Effects of cigarette smoke exposure on a mouse model of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory autoimmune disease associated with genetic and environmental factors. Cigarette smoking is harmful to health and may be one of the risk factors for MS. However, there have been no systematic investigations under controlled experimental conditions linking cigarette smoke (CS) and MS. The present study is the first inhalation study to correlate the pre-clinical and pathological manifestations affected by different doses of CS exposure in a mouse experimental autoimmune encephalomyelitis (EAE) model. Female C57BL/6 mice were whole-body exposed to either fresh air (sham) or three concentrations of CS from a reference cigarette (3R4F) for 2 weeks before and 4 weeks after EAE induction. The effects of exposure on body weight, clinical symptoms, spinal cord pathology, and serum biochemicals were then assessed. Exposure to low and medium concentrations of CS exacerbated the severity of symptoms and spinal cord pathology, while the high concentration had no effect relative to sham exposure in mice with EAE. Interestingly, the clinical chemistry parameters for metabolic profile as well as liver and renal function (e.g. triglycerides and creatinine levels, alkaline phosphatase activity) were lower in these mice than in naïve controls. Although the mouse EAE model does not fully recapitulate the pathology or symptoms of MS in humans, these findings largely corroborate previous epidemiological findings that exposure to CS can worsen the symptoms and pathology of MS. Furthermore, the study newly highlights the possible correlation of clinical chemistry findings such as metabolism and liver and renal function between MS patients and EAE mice.

1. Introduction

Multiple sclerosis (MS) is the most widespread neurological condition among young adults, with an average age of onset around 30 years. Common neurological manifestations of MS include optic neuritis, diplopia, sensory loss, limb weakness, gait ataxia, spasms, fatigue, pain, loss of bladder control, and cognitive dysfunction. Paralysis, vision loss, and deteriorating respiratory function can occur in rare, severe cases. In 2016, approximately 2.2 million people worldwide were estimated to have MS, corresponding to a prevalence of 30.1 cases per 100,000 population [110]. This is more than double the number of reported cases

since 1975. The full economic cost of managing MS is substantial. In the United States (US) alone, the charges associated with MS care have been reported to increase at rates of US$ 40 million and US$ 8 million a year, respectively [14]. Overall, a good understanding of the disease pathology and risk factors associated with MS is crucial for effectively battling this debilitating neurodegenerative disease.

MS is a chronic and progressive autoimmune disorder in which the body's immune system attacks myelin and oligodendrocytes, damaging the brain and spinal cord [63]. The exact etiology of the disease is unknown but it is thought to involve various genetic and environmental factors as well as viral infection (e.g., Epstein-Barr virus, human

herpesvirus, and nonspecific viral infections involving the upper respiratory or gastrointestinal system) and hormonal changes associated with, for example, pregnancy [15]. Among the environmental risk factors, cigarette smoking has emerged as a potentially important risk factor associated with MS [32,42,73,118]. Indeed, smoking causes serious diseases, and smokers have been reported to be approximately 1.5 times more likely to be diagnosed with MS than nonsmokers [32]. The risk of MS increases with cigarette smoking in a dose-dependent manner, with the risk increasing 2-fold for individuals exposed to a cumulative dose of >16 pack-years [31]. This smoking-related increase in risk reportedly remains unchanged for up to 5 years after smoking cessation; but, by 10 years post-cessation, the MS risk decreases to the same level as that of the general population, regardless of the prior cumulative dose [31]. The mechanism of the harmful effect of smoking on MS is unknown but might include direct toxicity in neural tissues and immunomodulation [97]. CS contains high concentrations of free radicals, such as hydrogen cyanide, nitric oxide, and carbon monoxide, which can induce demyelination lesions [30]. Furthermore, smokers have increased expression of Fas, a membrane receptor on B cells and CD4+ T cells, that signals for apoptosis in activated lymphocytes, which could overwhelm the scavenging capacity of the immune system and initiate immune responses preceding autoimmunity in susceptible individuals [30]. Taken together, published studies indicate that there might be significant advantages in stopping smoking even after onset of MS.

Although the epidemiological data on the effect of cigarette smoking on MS are important, there have been limited systematic investigations under controlled laboratory conditions to study a direct cause-and-effect relationship between CS exposure and MS development and prognosis. Among several animal models for MS, EAE is the most commonly used experimental model for studying pathogenesis and discovering pharmacological therapies for MS, and it has been used to test a number of compounds [16,20,23,78,96,117]. In addition, all the current US Food and Drug Administration (FDA)-approved immunomodulatory drugs are found effective to some degree in treating EAE, therefore it is a strong indicator that EAE is the preferred model to study autoimmune response aspects of MS and develop effective treatments for the human disease. Although animal models do not fully recapitulate all aspects of human MS conditions, animal models are still essential in understanding the complex interaction between a variety of immunopathological and neuropathological mechanisms, and lead to development of therapeutic strategies [62,86]. Induction of EAE has been successfully achieved in mouse and rat strains by using intact myelin protein or peptides [64, 107]. Therefore, in the present study, we evaluated the effect of CS exposure on the progression and severity of clinical symptoms, clinical pathology, and histopathology at different stages of disease progression in an EAE mouse model to assess the potential impact of CS exposure on MS.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (Murine Pathogen free™; InVivos Pte. Ltd., Singapore) aged 7–9 weeks were individually identified by subcutaneously implanted transponders and group-housed in an open-top cage (up to 8 mice per cage) with environmental enrichments (e.g., igloo and nesting materials) at 22 ± 2 °C and 30–70% relative humidity. Autoclaved softwood granulates (Lignocel® BK 8–15; Rettenmaier & Söhne, Rosenberg, Germany) were used as bedding. Gamma-irradiated pellet diet (2914C irradiated rodent diet; Envigo, Indianapolis, IN, USA) and sterilized drinking water were provided ad libitum except during CS exposure, when mice had access to water but not food. The light/dark cycle was 12 h/12 h, with the light period starting at 07:00 h. The mice were allowed to acclimatize to this home environment for at least 12 days before the experiment. The general condition and health of the

mice were assessed throughout the study, which included body weight measurement and group and individual observations. All animal experiments were approved by the Philip Morris International (PMI) Research Laboratories Institutional Animal Care and Use Committee (IACUC) under protocol no. 15051. The care and use of mice were conducted in accordance with National Advisory Committee for Laboratory Research guidelines and Assessment and Accreditation of Laboratory Animal Care requirements [1,67].
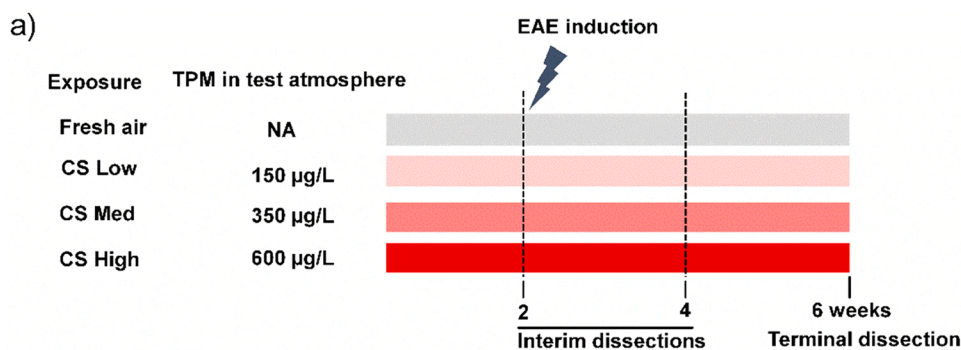
2.2. CS exposure

3R4F reference cigarettes (Center for Tobacco Reference Products, College of Agriculture, Food and Environment, University of Kentucky, Kentucky, USA) were used as a representative of the "full flavor" segment of the American market. Detailed 3R4F CS analytics can be found in Roemer et al. [87]. 3R4F reference cigarettes were smoked in accordance with the Health Canada Intense smoking protocol [29], which is based on International Organization for Standardization (ISO) standard 3308, with some modifications [41]. The CS was generated by using commercially available 30-port rotary (15 ports blocked) smoking machines (SM2000, Burghart Messtechnik GmbH, Wedel, Germany) equipped with a programmable dual-port syringe pump with active side-stream exhaust. The CS generated from the smoking machines was diluted with filtered conditioned air to obtain the respective target total particulate matter (TPM) concentrations.

