against an epitope at amino acids 163–192 as well as Glunomab, a monoclonal antibody that interacts with the lysine residue at position 178, blocks the tPA-mediated potentiation of NMDAR-mediated signaling and excitotoxicity in neurons by reducing the surface dynamics and clustering of extrasynaptic NMDAR (36–39). The therapeutic beneficit engendered by these GluN1 antibodies are not restricted to actions at neuronal NMDAR, with Glunomab shown to promote the maintenance of blood brain barrier integrity via actions on NMDAR expressed on endothelial cells (36, 37). Of note, in our own work we found GluN1 antibodies that interact with the glycine site on NMDAR expressed on platelets can inhibit platelet function and thrombus formation that could also contribute to limiting stroke-induced neuronal damage (31) suggesting any therapeutic benefit could occur through additive effects at multiple cell sites. Further investigation is required to understand the full spectrum of effects on therapeutic GluN1 antibodies including the impact on NMDAR-dependent processes such as learning and memory. GluN1-ATD antibodies have been reported to impair hippocampal-dependent spatial memory in rodents (35, 39, 40) although a later study suggested that the GluN1-ATD antibodies are not associated with cognitive or behavioral deficits (36).

Altogether, these data suggest that NMDA receptor location, and function, can be differentially modulated by GluN1 antibodies in a target-dependent manner with GluN1 immunotherapeutic benefit made feasible through strategic targeting to defined sites. What are the challenges for applying such an approach, for example, as a preventative treatment against stroke-induced damage in humans?

Challenges for a GluN1 Immunotherapy—the Role of GluN1 Autoantibodies in Health and Disease

In preclinical studies, GluN1 antibodies generated following immunization of naïve animals are presumed to be able to freely interact with their target site following passage into the brain. How the therapies would perform in humans with preexisting serum antibodies directed against the NMDAR that could directly compete for the same epitope targets (if present in sufficient quantities), is unknown. Serum GluN1 autoantibodies are found in healthy older adults and there is increased seroprevalence (>20%) in individuals affected by a wide-range of diseases including stroke, neuropsychiatric illnesses, and dementia (41–45), with a recent study suggesting GluN1 autoantibodies may be part of the normal autoimmune repertoire (46). The significance of these antibodies in contributing to functional outcomes in these conditions is an area of current investigation. Unlike NMDAR encephalitis that is primarily associated with the occurrence of IgG GluN1 antibodies, GluN1 IgA, and IgM antibodies are mainly found in non-specifically in healthy older adults and in disease conditions (44, 47). There are contradictory reports that GluN1 antibodies promote NMDAR internalization irrespective of immunoglobulin class and epitope, whereas other groups find these effects are only produced by NMDAR encephalitis-associated GluN1 IgG antibodies (44, 46, 48), suggesting further investigation into any possible pathogenic effects is required.

GluN1 autoantibodies in stroke have been associated with larger (45) as well as reduced lesion sizes after acute ischemic stroke (47). The discrepancy between these findings could depend antibody titer as well as the health of the BBB. Using apolipoprotein E4 (APOE4) carrier status as a marker for a leaky BBB, the presence of preexisting serum GluN1 autoantibodies at the time of acute ischemic stroke was associated with reduced infarct sizes in individuals with an intact BBB (APOE4 +/+), however lesion sizes appeared to be the largest in APOE4 carriers with a compromised BBB (47). We speculate these findings are in line with the neuroprotection observed in rodent stroke models with an intact BBB at the time of insult (12, 36). Whether our glycine binding site targeting GluN1 antibodies promote maintenance of BBB integrity like GluN1-ATD antibodies is unknown (37). Recent data has indicated GluN1 antibody seropositivity was not associated with any long-term functional benefit at 1 year following stroke (49) but further studies are required to examine whether therapeutic benefits might be found in specific patient subgroups such as APOE4 non-carriers.

There are many outstanding questions. Whether a GluN1 immunotherapy could counteract or override any possible pathogenic effects produced by GluN1 autoantibodies or help boost the neuroprotective capability of endogenous antibodies at multiple levels including modulating NMDAR signaling at neurons, maintaining BBB health, and function remains to be determined.

Delivery Challenges for CNS Immunotherapeutics

Another key challenge is whether sufficient amounts of antibody as one of the key drawbacks of immunotherapies for CNS disorders is the low efficiency of delivery into the brain. The BBB strictly regulates the entry of molecules including therapeutics, immune cells, and immune mediators from the systemic circulation into and out of the brain. Osmotic or chemical disruption of BBB integrity can facilitate delivery of therapeutics into the brain but the lack of specificity for the therapeutic biologic agent is problematic. Alternative methodologies have exploited the properties of endogenous BBB receptor-mediated transporters responsible for the passage of endogenous large molecules such as insulin, transferrin, insulin-like growth factor, and leptin into the brain. These circulating proteins bind to their cognate receptors on the luminal surface of the endothelial cells lining the BBB. Upon binding, the receptor–ligand complex is internalized into the endothelial cell

by receptor-mediated endocytosis where the ligand molecule is transported across the abluminal membrane of the endothelial cell into the brain. Molecular Trojan horses that are engineered to carry peptides or proteins ligands that target receptor mediated transport systems (e.g., receptor-binding sequences of insulin) or monoclonal antibodies that specifically target transferrin and insulin receptors have been shown to be effective in facilitating delivery of various therapeutic proteins into the brain (50, 51). Progress in antibody engineering has led to the generation of different antibody configurations including the artificial bispecific antibody that combine two antigen-recognizing components into a single construct. Bispecific antibodies could also act as scaffolds to deliver therapeutic antibodies into the brain by incorporating one arm with specificity against a BBB receptor-mediated transport receptor that facilitates passage across the BBB and the therapeutic arm that produces the pharmacological effect (52). Use of these technologies coupled with site-specific targeting of the GluN1 could be explored in future studies if required.

AUTHOR CONTRIBUTIONS

DY wrote the paper and conceived this work.

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Mir106b-25 and Mir17-92 Are Crucially Involved in the Development of Experimental Neuroinflammation

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MicroRNAs (miRNAs) are single-stranded RNA that have key roles in the development of the immune system and are involved in the pathogenesis of various autoimmune diseases. We previously demonstrated that two members of the miR106b-25 cluster and the miR17-92 paralog cluster were upregulated in T regulatory cells from multiple sclerosis (MS) patients. The aim of the present work was to clarify the impact of miR106b-25 and miR17-92 clusters in MS pathogenesis. Here, we show that the mice lacking miR17-92 specifically in $CD4^+$ T cells or both total miR106b-25 and miR17-92 in $CD4^+$ T cells (double knockout) are protected from Experimental Autoimmune Encephalomyelitis (EAE) development while depletion of miR106b-25 only does not influence EAE susceptibility. We suggest that the absence of miR106b does not protect mice because of a mechanism of compensation of miR17-92 clusters. Moreover, the decrease of neuroinflammation was found to be associated with a significant downregulation of pro-inflammatory cytokines (GM-CSF, IFNγ, and IL-17) in the spinal cord of double knockout EAE mice and a reduction of Th17 inflammatory cells. These results elucidate the effect of miR106b-25 and miR17-92 deletion in MS pathogenesis and suggest that their targeted inhibition may have therapeutic effect on disease course.

Keywords: MicroRNAs, experimental autoimmune encephalomyelitis, multiple sclerosis, IL-17, Th17, miR106b-25, miR17-92

INTRODUCTION

miRNAs take part in the regulation of immune processes not only during innate and adaptive immune system development but also in its homeostasis, as well as in disease, regulating immune cell functions (1–3) and cytokine expression (4, 5). We analyzed in the past, the miRNA expression profile in regulatory T cells (Tregs) from relapsing-remitting multiple sclerosis patients (RR-MS) (6). We identified 23 microRNAs differentially expressed in MS Tregs as compared to Tregs from healthy controls. In particular, we found two members of the miR106b-25 cluster (miR93, miR106b) and two members of the miR17-92 paralog cluster (miR-19a and miR-19b) upregulated

in Treg cells from MS patients (6). Further, the over-expression of miR17-92 in lymphocytes induces lymphoproliferative disease and autoimmunity in mice (7).

The miR106b-25 cluster (in particular miR-25 and miR-106b) over-expression can silence two important effectors of the TGF-β signaling pathway: the cell cycle inhibitor CDKN1A (p21) and the pro-apoptotic gene BCL2L11 (BIM) (8). Several results suggest that miR 106b-25 and miR17-92 clusters cooperate in inactivating the TGF-β pathway (8). TGF-β is an important immunomodulatory cytokine involved in the maintenance of self-tolerance and T cell homeostasis (9). miR17-92 and miR106b-25 clusters are ubiquitously expressed (10). During lymphocyte development, miR17-92 miRNAs are highly expressed in progenitor cells, expression levels decreasing 2- to 3-fold upon lymphocyte maturation (10, 11). miR17-92 regulates B- and T-cell development and its absence results in enhanced proliferation and survival of B- and T-cells. This is apparently due to increased expression of two target genes, namely the apoptosis facilitator BCL2L11 (BIM) and the tumor enhancer phosphatase and tensin homology (PTEN). PTEN is an inhibitor of the PI3K pathway promoting cell cycle progression and inhibiting apoptosis by negatively regulating the transcription of BIM (11). Members of this cluster clearly cooperate in the context of TGF-β signaling. miR-17 and miR-20a, for example, target directly the TGF-β receptor II (TGFBRII), while miR-18a targets other two members of the TGF-β signaling pathway, namely Smad2 and Smad4 (12–14). BIM and p21 are two mediators of the effects downstream to TGF-β activation. The host gene for the miR-106a/25 cluster, Mcm7, is down regulated during endoplasmic reticulum related stress, by the activation of transcription factor 4 (Atf4) and nuclear factor-erythroid-2-related factor 2 (Nrf2). This causes down-regulation of miR-106b/25 and repression of BCL2L11, and consequently apoptosis (7). Functional redundancy of homologous miRNAs having similar expression patterns may explain the lack of an obvious phenotype in mice deficient for miR106b-25, whose function is apparently largely compensated by miR17-92. Constitutive deletion of the two clusters, on the other hand, is embryonically lethal (10). We induced experimental autoimmune encephalomyelitis (EAE) in mice constitutively deleted for the miR106b-25 cluster, in mice lacking the miR17-92 in $CD4^+$ T cells, and in mice with both mutations, to try to dissect the contribution of these miRNA families to neuroinflammation.

