

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**DETERMINING THE ROLE OF *CCL4* GENE IN MULTIPLE
SCLEROSIS**

FATMANUR AKPUNAR
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CCL4 GENİNİN MULTİPL SCLEROZDAKİ
ROLÜNÜN BELİRLENMESİ

FATMANUR AKPUNAR
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

Multiple Sclerosis (MS) is a chronic neuroinflammatory disease of the central nervous system (CNS) that effects approximately 2.5 million people worldwide. It causes serious physical disabilities in patients with the symptoms of sensory and visual disturbances, motor impairments, fatigue, pain, and cognitive deficits. Like many neurodegenerative diseases, there are key questions that are unanswered yet. Although there are widely performed experiments in order to understand the pathology of MS, the clinical applications are limited and it is important to develop new biomarkers for diagnosis. The C-C motif chemokine ligand 4 (CCL4) gene is one of several cytokine genes that are clustered on the q-arm of chromosome 17. Cytokines are a family of secreted proteins that function in inflammatory and immunoregulatory processes. There are some recent studies that demonstrate CCL4 may have a role in MS. In our experiment, we aimed to show its role in mice by knocking-down the CCL4 gene. The animal model of MS, Experimental autoimmune encephalitis (EAE), was induced with Proteolipid protein (PLP) on 24 female, 8-week old Balbc mice, and intraperitoneal shRNA-LV injection was done 2 hours before PLP immunization. Clinical score and weight (every 2 days) were performed for 20 days. ELISA results demonstrated that immunization performed succesfully. Stainings of brain and spinal cord sections proved pathological evidence of MS model such as BBB leakage and cell infiltration in the lesions. %Weight change of sham and scrambled shRNA were significantly higher then PLP and CCL4 groups. The CCL4 silenced group showed significantly higher scores as PLP group showed a recovery decrease at day 16 even though inverted screen test at day 16 provides PLP group strength significantly lower then CCL4 group. All these results points out to the possible role of CCL4 in MS pathophysiology and future studies are granted.

Key Words: MS, CCL4, PLP-EAE model, Gene Silencing, shRNA LV.

ÖZET

Multipl Skleroz (MS), dünya çapında yaklaşık 2,5 milyon insanı etkileyen merkezi sinir sisteminin (CNS) kronik nöroinflamatuvar bir hastalığıdır. Duyusal ve görme bozuklukları, motor bozukluklar, yorgunluk, ağrı ve kognitif yetersizlik semptomları olan hastalarda ciddi fiziksel sakatlıklara neden olur. Birçok nörodejeneratif hastalık gibi, henüz cevaplanmamış kilit sorular mevcuttur. MS patolojisini anlamak için yaygın olarak yapılan deneyler olmasına rağmen, klinik uygulamalar sınırlıdır ve tanı için yeni biyobelirteçlerin geliştirilmesi önemlidir. C-C motifli kemokin ligandı 4 (CCL4) geni, kromozom 17'nin q-kolunda kümelenmiş birkaç sitokin geninden biridir. Sitokinler, enflamatuar ve bağışıklık düzenleyici süreçlerde işlev gören bir salgılanmış protein ailesidir. CCL4'ün MS'de bir rolü olabileceğini gösteren bazı yeni çalışmalar vardır. Çalışmamızda, CCL4 genini susturarak farelerde rolünü göstermeyi amaçladık. MS'nin hayvan modeli, Deneysel otoimmün ensefalit, 24 dişi, 8 haftalık Balbc faresi üzerinde Proteolipid proteini (PLP) ile indüklendi ve intraperitoneal shRNA-LV enjeksiyonu, PLP ile immünizasyondan 2 saat önce yapıldı. Klinik skor ve ağırlık (2 günde bir) 20 gün boyunca ölçüldü. ELISA sonuçları, başarılı bir şekilde gerçekleştirilen PLP immünizasyonunu gösterdi. Beyin ve omurilik boyamalarında, lezyonlarda BBB sızıntısı ve hücre infiltrasyonu gibi MS modelinin patolojik kanıtları tespit edildi. Sham ve scrambled gruplarının % ağırlık değişimi, PLP ve CCL4 gruplarından anlamlı ölçüde daha yüksek bulundu. Yapılan davranış analizinde PLP grubunun sahip olduğu motor becerilerdeki gücü daha az bulunmasına karşın, PLP grubunda 16.günde CCL4 ile susturulmuş gruba göre anlamlı bir skor azalışı ve iyileşme vardı. Bütün bu bulgular CCL4'ün MS de var olabilecek potansiyel rolü için gelecekte yapılacak çalışmaları desteklemektedir.

Anahtar Kelimeler: MS, CCL4, PLP-EAE model, Gen Susturma, shRNA LV.

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
MS	: Multiple Sclerosis
CNS	: Central Nervous System
RRMS	: Relapsing-remitting multiple sclerosis
CCL4	: C-C motif chemokine ligand 4
CCR5	: C-C motif chemokine receptor type 5
CSF	: Cerebrospinal Fluid
PLP	: Proteolipid protein
ELISA	: Enzyme-linked immunosorbent assay
RT-PCR	: Reverse transcription polymerase chain reaction
EAE	: Experimental autoimmune encephalomyelitis
PTX	: Pertussis Toxin
shRNA LV	: Short hairpin RNA
PFA	Paraformaldehyde
ddH ₂ O	: Double distilled water
FBS	: Fetal Bovine Serum
PBS	: Phosphate buffered saline
RPMI	: Roswell Park Memorial Institute
RBC	: Red blood cell
TBM	: Tetramethylbenzidine
HCl	: Hydrogen chloride
DMSO	: Dimethyl Sulfoxide
TAE	: Tris-acetate-EDTA
CFA	: Complete Freund's Adjuvant
DPBS	: Dulbecco's phosphate buffered saline
OCT	: Optimum cutting temperature
PBMC	: Peripheral blood mononuclear cell

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1. INTRODUCTION

Multiple sclerosis [MS] is an inflammatory neurodegenerative disease, causing the formation of lesions because of demyelination of the central nervous system which effects 2.5 million people in the world with a higher frequency in women [1,2]. Pathological changes of MS can be defined as 4 facts; inflammation, demyelination, axonal loss or impairment and gliosis as astrocytic response [1,3]. MS can effect various sides of the central nervous system (CNS) such as the brain, spinal cord, and the optic nerves [4]. Lesions may present in both white matter and the grey matter of the brain, and are often located around the ventricles, the optic nerve, the brain stem, and the spinal cord [1].

Heterogeneity in the symptoms depends on theseverity of the inflammation and location of the affected neurons. Most common symptoms are impairment in vision, balance control, muscle control, and other essential body functions. Most common subtype of the MS which effects 85% of the patients is relapsing-remitting MS (RRMS) [5]. MS attacks are called as relapse and following recovery as decrease in the severity of the symptoms is called as remission [6]. This relapsing-remitting period can be followed by a progressive phase and this subtype is called secondary progressive MS. The other type showing disease progress with no relapsing from the beginning and called as primary progressive MS [7](Figure 1).

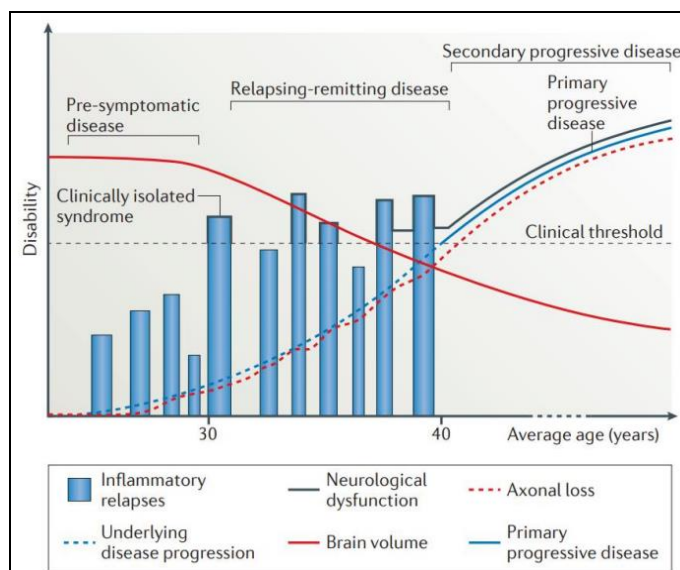


Figure 1: Types of MS.

