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Investigating mechanisms underlying the development of paralysis symptom in a model of MS

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Abstract

Multiple sclerosis (MS) is an autoimmune neurodegenerative disorder with approximately 80 % of patients suffering from pain and 50 % from paralysis. Using a rodent model for MS, experimental autoimmune encephalomyelitis (EAE), researchers have predominately investigated paralysis/motor disease as the clinical symptom of EAE with fewer studying MS/EAE pain. However, in EAE, all mice exhibit a pain like phenotype and only a subset progresses to paralysis. Despite extensive research characterizing the disease pathology, the etiology that contributes to the range of pain and motor symptom occurrence in MS remains understudied. This is the first study to dissect MS symptom pathophysiology, using the non-PTX EAE model, in mice that experience mechanical hypersensitivity (pain-like phenotype) with and without paralysis. We found that mechanical hypersensitivity experienced by mice with or without paralysis is comparable between the two groups, irrespective of sex. In addition, there is a significant increase in the activation and infiltration of immune cells, demyelination, and heightened protein expression of B cell chemoattractant CXCL13 within the spinal cord of mice exhibiting mechanical hypersensitivity and paralysis, compared to mice only experiencing mechanical hypersensitivity.

Keywords

Multiple sclerosis (MS); Experimental autoimmune encephalomyelitis (EAE); Mechanical hypersensitivity; Paralysis; Demyelination; Inflammation; Leukocytes

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CRediT authorship contribution statement

Gupta Shruti: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Arnab Sreejita:** Methodology, Investigation. **Silver-Beck Noah:** Methodology, Investigation. **Nguyen Kayla L.:** Writing – review & editing, Visualization, Formal analysis. **Bethea John R.:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2025.111275.

1. Introduction

Multiple sclerosis (MS) affects 2.3 million people globally with approximately 3 times more women diagnosed than men (Voskuhl et al., 2020; Harbo et al., 2013). To study MS, experimental autoimmune encephalomyelitis (EAE) is a commonly used mouse model sharing key pathologies with MS such as demyelination, neuroinflammation, and axonal damage (Glatigny and Bettelli, 2018; Gold and Wolinsky, 2011; Constantinescu et al., 2011; Robinson et al., 2014; Gajofatto and Benedetti, 2015; Procaccini et al., 2015).

The two most prominent symptoms of MS are chronic neuropathic pain (CNP) and paralysis. Approximately 50–86 % of patients suffer from CNP and 36–85 % suffer from motor impairments/paralysis (Krementsov et al., 2014; Gustavsen et al., 2021; Murphy et al., 2017, 2020; Truini et al., 2013; O'Connor et al., 2008; Olechowski et al., 2009; Khan and Smith, 2014; Duffy et al., 2018). Patients also experience a range of other debilitating symptoms which include vision impairment, bladder and bowel dysfunction, cognitive deficits, and fatigue (Tian et al., 2013; Doshi and Chataway, 2016; Constantinescu et al., 2011; Rolak, 2003; Rommer et al., 2019; Zhang et al., 2021). These symptoms can manifest either in isolation or in combination with one another (Huang et al., 2017; Rolak, 2003; Rommer et al., 2019; Zhang et al., 2021).

Symptom variation in MS is not only a noteworthy finding but also presents a significant challenge in the field. Owing to the symptom variability, accurate MS diagnosis relies heavily on MRI. Moreover, no treatments can address the full spectrum of MS symptoms (Blumenthal, 2006; Wildner et al., 2020). Unfortunately, still the underlying etiology between different symptom distribution in MS remains understudied while focus on disease pathophysiology (Dobson and Giovannoni, 2019; Yamout and Alroughani, 2018; Constantinescu et al., 2011) and paralysis as the main MS/EAE symptom (Bittner et al., 2014; Dal Canto et al., 1995; Giralt et al., 2018), remains the focus of most studies.

Our research represents a pioneering effort to compare the pathophysiology associated with the two most prevalent symptoms of MS, paralysis and pain, using the non-PTX EAE model. We studied the pathological changes occurring in the central nervous system (CNS) of EAE mice that experienced paralysis and mechanical hypersensitivity and compared the findings to the pathology observed in the CNS of the same EAE cohort, who only experienced mechanical hypersensitivity. Our results indicate that increased immune cell infiltration in the CNS leads to increased CNS damage and the emergence of new EAE symptoms.

2. Materials and methods

2.1. Mice

8–10 weeks old male and female wild-type C57BL/6 J (#000664) mice were purchased from the Jackson Laboratories and were housed in a 12-h light/dark cycle and given access to food and water ad libitum. Mice were acclimated to the animal facility for one week prior to the beginning of all experiments. All studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

2.2. nPTX EAE induction

Mice were injected with a subcutaneous immunization containing 100 μg of MOG35–55 peptide emulsified with complete Freund's adjuvant (CFA; InvivoGen, San Diego, California) and supplemented with 200 μg of heat-inactivated Mycobacterium tuberculosis H37Ra (9, 12). Injections were subcutaneously administered in the posterior right and left flanks at a volume of 50 μL each. A booster containing the same emulsion was administered 1 week following the first injection.