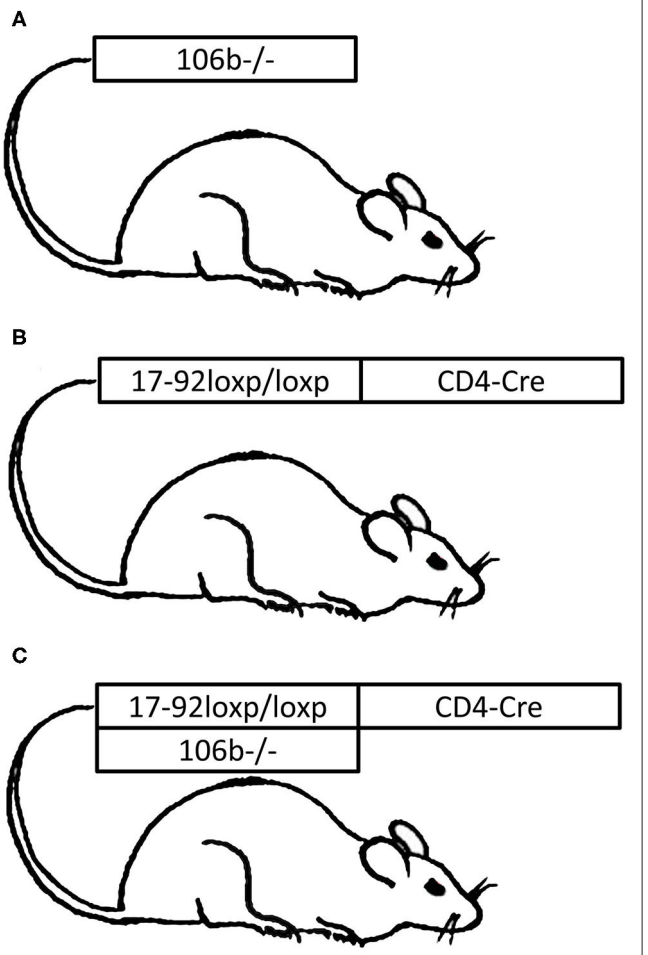

FIGURE 1 | Graphic representation of the three mutant strains used for the EAE experiments. Experimental Autoimmune Encephalomyelitis (EAE) was induced in mice constitutively deleted for **(A)** miR106b-25 cluster ($106b^{-/-}$ mouse), **(B)** miR17-92 in CD4+ T cells ($17\text{-}92^{-/-}$ mouse), or in mice with **(C)** both mutations ($106b^{-/-}17\text{-}92^{-/-}$ mouse). miR17-92 was conditionally deleted only in CD4+ T cells because mice deficient for miR-17-92 die shortly after birth (10).

RESULTS

Mice Lacking Mir17-92 or Both Mir17-92 and Mir106b Are Protected From Clinical Signs of Eae

We used the mutant strains depicted in **Figure 1**. Since mutant mice colonies have been maintained in heterozygosity, littermates have been used as appropriate WT controls. All mice were on the C57BL/6 background and we therefore induced EAE by MOG_{35-55} immunization. As shown in **Figure 2**, the absence of both miR106b and miR17-92, almost abolished susceptibility to EAE, with 90% of mice remaining disease free, and 10% displaying very mild and transient clinical signs (**Figures 2A–D**). The absence of miR17-92 only resulted in a mild disease with 50% incidence, while mice lacking only miR106b did not differ from wild type control mice, displaying full-blown EAE (**Figures 2A–D**).

Mice Lacking Mir17-92 or Both Mir17-92 and Mir106b Are Protected From Pathological Signs of Eae

Clinical findings in mice lacking miR17-92, or miR17-92 and miR106b, are paralleled by neuropathological findings. Indeed, the number of inflammatory infiltrates (**Figures 3A,D,G,J**), demyelination (**Figures 3B,E,H,K**) and axonal damage

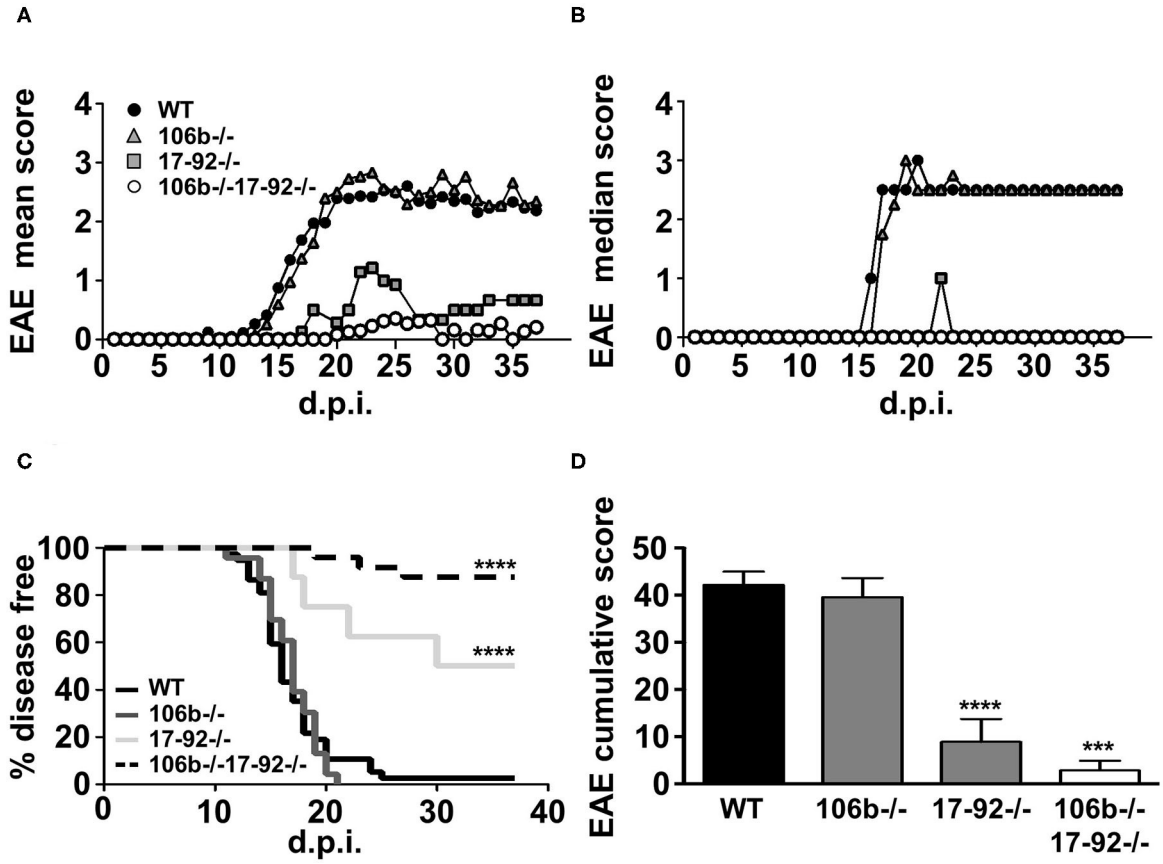

FIGURE 2 | Mice lacking miR17-92 or both miR17-92 and miR106b are protected from clinical signs of EAE. WT ($n = 41$), 106b$^{-/-}$ ($n = 29$), 17-92$^{-/-}$ ($n = 8$), and 106b$^{-/-}$17-92$^{-/-}$ ($n = 25$) mice were immunized to induce EAE. Clinical signs of EAE were monitored daily until day 37 post-immunization. Mean **(A)** and median **(B)** clinical score and disease incidence **(C)** were assessed for each group. The Log-Rank (Mantel Cox test) was used for the comparison of EAE incidence rates between the different groups. EAE was evaluated as cumulative score using Mann Whitney test **(D)**. Error bars indicate mean ± SEM. ***$P \leq 0.001$ and ****$P \leq 0.0001$.

(**Figures 3C,F,I,L**) in the spinal cord were significantly decreased (**Figures 3M–O**) in mice lacking miR17-92 or both miR17-92 and miR106b, while in mice lacking only miR106b tissue damage was similar to that in control mice (**Figures 3D–F,M–O** and **Figure S1**).

Absence of Mir106b Is Compensated by Mir17-92 Overexpression but Not Viceversa

We hypothesized that the absence of any alteration of the disease phenotype in miR106b$^{-/-}$ mice might be due to compensation by the miR17-92 cluster. This may occur functionally or also due to overexpression. We therefore analyzed, by RT-PCR, the expression of the miRNAs from the two clusters in CD4^{+} T cells purified from the spleen of naïve mice. We found that miRNAs from the miR17-92 cluster were up-regulated in mice lacking the miR106b cluster (**Figure 4A**), while the opposite did not occur (**Figure 4B**). miRNAs levels in double deletion mutant mice are shown for comparison (**Figure 4C**). When we measured mRNA levels of classical targets for miR17-92 and miR106b however, namely p21, BIM, and STAT3, in brain, spinal cord, lymph nodes, and spleen, we did not find any significant difference, with the exception of a slight decrease of BIM and STAT3 in the spinal cord of EAE mice deleted for miR17-92 and miR106b (**Figure S2**).

Pro-inflammatory Cytokine Expression Is Decreased in Eae Mice Lacking Mir17-92 and Mir106b

We next examined the expression of cytokines known to drive neuroinflammation in the spinal cord of EAE mice. We found that GM-CSF (**Figure 5A**), IFNγ (**Figure 5B**), and IL17 (**Figure 5C**) mRNA levels are significantly reduced in mice deleted for miR17-92 and miR106b as compared to mice lacking only miR106b or WT EAE mice. Mice lacking only miR17-92 display a non-significant decrease of these mRNAs, coherent with the disease phenotype which is in between the double deletion mutants and the miR106b and the WT EAE mice.