The potential causes of the disease can be genetic (HLA and non-HLA associated genes) or environmental immune triggers such as herpesviruses, vitamin D, obesity, smoking and night shifts [8] (Figure 2). The biggest question in the MS pathology is that the disease triggers inflammation in the CNS or periphery first. The peripheral activation of autoreactive T cells by molecular mimicry of CNS autoantigens cause entry of pathogenic CD4⁺ T Helper 1 cells and T helper 17 cells from lymph nodes to the circulation [2]. Selective lymphocyte entry into the central nervous system and crossing the blood brain barrier is indicative of an lesion [9]. Infiltration and activation of specific pathogenic autoreactive T cells, B cells and macrophages in CNS lesions are main components of MS immunopathogenesis, which is mediated by the interaction of different chemokines [10,11]. The superfamily of secreted proteins known as chemokines mediates chemotaxis [12].

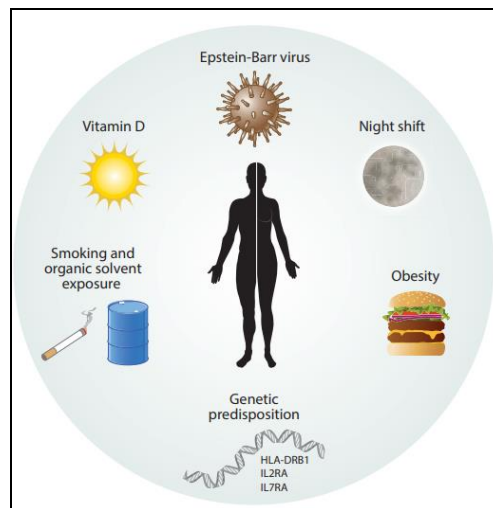


Figure 2: Causes of MS.

Inflammatory cytokines such as C-C motif chemokine ligand 4 (CCL4) have a function in the pathophysiology of neurologic disorders such as MS [13]. CCL4, also known as macrophage inflammatory protein 1 (MIP-1), is selective for CCR5 receptors found on chromosome 17 [11]. The chemokine CCL4 is a chemoattractant for monocytes, natural killer cells, and other immune cells [14,15,16]. The first study which shows the relation of CCL4 and MS was performed by checking mRNA level of CCL4 in MS lesions. They specifically showed presence of CCL4 in demyelinating brain tissues of MS patients. Strong CCL4 immunoreactivity is

detected in phagocytic parenchymal macrophages [13]. It is also demonstrated that higher concentration of CCL4 in serum cerebrospinal fluid (CSF) detected in RRMS patients compared to healthy persons. The levels of this chemokine correspond with the length of the sickness and the extended disability status scale score. Consequently, CCL4 may have a potential to be a biomarker for MS [15]. Another recent study also focused on effects of CCL4 on blood brain barrier endothelial cells and showed that CCL4 cause barrier disruption in neurovascular endothelium and induces inflammatory signaling [17].

Our unpublished data also shows one of the MIP1 protein CCL4L2 gene expression level in the B cells of RRMS patients are significantly changed compared to healthy humans.

The current applications of immunomodulatory drugs can decrease the immune cell activity and relapsing frequency but cause side effects. Even though successful effects of therapeutic approaches found in diseases associated T-cell mediated inflammation, the application of the treatment to MS patients had no effect or caused worsened disease progression [7]. It is important to enlightening the role of CCL4 in the pathology of MS for further diagnosis and treatment applications.

2. MATERIALS & METHODS

2.1. Experimental Outline

2.1.1. Aim of the Project

The study's goal is to determine the involvement of the CCL4 gene in MS. To determine the impact, the gene is silenced using short hairpin RNA (shRNA) carrying lentivirus particles, and Experimental autoimmune encephalitis, most common animal model for MS is performed by injecting Proteolipid protein (PLP), the CCL4 silenced mice are compared with the MS group to see whether there are any variations in behavior, immunological response, and immunopathology.

2.1.2. Animals and Experimental Design

24 female Balbc mice (8-12 weeks old) produced at Koç University Experimental Animal Production Center were used in the experiments as the groups are indicated in the TABLE 1. Animals were housed seven or eight per cage. They were placed in a quiet and temperature and humidity controlled room ($22 \pm 2^\circ\text{C}$ and $60\% \pm 5\%$, respectively) in which a 12/12 h light–dark cycle was maintained (07:00 h–19:00 h light). Food and water were available ad libitum. All experiments were performed at the same time and in the light period. All procedures in the study were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA) and the Declaration of Helsinki. Koç University ethical committee approval is also obtained (2022.HADYEK.011).

There are four groups in the project: Sham, PLP, Scrambled and CCL4. The general procedure in animal part has 2 applications; intraperitoneal injection of shRNA containing lentivirus solution or saline as gene silencing part and injection of EAE chemicals for MS modeling. Sham group received saline and non-PLP containing EAE chemicals as control where the PLP the active substance for immunization in EAE model. PLP group received first saline then PLP containing EAE chemicals as MS modeled without gene silencing. Scrambled group received

shRNA lentivirus which is not targeting CCL4 gene and then EAE (Experimental autoimmune encephalomyelitis) chemicals. The last group received CCL4 targeting shRNA lentivirus particles and EAE chemicals as silenced EAE group in the project (TABLE 1).

Table 1: Experimental groups.

Group No	Number of Animal	Group Name	Applications
1	n=6	Sham	Saline (i.p.) + Sham (CFA + PTX)
2	n=6	PLP	Saline (i.p.) + PLP EAE (PLP + CFA + PTX)
3	n=6	Scrambled	Scrambled Lentivirus (i.p.) + PLP EAE (PLP + CFA + PTX)
4	n=6	CCL4	CCL4 Lentivirus (i.p.) + PLP EAE (PLP + CFA + PTX)

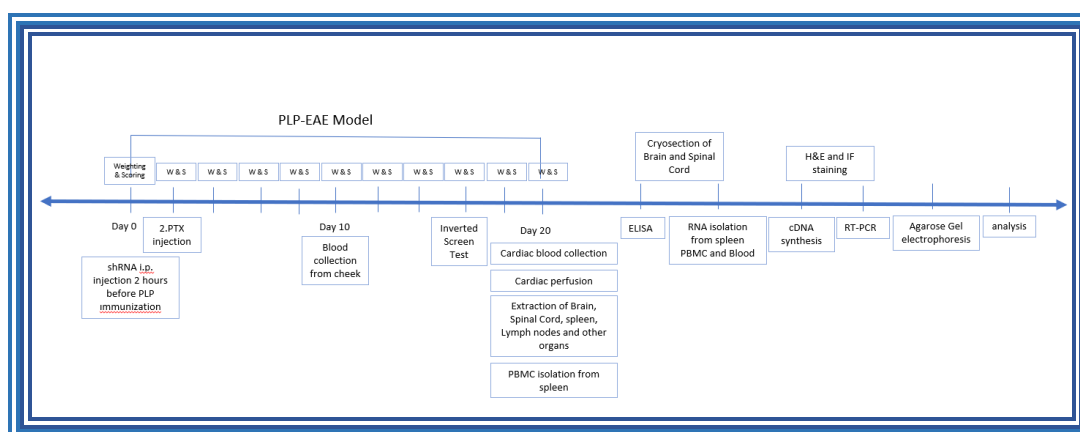


Figure 3 : Experimental Design. The experiments can be divided into two as in vivo part and molecular/histological part. Firstly gene is silenced and EAE is modeled and monitored during 20 days by checking weight change and intensity of the symptoms are scored. Then the blood and the organs are collected for molecular and histological part.