To achieve a similar mean baseline body weight across the groups at the start of the study, 8–10 mice were allocated to each treatment group (Supplemental Table 1) on the basis of body weight using system generated randomization sequence. Mice immunized with myelin oligodendrocyte glycoprotein amino acids 35–55 (MOG_{35-55}) (EAE mice) and those not immunized (naïve control mice) were both exposed to fresh air or CS (Fig. 1a and Supplemental Table 1). During the first week, the mice were exposed to fresh air (sham) or a low, medium (med), or high concentration (150, 350, or 600 μg TPM/L, respectively) CS for incremental durations by following a 7-day time-adaptation regimen (Fig. 1b). The three concentrations were chosen on the basis of prior in-house studies that examined the tolerability and potential biological effects of CS in the same strain of mice. After the adaptation period, all mice were exposed to either fresh air or respective concentrations of CS for 4 h per day, 5 days a week, by following an interrupted exposure regimen with fresh air breaks (Fig. 1b). During the week preceding necropsy, the mice were exposed for 7 days a week (instead of 5 days a week) to ensure that they were exposed to CS for at least 2 days prior to necropsy. The mice were exposed in a 24-cage whole-body exposure chamber (WBEC, 800 L, manufactured in-house by Philip Morris Research Laboratories Leuven, Belgium), and up to 8 mice were housed and exposed per cage during exposure.

To characterize the test atmosphere and verify the reproducibility of smoke generation, the concentrations of TPM, nicotine, and aldehydes, flow rate through the exposure chamber, and particle size distribution were tested in samples from the breathing zone of the exposure chambers (Supplemental Tables 2–4). TPM concentrations were determined four times daily for all exposure chambers and maintained within ± 10% of the target concentrations. Nicotine and CO concentrations were also measured (Fig. 1c).

2.3. CS uptake

Dosing dosimetry was calculated in accordance with a previously defined equation and parameters, assuming an inhalation fraction of 1 and average body weight of 20 g [8]. On the basis of this calculation, the estimated inhaled nicotine doses were determined to be approximately 2.25, 5.69, and 10.06 mg per kg per day for CS Low, Med, and High, respectively. To demonstrate CS uptake by the mice, nicotine and five major nicotine metabolites were analyzed in 24-h urine samples, which were collected from mice placed in metabolic cages during CS exposure

Fig. 1. CS exposure design. (a) Mice were exposed for 2 weeks before EAE induction and sacrificed at different time points for tissue collection. n = 8 mice per group, except n = 10 for CS Low, Med, and High at 6 weeks (Supplemental Table 1). (b) A schematic diagram of the time-adaptation regimen during the first week of exposure and the interrupted exposure regimen are shown. Mice were exposed to 1-h blocks of CS exposure, separated by 30-min or 1-h fresh air breaks. Black boxes represent CS exposures. White boxes represent fresh air exposures. (c) Average concentrations of TPM, CO, and nicotine in the test atmospheres are indicated as mean ± SD. Numbers in brackets represent the number of means for values collected daily (N). CS: cigarette smoke; EAE: experimental autoimmune encephalomyelitis; TPM: total particulate matter; CO: carbon monoxide; SD: standard deviation.

and overnight on study days 9 and 12. Further details of urine sample collection and analyzes are available in the Supplemental material. The recovery of total urinary nicotine metabolites was concentration-dependent and consistent with the target exposure concentrations in the test atmospheres (Supplemental Fig. 1a). The relative levels of the five major nicotine metabolites were similar across all groups; therefore, there were no differences in nicotine metabolic rate or uptake among the different CS groups (Supplemental Fig. 1b).

2.4. EAE induction and health monitoring

EAE was induced by using a commercially available kit in accordance with the manufacturer's instructions (EK-2110, Hooke Laboratories Inc., Lawrence, MA, USA). In brief, after a 2-week adaptation period to CS exposure, mice were immunized at the start of the 3rd week by a single subcutaneous (s.c.) injection of 100 μL MOG_{35-55}/Freund's complete adjuvant (CFA) emulsion into each flank (100 μg/100 μL MOG_{35-55} per site; i.e., 200 μg total MOG_{35-55} per mouse), and an intraperitoneal (i.p.) injection of 100 μL pertussis toxin (PTX) (80 ng/100 μL per mouse). The emulsion contained 5 mg killed *Mycobacterium tuberculosis* H37Ra in each mL of emulsion prepared using a propriety method and checked for quality by the manufacturer. On the following day, the mice were injected again with the same dose of PTX. The adaptation period was included in this study to reduce the stress associated with the initial CS exposure and to better understand the pharmacological effects of CS exposure on EAE. The mice were not exposed on the day of EAE induction; the exposure resumed the next day, and the time loss was compensated for on a weekend. The mice were sacrificed at different time points to understand the disease progression (Fig. 1a).

After EAE induction, the mice were weighed and observed daily for signs of EAE development. Naïve control groups (non-immunized mice) were weighed twice a week and examined at least once a week for clinical symptoms. EAE clinical signs were scored daily on a scale of 0–5 with 0.5 increments (Supplemental Table 5) [38,116]. Mice with clinical scores above 1 were transferred to another cage to prevent them from being injured by the unaffected cage mates and group-housed with other affected mice whenever possible. Enrichment and special care were provided as indicated in Supplemental Table 5. A weight loss of 25% or more from baseline (measured on day 0 of EAE induction; study day 15) was considered a humane endpoint for the affected mice. Mice declared moribund were immediately euthanized, and blood and tissue samples were collected. During the study, three EAE mice (two from the CS Low and one from the CS High groups) lost ≥ 25% of their baseline weight by study days 32 (post-immunization day 17) and 36 (post-immunization day 21) and were terminated. All immunized mice were included for study endpoint analyses, except the ones declared moribund. None of the EAE mice reached the maximum score for a humane endpoint (i.e., a score of 4 for approximately 24 h).

2.5. Biological sample collection

To understand the pathological progression in the spinal cord, mice were terminated before EAE induction (week 2), at the onset of EAE symptoms (week 4), and at the end of the study (week 6) (Fig. 1a). The mice were anesthetized with an intraperitoneal injection of pentobarbital solution (Valabarb, Jurox Animal Health, NZ) and terminal serum samples were collected for clinical chemistry analyses. Following blood collection, the mice were transcardially perfused with cold saline (0.9% sodium chloride, B. Braun, Melsungen, Germany). Tissue collection was performed on a cold surface decontaminated with RNaseZap® wipes

(Invitrogen™, Thermo Fisher Scientific, MA, USA). Once dissected, the brain was cut longitudinally into two halves along the midline. The right hemisphere was immersion-fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for approximately 3 h and then subsequently transferred to and stored in 15% sucrose at room temperature for histological analyses. The spinal cord was flushed from the spine with cold PBS by using a syringe and divided into three segments (cervical, thoracic, and lumbar). The cervical and lumbar portions were immersion-fixed in 4% PFA in PBS at room temperature for approximately 3 h and subsequently transferred to and stored in 15% sucrose at room temperature for immunohistochemistry analyses. The thoracic region of the spinal cord was snap-frozen in liquid nitrogen and stored at ≤ − 70 ℃ for Luminex analysis (refer to Supplemental material).

2.6. Brain and spinal cord pathology evaluation

Brain samples were processed and analyzed by QPS GmbH (Grambach, Austria). Spinal cord samples were processed and analyzed by PsychoGenics, Inc. (Paramus, NJ, USA). In brief, fixed brain and spinal cord samples were embedded in optimal cutting temperature (OCT) medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) in cryomolds and snap-frozen in dry-ice-cooled liquid isopentane. Brain samples were cut into 10-μm sagittal sections and spinal cord (cervical and lumbar regions) samples into 10-μm transverse sections with a Leica CM1950 cryotome (Leica Biosystems AG, Wetzlar, Germany) by following a uniform systemic random protocol. Six levels of brain sections and three levels each of cervical and lumbar spinal cord sections were analyzed. Initial assessment of the brain sections by hematoxylin and eosin staining revealed a limited number of noticeable lesions in some sections, and the differences between the treatment groups were not observable (data not shown); thus, no further analyzes were conducted for the brain. This observation is in agreement with those from previous studies showing limited central nervous system (CNS) infiltrates and no evidence of brain lesions in MS mouse models [20,54]. We have also observed that EAE induction, which induces more severe clinical symptoms, does not necessarily induce brain pathology and primarily affects spinal cord pathology, particularly in the lumbar region (unpublished data). Spinal cord sections were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and FluoroMyelin™ Green Fluorescent Myelin Stain (Thermo Fisher Scientific Inc., Massachusetts). The stained sections were imaged on a Zeiss AxioScan Z1 slide scanner microscope equipped with Colibri II LED illumination (Carl Zeiss AG, Oberkochen, Germany) and an Orca Flash 4.0 B&W camera (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan).