2.3. Behavioral testing

2.3.1. Mechanical sensitivity test—von Frey testing was used to determine mechanical hypersensitivity as an indicator of a pain-like phenotype in mice. Mice were placed in individual plexiglass chambers elevated on a mesh-wire platform and allowed to acclimate for 40–60 min prior to testing with the experimenter blinded to the groups. Using the up-down method (Chaplan et al., 1994), plantar surface of the hind paw was stimulated with von Frey filaments (Touch-test sensory evaluator) of varying diameters (force range:.02g-2g). This was performed for a series of 5 trials following the first positive response to the filament. To identify a positive response, the physical response to the stimulus (such as toe splaying and hind paw withdrawal) needed to be accompanied with a cognitive display of pain awareness (such as looking at the hind paw, hind paw licking, avoidance behavior and/or tucking the tail under hind paw etc., (Murphy et al., 2020)). Both left and right hind paws were averaged per mouse for each timepoint. The baseline withdrawal threshold of the mice was evaluated once, one week before EAE immunizations. Subsequent testing was done weekly until experiment termination. EAE symptoms manifests as ascending paralysis characterized predominately by unilateral hindlimb paralysis. Despite the onset of partial paralysis in mice, a noticeable level of reactivity to stimuli persists while evaluating mechanical hypersensitivity. Consequently, the criteria for identifying a positive response in paralysis mice remained unchanged.

2.3.2. Motor scoring—Mice were evaluated daily to measure motor function using an open-field testing paradigm. A score from 0 – 6 was assigned to each mouse based on the following criteria: 0 – no paralysis, 1 – 50 % loss of tail tone, 2 – fully flaccid tail, 3 – bilateral hind-limb paralysis, 4 – forelimb paralysis, 5 – moribund, 6 – death (Murphy et al., 2020). Groups with a motor score of ≥ 1 are labeled as "paralysis" and groups with a score of 0 are labeled as "non-paralysis".

2.4. Tissue collection

2.4.1. Whole tissue protein extraction—At 30 days post-immunization (DPI), mice were deeply anesthetized with Ketamine (215 mg/kg) and Xylazine (43 mg/kg) and subsequently perfused with 1x PBS. Cortex and lumbar spinal cord were dissected and immediately stored on dry ice. Tissue was homogenized in RIPA buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 1 % NP-40, 0.1 % Na Deoxycholate, 0.1 % SDS, 140 mM NaCl) supplemented with protease (Santa Cruz) and phosphatase inhibitors (Biovision) and incubated at 4 degrees for one hour. The samples were centrifuged for 15 mins at

14,000RPM and the supernatant was transferred into separate tubes from which protein concentrations were determined using the DC protein assay (Biorad).

2.5. Western blot

Protein extracts were run on 8–15 % sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Turbo blot, Biorad). Membranes were blocked for 2 h in 5 % non-fat milk (BioRad) in 1x TBS-T. Primary antibodies (supplementary material, table 1) were diluted in blocking solution and incubated at 4 °C overnight. All primary antibodies were followed by incubation with horseradish peroxidase-conjugated species-specific secondary antibodies along with B-actin (hFAB Rhodamine anti-Actin, BioRad 12,004,164). Proteins were visualized with a chemiluminescent substrate (West Pico, ThermoFischer Scientific) and band intensity was quantified using FIJI software (Rasband W, NIH). Proteins were normalized against B-actin.

2.6. Immunohistochemistry

Mice were transcardially perfused at 30 DPI with 4 % paraformaldehyde (Sigma-Aldrich) in 1x PBS pH 7.4. Dissected brains and lumbar spinal cord were post-fixed in 4 % PFA overnight and subsequently dehydrated in 25 % sucrose for at least 48 h at 4 °C. Tissue were embedded in O.C.T. (Optimal cutting temperature, Tissue-Tek®) and stored at −80 °C until sectioning. Brains and spinal cord were cryo-sectioned at 20 μm and were collected on super frost plus charged slides (Fisherbrand). Prior to staining, slides were acclimated to room temperature for 10 mins and placed in ice cold acetone for 3 mins followed by ice cold methanol for 7 mins. Sections were incubated in 0.1 % Triton-x100 in diH2O for 10 mins at RT. Sections were then blocked in 1 % BSA/10 % normal goat serum/0.3 % Triton-x100 in 1x PBS for 1 h at RT. Sections were then probed for the following primary antibodies: myelin basic protein (Rat anti-MBP, 1:100, Millipore), Iba1 (Rabbit anti-Iba1, 1:250, Abcam), and NF200 (mouse anti-N0142, Sigma, 1:100) diluted in blocking buffer and incubated overnight at 4 °C. Sections were then washed 3 times for 10 min in 1x PBS. Host-species specific secondary antibodies (Invitrogen, goat Alexa 488) were added at 1:500 in 1 % NGS/PBS for 1 h at RT. Three washes for 10 min in 1x PBS were repeated and Hoechst diluted at 1:10,000 was added for 10 mins at RT. Slides were washed and mounted with fluoromount and coverslips (Electron Microscopy, Fluoro-Gel with TES Buffer). Images were acquired using Leica LSM700 confocal microscope at a magnification 20x and were analyzed with FIJI.

2.7. Flow cytometry

At 30 DPI, mice were perfused with ice cold 1x PBS. Spleen and spinal cord were dissected and stored in ice cold RPMI buffer (Thermofisher, 11,875,093) and 1x HBSS (Thermofisher, 14,175,095) respectively.