In mice, PLP - induced EAE was modeled to mimic RRMS symptoms. Blood is collected on days 10 and 20 for detection of the level of PLP in blood serum by ELISA. At day 20, spleens and lymph nodes are collected for cell isolation to monitor gene expression by RT-PCR to validate gene silencing successfully done by injection of shRNA containing lentivirus particles. Brains and spinal cords are

collected at day 20 for immunohistochemistry or immunofluorescence stainings. All the experiments are summarized on a time line in Figure 3.

2.2. PLP-Induced EAE Model

To produce the EAE model that mimics the RRMS disease subtype, PLP antigen was injected into Balbc mice to immunize. PLP139-151 (Peptides International, Louisville, KY, #PLP-3812-PI) was dissolved at 1 mg/mL in PBS. With a total volume of 200 μ l, 100 μ g of PLP139-151 peptide was emulsified with Complete Freund's Adjuvant (CFA) (1:1) containing 533 g of inactivated Mycobacterium Tuberculosis and injected subcutaneously (s.c.) into four locations as of isoflurane-anesthetized balbc mice. In addition, 250 ng of Pertussis Toxin (PTX) was administered two times via i.p. injections to each mouse 1 and 48 hours after immunization, as illustrated in Figure 4 [18] and chemical informations are given in Table 2.

Table 2: Injected dosage of the EAE chemicals, PLP,CFA and PTX.

Compound	Stock Concentration	Injected Dose	Total Volume	Ratio, Administration
PLP	1 mg/mL	100 μ g	100 μ l	1:1, s.c.
CFA	5.33 mg/mL	533 μ g	100 μ l	
PTX	5 μ g/mL	250 ng	50 μ l	i.p.

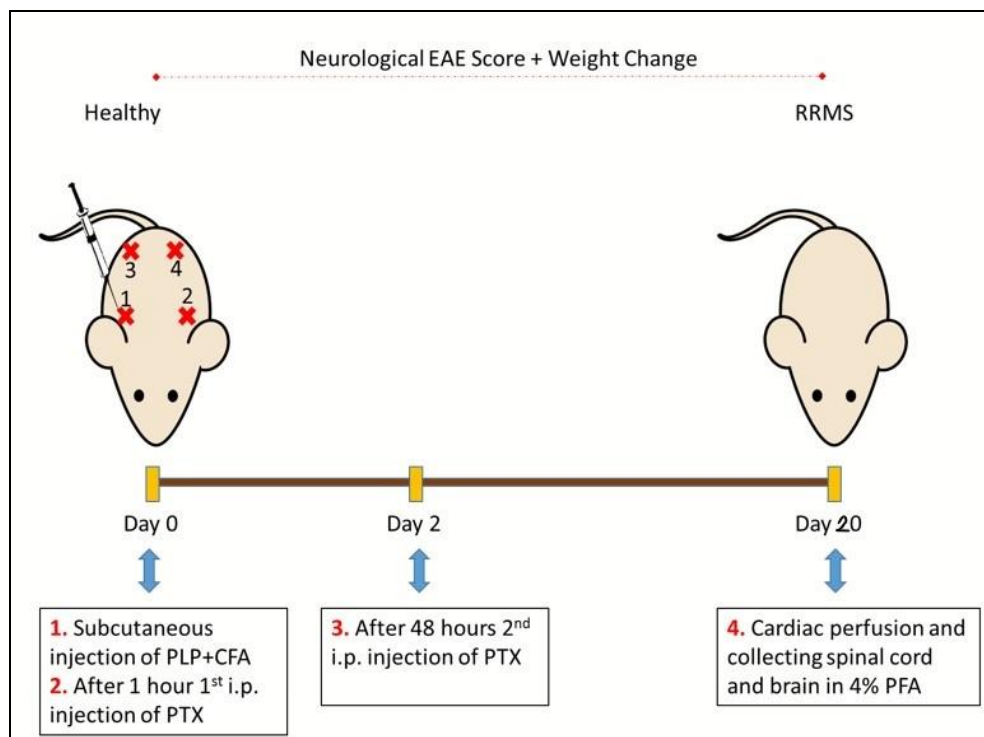


Figure 4 : Steps of inducing PLP-induced EAE model.

Every two days, the body weight and clinical EAE score were monitored. Clinical scoring performed according to given symptoms in Figure 5. At day 10, blood is collected from the cheek for ELISA.

Clinical score	Clinical symptoms
0	Mouse shows no symptoms of disease (asymptomatic)
1	Mouse has a limp tail (complete flaccidity, absence of curling at the tip) or hind limb weakness (waddling gait, mouse's hind limbs fall through the top of a wire cage), not both
2	Mouse has both a limp tail and shows hind limb weakness
3	Mouse has partial paralysis of the hind limbs (can no longer maintain posture of the rump, but can still move one or both limbs to an extent)
4	Mouse shows complete hind limb paralysis (complete loss of movement of the hind limbs, all movement is the result of the mouse dragging on the forelimbs) ^a
5	Moribund (death caused by EAE), mice are euthanized for humane reasons

Figure 5: Detailed MS scoring scheme.

2.3. Gene Knockdown by shRNA

Purchased CCL4 (MIP-1 β) shRNA mouse Lentiviral Particles (sc-45996-V, Santa Cruz Biotechnology) are a mixture of highly concentrated, transduction-ready viral particles carrying 3 target-specific constructs encoding 19-25 nt (plus hairpin) shRNA targeted for long term inhibition of the gene expression whereas control shRNA Lentiviral Particles (which is named as scramble in the experiment) (sc-108080, Santa Cruz Biotechnology) includes an shRNA construct encoding a sequence that will not result in the particular destruction of any known cellular mRNA. 200 μ l of frozen stocks containing 1.0×10^6 infectious viral units (IFU) in Dulbecco's Modified Eagle's Medium with 25 mM HEPES at pH 7.3 stored at -80.

At Day 0, each animal received i.p. 100 μ l shRNA LV solution (10 μ l stock + 90 μ l saline) which contains 50.000 IFU of virus, 2 hours before PLP immunization. Replication-incompetent lentiviral particles are engineered to self-inactivate following transduction and integration of shRNA constructs into the genomic DNA of target cells.

2.4. Preparations of Cryosections

At 20 day post-immunization, mice were sacrificed with an overdose of Ketamin (Ketasol, Richter Pharma AG) / Xylazine (Bayer, Rompun) and cardiac perfusion was performed immediately after cardiac blood collection. Their brains, spinal cords, spleens and lymph nodes were removed, brain and spinal cord tissues were incubated in 4% PFA at 4°C for at least 24 hours and in 10%, 20%, and 30% sucrose (Caisson #SO11) solutions until the tissues settled to the bottom of the falcon tube for each sucrose concentration before being cryopreserved. Tissues were frozen at -80 °C using an optimum cutting temperature (OCT, Leica, 14420108926) compound and sectioned into three parts as shown in Figure 6 by using a Cryotome instrument (Leica CM 1950). 10 μ m thick frozen brain slices were collected on polylysine-coated positively charged glass slides and stored at 4°C until stainings.

1 Liter of %4 PFA: In around 400 milliliters of water, 40 grams of paraformaldehyde have been dissolved. The solution was heated to between 58 to 60 degrees Celsius. The paraformaldehyde solution is clarified by adding NaOH

droplets until the solution became clear. Lastly, phosphate buffer stock was added and cooled. The pH is adjusted to 7.4, and then the solution is sterilized by a vacuum filter system. 4 Liters of 0.2M Phosphate Buffer : In 4 Liters ddH₂O, 17.66g Sodium Phosphate Monobasic and 90.03g Sodium Phosphate Dibasic Heptahydrate were added and the pH was adjusted to 7.4.

