ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2016; 15(2):145-155.

Immunomodulatory Effect of Chymotrypsin in CNS Is Sex-independent: Evidence of Anti-inflammatory Role for IL-17 in EAE

Ameneh Ghaffarinia[1], Shahram Parvaneh[1], Cyrus Jalili[2], Farhad Riazi-Rad[3], Somayeh Yaslianifard[4], and Nafiseh Pakravan[4]

[1] Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[2] Department of Anatomy, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran
[3] Department of Immunology, Pasture Institute of Iran, Tehran, Iran
[4] Department of Microbiology and Immunology, Medical School, Alborz University of Medical Sciences, Karaj, Iran

Received: 10 October 2015; Received in revised form: 8 December 2015; Accepted: 12 December 2015

ABSTRACT

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are inflammatory autoimmune diseases of the central nervous system. Chymotrypsin is a serine protease with immunomodulatory effect in the peripheral organs. We previously demonstrated the immunomodulatory effect of chymotrypsin in ameliorating the EAE in female Lewis rats. However, there are sex-based differences in the immune system, drug activity, and CNS structure and composition. In addition, female gender is a better prognostic indicator of MS and males are more severely affected by EAE than females. Consequently, gender may have an important impact on therapeutic effect. Therefore, in this study we investigated the anti-inflammatory effect of chymotrypsin in male Lewis rat model of EAE.

The disease was induced in male Lewis rats and the animals were evaluated for weight loss and clinical signs for 14 days. Intra-CSF injection of chymotrypsin was done on day 7 and expression of mRNA for IFN-γ, IL-4, IL-17, and FoxP3 in brain, spinal cord and deep cervical lymph node were determined using a two-step real-time PCR.

Administration of 0.2mg/ml chymotrypsin ameliorated the disease by decreasing IFN-γ and increasing expression of IL-4 and IL-17 at the inflammatory foci. This is consistent with anti-inflammatory effect of IL-4 and IL-17 at high concentrations.

We conclude that Immunomodulatory affect of chymotrypsin in CNS is sex-independent. Our result also provides more evidence on the anti-inflammatory role of IL-17. However more research is needed to elucidate the underlying immunomodulatory role of chymotrypsin and how to increase its beneficial effect by modification of dosage and/or regimen of administration.

Keywords: Anti-inflammatory; Chymotrypsin; CNS; Experimental autoimmune encephalomyelitis; Immunomodulation; Interleukin-17; Male

Corresponding Author: Nafiseh Pakravan, PhD;
Department of Microbiology and Immunology, Medical School, Alborz University of Medical Sciences, Karaj, Iran.

Tel: (+98 26) 3431 4400, Fax: (+98 26) 3430 2090, E-mail: nafiseh.pakravan@gmail.com

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) and served as an animal model of multiple sclerosis (MS).[1] EAE was previously regarded a Th1 cell-medicated disease. However, it was later demonstrated that Th1 response-deficient mice which lack molecules, such as IFN-γ, IFN-γR, or IL-12p35 develop severe EAE. These observations put doubt on the necessity of Th1 cells in pathogenesis of this autoimmune disease.[2,3] These findings raised the possibility that another T cell population may play a role in CNS autoimmune pathogenesis, as well as the IFN-γ–producing T cells.

It has been shown that differentiation of Th17 cells is induced when naïve T cells are stimulated in the presence of IL-6 and TGF-β1 signaling and maintained by the presence of IL-23. Deficiency of IL-17, a Th17 cytokine, confers resistant to EAE. In addition, depletion of IL-17-producing cells results in a severe disease and treatment of mice with a neutralizing anti-IL-17 mAb suppresses CNS autoimmune inflammation.[4] A number of prophylactic and therapeutics immunotherapies have shown that neutralization of Th17 responses and anti-inflammatory therapies lead to EAE amelioration .[5-8] However, timing and/or concentration of cytokines from these responses may have opposing outcomes.[9,10]

Some enzymes have been shown to have anti-inflammatory effect on EAE.[11-15] Chymotrypsin is a serine protease whose inhibition was demonstrated to affect inflammatory state of neutrophils *in vitro*.[16] There have also been reportedregarding anti-inflammatory effect of chymotrypsin in the peripheral organs.[13,16-20] Chymotrypsin performs proteolysis by cleaving peptides at the carboxyl side of tyrosine, tryptophan and phenylalanine. It also hydrolyzes other amide bonds over time, especially those with leucine-donated carboxyls. It is resistant to stomach acidity, and exerts its anti-inflammatory effect systemically.[21-23] We previously demonstrated that intra-CSF administration of chymotrypsin ameliorated EAE in female Lewis rat (24). However, the result cannot be extended to include male as well. The reason is that there are sex-based differences in treatment response and CNS composition, structure, and autoimmunity.[25-35]