2.7.1. Spleens—Cells were dissociated through a 40 μm cell strainer and collected in 10 ml of RPMI buffer. Splenocytes were harvested by centrifugation (300 g, 5 min) and washed once with 10 ml MACS buffer and were allowed to incubate for 60 min with fluorescence-labeled antibodies against surface antigens (supplementary material, table 2) at 1:100 concentration. Activation status was marked by MHCII+ signal. Cells were then

washed with MACS buffer by centrifugation (300 g, 5 min) and incubated for 30 min in fixation buffer (Miltenyi Biotech, Bergisch-Gladbach). Cells were washed again (300 g, 5 min) and collected in MACS buffer.

2.7.2. Lumbar spinal cords—Cells were dissociated through a 70 μm cell strainer and collected in 10 ml MACS buffer (PBS, 0.5 % BSA, 2 mM EDTA). Cells were harvested by centrifugation (300 g, 10 min) and incubated with Myelin Removal Beads II (Miltenyi Biotech) for 15 min at 4 °C. The cell/bead mixture was then washed with MACS buffer by centrifugation (300 g, 10 min) and myelin was removed by magnetic separation via LD columns (Miltenyi Biotech). Eluted cells were washed once in MACS buffer and surface antigens were stained using fluorescence-labeled antibodies at 1:100 concentration for 60 min. Microglia were identified as $CD45^{Low}/CD11b^{+}$ whereas macrophages were identified as $CD45^{High}/CD11b^{+}$. Cells were then washed with MACS buffer by centrifugation (300 g, 5 min) and incubated for 30 min in fixation buffer (Miltenyi Biotech, Bergisch-Gladbach). Cells were washed again (300 g, 5 min) and collected in MACS buffer. Single stained compensation beads were used for compensation control. Data were acquired using BD Fortessa (BD Biosciences, San Jose, CA) and analyzed with FlowJo (TreeStar, Ashland, OR).

2.8. Statistics

Data are presented as mean ± standard error of the mean (SEM) of *n* animals. All data analyses were performed using GraphPad Prism software (versions 7.0.0–9.4.1). Statistical analyses were performed with either student's T-test or one-way-analysis of variance (ANOVA), with Tukey's test to adjust for multiple comparisons among more than two groups. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. EAE immunization induces comparable mechanical hypersensitivity across all mice, while motor impairment is experienced by only a subset of immunized mice, irrespective of sex

EAE is used to investigate neuropathic pain in MS due to its ability to recapitulate pain phenotypes such as mechanical hypersensitivity, thermal hyperalgesia etc (Aicher et al., 2004; Svendsen et al., 2005; Maguire et al., 2021; Grace et al., 2017). In our EAE immunized cohort of 70 animals (35 males and 35 females), all 70 experienced mechanical hypersensitivity (Fig. 1 A, B). Previous studies from our lab have demonstrated after EAE immunization, mechanical hypersensitivity developed after 8 DPI is due to EAE rather than transient effects of CFA (Murphy et al., 2020). Withdrawal thresholds were significantly reduced in all mice by > 50 % starting at 8 DPI. However, by DPI 30 only 35 % of the cohort developed paralysis (12 out of 35 in each sex, Fig. 1 D, E) and presence of paralysis had no effect on mechanical hypersensitivity experienced by EAE males and females (Fig. 1 G, H). In addition, the onset and progression of both the EAE symptoms were independent of sex (Fig. 1 C, F).

3.2. Demyelination, microglial/macrophage activation, and axonal damage is more prevalent in EAE mice with paralysis

Demyelination and neuroinflammation are two common hallmarks of disease shared by both MS and EAE (Constantinescu et al., 2011; Robinson et al., 2014; Khan and Smith, 2014; Compston and Coles, 2008; Murphy et al., 2020). We investigated if the differential onset of the paralysis symptom in the EAE cohort was dependent upon the neuroinflammatory stress induced in the CNS after EAE immunization. Since there were no sex differences in the onset and the severity of mechanical hypersensitivity and paralysis in our EAE paradigm, we combined males and females for all our future analysis. Lumbar region of the spinal cord (Fig. 2) and cortex (supplementary figure 1) were isolated at DPI 30 from our EAE mice along with age matched naïve animals and were probed for myelin associated proteins (MBP, CNPase), microglial/macrophage activation (IBA1), and axonal damage (NF200, SMI312). In the spinal cord, CNPase protein expression was significantly reduced in the paralysis group compared to naïve and the non-paralysis group (Fig. 2A). In support of this data, the intensity of the MBP staining of the lumbar spinal cord was also reduced in the paralysis group as compared to the non-paralysis and the naive group (Fig. 2B). In cortex, we used the LUT filter to analyze the myelin expression in the corpus callosum (CC) region by assessing its thickness using MBP histological stain. The thickness of CC region was reduced in the paralysis group as compared to the non-paralysis and the naïve group (supplemental figure 1 A).

Macrophages/microglia are known to increase the production of proinflammatory mediates such as TNF in response to demyelination (Kempuraj et al., 2016). To determine if there is a marked increase in the microglial/macrophage activity, as an indicative of neuroinflammation, we analyzed for Iba1 protein expression. In the spinal cord and the corpus callosum, Iba1 protein expression was significantly elevated in the paralysis group as compared to the naïve and EAE non-paralysis group mice (Fig. 2C and supplemental figure 1B). Representative histological stain of Iba1 in the spinal cord and in the CC also show a marked increase in its expression (Fig. 2D and supplemental figure 1 C).

