Profiling blood-based brain biomarkers and cytokines in experimental autoimmune encephalomyelitis model of multiple sclerosis using single-molecule array technology

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Abstract

Experimental autoimmune encephalomyelitis (EAE) remains a widely used pre-clinical animal model to study multiple sclerosis (MS). Blood-based cytokines and CNS biomarkers are increasingly used as predictors of neurodegeneration, disease activity, and disability in MS. However, there exists variation in animal model characterization and disease course across animal strains/studies due to understudied confounding factors, limiting the utility of these biomarkers to predict disease activity in EAE. In this study, we investigated the profile of blood-based analytes including, cytokines (IL6, IL17, IL12p70, IL10, and TNFα) and neural markers (NFL and GFAP) in the plasma of relapsing-remitting (RR) (SJL) and chronic (B6) models of EAE during different phases (acute, chronic, and progressive) of disease course using ultrasensitive single molecule array technology (SIMoA, Quanterix), which can detect ultra-low levels of a wide range of analytes. NFL showed a substantial increase during post-disease onset at peak, chronic, and progressive phases in both RR SJL and chronic B6 models of EAE. The increase was markedly pronounced in the chronic B6 model. The leakage of GFAP from CNS into the periphery was also higher after disease onset in EAE, however, it was highest during the acute phase in B6. Out of all cytokines, only IL10 showed consistently lower levels in both models of EAE along the disease duration. We report that NFL, GFAP, and IL10 may be more useful predictors of disease activity and neurological outcome in EAE, which would make them potential candidates for use as surrogate markers for preclinical testing of therapeutic interventions in MS.

Introduction

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system (CNS), characterized by immune-mediated damage to the insulating myelin sheath, resulting in progressive demyelination and neurodegeneration. Experimental autoimmune encephalomyelitis (EAE) primarily remains the most widely used pre-clinical animal model of MS (Gold et al., 2006; Steinman and Zamvil, 2005; 2006; Farooqi et al., 2010; Bjelobaba et al., 2018). Despite certain inherent limitations, it has revolutionized the development of potential treatment options for MS (Steinman, 2009; Steinman et al., 2023). It represents a complex heterogeneous model depending on the disease induction methods adding variability to disease pathogenesis, making it an imperfect but approximate model to study MS. It is worthwhile to mention here that its use entirely depends on the scientific question to be addressed and the feasibility of translation. There are two widely used models to approximate relapsing-remitting (RR) and chronic disease phenotype of MS; SJL based biphasic model RR-EAE and monophasic B6-chronic model (Baker and Amor, 2015; 2014; Amor and Baker, 2012; Miller et al., 2007). The disease is induced in EAE through an immune-mediated response to CNS components by active immunization with self-antigens to myelin, making it an inflammatory model (Constantinescu et al., 2011; McCarthy et al., 2012). The CNS antigens targeted are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or proteolipid protein (PLP) injected in the form of an emulsified solution in complete Freund's adjuvant (CFA).

EAE is characterized by impaired inflammation resolution accompanied by dysregulation of cytokine profile, immune cell infiltration into CNS, disruption of the blood-brain barrier, neuroinflammation, and neuroaxonal pathologies including demyelinating lesions and gliosis (Kipp et al., 2017). Although the precise mechanism of EAE pathogenesis is still not clear, the pathogenic cascade mainly involves the production of cytokines by immune cells throughout the disease course ultimately compromising BBB integrity and tissue damage (Constantinescu et al., 2011). The measurement of blood-based markers is of growing importance for assessing disease activity in MS and its animal model EAE (Birmpili et al., 2022). Neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), and cytokines (IL17, IL6, IL10, TNF-α, IFN-γ, and IL12) are some of the emerging biomarkers in determining the inflammatory disease activity, disease progression, treatment response, and prognosis in MS (Jahan-Abad et al., 2020; Kuenz et al., 2007; Göbel et al., 2018; Imitola et al., 2005; Palle et al., 2017). The leakage of NFL and GFAP in plasma/serum is particularly used to monitor neuroaxonal damage in MS, yet there are limited studies in its preclinical animal model EAE (Jahan-Abad et al., 2020; Aharoni et al., 2021; Gnanapavan et al., 2012; Brummer et al., 2023).

Given the urgent need for blood-derived biomarkers for MS, the inception of highly sensitive immunoassays serves as the bedrock. The conventional methods of measuring blood biomarkers have certain limitations due to their low sensitivity and inconsistency across reports showing profiles of neural markers and cytokines in preclinical models (Kuhle et al., 2016). The comparisons across profiling studies of EAE often lack reproducibility due to variability across mice and underlying confounding factors such as the extent of inflammation or demyelination, and the use of low sample size (Aharoni et al., 2021). The levels are often impacted by the degree of inflammation and extent of CNS damage which varies from mouse to mouse. The biological matrix used for profiling blood-based analytes also contributes to the variability. With an increase in neuroprotective or reparative therapeutic strategies, potential therapeutics must be evaluated in EAE through quantitative neuropathology. We applied SIMOA (SIngle MOlecule Array) as this is the emerging ultrasensitive technology based on a bead-conjugate immunocomplex with potential to detect the analytes which are present in ultra-low levels (femtomolar range) and below detection limits for other conventional assays (Cohen et al., 2020; Lasseter et al., 2020; Revendova et al., 2022; Pafiti et al., 2023). It considers an average number of enzymes per bead (AEB) as the unit of measurement. Several different biomarkers could be detected in a single

experiment in various sample types with singleplex and multiplex assays. According to Quanterix, this digital platform can precisely measure analyte levels in human blood samples with 1,000 times greater sensitivity than conventional measurements. This means subtle changes can be captured earlier and less invasively than other approaches. In this study, we aimed to further investigate the outcome of EAE manifestations in the form of disease severity parameters and blood-based profile of NFL, GFAP, and cytokines during disease duration. Taken together, we propose the application of SIMOA as a valuable approach to indirect assessment of disease pathology and CNS damage in EAE.