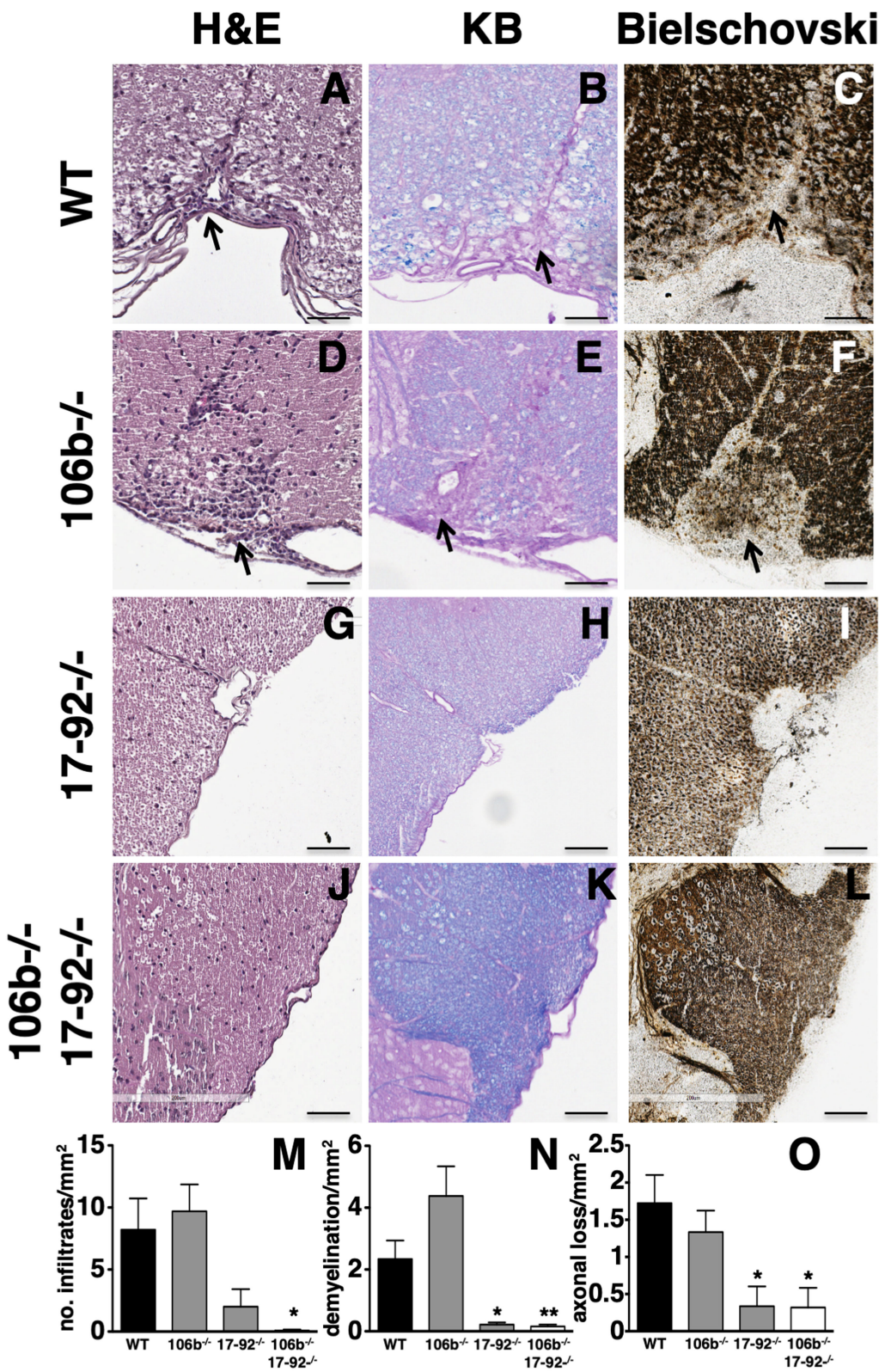

FIGURE 3 | Lack of miR17-92 or both miR17-92 and miR106b protects mice from pathological signs of EAE. Neuropathological analysis of infiltrates (Hematoxylin and Eosin; **A,D,G,J**), demyelination (Kluver Barrera; **B,E,H,K**), and axonal loss (Bielschowsky; **C,F,I,L**) in the spinal cord. Scale bar = 100 μm. Deletion of miR17-92 or both miR17-92 and miR106b is associated to a significant decrease of number of infiltrates **(M)**, demyelination **(N)**, and axonal damage **(O)**. Data are shown as mean ± SEM. Statistical significance was determined by Mann Whitney test. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

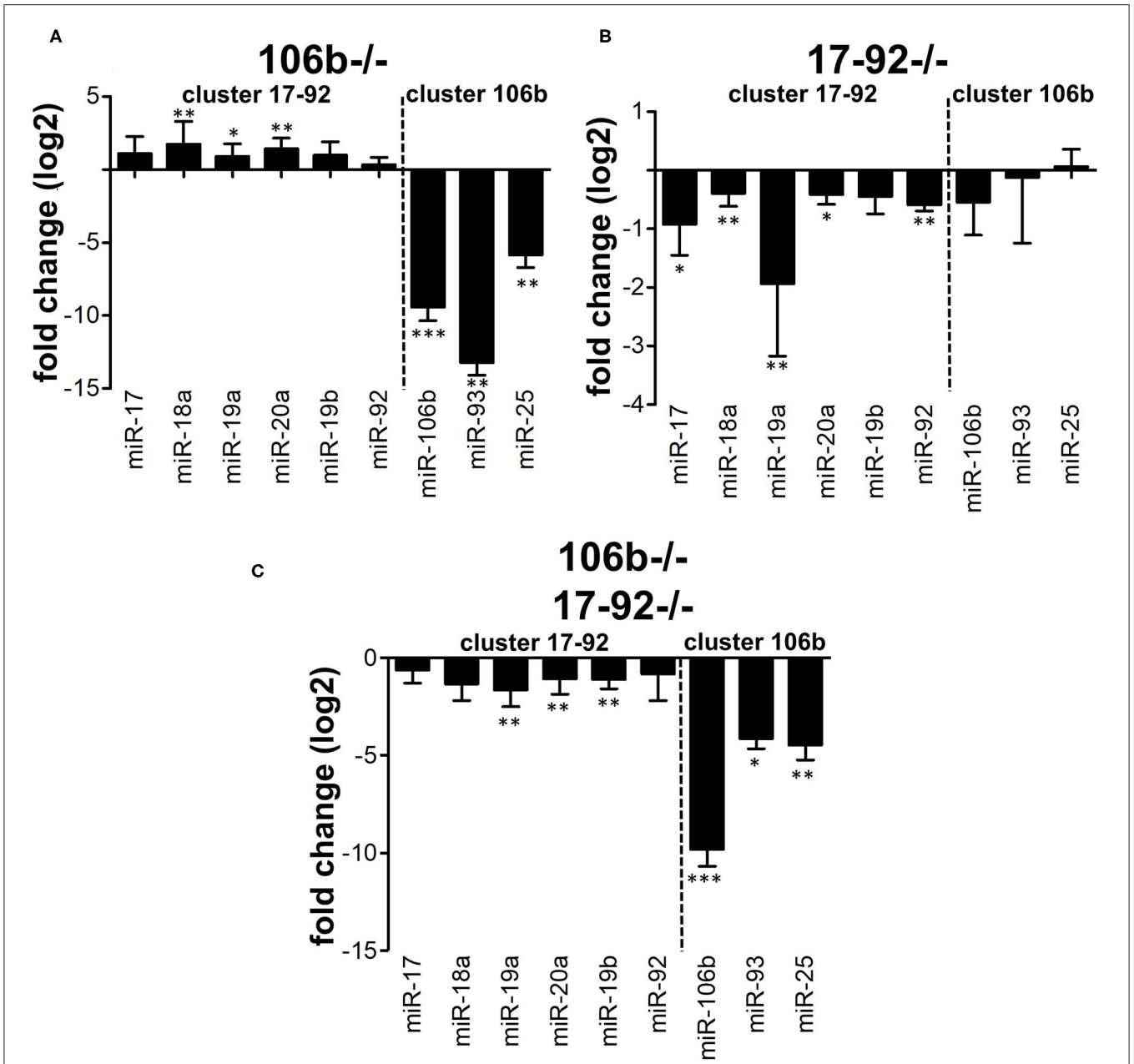

FIGURE 4 | Compensation between miR106b and miR17-92 family clusters. Relative expression (log_2 fold change vs. WT mice) of the 17-92 and 106b family clusters was measured by real time RT-PCR in CD4+ T cells isolated from the spleen of naive $106b^{-/-}$ **(A)**, $17\text{-}92^{-/-}$ **(B)**, and $106b^{-/-}17\text{-}92^{-/-}$ **(C)** mice. Data are shown as mean ± SEM. miRNA of the two different clusters (miR 106b-25 and miR 17-92 clusters) are divided by a dotted line. * <0.05; ** <0.01; *** <0.001 Mann-Whitney.

Th17 Cells Are Decreased in Mir17-92 Knock Out Mice

The reduction of pro-inflammatory cytokines may merely reflect the decrease of inflammatory cell infiltration in the CNS and the consequent decrease of neuroinflammation. To investigate what cell type is more affected in its development by the absence of miR17-92 and miR106b we therefore induced EAE in mice lacking miR106b, or miR106b and miR17-92, and WT mice and harvested spleens 37 days later. We isolated total splenocytes and re-stimulated them with the nominal antigen at escalating doses, and by intracellular staining followed by flow cytometry we measured the absolute numbers of Tregs, Th1, and Th17 cells. We found no differences in Tregs (**Figure 5D**), a significant decrease of Th1 cells in both single miR106b and miR106b and miR17-92 double deletion mutant mice as compared to WT mice (**Figure 5E**), but a significant decrease in Th17 cells specifically in miR106b and miR17-92 double deletion mutant mice (**Figure 5E**).

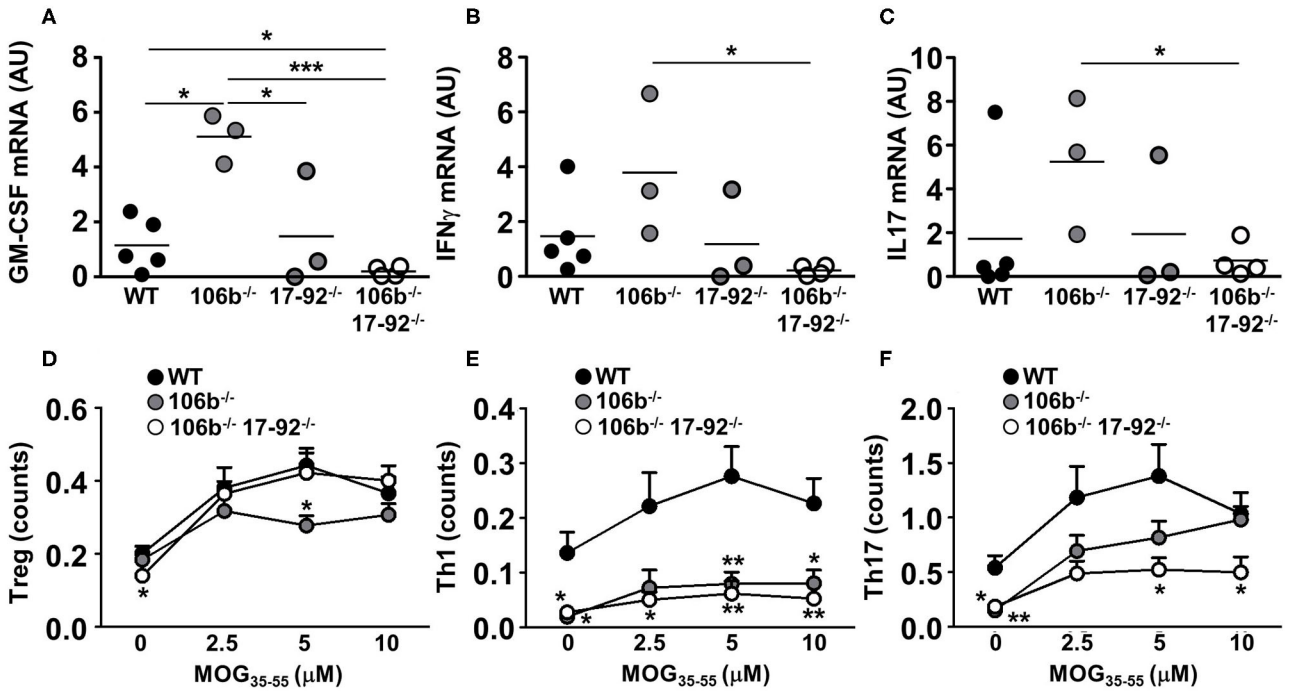

FIGURE 5 | Reduction of pro-inflammatory cytokines in mice lacking miR17-92 and miR106b. **(A–C)** mRNA levels of GM-CSF, IFNγ, and IL17 were measured in the spinal cord by real time RT-PCR. Data are shown as arbitrary units (AU). **(D–F)** Total splenocytes were isolated from mice spleen ($n = 5$ for each group) 37 days after EAE induction. Cells were re-stimulated for 3 days with increasing amounts of MOG_{35-55} and the absolute count of Treg, Th1, and Th17 cells (% singlets on 20,000 cells X % Treg or Th1 or Th17 on singlets/100) was measured by flow cytometry. Statistical significance was determined by Mann Whitney test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

DISCUSSION

miRNAs have been demonstrated to regulate several processes in the development and function of the immune system and the alteration of miRNA homeostasis was shown to lead to the dysfunction of immune responses causing autoimmune diseases such as MS.

In our previous studies, we analyzed miRNA expression profile in Tregs from relapsing remitting MS patients and we identified two members of the miR 106b-25 cluster (miR93, miR 106b) and two members of the miR17-92 paralog cluster (miR 19a and miR 19b) as increased in Tregs from relapsing remitting MS patients compared to healthy controls (6).