The number, density, and size of lesions were quantified within the ventral white matter of the spinal cord. A lesion in this study is defined as an area with increased cell density, which is indicative of increased inflammatory infiltrates, as demonstrated in previous studies [2,3]. A stepwise, automated approach using Image Pro Premier v9.3 (Media Cybernetics, Inc., Rockville, MD, USA) was developed to more accurately and objectively determine the extent of the spinal cord lesions (Supplemental Fig. 2). The left and right sides were delineated separately. Any changes in DAPI stain clustering were assumed to represent local cell infiltrations forming spinal cord lesions as reported previously [66]. Among the various parameters for determining spinal cord pathology by automated quantification, the total number of lesions was the most robust endpoint for determining the treatment-dependent effects and is reported in the Results section. We have also assessed myelination differences using a manual scoring method based on the following scoring criteria: 0 = 0%; 1 = 0–20%; 2 = 20–40%; 3 = 40–60%; 4 = 60–80%; and 5 = 100% demyelination (summarized in Supplemental Fig. 3). The entire white matter area of the spinal cord was taken into consideration for the manual scoring. To ensure the integrity of the sections and quality of staining, quality control (QC) checks of sections and images were performed in a blinded (to the treatment conditions) manner before statistical analyses. The sections were scored for right and left sides separately, and only sections that passed the QC check were included in the statistical analyses. Thus, for each mouse, there are a maximum of 12 data points each for the lumbar and cervical sections (i.e., 2 sides × 6 sections per spinal cord region).

2.7. Statistical analyses

Valid statistical analysis requires both proper statistical methodologies and sound statistical design. The former ensures correct estimation and proper interpretation of the statistical results, while the latter is critical to validate the statistical results, ensure statistical power, and remove bias at all levels. In our study, the experimental units (mice) were randomly allocated to the experimental treatments (study groups) on the basis of similar initial/baseline body weight distribution across groups. Randomization removed bias while ensuring that any observed differences among the experimental groups were not due to body weight differences. Furthermore, the sample size of 8–10 mice per group is sufficiently large to expect a reasonable statistical power for group comparisons [5]. The sample size also allowed us to use a panel of standard and innovative statistical methods for analysis of study endpoints.

For body weight and clinical scores, descriptive statistics were calculated for all treatment groups and all indicated time points. All mice were analyzed up to study day 44 (post-immunization day 29), when the terminal necropsy started. Mice declared moribund were not included in the statistical analysis for the days after death. Body weight and clinical scores from only the 6-week groups were used for the analysis to ensure a complete longitudinal dataset. Intrasubject variability in both body weight and clinical scores occurred naturally between consecutive days and was taken into consideration for investigating intergroup differences. To ensure more reliable and robust analysis, a semi-parametric generalized additive model (GAM) with smooth curves adjusted over time and fixed effects for exposure groups and random effects for intrasubject variation was applied [90]. The semi-parametric GAM was used to optimize the benefits while mitigating the drawbacks of running parametric or non-parametric analyses. The model takes advantage of the flexibility of non-parametric analysis while enhancing statistical power, which is often criticized in a non-parametric model [59,102]. Clinical scores were analyzed by the same procedure, with the exception that a GAM with zero-inflated gamma response was used to fit the high number of zero responses observed (particularly prior to EAE induction). The estimated curves over time obtained from the GAM model were used for overall comparison. This statistical model provided two different types of estimates for the clinical scores: (1) the probability of "good clinical results" and (2) the clinical severity or evolution over time. "Good clinical results" refer to the estimates of the effect of CS exposure on the probability of the clinical scores being higher than zero over time and pairwise comparisons between exposure groups. The second estimate included the evolution of clinical severity over time for the EAE groups. In addition, survival time was analyzed by the Kaplan–Meier non-parametric method for estimating the survival curves for each group, and the log-rank test was used for testing differences between the groups.

For spinal cord pathology analysis, raw data were aggregated so that one observation was reported per animal per spinal cord region (lumbar or cervical). Negative binomial regression was used instead of Poisson regression [108], because the numbers of lesions were highly variable despite the data aggregation. Because of the skewed data distribution, clinical chemistry data were transformed to a logarithmic scale to correct for heteroscedasticity (data variability dependent on data averages). For both spinal cord pathology and clinical chemistry data, two-way analysis of variance for equal means across all treatment groups was performed at the 5% significant level. Once the null hypothesis of equal means was rejected, the main effects and interactions

were assessed. The post-hoc paired student t-test was then used to test the group differences.

To determine a possible correlation between clinical symptoms and spinal cord pathology, the clinical score profiles across days were studied for each study subject. The highest clinical score (reflecting disease severity) was then recorded together with the total number of lesions for each experimental subject. The recorded data pairs (disease severity and histopathology) were then used to estimate the Spearman's rank correlation coefficient and test the hypothesis of being equal to zero. For all statistical analysis, $p \leq 0.05$ was considered significant.

3. Results

3.1. General health

There was no CS exposure-related mortality throughout the study, and the frequent in-life observations performed shortly after each exposure day revealed no abnormality, where the mice were observed to exhibit typical behaviors (e.g., exploration in home cages and normal righting reflex). Abnormal touch responses (e.g., high-pitched vocalization or aggression upon handling) were observed in some mice regardless of exposure. All immunized mice developed clear reddish bumps at the injection site 2–4 days after the injection, which was a sign of local immune response to MOG_{35-55}/CFA. Approximately 50–55% of the cohort developed skin ulcers at the site of injection within 1–6 days of immunization; the ulcers eventually resolved in the majority of the mice (reduced to ~ 23% in week 6) without veterinary intervention.

However, one mouse was declared moribund on study day 39 (post-immunization day 24) because of a worsening wound and was excluded from the final analysis of the study.

3.2. Body weight

On the day of EAE induction (study day 15), the body weights of the mice were measured and recorded as baseline values (Fig. 2a). Subsequently, the body weights were normalized against baseline values to monitor any changes (Fig. 2b and c). There were main and interaction effects of EAE and CS exposure on body weight. The main effect of EAE indicated that the EAE mice had lower body weight than naïve control mice. The interaction effect indicated that the effect of CS also depended on whether or not the mice had EAE. The body weights of fresh air-exposed naïve control mice steadily increased over time so that the body weights of naïve control mice exposed to medium and high concentrations of CS were significantly lower from approximately study day 30 onwards in comparison ($p \leq 0.05$; Fig. 2b and d). Such CS-dependent retardation of body weight gain indicated a direct relationship between body weight and CS exposure. In contrast, the body weights of EAE mice fluctuated over time, unlike those of naïve control mice, reflecting the effect of a complex interaction between EAE and CS on body weight. All EAE mice exhibited approximately 5% transient weight loss 1 day after EAE induction. The body weights then stabilized or increased to above baseline levels (Fig. 2c). This was followed by a downward trend (varying between 5% and 10%) approximately 10 days after EAE induction. Subsequently, fresh air-exposed EAE mice gained body weight
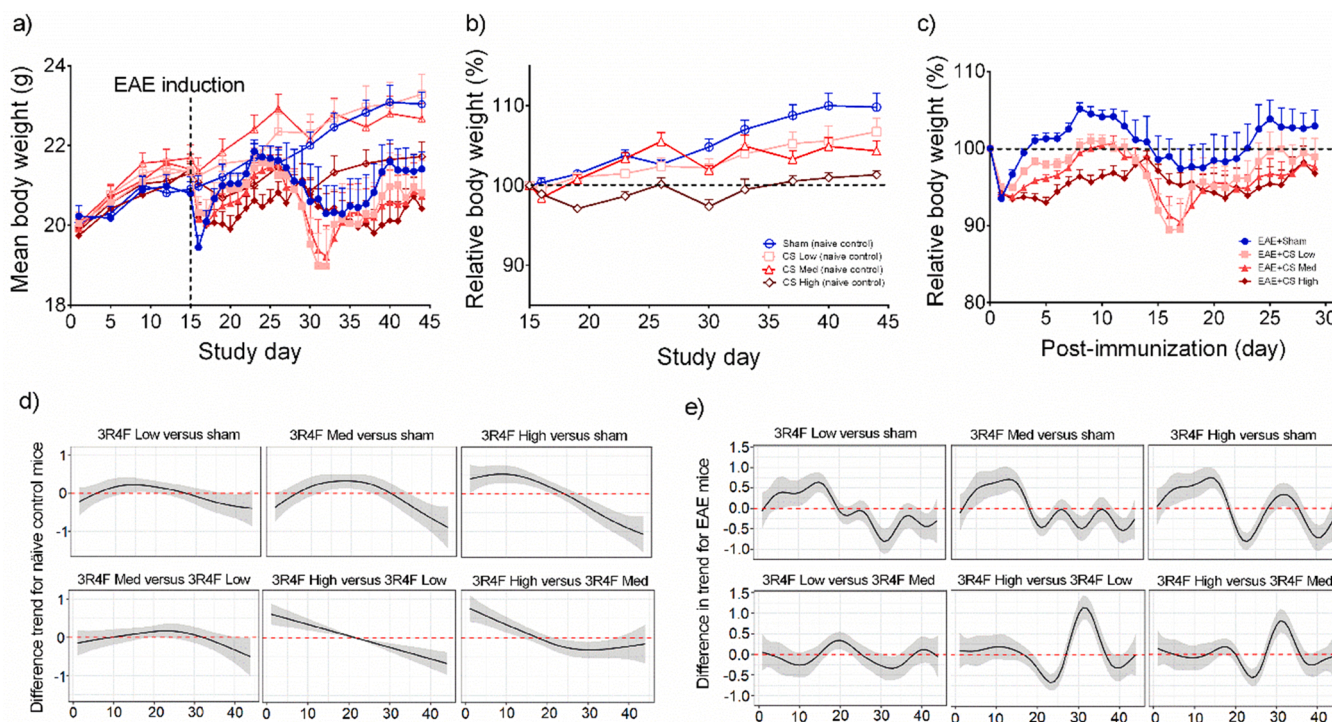