Demyelinated axons are succumbed to the pro-inflammatory environment in MS/EAE which leads to axonal damage and eventually neuronal death (Lee et al., 2014; Arnon and Aharoni, 2009). To investigate axonal damage, we analyzed the expression of SMI312 and NF200. SMI312 expression was significantly reduced in the spinal cord of EAE paralysis group as compared to the non-paralysis and the naïve group (Fig. 2E) and a trend in its decreased expression was observed in the cortex as well (supplementary figure 1). Histological stain of NF200 in the lumbar spinal cord displays increased axonal fragmentation in the paralysis group as compared to the naïve and the non-paralysis group (Fig. 2F).

3.3. There is increased CNS infiltration of peripheral immune cells in motor diseased EAE mice

Infiltration of peripheral immune cells into the CNS is known to contribute to the deleterious effects of EAE induction (Rezai-Zadeh et al., 2009; Rossi and Constantin, 2016). To investigate the potential of infiltrating immune cells for differential onset of the paralysis symptom in EAE mice, we analyzed both spleen (for peripheral analysis) and spinal cord

tissue (for CNS analysis) via flow cytometry. As EAE is an ascending paralysis disorder, we anticipated discernible alterations within the spinal cord at DPI 30. There were no differences in total leukocyte (CD45 +) presentation in the naïve, non-paralysis and the paralysis groups in both spleen and SC (Fig. 3A). There was no difference in the percentage of CD45 +CD4 + helper T cells between the three groups in the spleen, however, their percentage significantly increased in the spinal cord of the paralysis group as compared to the non-paralysis and the naive group (Fig. 3B). There was a significant decrease in the CD45 +CD8 + cytotoxic T cells in the spleen of the paralysis and the non-paralysis group as compared to the naïve but their percentages were not different between the three groups in the spinal cord (Fig. 3C). There was no difference in the CD45 +B220 + B cell percentages between the three groups in both spleen and spinal cord (Fig. 3D) but activated CD45 +B220 +MHCII+ B cell were significantly increased in the spleens of both the EAE groups as compared to the naïve group and there was a significant increase in their percentage in the spinal cords of the EAE paralysis group as compared to the non-paralysis group (Fig. 3E). There was no difference in the CD45highCD11b+ percentage of macrophage population between the EAE paralysis and the non-paralysis group in both spleen and the spinal cord (Fig. 3F). However, there was a significant increase in the percentage of CD45highCD11b+MHCII+ activated macrophages in the spinal cord of the paralysis group as compared to the non-paralysis group (Fig. 3G). Similarly, there was no difference in the CD45lowCD11b+ microglia of the spinal cord between the paralysis and the non-paralysis group (Fig. 3H) but a significant increase in the CD45lowCD11b+MHCII activated microglia in the paralysis group was seen as compared to the non-paralysis group (Fig. 3I).

3.4. EAE paralysis mice have increased B-cell recruiting chemokine expression

Due to emerging evidence on the importance of B-cells in MS pathogenesis and the therapeutic promise demonstrated by anti-CD20 B-cell depleting antibodies in MS and EAE, we wanted to explore the potential mechanism for our observed increase of spinal B-cell infiltration (Jasmin Ochs et al., 2022; Weber et al., 2010; Monson et al., 2011; Alcalá et al., 2018). At DPI 30, spinal cord tissue from the EAE paralysis and the non-paralysis mice along with age matched naïve mice was isolated and prepared for western blotting. We quantified the mRNA and protein expression of CXCL13 (B cell chemoattractant) in the spinal cord and found that its expression was significantly greater in the EAE paralysis group compared to non-paralysis (Figs. 4A, B).

We next investigated the major cytokines secreted by CD4 + T cells, activated macrophages and microglia, as their infiltration was increased in the spinal cord of the paralysis mice as compared to the non-paralysism mice. At DPI 30, we saw no change in the protein expression of TNF, IL1B, and IL-6 in the spinal cord between the paralysis and the non-paralysis group (Fig. 4 C-E respectively).

4. Discussion

Despite the longstanding use of EAE to understand MS pathology, research has yet to investigate the distinct presentation of major symptoms in MS. Investigating differential

symptom presentation could provide valuable insights regarding the underlying pathology of the diverse clinical features seen in MS and allow for future development of targeted therapeutics with enhanced efficacy.

We investigated the differences between the pathophysiology of mechanical hypersensitivity and motor disease in EAE and found a significant increase in CNS demyelination, neuroinflammation, and axonal damage among mice experiencing paralysis and mechanical hypersensitivity, versus those with only mechanical hypersensitivity. This difference may be attributed, at least in part, to the increased infiltration of activated immune cells within the CNS of paralysis mice. Activated immune cells secrete inflammatory cytokines and chemokines which increase demyelination and neuroinflammation in EAE (Rostami and Ciric, 2013; Yao et al., 2014; Kaskow and Baecher-Allan, 2018; Onishi and Gaffen, 2010; Steinman, 2007).