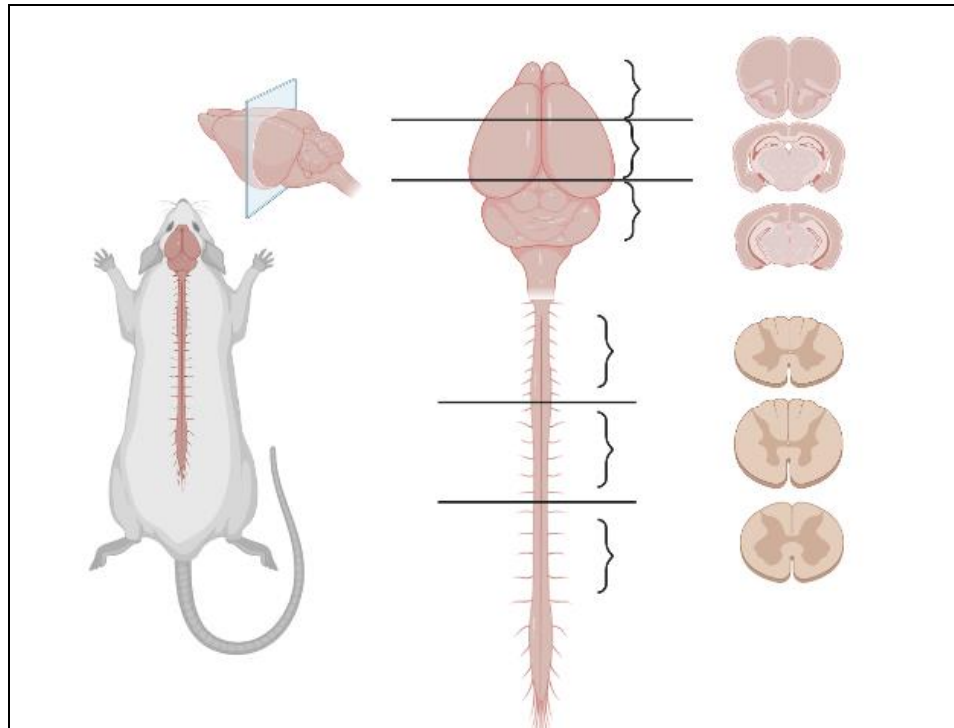


Figure 6: Preparation of Cryosectioning tissues.

2.5. Hemotoxylen and Eosin Staining

The slides were submerged in distilled water for one minute to eliminate any leftover tissue or cryosectioning debris before being dried with paper towels to remove excess water from the tissues. The slides were incubated in Mayer's Hemalum solution (Merck, 109249) for 5 minutes before being rinsed with running tap water to remove excess staining. The slides were then submerged for 5 minutes in PBS. The slides were then treated with an Eosin Y-alcohol solution (Shandon, 6766007) for 30 seconds, dipped in 70%, 90%, and 100% ethanol for two minutes each, and finally Xylene for two minutes. During the last phase, Entellan was used to

cover the tissues. Hematoxylin and Eosin (H&E) images were obtained for further examination using light microscopy (Zeiss Axio).

2.6. Immunofluorescence Stainings

Initially, the slides were repeatedly immersed in distilled water until all residue and OCT had been removed. Following this, the slides were treated for 5 minutes at room temperature with methanol to permeabilise the cell membranes. Afterwards, the slides were cleaned three times with Dulbecco's phosphate buffered saline (DPBS, Gibco, #14190-094). On each tissue sample, Super Block (Thermo Scientific™, PI37535) solution was administered. Each tissue was tagged with a liquid blocker using a PAP pen. Following these preparations, the slides were incubated for one hour at room temperature while a wet paper towel was placed in each box to maintain humidity. Following the removal of the super block solution, the appropriate primary antibody (diluted in Super Block solution) was applied to the samples and incubated overnight at 4°C. After removing the antibody solution, the slides were submerged three times for one minute in DPBS. The sample was then incubated for two hours at 37 degrees Celsius with the secondary antibody, which had been diluted in superbloc solution. The secondary antibody was then eliminated, and the slides were washed three times for one minute in DPBS. In the subsequent step, the cells were either mounted with mounting media containing DAPI (Abcam) or stained with another primary antibody for double labeling. The slides were covered with a cover glass that was secured with clear nail varnish. Leica DMI8 LCI microscope was used to take fluorescence pictures, and Leica LASX software was utilized to process the images. Product Information of the antibodies listed in APPENDIX A.

2.7. ELISA

Bloods collected at day 10 and Day 20 centrifuged at 500 g for 1 min and serums are transferred to Nuclease free tubes and stored at -20. 96-well microtiter ELISA plates were coated with PLP antigen (0.5 µg in 100 µL each well) in PBS and incubated at 4°C overnight. (The antigen stock for PLP is 1 mg/ml. 48 µL of stock in

9600 μ L PBS). Once washed with 0.5% Tween20-PBS (2.5 mL Tween-20 in 500 mL PBS) and incubated for 2 hours at room temperature in 2% FBS-PBS (49 mL PBS and 1 mL FBS). Diluted mice serums (1/1000) added as duplicate to emptied wells with blanks (2% FBS-PBS) and incubated at 37°C for 90 minutes. Five times washed with 0.5% Tween20-PBS. Anti-mouse IgG conjugated with HRP (1:5000 in 2% FBS-PBS) added and incubated at 37°C for 90 minutes. Again five times washed with 0.5% Tween20-PBS. Incubated for 10 minutes with 100 μ L of Tetramethylbenzidine (TMB) solution (#SLCC950, Sigma). Reaction is stopped by the addition of 100 μ L of 1M HCl. Absorbance was measured at 450 nm, using (Synergy H1, microplate reader) plate reader.

2.8. Splenocyte Isolation from Spleen

Fresh spleens collected from mice on day 20 were kept on ice for 2-3 hours. Spleens were divided into seven to ten pieces according to their size, and each piece was pressed between two microscope slides. Mashed tissues were collected with 5 mL of cold Roswell Park Memorial Institute (RPMI) medium and filtered through a 70 micrometer cell strainer. Tubes were topped up to 10 mL with wash buffer (RPMI + 2% FBS) and centrifuged for 5 min at 2000 RPM (settings, 10 accelerated, 10 decelerated). After removing the supernatant, the pellet was resuspended in 3 mL of 1X Red Blood Cell (RBC) lysis buffer (10X RBC lysis buffer (420301) + 9 mL dH₂O) and incubated at room temperature for 5 minutes. Then topped up to 10 mL with wash buffer and centrifuged at the same settings. After the supernatant was thrown away, the pellets were kept at -80°C until RNA isolation.

2.9. Cell Isolation from Lymph Nodes

Prior to sacrifice, tubes containing about 5 mL of 10% FBS-RPMI were prepared for each mouse. Mice lymph nodes were homogenized mechanically on a petri plate, and cells were collected in a volume of roughly 10 mL, filtered through a 40 μ m cell strainer and centrifuged at 4°C at 1800 rpm. The supernatant was discarded, and 1 mL of PBS was used to dissolve the pellet for washing. Centrifuged at 1800 rpm and 4°C. The pellet was dissolved in 1 mL of FBS, and the cells were

counted using the dye trypan blue. Maximum of 15 million cells/ml were frozen in 20% of DMSO-FBS (Dimethyl sulfoxide-Fetal Bovine Serum) serum per cryotube. Until RNA isolation, cells were kept in liquid nitrogen.

2.10. RNA Isolation and RT-PCR

RNA isolation of Peripheral blood mononuclear cell (PBMC) isolated from spleen, lymph node cells and total blood (Zymo MiniRNA isolation Kit), cDNA synthesis (Bio-RAD cDNA synthesis Kit) and real-time qPCR (Qiagen Quantinova Sybr Green PCR kit) were performed according to manufacturer's protocol. The designed primer sequences are listed in APPENDIX B. RT-PCR performed on Roche, LightCycler 480 II and $2^{-\Delta\Delta Ct}$ formulation was used when calculating the relative fold differences. The cycle settings are listed in APPENDIX C.

2.11. Inverted Screen Test

The Inverted Screen test measures motor strength and coordination. Untrained mice are put in the middle of the 30 cm x 30 cm square wire screen.



Figure 7: Application of inverted screen test.

The rod is then moved 180 degrees to position the mouse at the bottom of the panels. The falling time is recorded twice. Each trial was restricted to 5 minutes (Figure 7).