Fig. 2. Effects of EAE and CS on body weight. (a) Average body weights of the EAE and naïve control mice in all exposure groups are shown. The body weights of the mice were normalized to their respective weights on study day 15 and presented as percent change for the (b) naïve control mice and (c) EAE mice. Study day 15 corresponds to the first day of EAE induction (equivalent to day 0 post-immunization). Data presented as mean ± SEM. For naïve control mice, sham = blue lines with blue open circles (○), CS Low = pink lines with open squares (□), CS Med = red lines with open triangles (△), and CS High = brown lines with open diamonds (◇). For EAE mice, sham = blue lines with solid circles (●), CS Low = pink lines with solid squares (■), CS Med = red lines with solid triangles (▲), and CS High = brown lines with solid diamonds (◆). GAM analyses of the body weights of (d) naïve control mice and (e) EAE mice exposed to fresh air (sham) or three concentrations of CS (Low, Med, High) are shown. The black lines are average differences in body weight from pairwise comparison between the groups indicated in the headers. Grey bands represent 95% confidence intervals. The red dotted lines are set at zero to represent the body weight of the second group in each comparison (e.g., for CS Low vs. sham analysis, sham is set at zero). Note: Data from similarly exposed subgroups (e.g. 2-week, 4-week, and 6-week) were combined for presentation in (a), (b), and (c), while only 6-week groups were included in (d) and (e), as described in the Materials & Methods. n = 8 mice per group, except n = 7 in the 6-week CS Low, n = 10 in the 6-week CS Med, and n = 9 in the 6-week CS High groups (Supplemental Table 1). CS: cigarette smoke; EAE: experimental autoimmune encephalomyelitis; SEM: standard error of mean; GAM: generalized additive model.

faster than any of the CS-treated EAE mice. The body weight of all CS-treated EAE mice fluctuated below the baseline levels (Fig. 2c and e).

3.3. Clinical symptoms and prevalence

Mice affected by EAE showed clinical symptoms that commonly started from the tail and progressed to the hindlimbs and forelimbs. Owing to the fact that there was a minimal clinical score reported in the EAE groups during week 4, which approximately corresponded to disease onset, disease severity was statistically analyzed for the 6-week subgroups only. The survival probability was the same in all treatment groups ($p = 0.180$). The median disease onset of clinical symptoms in the sham and CS Low, Med, and High groups were days 19, 15, 18, and 25 post-immunization as analyzed by using non-parametric Kaplan–Meier estimator and log-rank tests, respectively (Fig. 3b). The onset difference was statistically significant only between the CS High and Low groups ($p = 0.022$). The highest daily average clinical score was 1.5 on post-immunization day 17 in the CS Low group, followed by 1.4 on post-immunization day 24 in the CS Med group and 1.1 and 0.75 on post-immunization day 21 in the sham and CS High groups, respectively (Fig. 3a). Similarly, the mean maximum clinical score was similarly higher in the CS Low (1.9) and CS Med (1.8) groups than in the sham (1.25) and CS High (1.1) groups (Fig. 3a insert). Statistically, both

CS Low and Med groups had significantly higher disease severity scores than the sham and CS High groups (Table 1). In terms of disease severity, EAE mice exposed to the low and medium concentrations of CS were indistinguishable ($p = 0.773$). Exposure to CS at the high concentration did not affect the overall disease severity relative to sham exposure ($p = 0.880$). The four animals that were declared moribund because of weight loss or severe wounds (three in the CS Low and one in the CS High groups) were excluded from the analysis for the days after death. Exclusion of these data was responsible for a sudden drop in clinical scores on post-immunization days 18 and 25 in the CS Low group and days 22 and 25 in the CS High group. The disease progression was similar in all EAE groups, as indicated by the parallel GAM curves (Fig. 3c). In addition, the clinical score correlated well with body weight changes over time (Fig. 3d).

No significant effect of CS exposure was observed relative to sham exposure in EAE or naïve control mice in terms of overall probability or global prevalence of disease, which was defined in this study as the proportion of mice with disease symptoms on at least one day during the study (Fig. 4 and Table 1). Relative to sham exposure, CS exposure had no effect on the incidence rate, defined as the number of cases over the total number of mice per day. Only the CS High group showed a significant reduction in the daily incidence rate relative to the CS Low group but not relative to the sham or CS Med groups (Table 1).
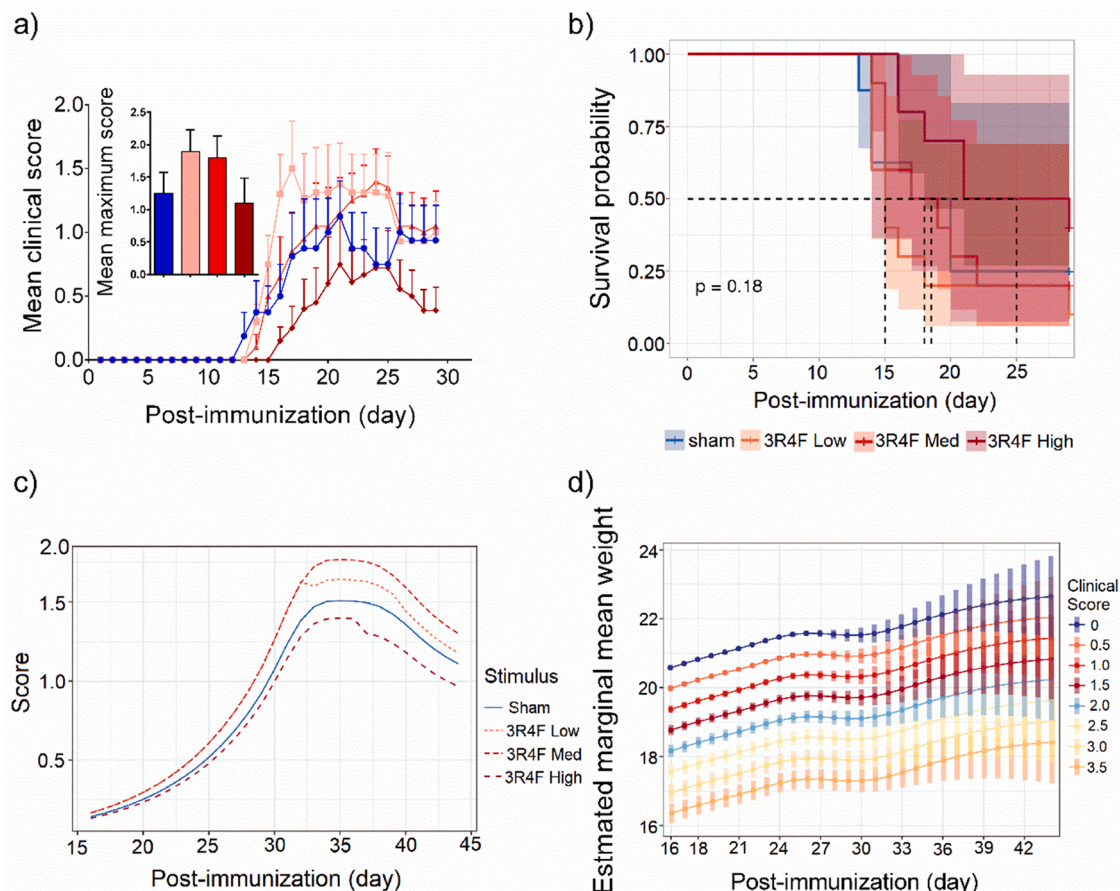

Fig. 3. Clinical score development in EAE mice exposed to various concentrations of CS. (a) Mean daily clinical scores for EAE mice are shown as mean ± SEM. Sham = blue lines with solid circles (●), CS Low = pink lines with solid squares (■), CS Med = red lines with solid triangles (▲), and CS High = brown lines with solid diamonds (◆). The insert shows the mean maximum clinical scores of the EAE mice. The color coding is the same as that for the mean clinical scores. (b) Clinical onset was analyzed by using non-parametric Kaplan–Meier estimator and log-rank tests. The shaded areas represent 95% confidence intervals for the survival probability of the respective groups. The p value indicated in the graph is the overall statistical significance among all groups and was not significant. (c) Disease progression and severity were analyzed by using a GAM. There were no differences in disease progression among any of the groups, as indicated by the non-crossing, parallel slopes. (d) The clinical score progression correlated well with weight loss over time. Sham = blue lines, CS Low = pink lines, CS Med = red lines, and CS High = brown lines. n = 8 mice per group, except n = 7 in the 6-week CS Low, n = 10 in the 6-week CS Med, and n = 9 in the 6-week CS High groups (Supplemental Table 1). CS: cigarette smoke; EAE: experimental autoimmune encephalomyelitis; SEM: standard error of mean; GAM: generalized additive model.