The influence of miR 17-92 cluster in Treg cell differentiation was demonstrated in MS also in a study of miRNA profiling. Nineteen miRNAs (including miR18a that belongs to miR 17-92 cluster) were found differentially expressed in naive CD4 T cells multiple sclerosis patients and predicted to target TGFβ. These miRNAs negatively regulated the TGFβ pathway (TGFBR1 and SMAD4 were significantly reduced in patients with multiple sclerosis), resulting in a decreased capacity of naive CD4 T cells to generate regulatory T cells (15).

Moreover, in a mouse model for Alzheimer's disease, miR 106b was demonstrated to influence TGF-β signaling through the direct inhibition of the TGF-β type II receptor (TβR II) translation indicating TβR II as a functional target of miR-106b (16). Starting from these evidences we investigated the role of miR17-92 and miR 106b-25 clusters in neuroinflammation in a mouse model of multiple sclerosis, EAE, and found that the absence of 17-92 inhibits EAE development. We can hypothesize that single (17-92$^{-/-}$) and double (miR-106b-25$^{-/-}$miR-17-92$^{-/-}$) knockout mice were protected from clinical signs of EAE due to the alterations of immune responses already described in literature, such as lymphoproliferation (10), antigen presentation (17), and Th17 differentiation (18), potentially affecting the induction of the experimental autoimmune disease *per sé*.

As expected, we found a significant decrease of GM-CSF level in the spinal cord of double knock out mice. GM-CSF is a well-known pro-inflammatory cytokine essential for development and progression of EAE (19, 20). GM-CSF reduction in double knock out mice, which are protected from EAE development, is in line with previous findings. Indeed, GM-CSF is required by CCR2^{+} monocytes to initiate tissue inflammation and the deletion of GM-CSF receptor in this cell subset induces EAE resistance (21). Moreover, overexpression of GM-CSF leads to the invasion and expansion of inflammatory myeloid cells into the brain and is sufficient to induce spontaneous CNS inflammatory disease (22). We found GM-CSF mRNA significantly upregulated in spinal cords of 106b-25$^{-/-}$ EAE mice. GM-CSF is not an obvious target of miR 106b-25, thus this finding may be secondary to the immune dysregulation in these mice.

We show here, however, that also IL-17 and Th17 cells, which are known to drive neuroinflammation, are importantly reduced in mice lacking both miR17-92 and miR106b as compared to mice deleted only for miR106b or WT EAE mice. We therefore hypothesized that this reduction may inhibit effector functions in the target tissue, and constitute a more specific inhibition of neuroinflammation.

Pathological evaluation of spinal cord from EAE mice confirmed our clinical findings. We found that deletion of miR17-92 or both miR17-92 and miR106b was associated to a significant decrease of inflammatory infiltrates, demyelination, and axonal damage. Clinical and pathological analysis of EAE mice showed that there were no differences in miR 106b$^{-/-}$ compared to wild type mice. Our RT-PCR data may suggest that this was due to function redundancy among miRNAs family clusters since miR17-92 and miR 106b-25 share the same set of target genes (10). While miR17-92 compensates for the absence of miR106b-25 in terms of increased expression, the opposite is not true.

Although we demonstrated a protective role for miR17-92 deletion we did not find the plausible targets of 17-92 that mediate this process. Quantification of the three major miR17-92 and miR 106b 25 targets (STAT 3, BIM, and P21) did not show any significant statistical difference in their levels among the different mice strains.

The study of Liu et al. (18) suggests a possible mechanism for miR17-92 cluster in Th17 differentiation. Indeed miR-19b and miR-17 enhances Th17 polarization by repressing the expression of Phosphatase and Tensin Homology (PTEN) and inhibiting Ikaros Family Zinc Finger 4 (IKZF4), respectively.

Our work underlines the relevance of the miR17-92 cluster for the development of experimental neuroinflammation, possibly for the inhibition of specific pathways, such as Th17 cells, crucial for EAE initiation. Indeed, we had found an imbalance of miR106b-25 and miR17-92 clusters also in human MS, suggesting that miR106b-25 and miR17-92 clusters may represent a plausible therapeutic target in human neuroinflammatory diseases.

MATERIALS AND METHODS

Mice

All animal experiments were done with permission from the Institutional Animal Care and Use Committee (IACUC). All mice were maintained under specific pathogen-free conditions in the animal facility at San Raffaele Scientific Institute.

miR-106b-25, miR-17-92 KO, and miR-106b-25/miR-17-92 DKO mice were kindly provided by Dr. Paolo Dellabona (San Raffaele Scientific Institute, Italy) and generated as described by Ventura et al. (10) using 6- to 8-weeks-old C57BL/6 female mice purchased from Charles River Laboratories (Calco, Italy).

Induction of Eae

C57BL/6 WT and mutant mice (8–10 weeks old) were immunized subcutaneously with 200 μg of MOG_{35-55} in Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 4 mg/mL heat-killed Mycobacterium tuberculosis (strain H37Ra; Difco, Florence, Italy). Each mouse was injected i.v. with Pertussis toxin (500 ng, List Biological Laboratories, Campbell, CA, USA) on the day of the immunization and 48 h later. Mice were weighed and scored for clinical signs daily. Clinical symptoms of EAE were classified as follow: 0 = no signs; 1 = tail paralysis; 2 = ataxia and/or paresis of hindlimbs; 3 = paralysis of hindlimbs and/or paresis of forelimbs; 4 = tetraparalysis; and 5 = moribund or dead. EAE mice were killed at 37 d.p.i for real-time PCR, histological evaluation and flow cytometer analysis.

Histological Evaluation

Pathological evaluation of spinal cord from EAE mice was performed 37 days post-EAE induction. Three mice per group were perfused through the left cardiac ventricle with saline plus EDTA 0.5 mM for 10 min followed by fixation with cold 4% paraformaldehyde, PFA (Sigma). Spinal cords from EAE mice were dissected out and post-fixed in 4% PFA overnight. Tissues were embedded in paraffin, sectioned and stained with Hematoxylin and Eosin, Kluver Barrera, and Bielschowsky to reveal perivascular inflammatory infiltrates, demyelinated areas, and axonal loss, respectively. Parameters were quantified on an average of 9 complete cross-sections of spinal cord per mouse taken at eight different levels. The number of inflammatory infiltrates were expressed as the number of infiltrates per mm^2, demyelinated areas and axonal loss were expressed as percentage per mm^2.

RNA Extraction

Total RNA from splenic CD4+ T (isolated from mouse spleen with CD4+T cell isolation kit, Miltenyi Biotec GmbH) was isolated with the miRvana kit (Life Technologies). For mRNA extraction from brain, spinal cord, lymph nodes, and spleen tissues were homogenized with 1 ml of TRIzol (Life Technologies, Paysley, UK) every 50–100 mg using an IKA Ultra Turrax rotor homogenizer (Sigma Aldrich, St. Louis, MO, USA). RNA was quantified by Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA).

Quantitative Real-Time Rt-Pcr

Reverse transcription of miRNA was performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). qRT-PCR was performed with TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes (Applied Biosystems). Values were normalized to snoRNA-202. The primers used in real-time RT-PCR experiments were as follows: miR 106b (Taqman Assay Applied Biosystems name: has-miR-106b; miRBase/Exiqon name: mmu-miR-106b; assay ID: 000442), miR 17 (has-miR-17; mmu-miR-17; 002308), miR 18a (has-miR-18a; mmu-miR-18a; 002422), miR 19a (has-miR-19a; mmu-miR-19a; 000395), miR 19b (has-miR-19b; mmu-miR-19b; 000396), miR 20a (has-miR-20a; mmu-miR-20; 000580), miR 25 (has-miR-25; mmu-miR-25; 000403), miR 92 (has-miR-92; mmu-miR-92a-3p; 000430), miR 93 (has-miR-93; mmu-miR-93; 001090), snoRNA-202 (assay ID: 001232).

For mRNA expression, RNA reverse transcription was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). STAT3 (Mm01219775 m1), BIM (Mm00437796 m1), p21 (Mm00432448 m1), GM-CSF (Mm00438328 m1), IFNγ (Mm01168134 m1), IL-17 (Mm00439618 m1) mRNA levels were measured by real-time RT-PCR using Taqman technology (Applied Biosystems, Invitrogen). PCR reactions were run on an ABI Prism 7500 Sequence Detection System. GADPH (4352339E) was used as a housekeeping gene. Relative changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method.

Flow Cytometry

Mouse-specific anti-CD3 (Pacific Blue conjugated, clone 500A2, catalog no. 558214), anti-CD4 (PerCP conjugated, clone RM4-5, catalog no. 553052), anti-CD25 (Phycoerythrin/Cy7, clone PC61, catalog no. 561780), anti-FoxP3 (Alexa Fluor 488 conjugated, clone MF23, catalog no. 560403), anti-IL17A (Alexa Fluor 647 conjugated, clone TC11-18H10, catalog no. 560184), and IFNγ (PE conjugated, clone XMG1.2, catalog no. 554412) mAbs were all purchased from BD Biosciences.

For intracellular staining total splenocytes were isolated 37 days after EAE induction and cells were re-stimulated for 3 days with increasing amounts of MOG35-55. After that cells were stimulated for 4 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (1:1,000, BD Pharmingen). Cell were stained with surface markers (CD3, CD4, CD25), permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set and stained for IL-17, IFNg, and FoxP3.

Samples were acquired on BD FACS Canto II flow cytometer and analyzed with FlowJo software.

The absolute count of Treg, Th1, and Th17 cells was measured as % singlets on 20,000 cells X % Treg or Th1 or Th17 on singlets/100.

Statistical Analyses

Differences between survival curves were calculated by Log-rank test (Mantel-Cox) while Mann-Whitney tests were used to evaluate differences between groups for non-parametric data. The results are expressed as means ± SEM. Differences are considered statistically significant when $p < 0.05$.

Statistical analyses were performed using GraphPad Prism version X (GraphPad Software, San Diego, CA, USA).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) and IRCCS Ospedale San Raffaele.

AUTHOR CONTRIBUTIONS

AF, MD, LC, CA, and GD performed the experiments. MF, PD, GC, and RF designed the experiments and analyzed the results. AM and RF wrote 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/fneur.2020.00912/full#supplementary-material

Figure S1 | One representative image of the whole section of spinal cord presented in **Figure 3**. Spinal cord sections were stained with Hematoxylin and Eosin, Kluver Barrera, and Bielschowsky to asses number of infiltrates, demyelination, and axonal loss.

Figure S2 | mRNA levels of classical targets for miR17-92 and miR106b. mRNA levels of p21, BIM, and STAT3 were measured in the brain, spinal cord, lymph nodes and spleen by real time RT-PCR. Data are shown as arbitrary units (AU). *$P < 0.05$ (Mann Whitney test).