4.1. There is equivalent mechanical hypersensitivity in EAE mice irrespective of motor deficits

Historically we have seen a range between 35 % and 60 % for the onset of motor deficits in the EAE mice (Murphy et al., 2020), which mirrors distribution of motor deficits among MS patients (Gustavsen et al., 2021). Demyelinating lesions disrupt action potential propagation and expose axons to a cytotoxic neuroinflammatory environment exacerbating damage and subsequent neuronal loss (Lee et al., 2014; Arnon and Aharoni, 2009), damage to the axons/neurons lead to neuropathic pain (Finnerup et al., 2021). Even though there was more extensive neural damage in the paralysis group as compared to the non-paralysis group, both groups experienced mechanical hypersensitivity at a similar intensity. Therefore, 40 %-65 % of the non-paralysis mice can still be utilized for understanding the pathophysiology of pain in MS.

4.2. Increased immune cell infiltration is observed in the CNS of EAE mice with motor deficits and mechanical hypersensitivity

Previous research has shown that in EAE, immune cells activated and circulating in the periphery migrate into the CNS. Furthermore, upon entering the CNS, these cells are reactivated by resident or infiltrating MHC-II peptide presenting cells (Furtado et al., 2008; Rossi and Constantin, 2016). These activated immune cells release neuroinflammatory cytokines and chemokines that further exacerbate inflammation and demyelination (Hemmer et al., 2002; Frohman et al., 2006; Inglese, 2006; O'Connor et al., 2001; Garg and Smith, 2015). At DPI 30, mice experiencing both sensory and motor deficits, had increased T helper cell, activated macrophages and microglia, and activated B-cell infiltration in their CNS. We also found a significant increase in CXCL13 protein expression in the spinal cord of the mice with paralysis

4.3. Implications of proinflammatory cytokines in EAE motor disease pathology

In MS and in EAE T helper cells undergo differentiation into Th1 and Th17 cell types which secrete TNF, and IL-6 (Lubetzki and Stankoff, 2014; Kaskow and Baecher-Allan, 2018). Additionally activated macrophages secrete IL-1b which exacerbates CNS pathology in MS/EAE (Chu et al., 2018; Jagessar et al., 2012). Despite the observed increase in CD4

+ T helper cells and activated macrophages within the spinal cord of the paralysis mice, we did not observe elevated levels of the aforementioned cytokines. This could be majorly attributed to the analysis being done at a chronic time point instead of acute. IL-1b, IL-6, and TNF are known to recruit immune cells to the CNS in earlier stages which leads to demyelination and neuroinflammation (Kaskow and Baecher-Allan, 2018; Chu et al., 2018).

4.4. Infiltrated activated B Cells can be crucial for paralysis symptom in EAE

Recently, anti-CD20 antibodies, that deplete B-cells, have gained attention as a potential therapeutic for MS (Jasmin Ochs et al., 2022; Weber et al., 2010; Monson et al., 2011; Alcalá et al., 2018). B-cells differentiate into plasma cells and produce myelin-specific antibodies, contributing to demyelination (Racke, 2008; Franciotta et al., 2008). Demyelination, in turn, further activates macrophages and microglia, leading to increased secretion of neuroinflammatory cytokines and reactive nitrogen species, which weakens the blood-brain barrier (Kempuraj et al., 2016; Chu et al., 2018; Benveniste, 1997; Jiang et al., 2014; Kaskow and Baecher-Allan, 2018; Zhu et al., 2010; Kunkl et al., 2020). This allows more T cells, macrophages, and even B cells to enter the CNS.

We found a significant increase in CXCL13 protein expression in the CNS of mice with paralysis and mechanical hypersensitivity as compared to EAE mice with mechanical hypersensitivity. CXCL13 is a known B-cell chemoattractant shown to drive B-cell lymphotoxin expression creating a positive feedback loop further generating CXCL13 (Rubio et al., 2020).

Increase in CNS CXCL13 will attract more B-cell infiltrates which will differentiate into plasma cells and will eventually heighten the immune cell burden. Our studies indicate the importance of activated B cell infiltration which could contribute to EAE paralysis. To isolate the role of infiltrated and activated B cell in the CNS specifically contributing to the paralysis symptom, studies with time point where paralysis onsets (DPI 17–20), need to be conducted. Moreover, anti-CD20 antibodies can be employed to understand its role in paralysis symptom contribution.

5. Conclusion

Even though MS symptoms are reported to varying extents, the physiology underlying the distinct presentation of multiple symptoms individually or in coexistence remains unknown. The variability in clinical features of MS makes diagnosis and disease management incredibly challenging forcing most doctors to rely on MRI scans for accurate diagnosis (Blumenthal, 2006; Wildner et al., 2020). In addition, there are no disease-modifying therapies in place that can address the multitude of symptoms experienced by MS patients. EAE has been used for a long time to understand the pathology behind MS, and to our best knowledge, we are the first one to compare the two most prevalent symptoms in EAE, pain and paralysis. We are also the first one to report the differential occurrence of pain and paralysis symptoms in the same set of EAE mice. Our findings suggest that as the disease burden increases, marked by heightened immune cell infiltration in the CNS, distinct EAE symptoms can emerge. Our study here opens the gateway for understanding the pathology of various MS symptoms which could be crucial for developing targeted therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability

Data will be made available on request.