Materials and Methods

EAE model induction

10-12 weeks old, female SJL and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in the pathogen-free animal facility of Henry Ford Health, Detroit, MI, according to the animal protocols approved by the IACUC. All the mice were housed with standard food and water ad libitum at a room temperature of 22 ± 2ºC under a 12:12 h light-dark cycle. For RR-EAE model, SJL mice were immunized on day 0 with a total 200 µl of antigen $PLP_{139-151}$ peptide (100 µg/mouse) via subcutaneous injections in the flank region emulsified in Complete Freund's Adjuvant (CFA) (Sigma Chemicals, St. Louis, MO, USA) supplemented with 4 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (400 µg; Becton, Dickinson and Company, Sparks, MD, USA) as described previously (Mangalam et al., 2013; Mangalam et al., 2016; Poisson et al., 2015). For the chronic model, EAE was induced in B6 mice on day 0 with a total of 200 µl of antigen MOG_{35-55} (200 ug/mouse) as described before (Mangalam et al., 2013; Mangalam et al., 2016; Poisson et al., 2015). Pertussis toxin (List Biological Laboratories, CA, USA) was administered via intraperitoneal (ip) injection (200 ng/200 µL) on days 0 and 2. In both models, one set of mice was injected with CFA without antigen/peptide to serve as control.

Clinical assessment of EAE models

For clinical evaluation of models based on the neurological deficits, the clinical score was monitored daily till the duration of study in a blinded fashion by measuring disease signs including paralysis according to the conventional grading system on a scale of 0-4 as described previously (Mangalam et al., 2013; Mangalam et al., 2016; Poisson et al., 2015). The onset of disease in EAE was considered on the day animals showed the first signs of disease. Disease peak was considered on the day when the EAE score was maximum and did not increase from the previous day (day 15–19 post-immunization). The chronic phase of the disease was considered after 20 days post-immunization if the EAE score decreased or was maintained concerning the disease peak. Weight loss was monitored after EAE induction. Change in weight during the disease course was evaluated every week, readings were taken twice a week per mouse.

Experimental groups and blood sampling

For disease monitoring and characterization of EAE models, mice were euthanized at different time points during the disease course. The time points selected for EAE profiling included day 17 (peak), day 30 (chronic), and day 45 (progressive) for RR-EAE (SJL) and day 15 (peak), day 20 (chronic), and day 45 (progressive) for chronic-EAE (B6). Mice were anesthetized with CO_2 and transcardially perfused with 1X chilled PBS. Control CFA mice were also taken at respective time points. For plasma analysis, blood samples from EAE and CFA groups were collected in purple-capped EDTA-vacutainer tubes. Plasma was separated from blood by centrifugation at 1500 rpm

for 10 min. The clear yellow liquid supernatant part was collected from the top and stored in respective tubes at -80°C till further processing. The secondary method of euthanasia for mice was either cervical dislocation or needle-induced pneumothorax. All the animal experiments in this study were performed as per the policies and guidelines of the IACUC committee at Henry Ford Health under the animal welfare assurance number D16-00090.

Single Molecule Array (SIMoA) Assay

To characterize the profile of neural markers and cytokines in EAE, we measured NFL and GFAP in plasma samples of CFA and EAE mice in both models (RR and chronic) with commercially available SiMoA™ Neuro 2-Plex B Advantage Kit (Product Number: 103520) (Quanterix, MA) on the SR-X analyzer according to manufacturer's instructions. Also, the cytokine profiling was performed using a mouse Cytokine 5-Plex Kit for IL6, TNFA, IL17, IL12, IL10 (Cat # 107-178-1-AB; Product Number: 85-0441) (Quanterix, MA) on the SP-X analyzer according to manufacturer's instructions. The calibrators supplied with kits were used as standards. The number of mice taken for each assay at a given time-point was 4. Samples were only loaded after centrifugation at 10,000 rpm for 10 min to prevent lipids present in the sample from interfering with the assay. For every 96-well plate-based assay, one batch of reagents was used to minimize the lot-specific variations as emphasized in the protocol instructions by Quanterix.

Data analysis

All values are presented as Mean ± SEM, and statistically significant differences were computed by Welch's t-test. Statistical significance in terms of the P-value is mentioned in the figure legends (and wherever applicable). The comparison between different time points across disease courses in both models was done using one-way ANOVA followed by Tukey's post-hoc test. All statistical analyses were performed with GraphPad Prism9. Statistical significance is indicated by an asterisk * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ with $n > 3$ for all variables.

Results

EAE disease course shows the classical trend in RR and chronic models

Disease signs in EAE appeared in both models (RR and Chronic) after 8–9 days post-disease induction (**Fig. 1A; Fig. 2A**) whereas the control CFA group did not show any neurological deficits. Both models reached the peak phase of the disease around 15–17 days. The clinical scores showed remarkable differences between the EAE and CFA at the pre-determined time points selected for profiling along disease duration. The clinical readout in form of a maximum score ($p<0.001$) (**Fig. 1C**) showed a mean score of 3.95 in the SJL model and average cumulative score ($p<0.0001$) was 64.1 (**Fig. 1B**). For B6 model, maximum score ($p<0.0001$) averaged to 3.7 (**Fig. 2C**) with mean cumulative score of 133.1 ($p<0.0001$) (**Fig. 2B**), suggestive of classical disease pattern in both models. Overall, the disease severity parameters indicated enhancement of neurological deficits with EAE.