Table 1
Summary of statistical analyses of clinical scores.

Comparison	Incidence rate[a] (Risk of disease)	Global prevalence[b]/ incidence	Onset[c]	Severity[d]
CS Low vs. sham	1.241	3.003	Day 19 vs. 15	1.232***
CS Med vs. sham	1.015	1.333	Day 19 vs. 18	1.248***
CS High vs. sham	0.605	0.500	Day 19 vs. 25	0.991
CS Low vs. CS Med	1.222	2.250	Day 15 vs. 18	0.987
CS Low vs. CS High	2.051*	6.000	Day 15 vs. 25*	1.244***
CS Med vs. CS High	1.678	2.667	Day 18 vs. 25	1.259***

Remarks:

Statistical significance: *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

[a] Number of cases over total number of mice per day; an incidence rate ratio lower than 1 is interpreted as showing lower incidence in the first group than in the second group, and the converse is true for values higher than 1.

[b] Number of mice that show the effect of the disease at least once throughout the study.

[c] Time at which the mice show an effect of the disease for the first time as analyzed by using non-parametric Kaplan-Meier estimator and log-rank tests.

[d] Degree of disease symptoms (expressed as clinical scores) over time; a value smaller than 1 indicates that the clinical score in the first group is lower than that in second group, and the converse is true for values higher than 1.

3.4. Histopathological findings

EAE mice had a greater number of spinal cord lesions in the lumbar and cervical areas than naïve control mice at week 4 ($p \leq 0.01$ for cervical) and week 6 ($p \leq 0.001$ for both lumbar and cervical) (Fig. 5c). CS exposure, in general, did not affect the number of lesions in naïve control mice. In EAE mice, both low and medium concentrations of CS caused an increase in the total number of lesions by nearly 3-fold in the cervical spinal cord region relative to sham exposure at week 6 ($p \leq 0.05$ for both concentrations). This effect was also observed in the lumbar part of the spinal cord, although it was not significant for CS Low exposure. Exposure to the high concentration of CS, on the other hand, did not affect the total number of lesions in the cervical or lumbar spinal cord region relative to fresh air exposure. The only exception was an increase in the total number of lesions in the cervical spinal cord at week 4 relative to fresh air exposure ($p \leq 0.05$). Lastly, Spearman's rank correlation analysis confirmed that there was a significant correlation between the clinical score and total number of lesions in both lumbar and cervical samples ($p \leq 0.001$). In line with the histopathological findings, EAE mice showed increased levels of signal transducer and activator of transcription 3 (STAT3), STAT5, and some cytokines (interleukin 17 A, C-X-C motif chemokine ligand 1 [CXCL1], and monocyte chemoattractant protein-1) in the thoracic spinal cord relative to naïve control mice ($p \leq 0.05$), suggesting increased inflammatory response in the spinal cord of EAE mice (Supplemental Table 6). Interestingly, the levels of CXCL1 (a chemokine which acts as a major neutrophil chemoattractant in regulating inflammatory responses) were first decreased in EAE mice before the onset of clinical symptoms when they were exposed to medium and high concentrations of CS; this effect, however, diminished afterwards. In general, chemokine CXCL1 levels were significantly increased in the EAE mice at week 6, independent of the stimulant used (CS). This observation emphasizes the functional role of CXCL1 in stimulating neutrophil migration within the CNS and its contribution to neuroinflammation and demyelination in EAE [26,113]. On the other hand, initial assessment of the brain sections by hematoxylin and eosin staining revealed a limited number of noticeable lesions in some sections, and the differences between the treatment groups were not observable (data not shown); thus, no further analyzes were conducted for the brain. Even though limited CNS infiltrates and brain lesions similar to ours were reported in MS mouse models [20,54], some recent advance analyses enabled further insights at the lesion formation in the brain [27,74,109].

3.5. Clinical chemistry profile

Serum samples were collected during necropsy and analyzed for various blood chemistry parameters, representing, for example, liver and renal function and energy metabolism (Fig. 6 and Table 2). In general, albumin levels were reduced by 8% and 4% at weeks 4 and 6, respectively, in EAE mice relative to naïve control mice (at equivalent exposure). In contrast, globulin levels were elevated by 7% and 6% at weeks 4 and 6, respectively, in EAE mice relative to naïve control mice exposed to the same CS concentrations. The major portion of total protein is albumin (approximately 70%), and the change in total protein was negligible (3%) at week 4 for the same comparison (EAE vs. naïve control). The enzymatic activity of alkaline phosphatase was reduced by 47% and 13% at weeks 4 and 6, respectively, in EAE mice relative to naïve control mice. A similar reduction in alanine aminotransferase activity (by 32% at week 4) was seen for the same comparison.
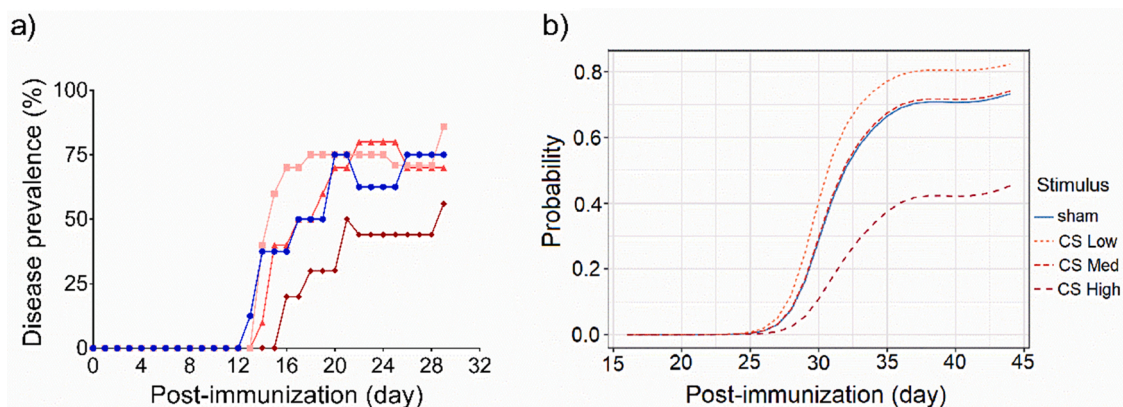

Fig. 4. Disease prevalence in EAE mice exposed to various concentrations of CS. (a) Descriptive data indicating the average daily probability of disease occurrence are shown (n = 7–10). Sham = blue lines with solid circles (●), CS Low = pink lines with solid squares (■), CS Med = red lines with solid triangles (▲), and CS High = brown lines with solid diamonds (◆). (b) GAM analysis was used to estimate the probability of disease curves over time. Disease prevalence was the lowest in EAE mice exposed to the high concentration of CS. Sham = blue lines, CS Low = pink lines, CS Med = red lines, CS High = brown lines. n = 8 mice per group, except n = 7 in the 6-week CS Low, n = 10 in the 6-week CS Med, and n = 9 in the 6-week CS High groups (Supplemental Table 1). CS: cigarette smoke; EAE: experimental autoimmune encephalomyelitis; GAM: generalized additive model.
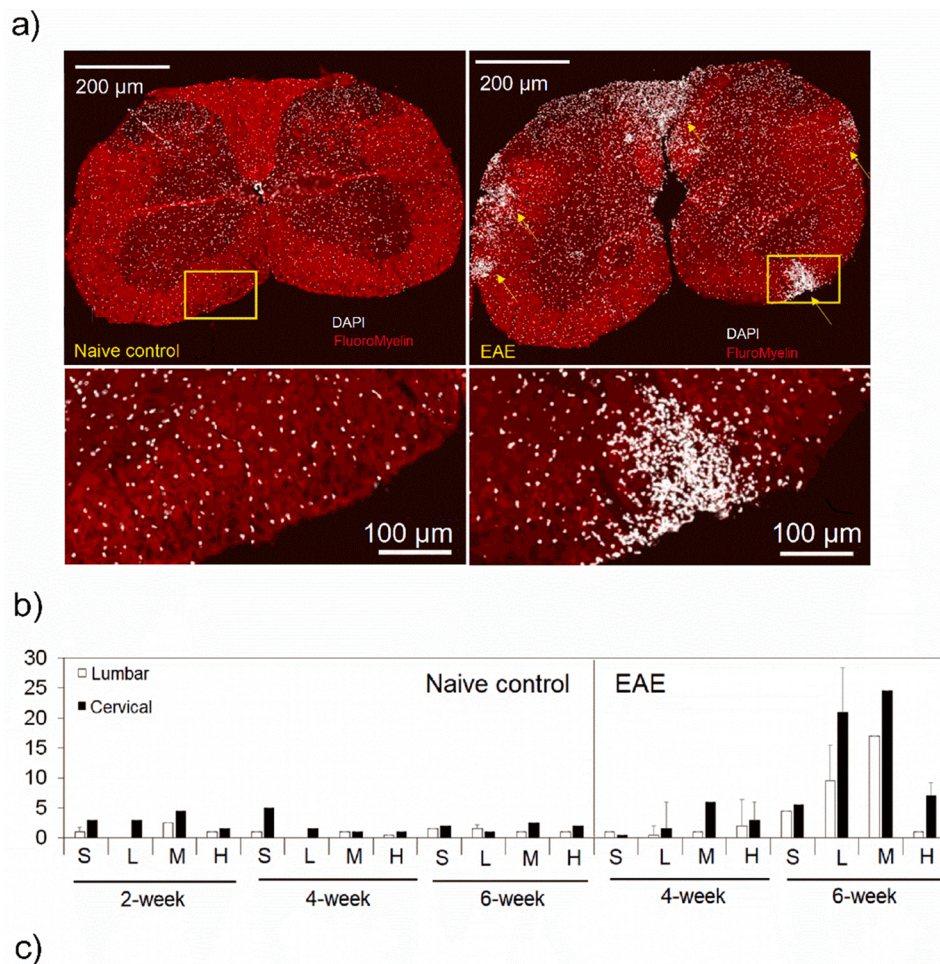
Fig. 5. Spinal cord pathology in EAE mice exposed to various concentrations of CS. (a) Representative images of spinal cord sections labeled with DAPI (white) and FluroMyelin (red) from naïve control (left) and EAE (right) mice are presented. (b) Total number of lesions are presented as the median ± MAD for naïve control and EAE mice exposed to different concentrations of CS. (c) Statistical analysis of total number of lesions in the spinal cord, indicating a general increase in the number of lesions in EAE mice exposed to the low or medium concentration of CS. Abbreviations: S = sham, L = CS Low, M = CS Med, H = CS High. Statistical significance: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. n = 8 mice per group, except n = 7 in the 6-week CS Low, n = 10 in the 6-week CS Med, and n = 9 in the 6-week CS High groups (Supplemental Table 1). CS: cigarette smoke; EAE: experimental autoimmune encephalomyelitis; MAD: mean absolute deviation; DAPI: 4′,6-diamidino-2-phenylindole.