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Further reading

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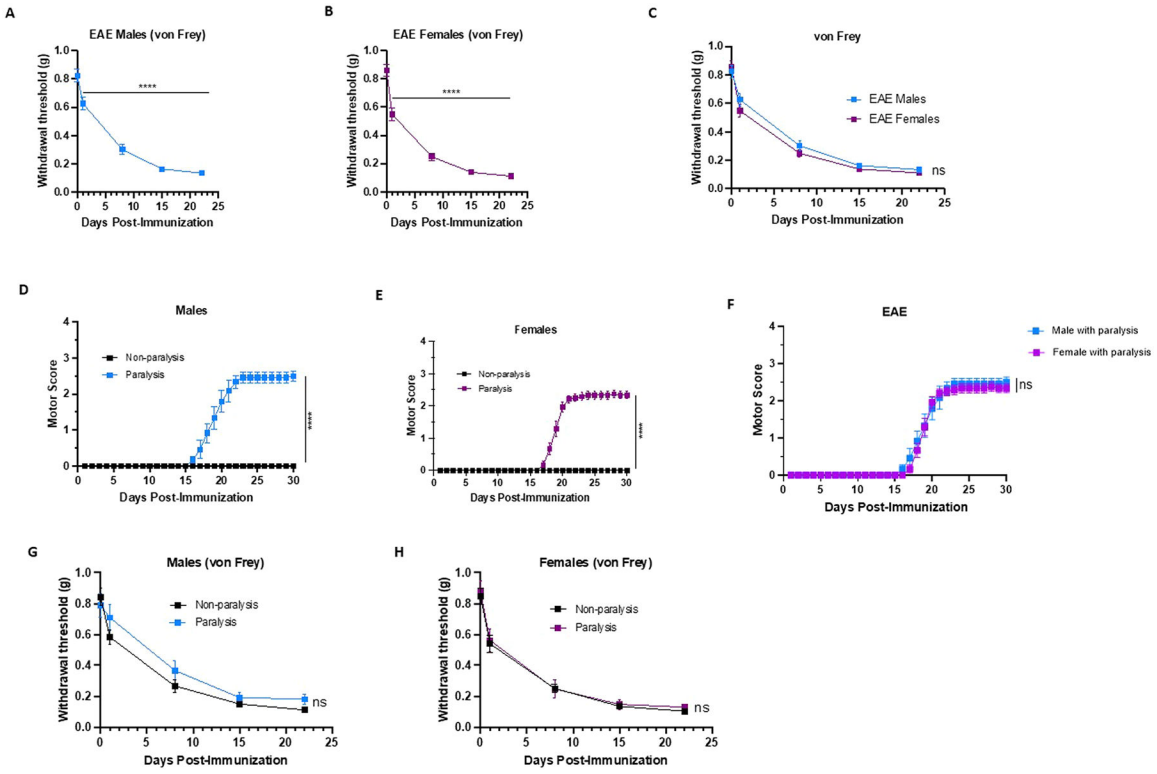

Fig. 1.

All EAE-immunized mice exhibit hypersensitivity irrespective of sex, with only a subset also experiencing sex-independent paralysis. Mice were immunized with MOG emulsified in CFA at DPI 0 and mechanical allodynia was evaluated weekly using Von Frey test over a period of 25 days. (A, B) 100 % of the immunized mice developed mechanical hypersensitivity irrespective of sex (n = 35; each sex). (C) There was no difference in withdrawal threshold between sexes after EAE immunization. (D, E) Clinical scores were evaluated daily to assess paralysis until DPI 30 and only 35 % of the EAE immunized mice developed paralysis in both sexes (n = 12 EAE paralysis, n = 23 EAE non paralysis; in both sexes). (F) The severity of paralysis was similar between male and female EAE mice (n = 12; each sex). No disparity in withdrawal thresholds was observed between EAE immunized groups experiencing non-paralysis and paralysis in both sexes, males (G) and females (H) (Non-paralysis n = 23, Paralysis n = 12; in both sexes). Data is represented as mean ± SEM, ****$p < 0.0001$.