EAE reduces body weight in both models

The physical effect of EAE was assessed through body weight changes in both models. As mentioned in the methods section, body weight was measured every week throughout the disease course. EAE mice showed loss of weight along disease duration post-immunization irrespective of disease remission or improvement in neurological deficits after the acute phase. There was a steady decline in the weight of mice in both models along with disease duration marking one of the manifestations of EAE. A significant reduction in body weight was observed in the EAE compared to CFA in both models (RR-EAE $p<0.001$; B6-EAE $p<0.0001$) (**Fig. 1D; Fig. 2D**). The

mean weekly weight remained 18.7 ± 0.24 g (**Fig. 1D**) for SJL mice compared to CFA with a mean weight of 21.41 ± 0.32 g and for B6 mice it was 18.5 ± 0.34 g compared to CFA with a mean weight of 21.83 ± 0.54 g (**Fig. 2D**). Our findings further confirm that as the disease progressed in both models, there is shift in weight distribution more towards the forelimbs to counterbalance for paralysis of the hindlimbs and tail, particularly in paralytic mice.

EAE increases NFL and GFAP leakage in peripheral circulation

Neuroaxonal damage in CNS was assessed by measuring the leakage of NFL and GFAP into the peripheral circulation during the disease course in EAE. The time points taken for the measurement were selected based on different phases of the disease course in EAE, categorized as peak, chronic, and progressive. The time-points are indicated in respective disease severity plots for RR-EAE (**Fig. 1A**) and Chronic-B6 (**Fig. 2A**). As shown in Fig. 3 and Fig. 5, SIMOA showed a significant increase in the plasma level of NFL and GFAP at all time-points in both models of EAE compared to CFA group. The mean value of NFL for the RR-EAE model in CFA was 29.83 ± 0.55 pg/ml compared to EAE with an average value of 284.53 ± 30.60 pg/ml across all time points when combined (**Fig. 3**). NFL showed maximum leakage with mean value of 326.42 pg/ml at day 17 (clinical score ~3-4) (**Fig. 3A**) in RR-EAE during peak phase of disease compared to other time-points (day 30 302.23 pg/ml $p<0.01$; day 45 224.94 pg/ml $p<0.05$) which showed slight decline but with substantial elevation compared to the control group; indicating pathological manifestations of EAE (**Fig. 3, Fig. 7A**). Overall, the NFL ranged in SJL model across all time-points between 60.43 to 899.25 pg/ml compared to CFA with range between 18.35 to 52.29 pg/ml which suggests elevated leakage of NFL in EAE (**Table 1**). However, there was no significant difference across disease stages in RR-EAE. Furthermore, the mean NFL level reached 653.83 ± 188.01 pg/ml for combined time-points in the EAE-B6 model compared to 16.57 ± 2.88 pg/ml with statistically significant elevation in EAE at day 15 (clinical score ~3-4) (mean value 533.5 pg/ml; $p<0.01$) and day 45 (mean value 1022.52 pg/ml; $p<0.05$) (**Fig. 5; Fig. 7B**). The NFL values were within the range between 92.47 to 1960.59 pg/ml compared to CFA with values between 9.53 to 26.11 pg/ml (**Table 1**).

Further, the mean plasma levels of GFAP in RR-EAE were 203.56 ± 173.60 pg/ml compared to 69.88 ± 41.37 pg/ml in CFA which reflects leakage in EAE even though it was statistically non-significant (**Fig. 3D**). The range of GFAP levels was 45.65 to 654.52 pg/ml in RR-EAE compared to 7.22 to 168.70 pg/ml in CFA (**Table 1**). For B6-EAE, when compared jointly across all time points, the average plasma levels of GFAP were 33.27 ± 7.92 pg/ml compared to 2.14 ± 0 pg/ml for CFA. The leakage was maximum at day 15 ($p<0.001$) with an average value nearing 42.98 pg/ml compared to 23.56 pg/ml for day 20 in EAE (**Fig. 5D, E**). The detection range for GFAP in the EAE-B6 model was 7.62 to 52.40 pg/ml in EAE compared to 0.00 to 8.17 pg/ml in CFA (**Table 1**). Taken together, NFL and GFAP show substantial leakage from CNS into the periphery in both models of EAE post-disease immunization.

EAE decreases the anti-inflammatory cytokine IL10 in the blood

IL10 has a crucial role in the pathophysiology of MS and its animal model EAE as it's one of the key cytokines involved in the regulation of immune response (Porro et al., 2020). In present study, the plasma level of IL10 showed significant reduction in EAE compared to CFA with mean level of IL10 in RR-EAE model nearing 3.76 pg/ml at day 17 ($p<0.01$) (**Fig. 4A**), 5.57 pg/ml at day 30 ($p<0.05$) (**Fig. 4B**) and 17.18 pg/ml at day 45 (**Fig. 4C**). The level of IL10 was lowest at day17 (clinical score ~3-4), peak phase of the disease compared to other time-points (**Fig. 4**). The mean concentration of IL10 was 8.83 ± 4.20 pg/ml for RR-EAE compared to CFA where it was 26.53 ± 8.59 pg/ml with detection range of 0.46 to 20.03 pg/ml in EAE and 15.73 to 95.88 pg/ml in CFA (**Table 1**). Similarly, assay results showed the same downtrend in the B6 model of EAE with a

mean concentration of IL10 in EAE as 6.44 ± 1.27 pg/ml and 23.96 ± 10.73 pg/ml in CFA, with a detection range of 1.92 to 9.78 pg/ml in EAE and 7.78 to 51.38 pg/ml in CFA (**Fig. 6; Table 1**). The decrease was maximum in EAE at day 45 (mean level 4.88 pg/ml; $p<0.01$) compared to day 20 (8.00 pg/ml; $p<0.05$). Altogether, IL10 showed a decrease in both models of EAE.