Comparison	Section	2-week Difference	4-week Difference	6-week Difference
EAE vs Naïve	Lumbar		1.65	7.17***
EAE vs Naïve	Cervical		2.12**	7.59***
CS Low vs sham	Naïve	Lumbar	0.08	0.34	1.08
CS Med vs sham	Naïve	Lumbar	0.89	0.19*	1.07
CS High vs sham	Naïve	Lumbar	1.43	0.27	1.07
CS Low vs sham	Naïve	Cervical	1.17	0.42	1.33
CS Med vs sham	Naïve	Cervical	1.63	0.38	0.97
CS High vs sham	Naïve	Cervical	0.66	0.39	0.90
CS Low vs sham	EAE	Lumbar		0.75	2.33
CS Med vs sham	EAE	Lumbar		0.84	3.22*
CS High vs sham	EAE	Lumbar		2.42	1.14
CS Low vs sham	EAE	Cervical		0.93	2.88*
CS Med vs sham	EAE	Cervical		1.67	2.93*
CS High vs sham	EAE	Cervical		3.47*	0.96

The effect of CS exposure on liver function-related biochemical endpoints (i.e., protein levels and alkaline phosphatase activity) was also detected when the data from mice exposed to fresh air (sham) and CS were analyzed together. For example, albumin levels were elevated by 6% in the CS Med group and by 9% in the CS High group at week 4, which was in line with the significantly higher total protein levels in these groups at week 4 (Table 2). Although globulin levels were decreased at week 6 in the CS High group, this decrease did not affect the total protein concentration, which supports the fact that globulin makes up a smaller proportion of total protein than albumin. In addition, CS exposure had different effects on EAE and naïve control mice. Naïve control mice exposed to the medium concentration of CS showed increased levels of total protein and globulin (8% and 14%, respectively), while EAE mice exposed to this concentration of CS had increased levels of total protein and alkaline phosphatase activity at week 4 (5% and 38%, respectively).
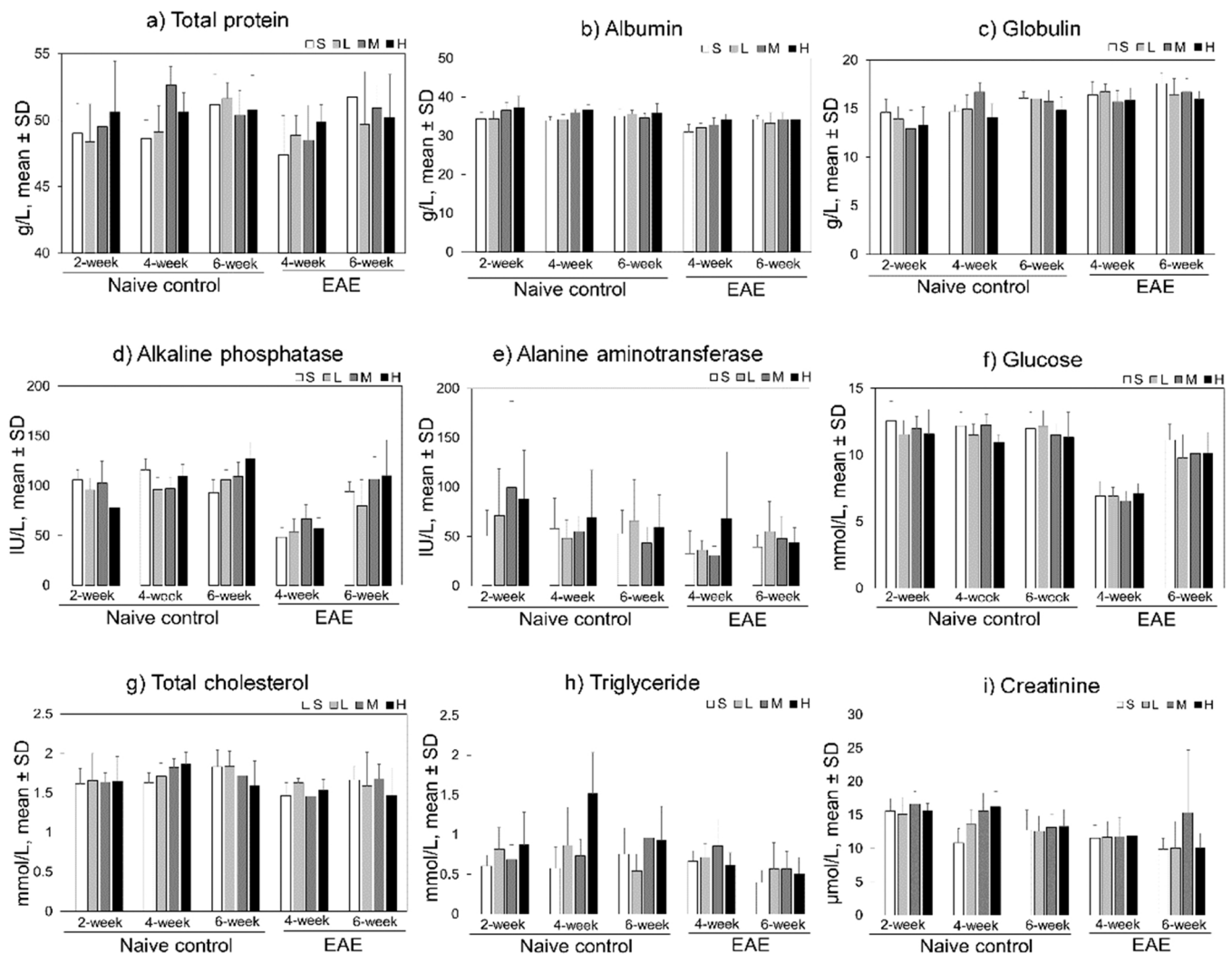
Fig. 6. Effects of EAE and CS on blood chemistry parameters. Levels or activities of serum biochemical markers (a) total protein, (b) albumin, (c) globulin, (d) alkaline phosphatase, (e) alanine aminotransferase, (f) glucose, (g) total cholesterol, (h) triglyceride, and (i) creatinine are presented as mean ± SD. Sham (S) = white bars, CS Low (L) = light grey bars, CS Med (M) = dark grey bars, CS High (H) = black bars. Statistically significant effects are summarized in Table 2. n = 8 mice per group, except n = 7 in the 6-week CS Low, n = 10 in the 6-week CS Med, and n = 9 in the 6-week CS High groups (Supplemental Table 1). CS: cigarette smoke; EAE: experimental autoimmune encephalomyelitis; SD: standard deviation.