2.12. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 9.3.1 (La Jolla, United States) using the non-parametric unpaired two-tailed students T-test (Mann-Whitney) for comparing two groups. Statistical symbols remark **= $P < 0.01$, *= $P < 0.05$ and ns= non-significant.

3. RESULTS

3.1. Weighting and Scoring,

3.1.1 % Weight Change Compared to the First Day

The weight change is calculated according to the % change referenced to the first day of each animals. The statistical analysis shows that Sham and Scramble groups showed similar pattern whereas PLP and CCL4 group also progress similarly ($p>0.05$). But the significant differences starts in the day of 4 between scramble and sham vs. PLP and CCL4 (Figure 8 and Table 3).

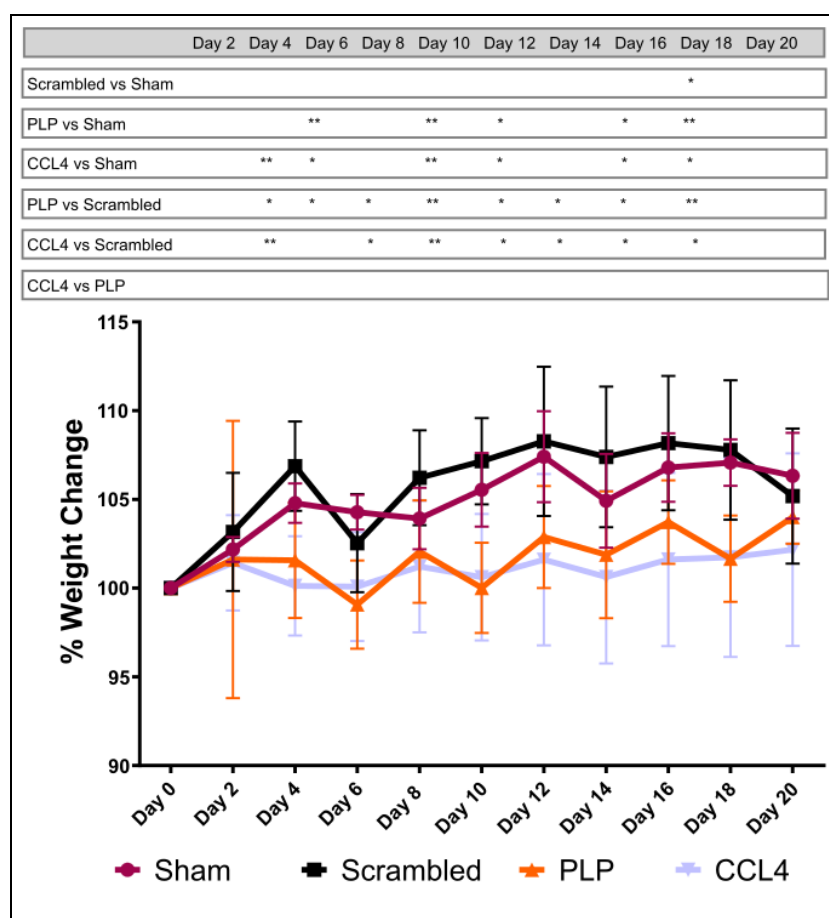


Figure 8: Percent weight change of the groups.

Table 3: Percent weight change, p values of the non-parametric t-test.

	Weight Change (%)		Weight Change (%)		Weight Change (%)		Weight Change (%)		Weight Change (%)		Weight Change (%)	
	Scrambled vs Sham		PLP vs Sham		CCL4 vs Sham		PLP vs Scrambled		CCL4 vs Scrambled		CCL4 vs PLP	
Days	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol
0	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns
2	0.6991	ns	0.6991	ns	0.6991	ns	>0.9999	ns	0.4848	ns	0.8182	ns
4	0.132	ns	0.132	ns	0.0022	**	0.026	*	0.0022	**	0.3939	ns
6	0.3939	ns	0.0043	**	0.0411	*	0.0411	*	0.132	ns	0.8506	ns
8	0.132	ns	0.2403	ns	0.132	ns	0.0152	*	0.026	*	0.6991	ns
10	0.3939	ns	0.0022	**	0.0043	**	0.0022	**	0.0043	**	0.5887	ns
12	0.5887	ns	0.026	*	0.0303	*	0.0411	*	0.0411	*	0.8182	ns
14	0.2403	ns	0.1797	ns	0.1797	ns	0.0411	*	0.026	*	0.8182	ns
16	0.3095	ns	0.026	*	0.026	*	0.0411	*	0.026	*	0.9372	ns
18	>0.9999	ns	0.0043	**	0.0152	*	0.0087	**	0.0455	*	0.5887	ns
20	0.5887	ns	0.0931	ns	0.0649	ns	0.4848	ns	0.4848	ns	>0.9999	ns

3.1.2 Scoring

EAE scores between sham and the other groups was not significant before the day of 6 ($p>0.05$). All the groups which received PLP immunization showed significant differences compared to the sham after day 6 ($p<0.01$). Compared to PLP group scrambled were different at day 8 and 10 ($p<0.05$) and for scrambled vs. CCL4 it is the 8.day ($p<0.05$) and between PLP and CCL4 day 16 ($p<0.05$) (Figure 9 and Table 4).

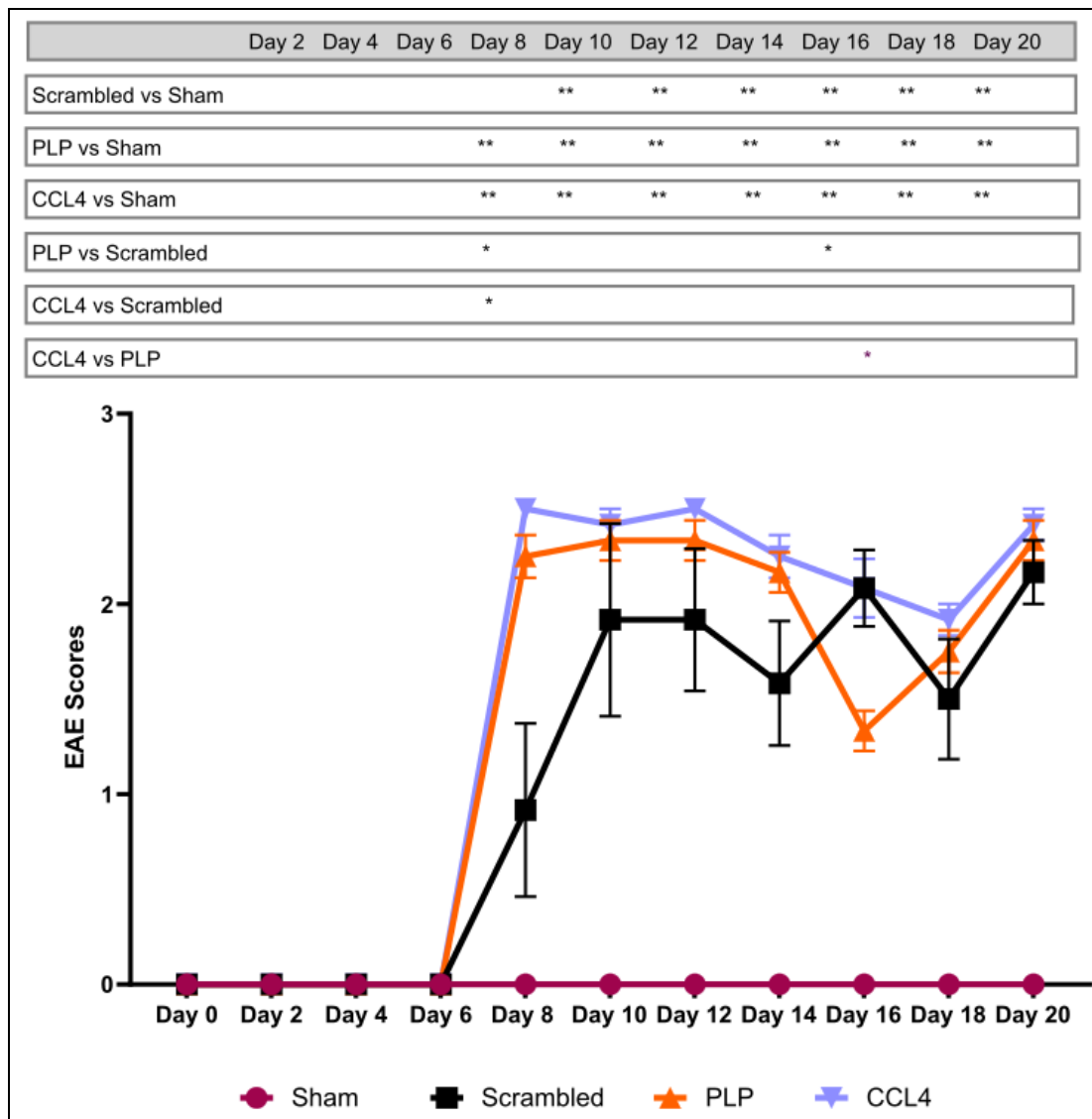


Figure 9: MS scores of the groups.