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Correlation Between Anti-Myelin Proteolipid Protein (PLP) Antibodies and Disease Severity in Multiple Sclerosis Patients With PLP Response-Permissive HLA Types

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The most prominent pathological features of multiple sclerosis (MS) are demyelination and neurodegeneration. The exact pathogenesis of MS is unknown, but it is generally regarded as a T cell-mediated autoimmune disease. Increasing evidence, however, suggests that other components of the immune system, particularly B cells and antibodies, contribute to the cumulative CNS damage and worsening disability that characterize the disease course in many patients. We have previously described strongly elevated T cell reactivity to an extracellular domain of the most abundant CNS myelin protein, myelin proteolipid protein (PLP) in people with MS. The current paper addresses the question of whether this region of PLP is also a target of autoantibodies in MS. Here we show that serum levels of isotype-switched anti-$PLP_{181-230}$ specific antibodies are significantly elevated in patients with MS compared to healthy individuals and patients with other neurological diseases. These anti-$PLP_{181-230}$ antibodies can also live-label PLP-transfected cells, confirming that they can recognize native PLP expressed at the cell surface. Importantly, the antibodies are only elevated in patients who carry HLA molecules that allow strong T cell responses to PLP. In that subgroup of patients, there is a positive correlation between the levels of anti-$PLP_{181-230}$ antibodies and the severity of MS. These results demonstrate that anti-PLP antibodies have potentially important roles to play in the pathogenesis of MS.

Keywords: antibody, myelin proteolipid protein, multiple sclerosis, disease severity, HLA type

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), which affects about 2.5 million people worldwide. Although the exact pathogenesis of MS is unknown, it is predominantly regarded as a T cell-mediated autoimmune disease directed against myelin antigens. However, increasing evidence suggests that other components of the immune system, in particular B cells and antibodies, may contribute to the cumulative CNS damage and disability that characterize the disease course.

The single most consistent laboratory finding in MS is the presence of oligoclonal immunoglobulin G (IgG) bands in the cerebrospinal fluid (CSF) but not in the serum; these signify intrathecal antibody production, persist for the lifetime of the patient (1), and strongly suggest involvement of antibodies in the immunopathology of MS. Because CSF drains into the blood, and activated B cells can freely enter and exit the CNS, such antibodies could also enter the circulation. Numerous published studies have reported antibodies directed against myelin proteins (2–12) or other CNS antigens (13–18) in CSF or serum of MS patients, but only a small number of these studies have attempted to determine whether these antibodies are potentially pathogenic (3, 7, 15, 18). In some cases, antibodies in MS patients may be merely an epiphenomenon; however, there are multiple examples in the non-MS literature of how antibodies targeting CNS tissues can be directly pathogenic (reviewed in (19)), and the chance that antibodies present in at least some patients with MS are pathogenic is relatively high.

One question that is often raised is that of the relevance of serum antibodies to pathology in the CNS, since antibodies and complement are normally excluded from the CNS by the blood-brain barrier (BBB). However, activated B cells can easily cross the intact BBB (20) and differentiate into plasma cells which secrete antibody intrathecally, and during acute inflammatory episodes of MS there is increased permeability of the BBB, allowing entry of antibodies and complement components from the blood (21, 22). Thus, antibodies in both the serum and the CSF are of potential relevance in MS.

We have had a long-term interest in the role of immune T cell reactivity directed against the most abundant component of CNS myelin, myelin proteolipid protein (PLP), and have previously shown that T cell reactivity to PLP in MS is restricted by certain HLA types (23). Patients carrying these HLA types make stronger T cell responses to PLP peptides, especially those within the second extracellular loop of PLP (residues 181-230), and are more likely to develop lesions in the brainstem and cerebellum (23). Interestingly, we also found in a mouse model of MS that the sites in which lesions developed depended not only on T cells, but also on the presence or absence of anti-PLP antibodies against the same region of PLP (24, 25). PLP is present right throughout the lamellae of compact myelin, including the outermost loop (26), and monoclonal antibodies directed against epitopes within the two extracellular domains of PLP (encompassing residues 30–60 and 181–230) can live-label cultured oligodendrocytes (27), confirming that these PLP epitopes are exposed on the surface of oligodendrocytes, which makes them potential targets of demyelinating antibodies. Furthermore, a recent study has shown that monoclonal anti-PLP antibodies directed against these same extracellular epitopes of PLP can also label neurons (28), most likely through cross-reactivity between PLP and the related M6 family of molecules that are expressed on both neurons and oligodendrocytes. Since the most prominent pathological features of MS are demyelination and neurodegeneration, anti-PLP antibodies therefore have the potential to play a role in both of these aspects of MS.

Since strong antibody responses are reliant on T cell help, the work presented in this paper set out to determine if patients who carry HLA types that allow a strong T cell response to PLP also allow elevated antibody responses to PLP, and to determine if there is any relationship between levels of anti-PLP antibodies and disease severity in MS.

MATERIALS AND METHODS

Patients and Controls

This study was approved by the Royal Brisbane and Women's Hospital Human Research Ethics Committee, and The University of Queensland Medical Research Ethics Committee. Up to 10 ml of blood was obtained from patients with MS [$n = 146$; 81 with relapsing-remitting MS (RR-MS), 38 with secondary progressive MS (SP-MS) and 27 with primary progressive MS (PP-MS)], a first demyelinating event suggestive of MS (clinically isolated syndrome (CIS); $n = 40$), patients with other CNS neurological disorders (OND; $n = 42$) and healthy individuals ($n = 54$). All patients with MS, apart from 4 PP-MS patients, met the 2010 revised McDonald criteria (29). None of the MS patients had received any immunosuppressant, immunomodulatory, or corticosteroid therapy in the 3 months prior to blood collection. Informed consent was obtained prior to blood collection. Five ml of the blood was allowed to clot at room temperature for 1–2 h, after which serum was collected. The other 5 ml of blood was used to extract genomic DNA for HLA typing. Demographics for individuals from whom blood was collected are shown in **Table 1**.

HLA Typing

Genomic DNA was prepared using NucleoSpin Blood DNA extraction kits (Macherey-Nagel, Düren, Germany) as previously described (23). Dynal low and high resolution SSP kits (Dynal Biotech, Thermo Fisher, Australia) were used to type for HLA-DR, -DQA, and -DQB alleles, following the manufacturer's recommended protocols. Results for MS patients were reported to the 4 digit level, when it was able to be determined; however, alleles are grouped at the 2 digit level for the analyses.

Human PLP and 50-mer PLP Peptide

Human brain tissue was obtained from the Queensland Brain Bank (part of the National Brain Bank Consortium) at the University of Queensland, and a total lipid extract was prepared using the method of Folch et al. (30). To prepare PLP, the total lipid extract was concentrated on a flash evaporator, precipitated with acetone, and the precipitate dried under nitrogen until all trace of the acetone was removed. The dried precipitate was then dissolved in a small volume of chloroform:methanol:acetic acid (2:0:0.03 v/v) and left at 4°C until the lipids (mainly cerebroside) rose to the top of the tube. The clear lower fraction was then subjected to gel chromatography through a 100 × 2.5 cm lipophilic LH-60 column at 1 atmosphere pressure, using chloroform:methanol (2:1) as the buffer. Elution of PLP from the column was monitored by measuring the absorbance at 280 nm. Fractions containing PLP were pooled and stored at 4°C in the dark until required. To convert PLP to a water soluble form, a small volume of PLP in chloroform:methanol in a shallow watch glass was diluted with an equal volume of chloroform:methanol:acetic acid (2:0:0.03 v/v), and then distilled

TABLE 1 | Demographics of patients and controls.

Group	*n*	% Female	Age median (range)	MS duration in years median (range)	MS severity score median (range)
Healthy control	54	84.3%	38 (22–57)	n/a	n/a
MS (All)	146	80.1%	45 (19–72)	9 (0.25–31)	5.39 (0–9.88)
RR-MS	81	83.7%	37 (19–57)	5 (0.25–23)	4.44 (0–9.81)
SP-MS	38	89.5%	50.5 (27–72)	20 (3–31)	5.74 (1.29–9.63)
PP-MS	27	59.3%	52 (37–64)	11 (1–28)	8.04 (2.65–9.88)
OND	42	42.9%	46.5 (18–65)	n/a	n/a
CIS	40	75.0%	38 (20–56)	n/a	n/a

n/a, not applicable.

deionized water (dH_2O) was added, one drop at a time, under a constant stream of nitrogen gas. Once the liquid no longer turned cloudy when each drop of dH_2O was added, 0.5 mL of dH_2O was added, and the PLP was dialyzed against 3 changes of dH_2O in 10,000 MW cutoff dialysis tubing. The protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce) and the sample was diluted to 1 mg/mL with dH_2O. This water soluble form was kept at 4°C for no longer than 1 week.

The 50 mer $PLP_{181-230}$ peptide containing 2 disulphide bonds (between cysteines residues at 200 and 219, and at 183 and 227), as occurs in the second extracellular loop of the native protein, was synthesized and checked for the presence of the correct two disulphide bonds as previously described (31). As this peptide has been shown to oxidize relatively rapidly, it was stored under nitrogen gas, and was prepared just prior to use by dissolving peptide in 0.2 M acetic acid to a concentration of 5 mg/mL.

ELISA for Anti-PLP Antibodies

Since PLP precipitates out of solution in the presence of salts, it was diluted to 10 μg/mL in dH_2O containing 25 μg/mL bovine serum albumin (BSA) and coated onto high protein binding Nunclon ELISA plates. Control wells were coated with 25 μg/mL BSA in dH_2O. For the 50 mer $PLP_{181-230}$ peptide, the 5 mg/mL stock solution was diluted to 5 μg/mL in bicarbonate buffer (pH 9.6) containing 25 μg/mL BSA for coating onto the ELISA plates. As negative control for the peptide-containing wells, ELISA plate wells were coated with the bicarbonate buffer containing 25 μg/mL BSA alone. Plates were blocked with 1% skimmed milk powder in PBS containing 0.05% Tween 20 (PBS-T-SM). Four dilutions (1/25, 1/50, 1/100 and 1/200) of a control serum sample (which was a pool of 5 MS sera with moderate to high reactivity in preliminary assays) diluted in PBS-T-SM were used on each plate to serve as a positive control to ensure consistency of peptide coating and to normalize results from one plate to another (see below). Each test serum sample was tested at a dilution of 1:40 in PBS-T-SM on three wells coated with the BSA alone, and three wells coated with the BSA + $PLP_{181-230}$ peptide. After 2 h at room temperature, plates were washed with PBS-T, and an alkaline phosphatase-conjugated anti-human polyvalent Ig secondary antibody was added to all wells of the plate for 2 h at room temperature. After washing 5 times with PBS-T and once with dH_2O, the substrate p-nitrophenyl phosphate (pNPP; Sigma) was added to each well, incubated for 15 min, and the reaction was then stopped with 3 N NaOH. The absorbance at 405 nm was read on a Tecan Spark 10 M Multimode plate reader. The absorbance of the wells containing BSA alone was subtracted from the absorbance of the wells containing BSA + $PLP_{181-230}$ for each sample, to give the PLP-specific absorbance. A semi-log XY standard curve was drawn using the 4 dilutions of the positive control serum sample on the x axis (log scale) and their absorbance values on the y axis, and the equation of the curve determined ($Y = \text{slope x } \log_{10}(X) + Y \text{ intercept}$). From that equation, *X* values for each test serum sample were calculated. The absorbance of the 1/100 dilution was normalized to 1 absorbance unit, which changes the *Y* intercept, but not the slope of the curve. From the equation of this normalized curve, the normalized *Y* values of the test samples were obtained (i.e., normalized $PLP_{181-230}$-specific absorbance values).