Additional changes induced by EAE included a reduction of 10–42% in serum creatinine, cholesterol, triglyceride, and glucose levels at weeks 4 and 6 relative to naïve control mice. In contrast, CS exposure caused an increase in the levels of triglycerides and creatinine at week 4 relative to fresh air exposure, but the differences were only statistically significant in CS High mice (increased by 58% and 23%, respectively; both $p \leq 0.05$). These CS exposure effects were mainly observed in naïve control mice, with the CS High group showing nearly 3-fold and 2-fold increases in the levels of triglycerides and creatinine, respectively, at week 4. In addition, cholesterol levels were also elevated at week 4 in naïve control mice exposed to the medium or high concentrations of CS. There were no statistically significant changes in these energy metabolism parameters or creatinine levels in EAE mice exposed to any concentration of CS. Lastly, only minor changes and no obvious trends were observed in serum electrolyte and mineral levels in all study groups (Supplementary Fig. 4).

4. Discussion

MS may be caused by various genetic and environmental factors, though identification of specific combinations of factors giving rise to disease remains elusive. In this study, we evaluated the effect of different doses of CS exposure on the development of MS in the EAE mouse model. As smoking is associated with an increased risk of MS [100,118], we performed the experiment using mice that were exposed to CS for 2 weeks prior to EAE induction in addition to being continuously exposed to CS during the disease course. Although animal models cannot fully recapitulate human conditions, our paradigm attempted to closely mimic what could be the risk and impact of CS exposure for the development of MS. Because MS affects women preferentially (with a female: male ratio approaching 3:1) and the differential susceptibility to EAE of male and female mice is well accepted [16,84], we used female C57BL/6 mice for our EAE model. The use of female mice also reduced the potential risk of experimental variability, which could have been introduced by aggression and penile prolapse in male mice at advanced stages of EAE.

In the present study, EAE symptoms typically included a limp tail and wobbly gait due to paresis of the limbs, progressing to paralysis of the hindlimbs or forelimbs in some severe cases, as previously reported [6]. The mice lost weight immediately after EAE induction; the body weight slowly increased to the baseline level, only to be reduced again around the onset of clinical symptoms. The body weight of most affected mice

Table 2
Statistically significant changes in serum biochemical markers.

Panel	Blood chemistry parameters	Changes due to EAE	Changes due to smoke exposure	Changes due to smoke exposure (naïve control)	Changes due to smoke exposure (EAE)
Liver function	Total protein	3% ↓ w4**	5% ↑ M w4** 5% ↑ H w4**	8% ↑ M w4***	5% ↑ H w4*
	Albumin	8% ↓ w4*** 4% ↓ w6***	8% ↑ H w2* 6% ↑ M w4** 9% ↑ H w4***		
	Globulin	7% ↑ w4*** 6% ↑ w6**	8% ↓ H w6**	14% ↑ M w4**	
	Total bilirubin				
	Alkaline phosphatase	47% ↓ w4*** 13% ↓ w6**	27% ↓ H w2***		38% ↑ M w4***
	Alanine aminotransferase	32% ↓ w4**			
	Aspartate aminotransferase				
Energy metabolism	Cholesterol	13% ↓ w4*** 10% ↓ w6*		12% ↑ M w4* 15% ↑ H w4*	
	Triglyceride	35% ↓ w6***	58% ↑ H w4**	275% ↑ H w4***	
	Glucose	42% ↓ w4*** 13% ↓ w6***			
Renal function	Creatinine	16% ↓ w4*** 16% ↓ w6**	23% ↑ H w4*	45% ↑ M w4** 51% ↑ H w4***	
	Urea				
Electrolytes	Chloride		2% ↓ M w4* 3% ↓ H w4*** 2% ↑ L w6 *		
	Sodium		1% ↑ L w4 * 1% ↑ M w4 * 1% ↑ H w4**		
	Potassium	8% ↓ w4*** 7% ↑ w6 *			
Minerals	Calcium		5% ↑ M w4*** 6% ↑ H w4***		
	Inorganic phosphate				

Remarks:

Up arrows represent percent increase and down arrows represent percent decrease relative to sham or naïve controls.

Abbreviations: S = sham; L = CS Low; M = CS Med; H = CS High; w2 = 2-week; w4 = 4-week; w6 = 6-week. Statistical significance: *$p \leq 0.05$, * *$p \leq 0.01$, and * **$p \leq 0.001$.

began to stabilize and increase after 23 days post-immunization, paralleling a slight decrease in clinical scores by 0.5 or 1 before the scores stabilized. These fluctuations in EAE progression are in agreement with previous reports [20,23,78,104]. In addition, mice exposed to low and medium concentrations of CS showed a general tendency towards more severe disease symptoms. This observation supports the epidemiological data indicating that smoking can increase the risk and worsen the prognosis of MS; it also supports the validity of using the rodent model of EAE under a CS inhalation regimen for examining the link between MS conditions and smoking [34,48,73,100,112]. The level of clinical symptoms observed may be considered on the mild side in the current study, which may be due to the use of inhalation exposure chamber. The same EAE induction paradigm, resulting in slightly higher clinical scores, was observed in a preliminary study without the inhalation exposure (data not shown). Nevertheless, the effects of EAE were clearly observable in this study, and the mild EAE in the fresh air-exposed animals allowed the detection of worsening effect of low and medium concentrations of CS without reaching humane endpoints. Therefore, the EAE condition was rather optimal for current study design with inhalation exposure.

The manifestation of spinal cord pathology coincided with the onset of clinical symptoms in this study. The number of cell clusters—likely representing infiltrating cells in spinal cord lesions, as previously reported by others [61,93,115]—was increased in EAE mice approximately 10 days post-immunization (week 4), and this became more evident at 4 weeks post-immunization (week 6). Exposure to low and medium concentrations of CS caused an increase in the number of lesions relative to sham or CS High exposure, which was consistent with the worsened clinical symptoms observed in the CS Low and Med groups relative to the sham and CS High groups. These observations suggest that

there is a good correlation between spinal cord pathology and clinical manifestation, as previously reported [23,104].

EAE mice exposed to the high concentration of CS did not show significant changes relative to fresh air-exposed EAE mice in disease onset, progression, severity, prevalence, or spinal cord pathology. The reasons for this apparent lack of effect might be manifold. It is possible that stress to the mice was a considerable factor in this study, and increased stress levels due to the aversive sensory experience associated with high concentration of CS could have had an impact on the outcome. We and others have observed that rodents typically show signs of stress related to CS exposure, exposure modality, as well as handling [21,81]. Whether increased stress have an impact on EAE might depend on the paradigms used for inducing the stress [12,17,24,76], but stress-induced elevation of blood corticosterone level has been correlated with reduced severity of EAE symptoms [25]. Thus, further investigation on the effect of stress on EAE development and progression is needed to improve our understanding of their interdependence in experimental conditions.

The effects of CS exposure on immunity are extremely complex, which may partially explain the observed dose-dependent effect of CS on the EAE pathology. Interestingly, a similar inverted U-shape concentration dependent effect of CS extract on the TNF-α and IL-8 release has been reported using a human macrophage cell line [22]. In fact, smoking has been linked to reduced T-cell responses and proliferation in vitro and in vivo [35,83,98]. More specifically, the effect of CS on systemic immune response has been reported to involve Th17-mediated immunomodulation of pulmonary inflammation, which, in turn, affects CNS autoimmunity relevant for the pathogenesis of MS and EAE [45,46]. Previous studies have shown that nicotine can also control immune responses through Th17 [43,56,72]. Thus, it is possible that the adaptive immune T-cell response modulated by nicotine in the lungs may at least

partially explain the effect of CS on EAE pathogenesis. Typically, CS promotes inflammation by inducing the production of pro-inflammatory cytokines in the airway [18]. However, nicotine was also shown to decrease IL-6, IL-8 and IL-10 production [4]. In fact, CS may alter the immune profile of macrophages and dendric cells in a variety of ways, some of which are contradictory [99]. The impact of CS in certain T cell subtypes are prevalence in blood and tissues, with smokers showing increased circulating CD3+ T and CD4+ T as well as Th17 lymphocytes counts, but where smoking was associated with increased numbers of CD8+, Th1 and Th17 cells in bronchoalveolar fluid only [99]. Mice exposed to CS recovered poorly from influenza pneumonia, which was associated with the reciprocal expression of IFN-γ and IL-17A in the lungs but not spleen or draining lymph nodes [37]. This is likely to be similar for other animal disease models. However, the plethora CS effects in the context of acute and chronic inflammation leaves an ambiguous understanding of the mechanisms underlying CS-induced modifications of innate and adaptive immunities [39,47,50,58,85,103].