Discussion

The current goal of MS biomarker research is to use highly sensitive platforms that would generate a specific biomarker profile to improve diagnosis and management of MS. SIMOA technology is a fully automated platform that detects analytes (mainly proteins) when bound to antibody-coated beads combined with high-resolution fluorescence imaging (Rissin et al., 2020). Currently, the two most promising biomarker candidates for assessing neural damage in MS are NFL and GFAP, however, results are inconsistent across studies, highly suggestive of the variability in the techniques used for their measurement as well as the heterogeneity across study cohorts used in studies. NFL represents the cytoskeletal component of neurons whereas GFAP is the cytoskeletal intermediate filament of astrocytes, and both serve as biomarkers of neuronal death, axonal degeneration, and astrogliosis (Yang & Wang, 2015). Blood NFL is a marker of neuro-axonal injury and is associated with relapses, EDSS worsening, lesions on MRI scans, and atrophy of both the brain and spinal cord in MS (Barro et al., 2018). NFL has been investigated as a potential prognostic and disease activity marker, with a potential relation between its leakage and the rate of neurodegeneration (Bridel et al., 2019). However, there are several limitations in the application of the NFL. It is a cytoskeletal protein that can be released because of any kind of brain injury, and it is not specific to MS, thus, any neurologic disease or injury can have confounding effects. It is more logical to have a panel of biomarkers by utilizing an ultrasensitive platform that would perhaps enhance the specificity, accuracy, and reproducibility in characterizing MS and post-treatment assessment.

CNS biomarkers, NFL, and GFAP are being used to monitor the pathophysiological and neurodegenerative manifestations in preclinical animal models of MS, and EAE. The present study covers both RR and Chronic-B6 models of EAE compared to already published reports where they have used only the B6 model (Gnanapavan et al., 2012; Aharoni et al., 2021). Given the reports show its association with impairment of blood-brain barrier integrity, immune cell extravasation, and CNS inflammation following the first demyelinating event in MS (Uher et al., 2021), we measured its plasma level in EAE. It is pertinent to mention that NFL leakage in EAE was more pronounced for the B6 model compared to SJL with no conclusive trend for kinetics across time points in both models which contrasts with the report of Aharoni *et al.,* where they have shown maximum leakage at peak of the disease followed by decline afterward as the disease progresses (Aharoni et al., 2021). However, with $n<3$ and variable across time points along disease duration, the significance of their data is compromised. Also, the findings reported by Gnanapavan *et al*., in B6 mice show quite an opposite trend which could be due to the application of non-SIMOA methods for NFL detection or batch variation in mice (Gnanapavan et al., 2012). In the present study, neurodegeneration in the form of neuroaxonal damage was confirmed by substantial leakage of NFL into peripheral circulation at day 17 (mean value 326.42 pg/ml) after disease induction in the RR model of EAE which overlaps with the appearance of maximum clinical score ~3-4 in mice. As the disease progresses, NFL level declines in EAE at other time points (day 30 and day 45) compared to the peak phase of the disease at day 17. Further, the average level of NFL was higher in the B6 model (653.83 ± 188.01 pg/ml) compared to SJL (284.53 ± 30.60 pg/ml) for combined time-points in both models with mean value in B6 nearing 1022.52 pg/ml. These findings further substantiate NFL as a reliable biomarker to assess disease activity in EAE. However, the dynamics of NFL in blood is a limiting factor making it tricky to link NFL leakage with a degree of neurodegeneration and extent of CNS damage.

Similarly, we found an increase in plasma GFAP post-immunization in EAE for both models with a maximum level detected in RR-EAE (203.56 ± 173.60 pg/ml). It is worthwhile to mention that GFAP was not detected by SIMOA at other time points mentioned in the study design as the readings for CFA and EAE in both models were reported to be zero by the assay, which raises the possibility that zero readings could be due to machine error or assay did not work for those samples or issue with bead-analyte binding. Given the sophistication of the SIMOA platform, it's worth mentioning here that sample processing was uniform across all batches due to which its impact on the data can be ruled out. Taken together, our findings for NFL and GFAP confirm the substantial leakage of CNS components into the periphery as a pathological manifestation of CNS damage in EAE.