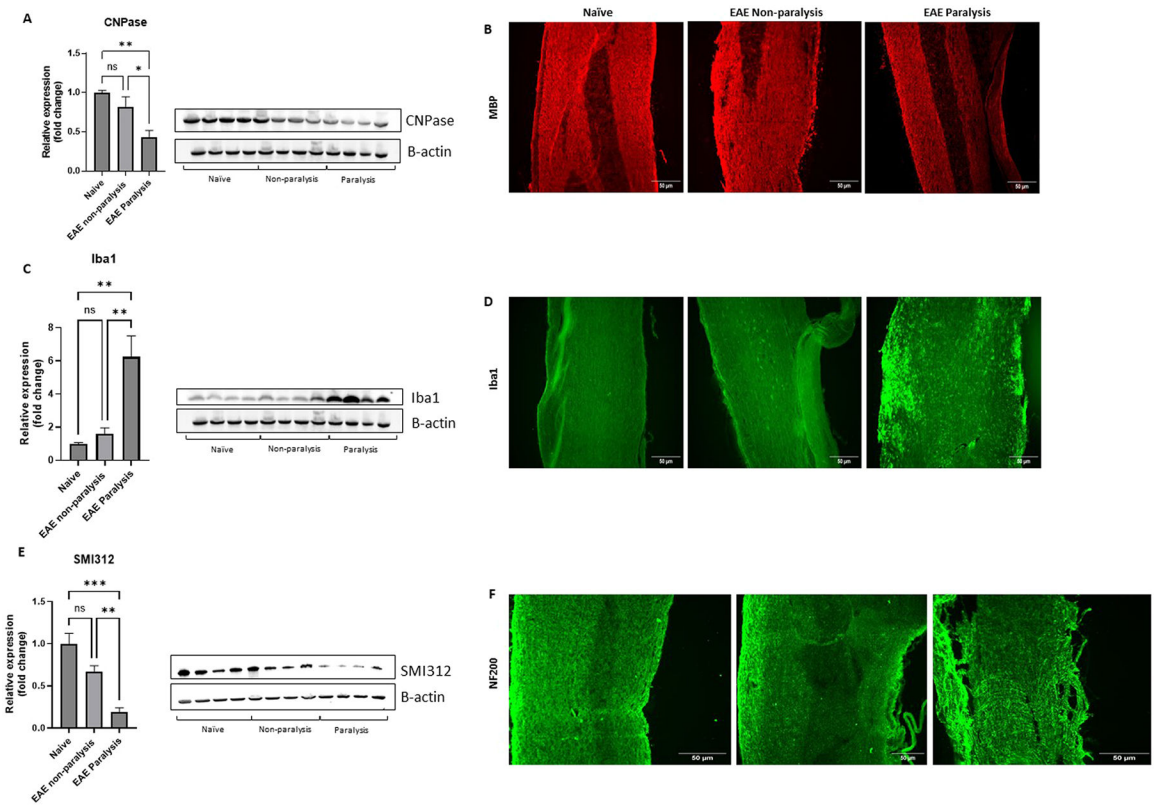

Fig. 2.

There is increased demyelination, neuroinflammation and axonal damage in lumbar spinal cord in EAE mice with paralysis. EAE immunized and age matched WT naïve mice were euthanized at DPI30 and lumbar spinal cord was processed for immunohistochemistry and western blot analysis. (A) There was a significant decrease in the CNPase protein expression in the EAE paralysis group as compared to the EAE non-paralysis group (n = 4/group, 2 males and 2 females, combined) and (B) a decrease in myelin basic protein (MBP) intensity was also observed by immunohistochemistry (n = 3/per group; 2 males and 1 female combined). (C) There was a significant increase in Iba1 protein expression in the paralysis group as compared to the non-paralysis group (n = 4/per group; 2 males and 2 females, combined), and (D) it was also observed by representative histological sections (n = 3/group; 2 males and 1 female combined). Axonal injury was analyzed using NF200 and SMI312, (E) there was a significant decrease in SMI312 (n = 4/group; 2 males and 2 females, combined) protein expression in the paralysis group as compared to the naïve and the non-paralysis group and (F) an increase in axonal fragmentation was observed by NF200 staining in the paralysis group (n = 3/per group; 2 males and 1 female combined). Data represent as mean ± SEM, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

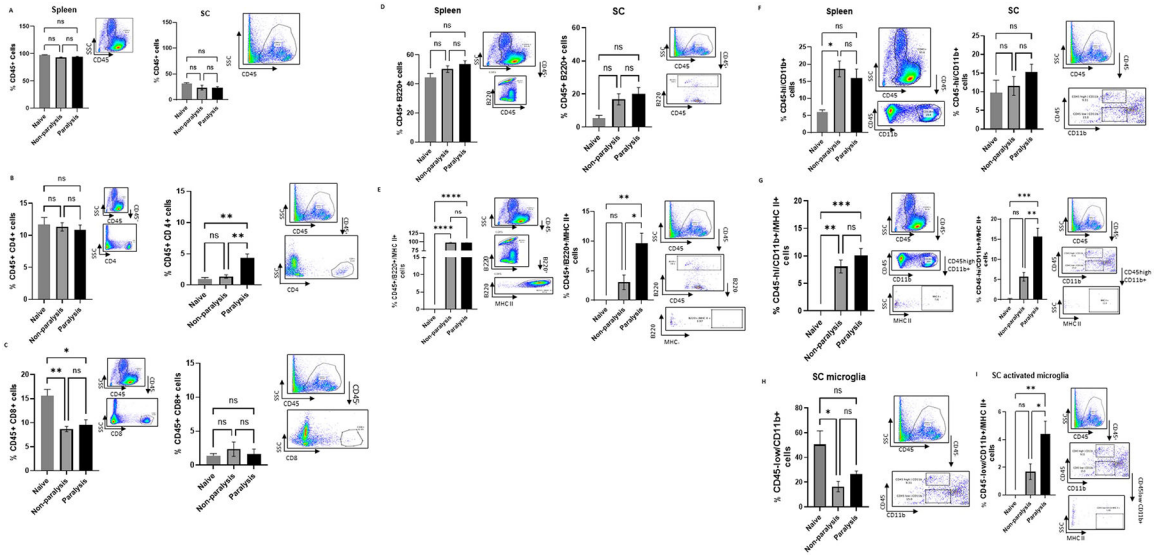

Fig. 3.