Table 4: Scoring of neurologic examination, p values of non-parametric t-test.

	EAE Score		EAE Score		EAE Score		EAE Score		EAE Score		EAE Score	
	Scrambled vs Sham		PLP vs Sham		CCL4 vs Sham		PLP vs Scrambled		CCL4 vs Scrambled		CCL4 vs PLP	
Days	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol	T-test P values	Statistical symbol
0	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns
2	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns
4	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns
6	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns	>0.9999	ns
8	0.0606	ns	0.0022	**	0.0022	**	0.0368	*	0.0152	*	0.1818	ns
10	0.0022	**	0.0022	**	0.0022	**	0.2987	ns	0.3853	ns	>0.9999	ns
12	0.0022	**	0.0022	**	0.0022	**	0.4069	ns	0.3312	ns	0.4545	ns
14	0.0022	**	0.0022	**	0.0022	**	0.1407	ns	0.0823	ns	>0.9999	ns
16	0.0022	**	0.0022	**	0.0022	**	0.0108	*	>0.9999	ns	0.0108	*
18	0.0022	**	0.0022	**	0.0022	**	0.1407	ns	0.0736	ns	0.5455	ns
20	0.0022	**	0.0022	**	0.0022	**	0.2424	ns	0.0801	ns	>0.9999	ns

3.2. Inverted Screen Test

At Day 16, inverted screen test latency is compared between PLP and CCL4 silenced grouped with Mann-Whitney Test and the differences was significant as p value is 0.0152 (Figure 10).

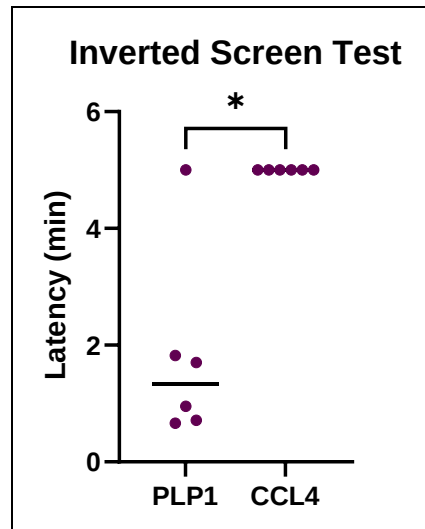


Figure 10: Inverted screen test analysis results.

3.3. ELISA

3.3.1. ELISA Day 10

The measurement of PLP antibody level which is produced against for PLP antigen in the blood serums collected at Day 10 done and the results showed that except scramble ($p>0.05$) PLP and CCL4 level increased significantly ($p<0.01$). The differences between Scramble vs PLP and CCL4 was also significant ($p<0.01$).

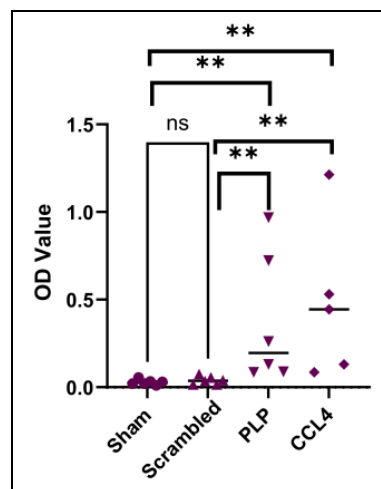


Figure 11: ELISA results of day 10.

Table 5: ELISA day 10 p values of non-parametric t-test.

	Elisa Day 10	
	T-test P values	Statistical symbol
Scrambled vs Sham	0.4091	ns
PLP vs Sham	0.0022	**
CCL4 vs Sham	0.0043	**
PLP vs Scrambled	0.0022	**
CCL4 vs Scrambled	0.0043	**
CCL4 vs PLP	0.7597	ns

There was no differences between PLP and CCL4 Groups ($p>0.05$) (Figure 11 and Table 5).

3.3.2. ELISA Day 20

The measurement of PLP antibody level which is produced against for PLP antigen in the blood serums collected at Day 20 done and the results showed that scrambled, PLP and CCL4 level increased significantly compared to the sham group ($p<0.01$). The differences between scrambled vs PLP ($p<0.05$) and CCL4 ($p<0.01$) was also significant. There was no significant differences between PLP and CCL4 Groups ($p>0.05$) (Figure 11 and Table 5).

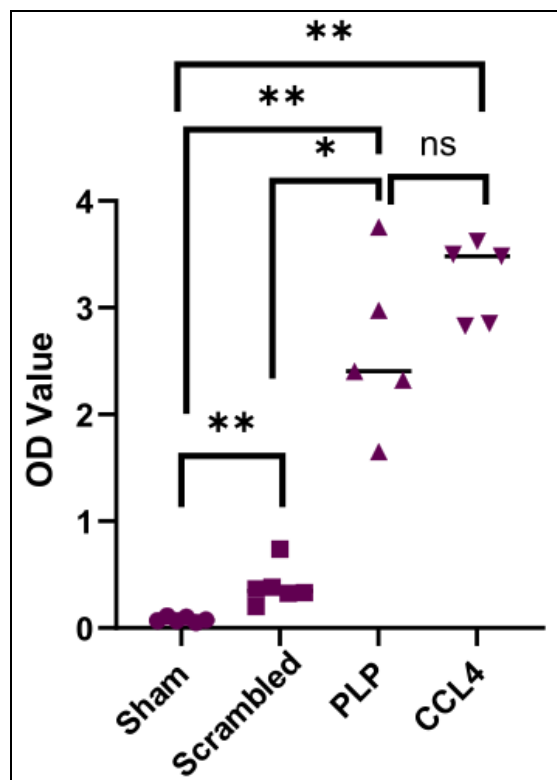


Figure 12: ELISA results of day 20.

Table 6: ELISA day 20, p values of non-parametric t test.

	Elisa Day 20	
	T-test P values	Statistical symbol
Scrambled vs Sham	0.0022	**
PLP vs Sham	0.0022	**
CCL4 vs Sham	0.0022	**
PLP vs Scrambled	0.0152	*
CCL4 vs Scrambled	0.0022	**
CCL4 vs PLP	0.3939	ns

3.4. RT-PCR

According to the RT-PCR results, spleen cells CCL4 expression differences could not be detected between sham, PLP, scrambled and silenced groups. But in the cells isolated from the lymph nodes, some animals from CCL4 silenced groups showed a decrease in the expression but this decrease was not significant ($p > 0.05$) (Figure 13).

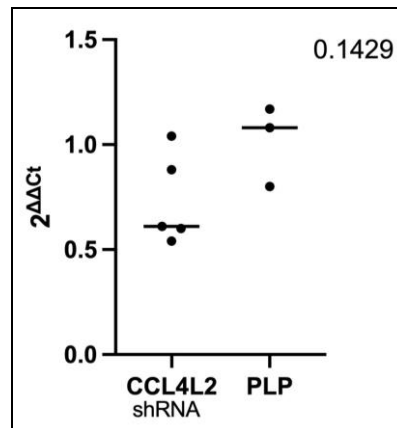


Figure 13: RT-PCR results of CCL4 gene expression in isolated lymph node cells.