Moreover, the pathogenic cytokines (IFNγ, IL17, TNFα, IL6, IL12p70, IL10) can also reflect the degree of inflammation in patients as MS therapies are largely aimed to lower the inflammatory response by modulating the levels of inflammatory markers (Wagner et al., 2020). Cytokine profiles have been established in various studies for adult and pediatric MS patients (Bhise et al., 2016; Chen et al., 2012; Chen et al., 2012; Imitola et al., 2005; Martins et al., 2011); however, this is far more complex as it involves an array of cytokines which show temporal effect in MS. Due to this complexity, targeting multiple variables including CNS damage biomarkers provides a more meaningful option for monitoring disease evolution. In the present study, the concentration of cytokines IL6, IL17, IL12p70, and TNFα was below the detection limit (conc. lower than the lowest calibrator) across all time points in most of the samples and that's why there was no conclusive data about that making it difficult to interpret the trend in EAE. However, we found a lower level of IL10 in EAE for both models with a mean of 8.83 ± 4.20 pg/ml in RR-EAE and 6.44 ± 1.27 in B6. Given the central role of IL10 in the pathophysiology of MS and other neurodegenerative diseases, its lower production in humans is considered a risk factor for MS (Porro et al., 2020; Vandenbark et al., 2001). IL10 also regulates inflammation-mediated CNS damage in EAE as evidenced by exacerbation of EAE in IL10 KO mice compared to mice where IL10 was over-expressed showing resistance to EAE (Bettelli et al., 1998; Cua et al., 1999). Overall, our findings indicate that IL10 is suppressed in EAE confirming its anti-inflammatory role in suppressing MS.

In conclusion, our data indicates that the plasma profile of CNS biomarkers (NFL and GFAP) and IL10 can be used for monitoring pathological manifestation during disease duration and testing therapeutic interventions in EAE. This is also confirmed by our prior study showing a protective effect of a pro-resolution lipid mediator maresin1 (MaR1) in EAE where SIMOA showed a lower level of NFL in the plasma of EAE treated with MaR1 as compared to the untreated group. This indicated that MaR1 could preserve neuronal and axonal integrity in EAE which prevents the release of CNS contents into the peripheral circulation. Further, MaR1 treatment elevated IL10 levels in EAE, showing IL10-mediated neuroprotective and inflammation-resolving effects of MaR1 (Zahoor et al., 2023). It is worth noting that we have also performed SIMOA assays in human patient samples which again confirmed the use of NFL and GFAP as biomarkers of disease activity in MS validating the consistency of SIMOA (Zahoor et al., 2022). Taken together, our results validate and confirm the utility of SIMOA as a robust analytical platform with higher sensitivity and can precisely detect small changes in samples, making it a highly valuable approach under clinical settings in both MS and EAE. At the same time, the inconsistency of data and variation across mice strains/batches due to unknown confounding variables cannot be ruled out. However, further prospective studies are warranted in large-scale samples to validate the utility of these markers in clinical practice.

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A Tribute and Thank You to my Dear Dad: Gone but never forgotten, forever and always.

“Death leaves a heartache no one can heal; love leaves a memory no one can steal”- Richard Puz.

Dear Dad, although you are no longer with me, I don’t know if I thanked you enough before you left this world. But here is a big thank you for being my biggest cheerleader, mentor, friend, and anchor in life’s stormy seas, who always supported its branches like a tree trunk. I am forever grateful to be your daughter. Fathers like you never die; they live through their daughters. You are loved beyond words and missed beyond measure; I LOVE YOU FOREVER MY HERO…

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Statements and Declarations

Ethics Approval The animal studies performed in this manuscript stand approved by the IACUC committee of Henry Ford Health. The article does not contain any studies with human participants.

Conflict of Interest The authors declare that they have no competing interests.