Furthermore, different constituents of CS have been reported to have pro- and anti-inflammatory effects in nonclinical studies that might differentially affect EAE disease outcomes depending on their concentrations [51]. For example, a previous study linked the delayed onset of EAE after CS exposure to the modifications of microglia via nicotinic acetylcholine receptors (nAChR) in SJL and C57BL/6 mice [20], indicating the importance of nicotine alkaloids in regulating the EAE outcome. In addition to nicotine alkaloids, several bioactive agents present in CS, such as nicotine, carbon monoxide, acrolein, and reactive oxygen species, have immunomodulatory effects [51]. For example, acrolein, a highly reactive and toxic β-unsaturated aldehyde capable of instigating and perpetuating oxidative stress, has been demonstrated to be a neurotoxin in MS and in the EAE model [65,75,92,105,106]. Interestingly, hydralazine, a known acrolein scavenger, can significantly improve behavioral outcomes and lessen myelin damage in the spinal cord of EAE mice [52]. Other toxic CS constituents, such as hydrogen cyanide and nitric oxide, have been shown to induce demyelination lesions in experimental studies [30]. In addition, CS also contains trace amounts of microbial cell components, including bacterial lipopolysaccharide, as well as tar and nitric oxide, which are known pro-inflammatory factors [51,70,106]. These and other CS constituents can induce inflammatory responses and potentially promote autoimmunity, which would be detrimental in MS. In contrast, although nicotine can suppress the innate and adaptive immune system and is not risk-free, it might also have therapeutic potential as a neuroprotective and anti-inflammatory agent for the nAChR (e.g. α7- and α9-nAChR subunits) could be the potential targets for modulation [20,55,98]. Several studies have reported that nicotine exposure significantly delays and attenuates inflammatory and autoimmune responses in the mouse EAE model, whether nicotine administration begins prior to, at the time of, or after immunization with myelin antigens for inducing EAE [20,23,68,72,96]. Nicotine may inhibit the spreading of myelin-reactive T cells from the periphery to the CNS, and the immunological effects induced by nicotine may also have contributed to the decrease in T cell proliferation and alteration in cytokine profiles in vitro and in vivo [82,96]. Therefore, further investigations on the effects of different CS doses and various CS constituents are warranted to understand the exact mechanisms responsible for smoking-mediated immunopathology and to address the lingering questions around its dual effects on immune responses.

It is noteworthy that this study is the first to report a wide range of serum biochemical markers for understanding the general physiological effects of EAE and CS exposure in a preclinical model of MS. Although a number of studies have reported the involvement of liver and renal function as well as an association of energy metabolism with MS, only a few have reported these data for the EAE mouse model thus far. In this study, we found that alkaline phosphatase activity and triglyceride and glucose levels in EAE mice were reduced by 35–40% relative to naïve control mice. Other clinical chemistry parameters were minimally

changed, often by less than 15% relative to the naïve control. How these changes translate into EAE clinical symptoms or pathology is yet unclear. Alkaline phosphatase, for example, is a ubiquitously expressed enzyme, present in many tissues, including bone, intestines, kidney, liver, and white blood cells. This enzyme is suggested to play a particularly important role in liver function and bone development [95]. In patients with MS, the activities of alkaline phosphatase can be increased or decreased depending on the biological sample analyzed [28,40,49,77]. Interestingly, alkaline phosphatase treatment of EAE mice can reduce the neurological signs of EAE if administered pre-symptomatically [40], indicating that the enzyme might have a potential regulatory role in EAE.

Leakage of the BBB is a common pathological feature in MS, whereby albumin—the most abundant protein in plasma—could gain access to CNS tissues, where it is exposed to an inflammatory milieu. Here, albumin can participate in protective mechanisms by becoming a target for oxidation and nitration reactions [53]. Furthermore, albumin binds metals and heme, thereby limiting their ability to produce reactive oxygen and reactive nitrogen species [53]. Serum albumin levels can also be reduced as a result of the liver switching from producing albumin to making more urgent proteins during inflammation [94]. Therefore, the decreased albumin levels in EAE mice relative to naïve control mice in our study might signify a general response of the body to inflammation. In addition, the reduction in albumin levels together with the reduction in alanine aminotransferase activity in EAE mice (relative to naïve control mice) could potentially indicate physiological responses to EAE inflammatory responses, in addition to the lower muscle mass, frailty, and risk of mortality reported in humans [19,89]. In the present study, we observed higher levels of serum liver function parameters (such as total protein and albumin) in CS-exposed naïve control mice than in sham mice. However, the extent of these changes were within the physiological ranges for C57BL/6 mice [9]. In addition, even though CS often has been linked to exacerbation of underlying liver diseases [33,57], alkaline phosphatase activity was only transiently altered in this study, without concentration-dependent changes.

Another biochemical observation that deserves attention is the change in cholesterol and triglyceride levels. Serum lipids, including cholesterol and triglycerides, are affected by MS. Cholesterol has been discussed mainly as a contributing factor for demyelination in the brain [7,13,69,71,101]. The serum concentrations of total cholesterol and triglycerides, in general, are elevated in patients with MS [91,111]. In our study, however, the levels of cholesterol and triglycerides were reduced by 10–13% and 35%, respectively, in EAE mice. The cause of this reduction might be related to the possible loss of muscle and fat mass, which could have jointly contributed to a reduction in body mass, as previously reported in EAE [7]. In addition, possible differences in food intake between the EAE and naïve control mice might have also caused variations in lipid profiles [60], as none of the mice were fasted prior to necropsy. It is also interesting to note that the metabolic parameters (i.e. cholesterol and triglyceride levels) were increased in naïve control mice following CS exposure, which further confirmed lipid accumulation in the plasma and liver in CS-exposed mice [10]. These findings are in contrast to the reductions in these levels previously reported in rats exposed to CS or nicotine in subchronic inhalation studies [36,80,114]. Inconsistencies in observations were also noted in other unpublished in-house data from different strains, which suggests that the duration of exposure or strain-specific differences can influence serum cholesterol levels.

Similarly, serum creatinine levels were also reduced by approximately 16% due to EAE induction in our study. Under normal circumstances, serum creatinine is a widely used marker in assessment of renal function. In fact, in MS, creatinine clearance can be reduced because of kidney failure, which can affect the levels of circulating creatinine [11,44,101]. A study on progressive MS had reported that the estimated glomerular filtration rate (eGFR) of patients was significantly lower than that of healthy individuals, revealing disruption in kidney function.

However, the serum creatinine levels of the patients were normal or lower than normal. In progressive MS, a high volume of muscle mass loss can lead to a decline in serum creatinine levels. Therefore, this effect can mask the real changes in eGFR [11,88]. In such a scenario, serum creatinine level might only be an indication of muscle weakening or deterioration [79,101]. In contrast, in our study, naïve control mice exposed to the high concentration of CS had higher creatinine levels, which might indicate the risk of renal functional deterioration instead of increased muscle mass. Therefore, serum clinical chemistry results should be interpreted with caution and be considered within a framework of the global physiology of the animals. In addition, our results showed only minor changes in serum electrolyte and mineral levels, suggesting a less critical role of these parameters in representing the physiological changes associated with EAE or CS exposure.

5. Conclusion

This is the first study to investigate and demonstrate the effects of different doses of CS exposure in a preclinical model of MS, with an extensive list of endpoints, including clinical symptoms, spinal cord pathology, and serum clinical chemistry. Our study has corroborated the findings of previous human studies by showing that CS exposure exacerbates clinical symptoms and spinal cord pathology. Furthermore, we have extended the correlation between MS and a mouse EAE model by using clinical chemistry parameters (e.g., cholesterol, triglycerides, and glucose levels, alkaline phosphatase and alanine aminotransferase activities, and creatinine level) that could be considered as cross-species biomarkers when investigating MS. To our knowledge, this is the first study that has attempted to make this bridge across multiple serum markers. We observed that a high concentration of CS does not have any observable effect on EAE. The reason for this dichotomy between the different doses of CS is unclear, but may be partially explained by some reports indicating inhibition of pro-inflammatory infiltrates into the CNS via the interplay between nAChRs and some tobacco alkaloids [20, 68,78]. The complexity of how various tobacco components could interact has been indicated in the works by Hedström showing a protective effect of moist snuff in contrast to the negative impact cigarette smoking has on MS [32]. Thus, further investigations are needed to decipher the specific combinatorial effects of the various key pro- and anti-inflammatory agents in order to understand the complex role of CS in MS. Another aspect requiring further attention is the effect of increased stress levels associated with CS exposure on EAE development and progression. The recognition of specific mechanisms by which CS affects host immunity is an important step towards elucidating mechanisms of cigarette smoking-associated diseases.

CRediT authorship contribution statement

JH conducted, analyzed, interpreted and summarized the in-life experiments, clinical observations, clinical chemistry endpoints, prepare the figures as well as authored this manuscript. **KK** coordinated, analyzed, interpreted and summarized the histopathology and Luminex analyses as well as authored this manuscript. **WX** established the EAE model in the test facility. **KL** and **BP** provided supervision and important intellectual content in study design, execution and reporting. **AK** and **LG** provided statistical evaluation. **MP** and **JH** provided overall supervision and important intellectual content.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors of this study are employees of Philip Morris International. Philip Morris International is the sole source of funding and sponsor of this research.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.03.032.

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