3.5. H&E and IF Staining of Brain

The brain sections are stained with H&E technique which allowed us to see cell accumulation especially around the ventricles. This cell infiltration is a hallmark of neuroinflammation in the brain. The sham group has no accumulation but the PLP-induced EAE modeled mice have the accumulation which is a validation of the successful PLP application and modeling of neuroinflammation.

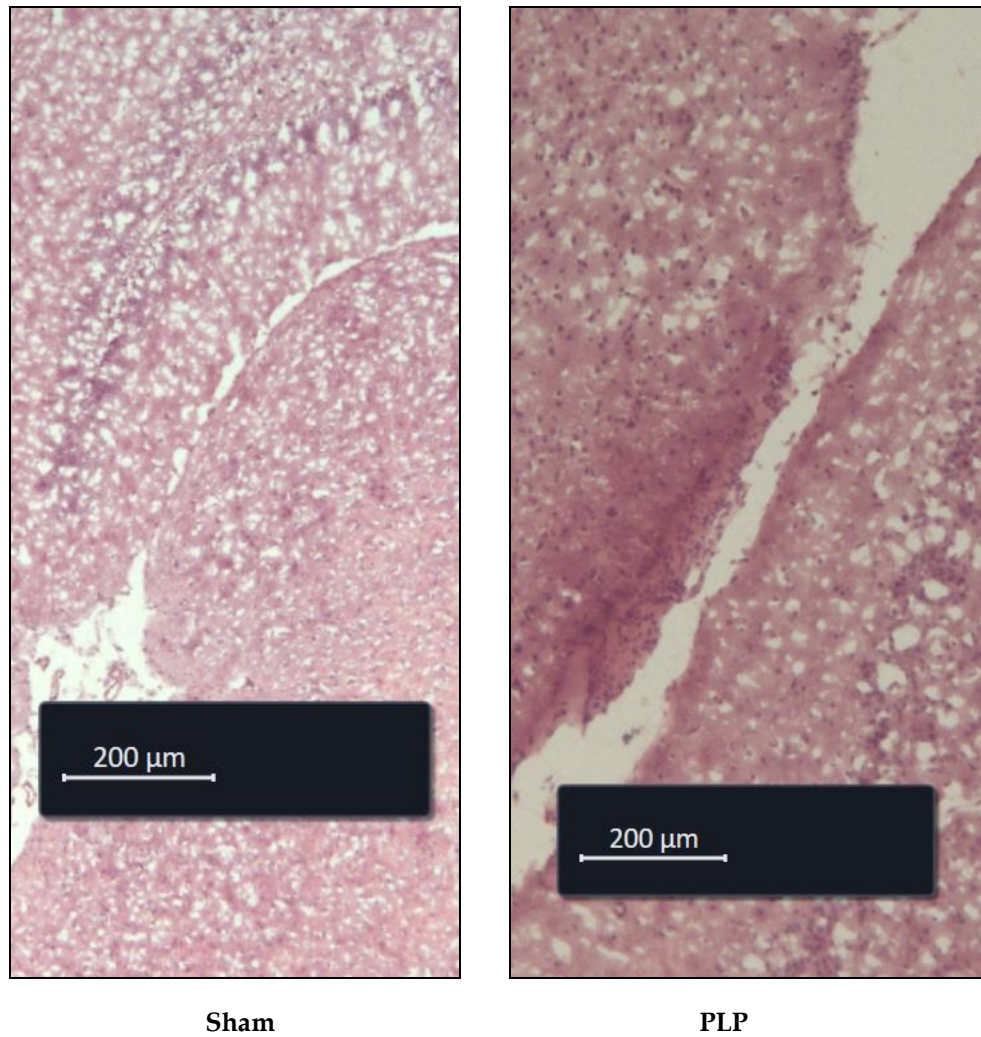


Figure 14: H&E staining of brain sections sham vs.PLP group. Compared with the SHAM group there are several small spots with accumulation of inflammatory cells in the lesion of PLP-induced animals on the left.

Albumin is a 60kDa protein found in the blood and normally can not pass to brain tissue. Leakage in the blood brain barrier can lead passing of this proteins through the CNS tissues. In the Albumin IF stainings of the brain sections of the sham groups, the vessels are observed with intense DAPI stainings(blue) and albumin (green) inside. But the PLP-induced EAE model groups shows albumin stainings around the vessels and in the brain tissue surrounding them.

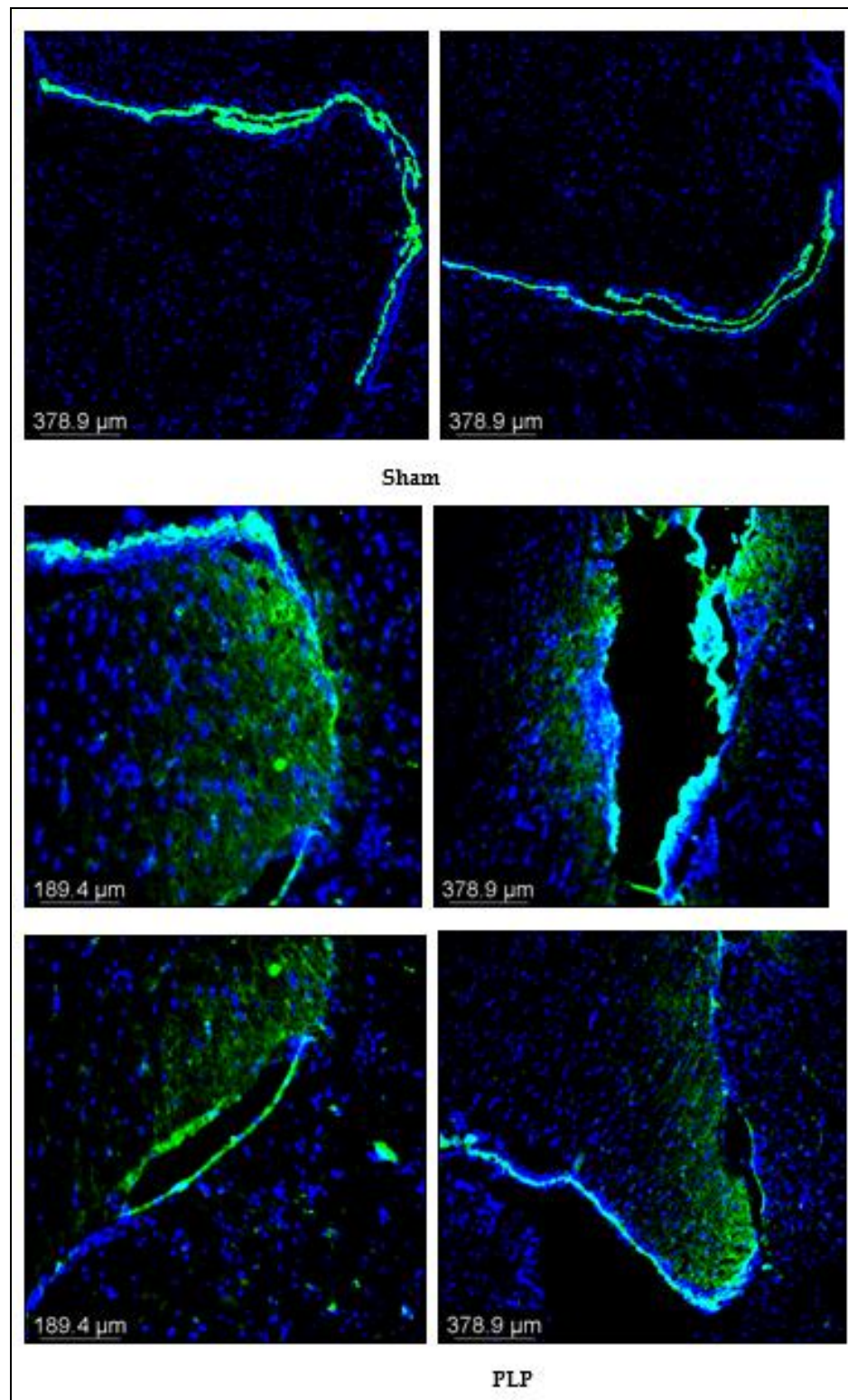


Figure 15: Albumin IF staining. Albumin staining in the brain sections of sham and PLP groups. Albumin (green) stains BBB leakage and DAPI (blue) stains cell nucleus.