Figures

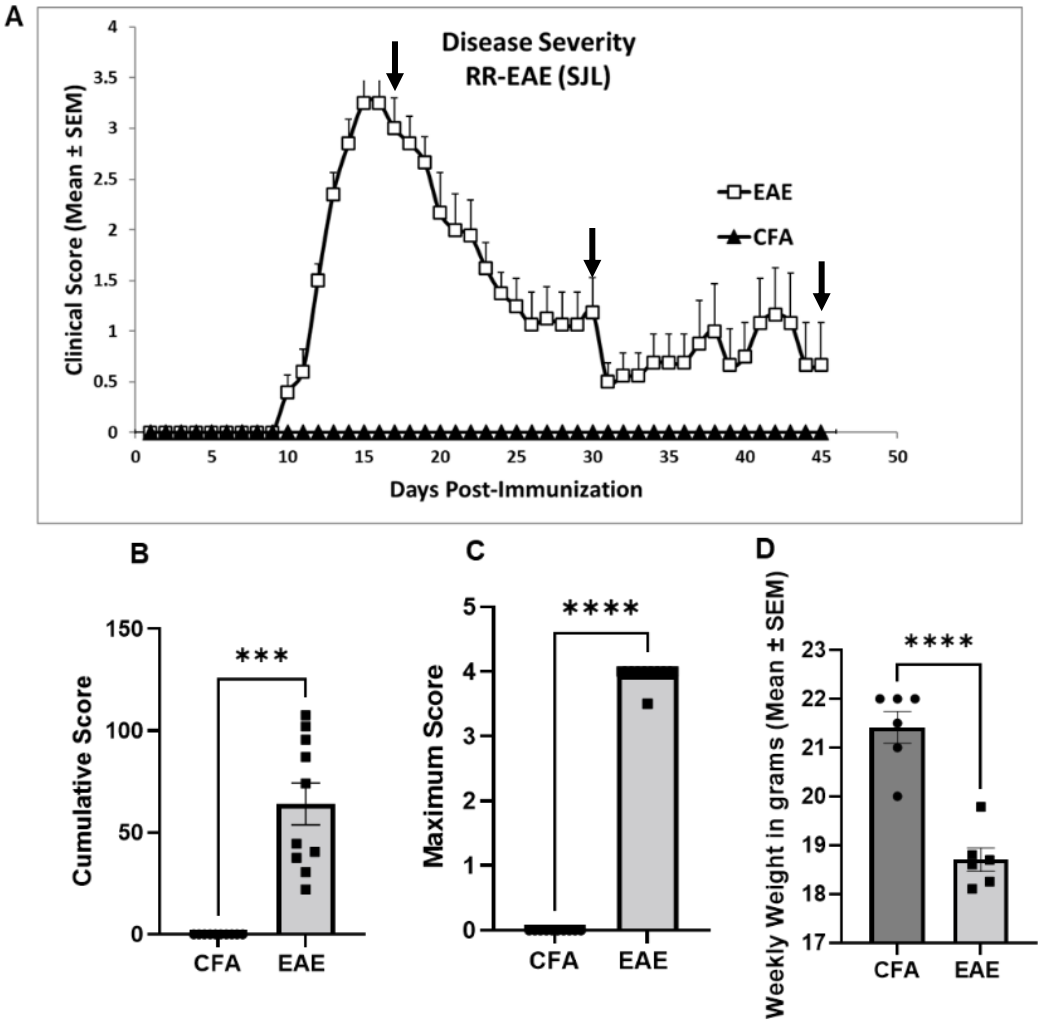

Fig 1. Representative disease severity parameters in RR (SJL) model of EAE.

(A) Clinical score plot in SJL mice consisting of experimental groups CFA and EAE during the disease course. **(B)** Cumulative score. **(C)** Maximum score. **(D)** The trend in weight changes over the disease course. ***p<0.001, ****p<0.0001 (as determined by Welch's t-test) CFA vs EAE. Data are shown as Mean ± SEM.

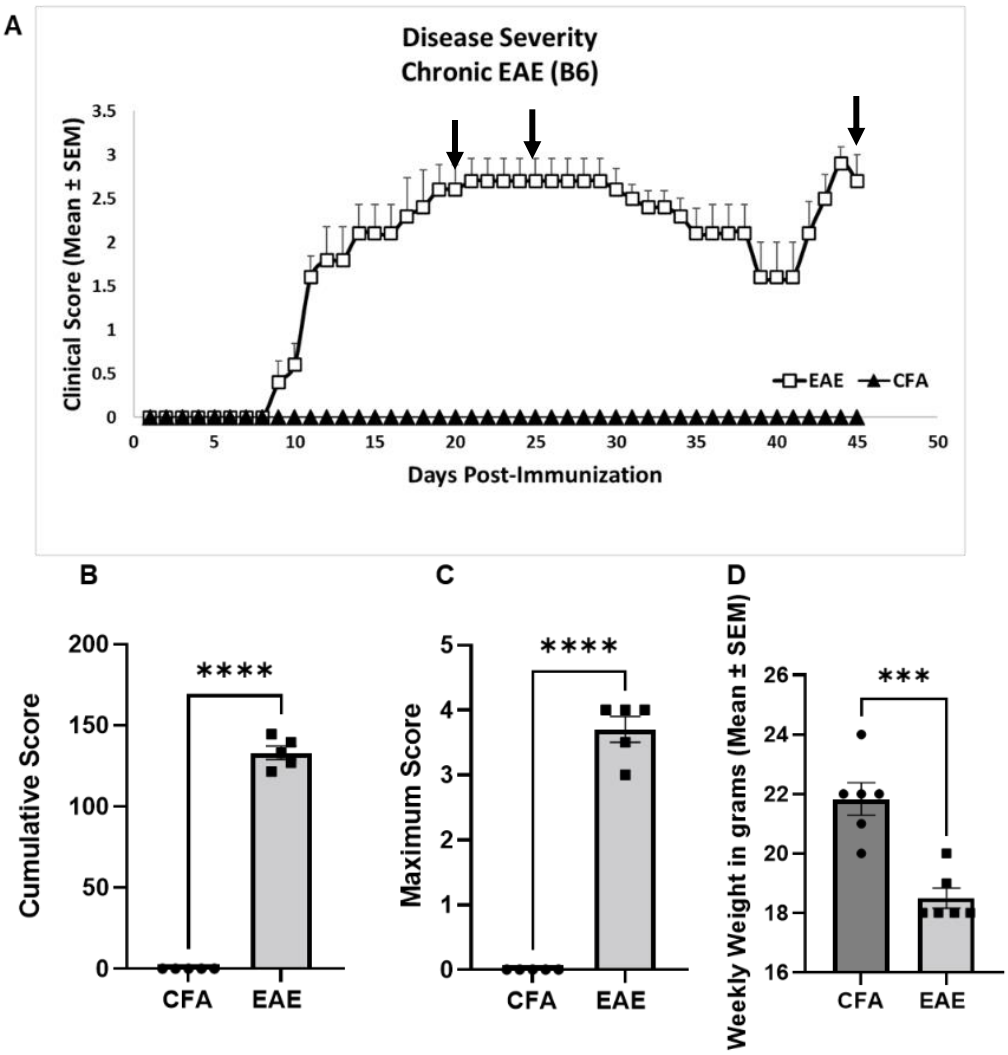

Fig 2. Representative disease severity parameters in chronic (B6) model of EAE.

(A) Clinical score plot in B6 mice consisting of experimental groups CFA and EAE during the disease course. **(B)** Cumulative score. **(C)** Maximum score. **(D)** The trend in weight changes over the disease course**.** ***p<0.001**,** ****p<0.0001 (as determined by Welch's t-test) CFA vs EAE. Data are shown as Mean ± SEM.