Isotyping

For isotyping of samples, the ELISA was repeated on serum samples that showed a detectable level of $PLP_{181-230}$ specific antibodies above the level of the 75% percentile of the healthy individuals. In this case, however, the secondary antibodies used were horse radish peroxidase (HRP) conjugated mouse antibodies specific for human IgG1, IgG2, IgG3, IgG4, or IgM antibodies (clones HP6070, HP6014, HP6047, HP6023 and HP6083, respectively, all from Invitrogen), which were used instead of the alkaline phosphatase-conjugated polyvalent human Ig antibody above. To detect the HRP-conjugated secondary antibodies, plates were incubated with o-phenylenediamine dihydrochloride (OPD) substrate (Sigma) for 15 min at room temperature, stopped with 2.5 M sulphuric acid, and the absorbance was read at 490 nm. Each sample was tested in triplicate on wells coated just with BSA and on wells coated with BSA + $PLP_{181-230}$ for the presence of each different IgG or IgM isotype. Data were reported as the isotype of antibody that gave the strongest $PLP_{181-230}$ specific response.

Immunolabelling of PLP-Expressing CHO Cells

A plasmid encoding wild-type human *PLP1* coupled to an mCherry tag was constructed as previously described (32). Dissociated CHO-K1 cells (10^6 cells in PBS) were transiently transfected via electroporation with 2 ng of the plasmid using the Amaxa II transfection device (Lonza, Basel, Switzerland),

following the manufacturer's recommended protocol. The transfected cells were plated in 8 well-chamber slides at a concentration of 10^5 cells/well. Two days following transfection, the cells were live-labeled with 1/20 dilution of patient serum at room temperature for 1 h. After 3 washes with PBS containing 2% fetal calf serum and 0.05% azide (PBS azide wash), cells were labeled with FITC-conjugated rabbit anti-human Igs (Dako, Agilent, Santa Clara, USA) at room temperature for 1 h. Slides were washed 3 times in PBS azide wash, and the chambers were removed. Finally, the slides were incubated for 15 min with PBS azide wash containing 1/30,000 dilution of DAPI, and then coverslips were mounted on the slides. Slides were viewed using a Zeiss Axio Imager M1 microscope fitted with an Axiocam 503 camera, and images acquired using Zen software (Zeiss).

Statistical Analysis

Statistical analyses were done using GraphPad Prism v 8.2.1. Data were first checked to determine if they were normally distributed. If so, 3 or more groups were compared using ANOVA, with Bonferroni correction for multiple comparisons. Data in this case are presented as mean ± SE of the mean. If data were not normally distributed, then the Kruskal-Wallis test with Dunn's multiple comparison test was used to determine statistical significance. In that case, data are presented as median and interquartile range. For correlations, since the data were from a non-parametric distribution, the Spearman correlation coefficient (ρ) was determined.

RESULTS

Comparing the Reproducibility of ELISA Results Using Human PLP or the $PLP_{181-230}$ Peptide

Whole PLP has been used as an antigen in various T cell and antibody studies, with varying levels of reproducibility and success, as previously discussed (33). One reason for this variability is the extreme hydrophobicity of PLP. It is difficult to get PLP into a water-soluble form, and, even then, the presence of salts tends to make it precipitate out of solution. We have previously shown that the major T cell response against PLP is directed against two overlapping peptides, $PLP_{184-199}$ and $PLP_{190-209}$, within the second extracellular loop of PLP ($PLP_{181-210}$), and that there is also an elevated antibody response to these peptides (23). However, the second extracellular loop of PLP normally contains 2 disulphide bonds, which would potentially produce conformational epitopes for antibodies that would not be able to be detected using the 2 overlapping peptides above. Therefore, we decided first to compare the reproducibility of antibody assays utilizing whole human PLP or a 50-mer PLP peptide covering this second extracellular loop, and synthesized with 2 disulphide bonds, to mimic the structure of PLP in the myelin membrane. Initially, serum samples from 82 MS patients were tested for reactivity against whole human PLP or the 50-mer peptide, with each sample tested in 3 independent assays. Each sample was scored as a positive (absorbance > 0.2 units) or negative (absorbance ≤ 0.2 units) response to human PLP and to $PLP_{181-230}$, and the proportion of samples that scored all positive or all negative in the 3 independent assays was determined. For whole human PLP, the same result was obtained in all 3 replicate assays only 48.8% of the time. In contrast, using the $PLP_{181-230}$ peptide, the same result was obtained in all 3 replicates 87.8% of the time, which was a marked improvement over the result with whole human PLP. In cases where a sample showed a positive response to the whole PLP protein, it also gave a positive result to the $PLP_{181-230}$ peptide in 92.9% of cases. In contrast, if the response to the whole protein was negative, the response to the peptide was negative in only 70% of cases. Thus, we believe that the peptide has better sensitivity for detection of the anti-PLP antibodies.

Antibodies That Bind to the $PLP_{181-230}$ Peptide in ELISA Can Also Live-Label PLP-Transfected Cells

To confirm that the anti-$PLP_{181-230}$ antibodies detected in the ELISA are also able to recognize whole PLP expressed in the cell membrane, CHO cells were transiently transfected with a mCherry-tagged PLP-expressing plasmid, and live-labeled 2 days later with sera from patients who showed strong, moderate, or no reactivity in the ELISA. There was a good correlation between the anti-$PLP_{181-230}$ reactivity of the serum samples in the ELISA and their ability to live-label PLP-transfected cells (**Figure 1**). The antibodies from patients with high or moderate antibody levels measured by ELISA showed strong membrane staining of the PLP-transfected cells, but not of non-transfected cells in the same slide, whereas sera that showed no anti-$PLP_{181-230}$ binding by ELISA did not label the PLP-transfected CHO cells. Therefore, the remainder of the assays in this paper were done using the $PLP_{181-230}$ peptide in ELISA for determining levels of anti-PLP antibodies.

Levels of Isotype-Switched Autoantibodies Specific for $PLP_{181-230}$ Are Increased in Patients With MS

Initial studies investigated the levels of antibodies specific for $PLP_{181-230}$ in patients with MS, OND patients and healthy individuals. There was a significant increase in the levels of anti-$PLP_{181-230}$ antibodies in MS patients, compared to the OND patients and healthy individuals (**Figure 2A**). Some OND patients did show elevated levels of anti-$PLP_{181-230}$ antibodies. The OND patients with an elevated antibody response to PLP in the top quartile of the OND group had a variety of CNS disorders, including epilepsy (3 patients), stroke (3 patients), and one each of CNS tumor and idiopathic intracranial hypertension.

Next, we looked at the isotype of the anti-$PLP_{181-230}$ antibodies. Sera from individuals who showed levels of antibodies at or higher than the 75th percentile of the healthy individual group were tested to determine the primary isotype of antibodies reacting with the PLP peptide. This included samples from 18 healthy individuals, 93 patients with MS, and 16 OND patients. Of interest, almost all of the positive samples from OND patients (13/16) and healthy individuals (16/18) contained IgM anti-$PLP_{181-230}$ antibodies only (**Figure 2B**). In contrast,

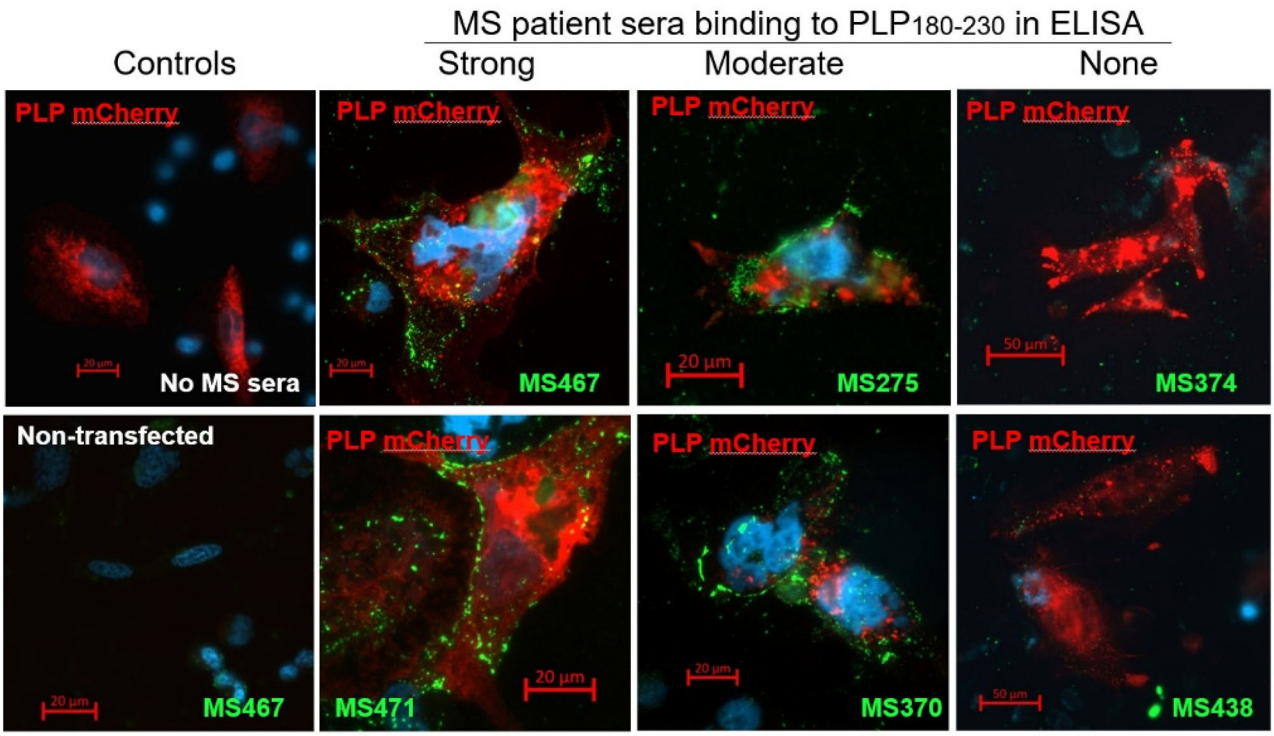

FIGURE 1 | CHO cells transiently transfected with an mCherry-PLP construct (so that cells expressing PLP fluoresce red) were live-labeled using MS patient sera that showed high (MS467 and MS471), moderate (MS275 and MS370) or no (MS374 and MS438) binding to the $PLP_{181-230}$ peptide in ELISA assays. Binding of patient sera to the transfected CHO cells was revealed by addition of a FITC-labeled anti-human Ig secondary antibody (green). Controls included wells with no MS sera added (top left image) and non-transfected cells in the wells to which MS sera were added (bottom left image showing non-transfected cells in the well in which MS467 serum was added). MS sera that showed high or moderate binding to the $PLP_{181-230}$ peptide in ELISA assays labeled the membranes of PLP-transfected CHO, whereas sera that showed no anti-$PLP_{181-230}$ binding by ELISA did not label the PLP-transfected CHO cells.