4. DISCUSSION

MS is an neuroinflammatory disease that chemokines has a key role for the progression of the disease. As the CCL4 protein concentration found higher in RRMS patients, the investigation of the potential of being a biomarker for diagnosis is aimed in the literature. EAE model is one of the excellent technique used to model MS in rodent models. In this project, PLP-induced EAE is modeled in Balbc mice and gene is silenced by intraperitoneal administration of shRNA containing lentiviral particles.

In order to validate PLP-EAE modeling success, ELISA is performed at day 10 and day 20 to measure PLP antibody level which is produced against for PLP antigen in the blood serums. In day 10, PLP and CCL4 groups were significantly higher than the sham but even if scrambled group received the same dosage with PLP and CCL4 group, the differences of Scrambled vs. Sham was not significant ($p>0.05$). At day 20 all the groups were significantly higher then sham group ($p<0.05$). Immunization of CCL4 silenced group was also higher than PLP and other groups but this differences was not significant ($p>0.05$). The data shows successfully modeled EAE in the project.

The percent weight change of the groups were compared and PLP and CCL4 groups Scrambledemonstrated significantly higher rate of change. The pattern in the scrambled group in weight change results were similar to sham group. Since they received PLP, the expected result was weight loss . But we got opposite result. The purchased scrambled lentivirus contains non CCL4 targeting shRNA but it is not clear what could it effects. The unexpected result could be due to the scrambled lentivirus may targeted some other gene or it produced immunological reaction. A validation is needed for the further studies. We planned to purchase scrambled RNA from another company and repeat the experiments.

According to the literature the best RRMS is modeled in SJL mice as the scores are determined from 0 to 5. In the literature also Balbc mice were used by injecting different dosage of PLP to induce RRMS model but with less severity of the relapses. In our experiments score scale was between 0 and 3 which can be explained due to the used mice strain used and dosage of PLP.

The MS attacks started at day 8 similarly in PLP and CCL4 groups and continued in a similar way until day 16, when PLP group showed significantly lower scores, which can be speculated as recovery (remission) time between day 14 and 18. CCL4 silenced group did not show a remission phase until 18th day and it was less severe than PLP group but this relaps did not reach statistical significance in scores when compared with PLP group. At day 18 both group has the score without differences and then their scores increased in the same ratio at day 20. It looks like silencing of CCL4 stabilized and postponed the MS severity but did not prevent the relapsing pattern.

The difference at day 16 was examined by application of inverted screen test. The strength of the PLP animals were significantly less than the CCL4 group even though PLP group has a recovery pattern in the scoring.

The silencing of the gene also tried to be proved by the RNA isolation of splenocytes isolated freshly from the spleens and lymph nodes. RT-PCR couldn't detect the expression difference between CCL4 shRNA treated group and PLP as a control in splenocytes because the isolated RNA amount were low. CCL4 expression in the lymph node cells measured and although some animals in CCL4 silenced group have lower expression, the differences between PLP and this group was not significantly different ($p>0.05$). This could be the reason of similarity in the scoring of PLP and CCL4 groups. The dosage of the injected shRNA for each animal decided as the highest recommended dosage of the company. But in the further studies higher dosage could be used to get more stable silencing or another brand can be preferred in studies of this gene.

Cryosections from brains and spinal cords stained with H&E and IF for Albumin (Figure 14 and 15). H&E staining showed cell infiltration in the brain sections in PLP applied group but not in sham. In the albumin staining, blood brain barrier leakage which is a hallmark for MS lesions also detected in PLP group sections. The changes in the brain histochemistry is another validation of successful EAE model in the project.

The most remarkable result of the study is behavioral scoring difference at day 16. It may demonstrate inhibition of recovery pattern in the CCL4 silencing. In future studies, 20 day experimental time frame can be extended to see the long term effects of gene silencing with an increased number of animals. Knock out and overexpression of the gene can be another goal for the future to discover the recovery

inhibition reasons. The genes that CCL4 interacting also can be checked by PCR and stainings for determining the pathways underlying this mechanism.

5. CONCLUSION

MS is an neuroinflammatory disease that chemokines has a key role for the progression of the disease. As the CCL4 protein concentration found higher in RRMS patients, the investigation of the potential of being biomarker for diagnosis is aimed in the literature. PLP-induced EAE is performed in 24 Balbc mice and gene is knockdowned by intraperitoneal administration of shRNA containing lentiviral particles. Model for PLP- induced EAE is validated by checking the level of PLP antigen in mice sblood serums by ELISA and both at day 10 and 20 the immune response for injected PLP was significantly higher than control group. Gene expression result showed decreased expression in CCL4 knockdowned group but not significant due to the injected dosage. Brain stainings showed inflammation like response due to the cell accumulation in H&E stainings and vascular integrity change as IF albumin stainings outside the vessels in all PLP-EAE modelled groups but not in control group. The pathophysiology of MS were performed and behavioral scoring difference at day 16 which is inhibition of recovery pattern in the CCL4 silencing is the most remarkable result of the experiment. It looks like silencing of CCL4 stabilized and postponed the MS severity but did not prevent the relapsing pattern. These results increases the presence of possible role of CCL4 in RRMS for the future studies. For a better understanding of the pahtology of MS related with CCL4 can be studied by knock out or overexpression studies both in vitro and in vivo.

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BIOGRAPHY

Fatmanur Akpunar went Beykoz Anatolian High School and received her Bachelor's degree at Molecular Biology And Genetics Department in Üsküdar University with 100% scholarship and 100% English. When she was a freshman she has started to work in neuropsychopharmacology research and application center and immunology lab in Marmara University Research Hospital. Her Project about effects of agmatin on caffeine sensitization on mice was supported by TÜBİTAK. She studied one term Molecular Biology at Umea University, Sweden with Erasmus+ mobility. She was in the brain team of Neurofilik group which a collaboration of university students from many department who aimed to be transfer neuroscientific knowled to the people from every age from 3 to 30. The workshops called as 'Brain on the stage' reach hunderds of students. That motivation lead the team hold seminars and symposiums. Together with this studies she was the president of Erasmus and International Students Club for a year to solve foreign student problems and providing a friendly social environment. She went to Uppsala University Neuropsychopharmacology center for 3 months Erasmus + Internship. After coming back, she started her master thesis in Gebze Technical University in the same department and she is conducting her theisis together with Prof.Dr.Yasemin Gürsoy Özdemir at Koç University Translational Medicine Reseach Center.

APPENDICES

Appendix A: Used Antibody List.

Tablo A1.1: Antibody Information.

Target	Primary Antibody (Catalog Number)	Ratio	Secondary antibody	Ratio
BBB Leakage	Goat anti-mouse Albumin (A90-234A)	1:50	Donkey anti-goat FITC	1:200

Appendix B: PCR Primer Sequences.

Tablo B1.1: PCR Primer Sequences.

Primer Name	Sequence (5'-3')
GAPDH-Forward	TGA CGT GCC GCC TGG AGA AAC
GAPDH-Reverse	CCG GCA TCG AAG GTG GAA GAG
CCL4-Forward	CCT CTC TCT CCT CTT GCT CG
CCL4-Reverse	TCT GTC TGC CTC TTT TGG TCA

Appendix C: PCR Cycle Settings.

Tablo C1.1: PCR Cycle Settings.

Step	Temperature (°C)	Time(min:sec)	Cycle
Denaturation	95	10:00	1
Amplification	95	15:00	35
	60	15:00	
	72	15:00	
Melting Curve Analysis	95	0:10	1
	65	0:05	1
	97	-	1
Cooling	4	∞	1