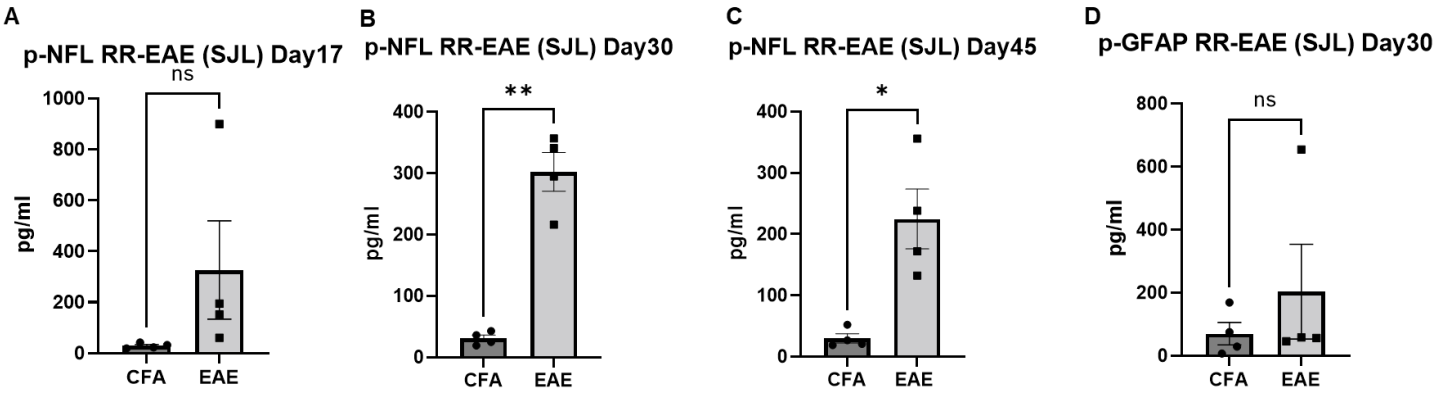

Fig 3. Profile of neural markers (NFL and GFAP) in plasma of RR-EAE (SJL).
Plasma level of NFL and GFAP at respective time points in CFA vs EAE. Values are shown in pg/ml.
*p<0.05, **p<0.01 (as determined by Welch's t-test) CFA vs EAE. Data are shown as Mean ± SEM.

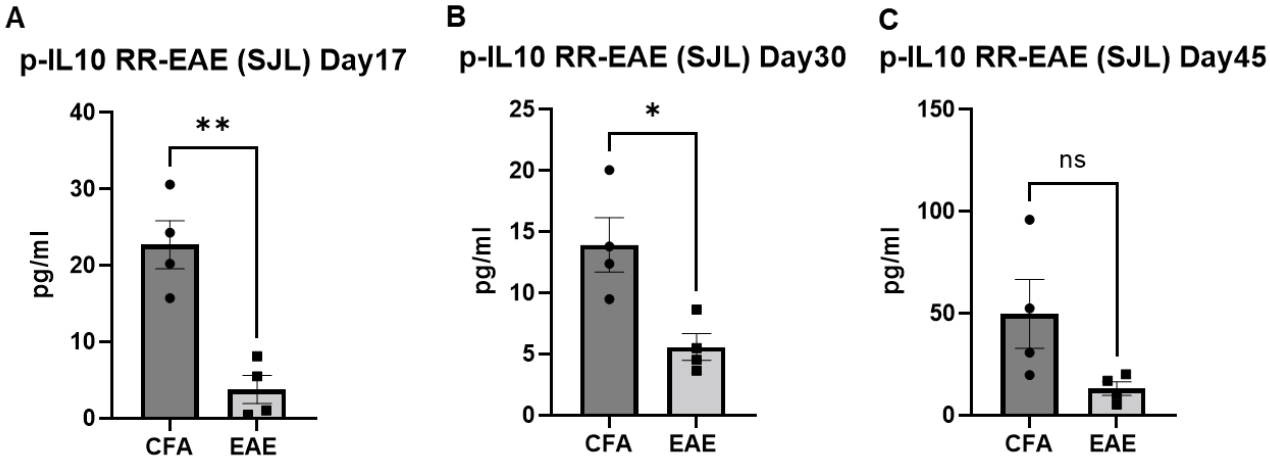

Fig 4. IL10 profile in plasma of RR-EAE (SJL).
Plasma level of IL10 at respective time points in CFA vs EAE. Values are shown in pg/ml.
*p<0.05, **p<0.01 (as determined by Welch's t-test) vs EAE. Data are shown as Mean ± SEM.