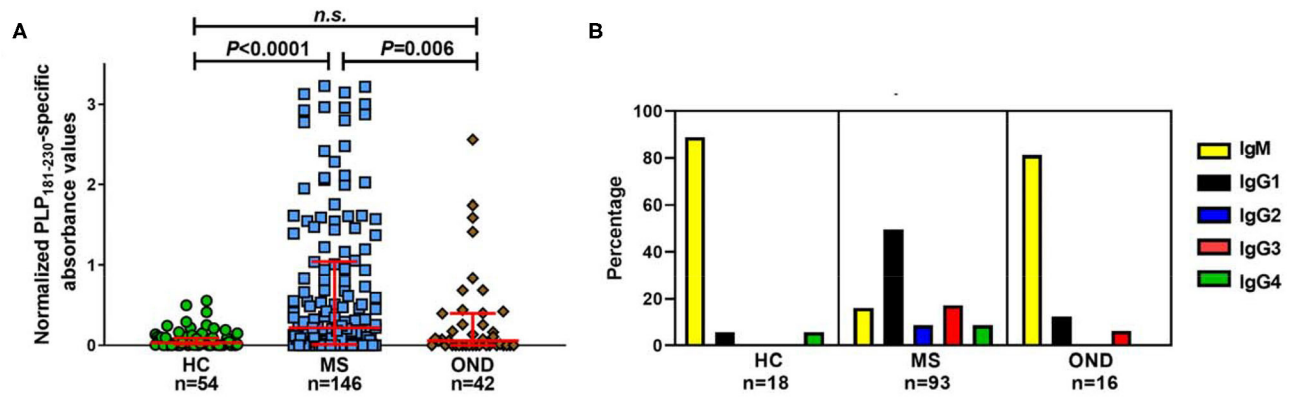

FIGURE 2 | Significantly elevated levels of isotype-switched anti-PLP antibodies are present in MS patients. **(A)** Reactivity in ELISA of healthy individuals' (HC), MS and OND patients' sera (all tested at 1/40 dilution) to $PLP_{181-230}$, synthesized with 2 disulphide bonds to mimic the structure of the second extracellular loop of PLP. Results are presented as the normalized $PLP_{181-230}$-specific absorbance values. Analyzed using Kruskal–Wallis test with Dunn's multiple comparison test. **(B)** Percentage of samples of each isotype from individuals with elevated (at or above the 75th percentile of the HC group) antibodies reactive to PLP. n.s., not significant.

the majority of the positive samples from MS patients contained isotype-switched anti-$PLP_{181-230}$ antibodies. IgG1 was the most common isotype for anti-$PLP_{181-230}$ antibodies from MS patients; however, several patients had high levels of IgG2, IgG3, or IgG4 antibodies, suggesting that the microenvironment in which the anti-PLP antibody producing B cells mature and class-switch can vary from patient to patient. The percentage of MS patients who had isotype-switched anti-PLP antibodies to any

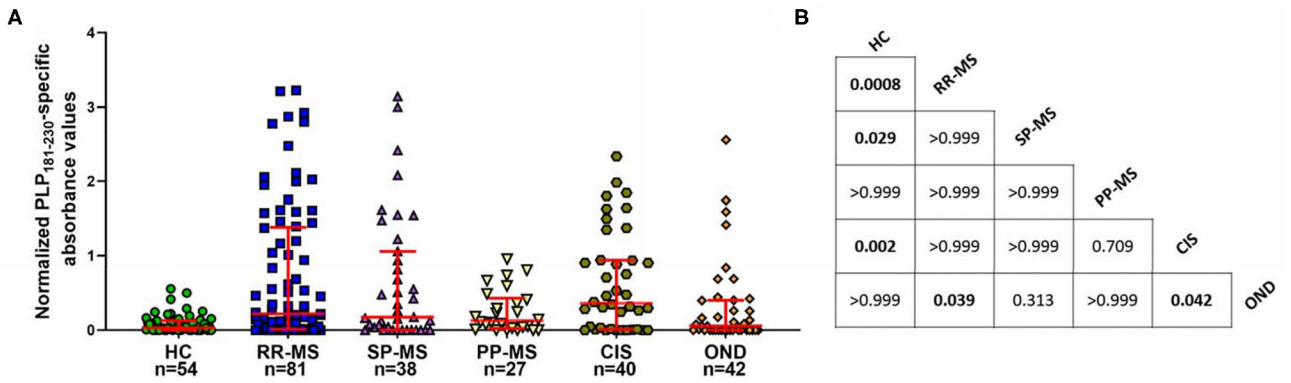

FIGURE 3 | (A) Comparison of anti-$PLP_{181-230}$ antibody levels in the different MS subgroups and in patients with CIS. PP-MS patients have lower levels of anti-$PLP_{181-230}$ antibodies than do other groups of MS patients or individuals with CIS. **(B)** Chart showing the *P*-values for comparison of all groups shown in **(A)** Significant differences are highlighted in bold. Analyzed using Kruskal-Wallis test with Dunn's multiple comparison test.

IgG subtype (83.9%) was highly significantly different from that of healthy controls (11.1%; $P = 1.8 \times 10^{-9}$) and that of OND patients (18.8%; $P = 2.0 \times 10^{-7}$) by χ^2 with Yates' correction.

Anti-PLP Antibodies Are Produced Throughout the Course of MS

Next, we investigated if there were differences between patients with different disease courses, and also if CIS patients had elevated levels of anti-$PLP_{181-230}$ antibodies. As shown in **Figure 3**, the levels of antibodies were elevated more in patients with RR-MS and SP-MS than in PP-MS, although this was not statistically significance. Most interestingly, approximately half of the CIS patients showed elevated antibody responses to $PLP_{181-230}$, suggesting that these antibodies are present from the early stages of disease. The anti-$PLP_{181-230}$ antibody levels were significantly different in RR-MS patients and CIS patients compared to both healthy individuals and OND patients, whereas the SP-MS patient antibody levels were only significantly different to healthy individuals, and PP-MS patient antibody levels were not significantly different to either the healthy individuals or OND patients.

Patients Carrying Certain HLA Types Are Much More Likely to Produce Elevated Levels of Anti-PLP Antibodies

Previously we have described that certain HLA class II alleles in particular alleles within the HLA-DRB1*04, DRB1*07 or DRB1*13 families, and the DQ8 serotype (which is in strong linkage disequilibrium with HLA-DRB1*04 alleles) correlate strongly with elevated T cell reactivity to peptides from the 184-209 region of PLP (23). The same PLP peptides appear to bind minimally to the MS-related HLA molecule DRB1*15:01 and to DRB1*03, which is also found commonly in Caucasian MS patients. Furthermore, they cannot induce disease in HLA transgenic mice expressing these alleles. However, $PLP_{175-194}$ can induce demyelinating disease in mice transgenic for HLA-DQB1*06:02, which forms the β chain of the DQ6 type that is in strong linkage disequilibrium with DRB1*15:01 (34). We reasoned that patients showing isotype-switched anti-$PLP_{181-230}$ antibodies might be more likely to also be able to make a strong T cell response to peptides in this region of PLP, as T cell help is likely required for the isotype switching to occur.

Levels of anti-$PLP_{181-230}$ antibodies were assessed on the basis of whether patient had HLA genotypes containing DRB1*03, DRB1*04, DRB1*07, DRB1*13, or DRB1*15:01, either in the

TABLE 2 | Relationship between HLA genotype of MS patients and antibody response to $PLP_{181-230}$.

HLA-DRB1 genotype	*n*	Response to $PLP_{181-230}$ median (IQR)¶	*P* vs. "other, other" genotype*
03, 15:01	13	0.027 (0–0.296)	0.608
03, other	13	0.076 (0–0.323)	0.550
		$P_{(03,15:01\ vs.\ 03,other)} = 0.930$	
04, 15:01	14	0.467 (0.050–1.945)	**0.007**
04, other	29	0.181 (0.064–1.467)	**0.013**
		$P_{(04,15:01\ vs.\ 04,other)} = 0.528$	
07, 15:01	12	0.709 (0.109–1.271)	**0.011**
07, other	14	0.640 (0.060-2.834)	**0.020**
		$P_{(07,15:01\ vs.\ 07,other)} = 0.981$	
13, 15:01	11	0.835 (0.104–1.951)	**0.006**
13, other	18	0.752 (0.087–1.480)	**0.009**
		$P_{(13,15:01\ vs.\ 13,other)} = 0.811$	
15:01, 15:01	19	0.884 (0.121–1.224)	**0.006**
15:01, other	18	0.108 (0.025–0.379)	0.281
		$P_{(15:01,15:01\ vs.\ 15:01,other)} = \mathbf{0.026}$	
Other, other	10	0.003 (0–0.243)	–

*"Other" indicates alleles other than DRB1*03, DRB1*04, DRB1*07, DRB1*13 or DRB1*15:01.*

¶*The P-value shown below the median and IQR for each pair of genotypes shows the significance of the comparison between those two groups.*

**Significant P-values are indicated by bold type.*

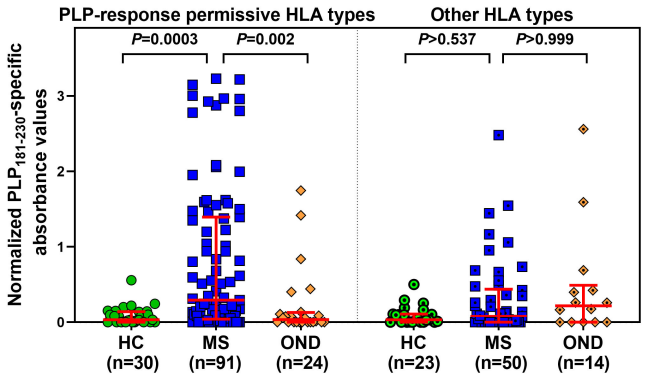

FIGURE 4 | Comparison of anti-$PLP_{181-230}$ antibody reactivity in individuals carrying PLP response-permissive HLA types vs. other HLA types. There were no significant differences between HC, MS, and OND groups in the patients carrying other HLA types.

presence or absence of DRB1*15:01 as the second allele (in order to assess whether the DRB1*15:01 was contributing to the response). Patients who carried none of these alleles are listed as "other, other" genotype. As shown in **Table 2**, the responses of samples from 146 MS patients and 20 CIS patients from whom HLA typing was available showed that patients carrying HLA-DRB1*04, DRB1*07, or DRB1*13 were significantly elevated compared to the "other, other" genotype, and that this was not dependent on the presence of DRB1*15:01, as there were no significant differences between (e.g.,) people with DRB1*04, DRB1*15:01 vs. DRB1*04, other genotype. Interestingly, the only patients who showed a difference between the presence and absence of DRB1*15:01 as the second allele were individuals who were homozygous for DRB1*15:01, who had elevated levels of anti-$PLP_{181-230}$ antibodies compared to those who had DRB1*15:01 and an allele other than DRB1*03, DRB1*04, DRB1*07, DRB1*13, or DRB1*15:01. It is likely that the elevated response in DRB1*15:01 homozygous individuals is due to increased expression of HLA-DQ6 in these patients, as studies in patients with narcolepsy have shown that allelic dosage of DQB1*06:02 is transmitted into significant changes in HLA-DQ6 heterodimer availability (35). Thus, these results show that PLP response permissive HLA types include DRB1*04, DRB1*07, DRB1*13, and homozygosity for DRB1*15:01. When the ELISA data from MS patients and controls shown in **Figure 2A** was assessed on the basis of carriage of these PLP response-permissive HLA types, there was still a highly significant difference between MS patients and controls for the PLP response-permissive HLA types, but not for those individuals who do not carry these HLA types (**Figure 4**). Most of the MS patients who did not carry the typical PLP response-permissive HLA types, but who showed higher levels of reactivity to PLP, carried DRB1*11 alleles. DRB1*11 is usually in linkage disequilibrium with DQ7 (DQB1*03), which is also expressed by many patients who carry DR4 or DR13 alleles. It may be that in some MS patients, the HLA restriction is actually through the HLA-DQ rather than HLA-DR molecules. It is notable that 2 OND patients who had high levels of anti-PLP antibodies but who did not carry typical PLP response-permissive HLA types both carried DRB1*10 alleles (which occur very infrequently in MS patients – only one MS patient in this study carried DRB1*10). Therefore, certain DQ and DRB1*10 alleles may represent additional PLP response-permissive HLA molecules, but that remains to be proven.