There is increased CD4 + T cell, activated B cell and macrophage infiltration, and a prominent increase in activated microglia in the lumbar spinal cord of the EAE mice with paralysis as compared to EAE mice without paralysis. EAE immunized mice were sacrificed at DPI 30. Splenocytes and immune cells from lumbar spinal cord (A) were isolated and percentages of CD4 + T cells (B), CD8 + T cells (C), B220 + B cells (D) B220 +MHCII+ activated B-cells (E), CD45hiCD11b+ macrophages (F), CD45hiCD11b+MHCII+ activated macrophages (G), CD45lowCD11b+ microglia in spinal cord (H), and CD45lowCD11b+MH-CII+ activated microglia in the spinal cord (I) were quantified using flow cytometry. Shown are representative quantification of the flow cytometry data and representative scatter plots with gating strategy from each tissue type (naïve n = 4; 2 males and 2 females, non-paralysis n = 9; 5 males 4 females, paralysis n = 10; 6 females 4 males, male and female data combined. Data represent mean ± SEM, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05).

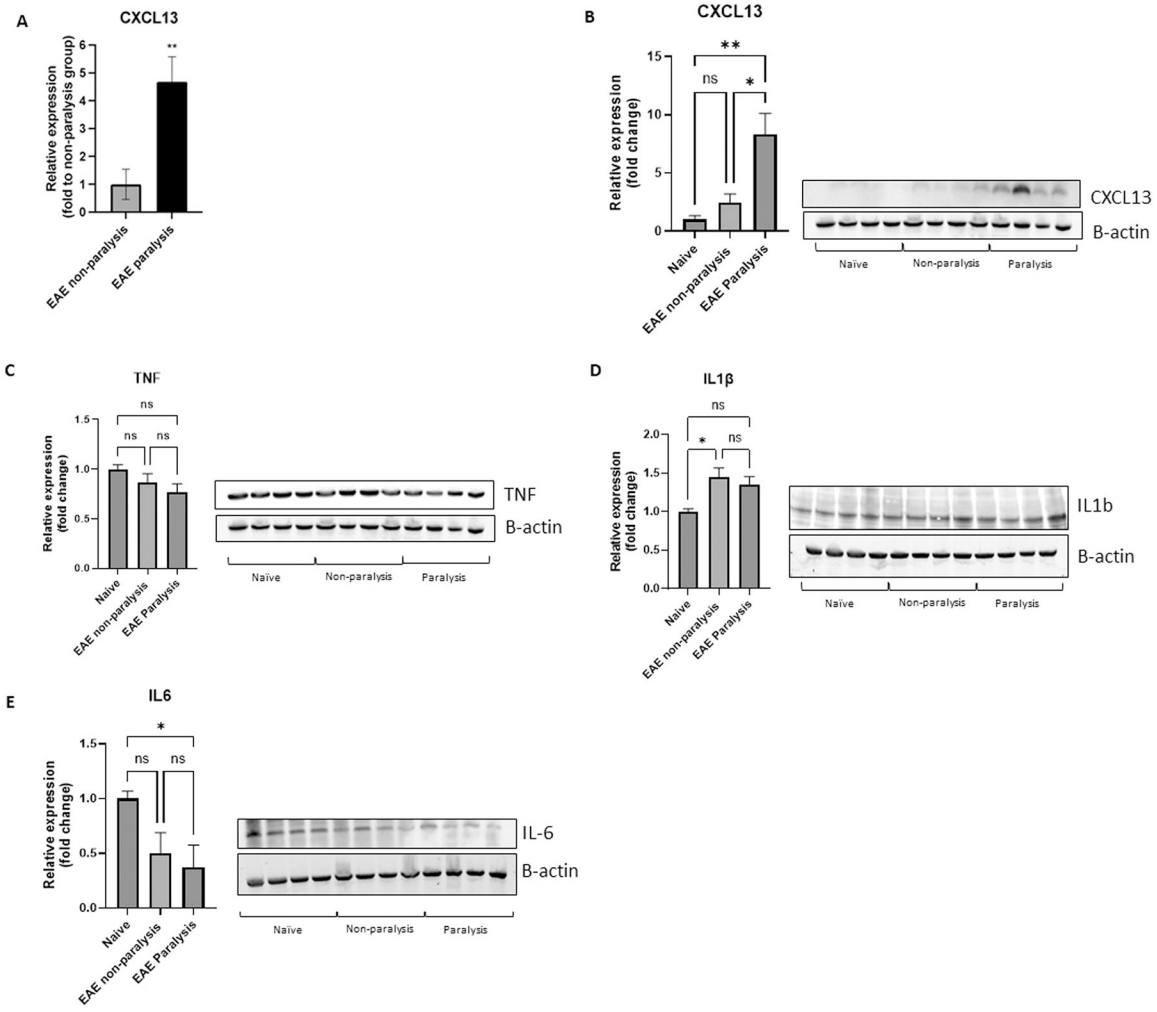

Fig. 4.

There is increased presence of CXCL13 chemokine in the lumbar spinal cord of EAE mice with paralysis as compared to EAE mice without paralysis. Lumbar spinal cord was isolated from EAE immunized and age matched WT animals and was processed for qPCR and western blot at DPI30. (A) Gene and (B) protein expression of CXCL13 was quantified using quantitative real time PCR and western blot respectively. Its expression was significantly elevated in the spinal cord of EAE mice with paralysis as compared to EAE mice without paralysis. We also studied the major implicated pro-inflammatory cytokines in the EAE pathophysiology. There was no difference in the protein expression of (C) TNF, (D) IL-1B, and (E) IL-6 between the paralysis and the non-paralysis group (n = 4/per group; 2 males and 2 females, combined. Data represent mean ± SEM, One-way ANOVA **p < 0.01, *p < 0.05).