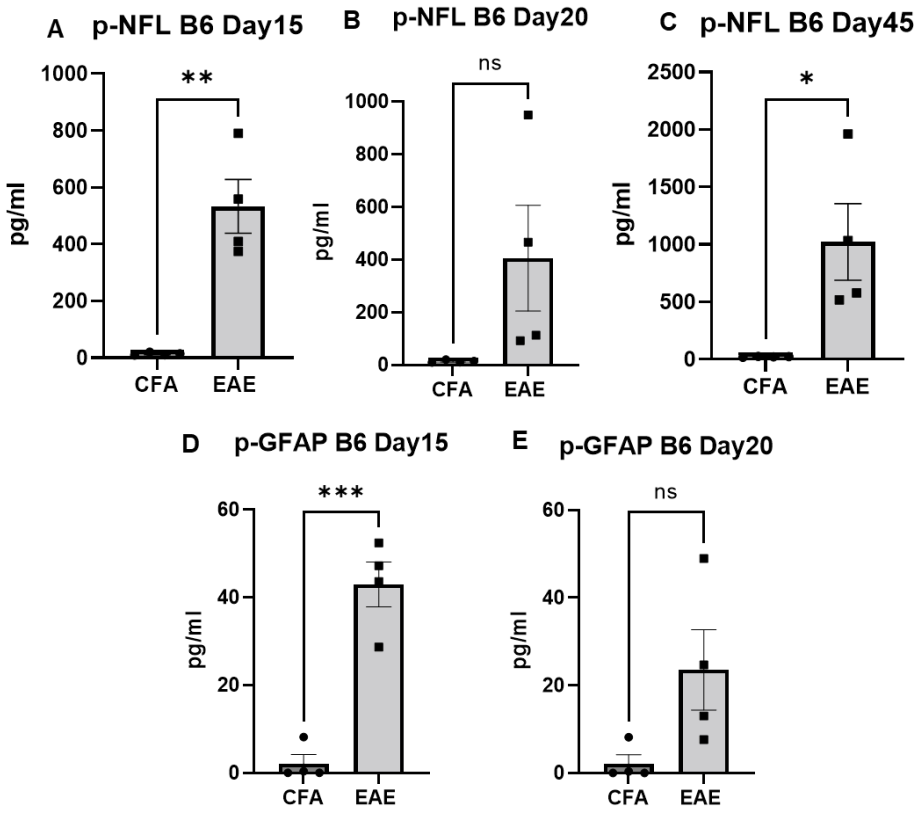

Fig 5. Profile of neural markers (NFL and GFAP) in plasma of chronic-EAE (B6).
Plasma level of NFL and GFAP at respective time points in CFA vs EAE. Values are shown in pg/ml.
*p<0.05, **p<0.01, ***p<0.001 (as determined by Welch's t-test) vs EAE. Data are shown as Mean ± SEM.

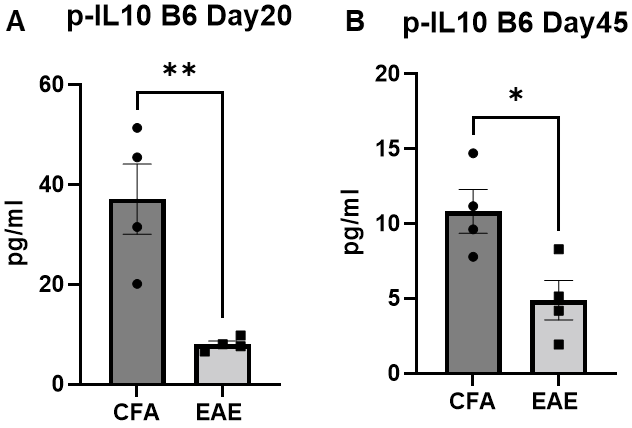

Fig 6. IL10 profile in plasma of chronic-EAE (B6).
Plasma level of IL10 at respective time points in CFA vs EAE. Values are shown in pg/ml.
*p<0.05, **p<0.01 (as determined by Welch's t-test) vs EAE. Data are shown as Mean ± SEM.

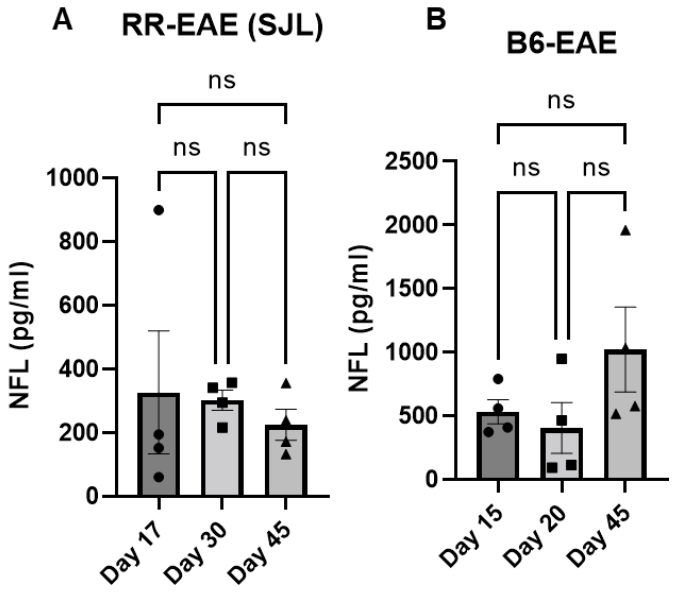

Fig 7. Plasma NFL concentration of EAE along disease duration.
(A) RR-EAE. **(B)** Chronic-B6. Values are shown in pg/ml as determined by one-way ANOVA, CFA vs EAE. Data are shown as Mean ± SEM.

Table 1. Comparative detection range of CNS biomarkers and cytokines in SJL and B6 models using SIMOA

Analyte	Conc range (SJL) (pg/ml)		Status in EAE	Conc range (B6) (pg/ml)		Status in EAE
	CFA	RR-EAE		CFA	Chronic-EAE	
NFL	18.35 to 52.29	60.43 to 899.25	Increase	9.53 to 26.11	92.47 to 1960.59	Increase
GFAP	7.22 to 168.70	45.65 to 654.52	Increase	0.00 to 8.17	7.62 to 52.40	Increase
IL10	15.73 to 95.88	0.46 to 20.03	Decrease	7.78 to 51.38	1.92 to 9.78	Decrease