Levels of Antibody Responses to $PLP_{181-230}$ Correlate With Disease Severity in RR-MS and SP-MS Patients Carrying PLP Response-Permissive HLA Types

The levels of anti-PLP antibodies were also correlated with the MS Severity Score (MSSS) (36), which uses disability and disease duration to rate disease severity. When all patients were included in the analysis, there was no significant correlation between the levels of anti-PLP antibodies and the MSSS (**Figure 5A**). However, as shown above, certain HLA types correlate with elevated levels of anti-$PLP_{181-230}$ antibodies. We therefore also analyzed the correlation between anti-PLP antibody levels in patients carrying the PLP response-permissive HLA types and the MSSS: in this group there was a significant correlation between the level of anti-PLP antibody and the MSSS ($P = 0.04$; **Figure 5B**). Interestingly, the majority of patients with low levels of anti-PLP antibody but high MSSS in this group had PP-MS (indicated by the red triangles in **Figure 5B**). When only RR-MS and SP-MS patients were included in the analysis, the correlation was much stronger ($P < 0.0001$; **Figure 5C**). The ρ value is relatively low (0.46); however, this may reflect differences in the disease state at the time of testing, with patients who were tested while in remission typically showing lower levels of antibody than those tested during an attack of MS. This data demonstrates that, in RR-MS and SP-MS patients who carry HLA types that allow autoreactivity to PLP to occur, anti-PLP antibodies show a correlation with disease severity. Furthermore, the finding that anti-PLP antibodies do not correlate with disease severity in patients with PP-MS would support the idea that the disease process in PP-MS is different from that seen in RR-MS/SP-MS.

DISCUSSION

In this paper, we have demonstrated that isotype-switched antibodies targeting $PLP_{181-230}$ are significantly elevated in the serum of patients with RR-MS and SP-MS, particularly in those carrying PLP response-permissive HLA types, and that there is a positive correlation between the levels of antibody and disease severity in these patients.

The use of the $PLP_{181-230}$ peptide, synthesized so as to fold with 2 disulphide bonds as occurs in PLP expressed at the oligodendrocyte cell membrane, significantly improved the reproducibility of the anti-PLP antibody results, compared to assays done using whole human PLP. A rabbit serum, raised against this 50-mer peptide, has been shown to be able to live label rat oligodendrocytes (37), in a pattern reminiscent of that seen when cells were labeled with the O10 monoclonal antibody, which recognizes a conformation-dependent epitope of the second extracellular loop of PLP (38). The PLP amino

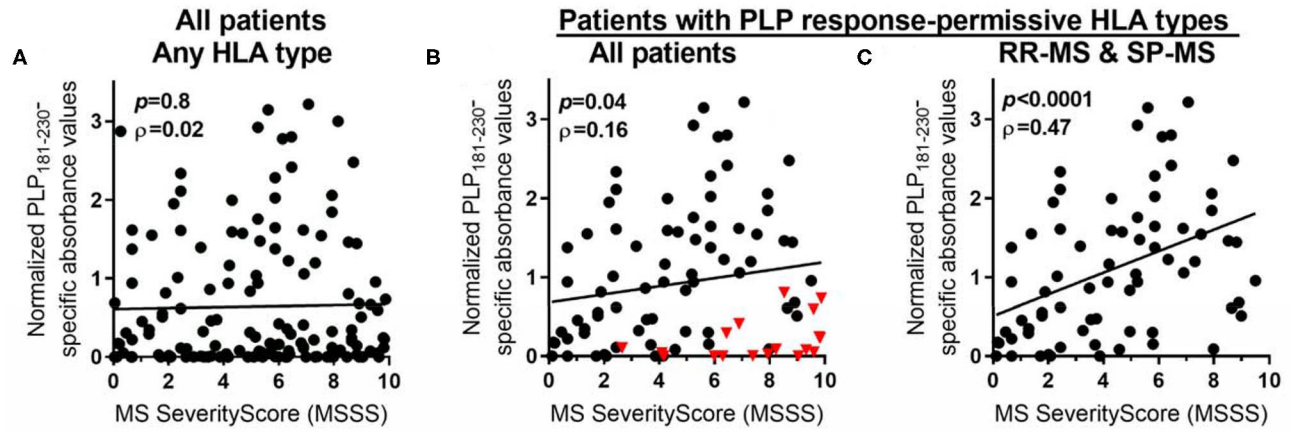

FIGURE 5 | Spearman rank correlation between levels of anti-PLP antibodies (as determined in **Figure 1**) and MSSS in: **(A)** all MS patients, irrespective of their HLA type; **(B)** all MS patients who carry PLP response-permissive HLA types (red triangles indicate PP-MS patients); and **(C)** RR-MS and SP-MS patients who carry PLP response-permissive HLA types. The *P*-value for each correlation and the Spearman ρ (rho) value are shown in each graph.

acid sequence is identical between human, mouse and rats, and therefore the fact that antiserum raised against the $PLP_{181-230}$ epitope could recognize rat oligodendrocytes suggested that antibodies of this specificity could also recognize human PLP. We have now shown in this paper than CHO cells, transfected to express human PLP, can be live labeled with antibodies that show elevated levels of reactivity to $PLP_{181-230}$ in the ELISA assays, suggesting that the antibodies of this specificity could be of potential functional relevance in humans.

Importantly for the synthesis of the $PLP_{181-230}$ peptide for testing of human antibody responses, this region of PLP contains two residues that differ between the human and bovine sequences, namely at residues 188 and 198. Many early studies on immune reactivity to PLP used bovine PLP as the antigen, and results from those studies were not particularly reproducible (33). However, it is not unexpected that difficulties arise when using whole PLP as an antigen in an ELISA or in other immunological assays, as, owing to its hydrophobicity and intolerance for solutions containing salt, the whole PLP molecule is not a suitable molecule for most of these assays. We have previously shown that there are elevated levels of antibodies against $PLP_{184-199}$ and $PLP_{190-209}$ in patients with MS compared to healthy individuals and OND patients (23), although the levels of antibodies were generally lower than those seen using the $PLP_{181-230}$ peptide. We suggest that this is due to the improved ability to detect antibodies against conformationally relevant epitopes of the second extracellular loop when using the $PLP_{181-230}$ peptide.

The finding that most MS patients had isotype-switched anti-$PLP_{181-230}$ antibodies, whereas those healthy individuals or OND patients with detectable levels of anti-$PLP_{181-230}$ antibodies generally had antibodies of the IgM isotype, suggests the presence of a PLP-driven immune process occurring in the MS patients. Testing of CSF for the presence of PLP-specific antibodies was not done in the current study, but will be a focus of future investigations.

In previous work, we have identified that ∼40–50% of MS patients can show elevated T cell proliferative responses to various epitopes of PLP, but that the response is directed against the second extracellular loop of PLP in most patients (23, 39, 40). Similarly in experimental animals, overlapping epitopes within the second extracellular loop of PLP form a cluster of immunogenic and encephalitogenic peptides for mice from many genetic backgrounds (41). We would therefore suggest that this second extracellular loop of PLP is the most likely target of disease-relevant autoreactivity in MS.

It is of interest to note that nearly half of the patients with a CIS suggestive of MS had elevated levels of anti-$PLP_{181-230}$ antibodies. There may therefore be some predictive power in studying these antibodies in early MS. However, as shown in this paper, the HLA type of the patients (and therefore the potential to develop strong T cell responses to PLP) also plays a role in whether or not antibodies develop. Previously we have shown that in a patient who carries a PLP response-permissive HLA type, highly increased numbers of PLP-specific T cells could be detected in both the blood and the cerebrospinal fluid (CSF) right from the earliest stage of disease (23). Therefore, it is likely that any predictive modeling of disease severity from early timepoints, based on the presence of anti-PLP antibodies, would need to take into account the HLA type of the patients.

There are many ways in which anti-PLP antibodies, particularly those targeting epitopes on the extracellular surfaces of oligodendrocytes or myelin, could potentially have an impact in MS, including mechanisms such as complement-mediated lysis, antibody mediated cell cytotoxicity, modulation of cell architecture, opsonization of myelin or myelin debris leading to increased activation of phagocytic cells (19). In a C3H/HeJ mouse model in which demyelinating disease can be induced by immunization with $PLP_{190-209}$, we have previously reported that mice that can make a T cell response, but not an antibody response, develop lesions in the brainstem, but not in the cerebellum, whereas in the presence of both T cells and antibodies

specific for $PLP_{190-209}$ there is development of lesions in both the brainstem and the cerebellum (23, 25). This suggests either that the anti-PLP antibodies can shift the sites of lesions, or alternatively that there is more severe disease when both the T cells and antibodies are present.

A recent study using several monoclonal antibodies specific for the first and second extracellular domains of PLP also suggests that antibodies against these regions of PLP might be able to cause damage to neurons (28). This study showed that the antibodies, specific for either PLP_{50-69} or $PLP_{178-191}$, could bind to cell surface proteins on neurons in human brain, and that, *in vitro*, the antibodies could inhibit neuronal differentiation and outgrowth of neurites. Preliminary findings suggested that the cross-reactivity between the anti-PLP antibodies and the neurons could be via the M6 proteins. M6a and M6b are glycoproteins belonging to the same gene family as PLP. They are involved in neuronal and axonal guidance, in an integrin-dependent fashion (42, 43). M6a is only expressed on neurons, but some M6b isoforms are also expressed by oligodendrocytes (44). The $PLP_{181-230}$ region has 50% sequence similarity to that of M6a and 72% similarity to M6b. Therefore, there is the potential that anti-$PLP_{181-230}$ specific antibodies produced by MS patients could bind to neurons and cause damage to them. Such effects could contribute to the apparent correlation between disease severity and the levels of anti-$PLP_{181-230}$ specific antibodies, as disease severity is, in large part, caused by underlying irreversible damage to neurons. Interestingly, PP-MS is usually thought to involve a greater degree of irreversible axonal damage and brain atrophy than are other forms of MS. However, we did not find any relationship between severity of PP-MS and levels of anti-$PLP_{181-230}$ antibodies. We have previously reported that patients with PP-MS carry a different complement of HLA alleles to those found in patients with RR-MS or SP-MS, in particular they are more likely to carry alleles with a negatively charged glutamic acid residue in pocket 4 of the antigen-binding site of the HLA-DR molecules (45). In addition, PP-MS patients show significantly lower T cell proliferation in response to $PLP_{184-199}$ or $PLP_{190-209}$ peptides compared to RR-MS and SP-MS patients (39). It is likely that these differences in the HLA molecules carried by PP-MS patients affects their ability to make effective antibody responses to $PLP_{181-230}$.

Overall, the results of this study suggest that anti-$PLP_{181-230}$ antibodies have potentially important roles to play in the pathogenesis of MS, particularly in patients with RR-MS and SP-MS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Royal Brisbane and Women's Hospital Human Research Ethics Committee and The University of Queensland Medical Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JG and MP contributed to conception and design of the study. JG extracted human PLP, performed all antibody experiments, and wrote the first draft of the manuscript. ET designed and synthesized the PLP peptide. MP recruited patients for the study and did clinical assessments. All authors contributed to manuscript revision, and read and approved the submitted version.

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