Sex-based different response to treatment is because of specific enzymes involved in drug metabolism.[25] In addition, adverse reactions to drugs may also occur due to sex-based differences in drug metabolism. Other factors, e.g. route of administration, may also be involved in the sex-based differences of treatment response.[26]

Structure and composition of CNS differs between males and females. Females have a lower percentage of cerebrospinal fluid and white matter which contains myelinated axonal fibers.[27-30] CNS autoimmunity is also influenced by the hormonal and genetic basis for sex differences. Accordingly, MS and EAE undergo a more rapid progression than females.[31-35]

As a result, the potential role of sex should be taken into account when optimizing therapeutic approaches of sexually dimorphic diseases in each clinical setting. In continuation of the previous work on EAE model of female Lewis rat (24), we further explored immunologic effect of intra-CSF administration of chymotrypsin on EAE model of male Lewis rats. In this study we attempted to find out if anti-inflammatory effect of chymotrypsin is sex-independent.

MATERIALS AND METHODS

Animal Breeding, EAE Induction, and Clinical Evaluation

Male Lewis rats were originally purchased from the Darou Pakhsh Company, Tehran, Iran. All animal, were locally bred and kept in light- and temperature-regulated rooms at the conventional animal department of Medical Biology Research Center of Kermanshah University of Medical Sciences. The animals were provided food and water *ad libitum*. All experiments were done according to Animal Care and Use Protocol of Kermanshah University of Medical Sciences, under No. 90276. Rats between 210-240gr were immunized subcutaneously, daily weighed, and clinical signs of disease were evaluated until day 14 after EAE induction according to the different signs and scores, as before.[24] Briefly, the animals were immunized subcutaneously with 50μg guinea pig spinal cord and 400μg *Mycobacterium tuberculosis* H3 RA RA (Difco Labs, Ditroit, MI) in complete Freund's adjuvant (CFA) (Difco, Germany). Clinical signs were evaluated as score 0 when no symptoms; score 0.5 when loss of tonicity of the distal portion at the tail or tail weakness; score 1 when complete tail paralysis; score 2 when mild paresis of hind limbs; score 3 when complete paralysis of one hind

limb; score 4 when bilateral hind limb paralysis; score 5 when complete paralysis (tetraplegia), urinary and/or fecal incontinence, moribund state, or death occurred. Rats with borderline scores were given a one half score.

Study Design, Experimental Group, and Intra-CSF Injection

EAE was induced on day 0 and the animals divided into four groups (6-7 animals in each group). Animals treated with 0.1 or 0.2mg/ml chymotrypsin were considered as test groups and two groups of rats were used as controls, including animals injected with saline and animals without injection. Since the results between saline-injected animals did not differ with the un-injected ones, only results of saline-injected are presented. The injected volume of saline alone or chymotrypsin (bovine pancreas Grade I/AppliChem, Darmstadt, Germany) at two concentrations of 0.1 and 0.2mg/ml was determined in our pilot study as 85- 100 microliter depending on the weight. Intra-CSF injection was performed between the last lumbar vertebra and the sacrum (L5-S1) using insulin syringe. Then, the animals were sacrificed, transcardially perfused with salineand consequently brain, spinal cord, and DCLN were removed and stored as explained before (24). It is important to mention that those animals that reached the score of 5 before day 14 post EAE induction were sacrificed and deleted from the study for ethical reasons.

RNA Extraction and Real-time PCR

Total RNA was extracted from each frozen brain, spinal cord, and deep cervical lymph nodes (DCLNs) using Trizol® Reagent from Invitrogen (Karlsruhe, Germany) according to a standard protocol. The quality and quantity of RNA concentrations were monitored Nano Drop 2000c Eppendorf (Hamburg, Germany). RNA was reversely transcribed using oligo-dt primers and M-MulV (Fermentas GMBH, St. Leon-Rot, Germany), according to the manufacturer's instructions. Expression of mRNA for β-actin, IFN-γ, IL-4, IL-17, and FoxP3 were determined using Rotor-Gene 6000™ (Corbett Research, Australia) thermocycler and SYBR®Premix Ex Taq™II Real Time PCR Master Mix (TaKaRa Co., Japan), according to the manufacturer's instructions. Each reaction contained 5µl master mix, 100 nM primers for β-actin, IFN-γ, IL-4, IL-17, FoxP3, and 1µl template cDNA.

The sequences of primers were forward 5'-aggccaaccgtgaaaagatg-3', and 5'-accagaggcatacagggacaa-3' for β-actin and forward 5'-ccacggagaacgagctcatc-3'and reverse 5'-gagaaccccagacttgttcttca-3' for IL-4, forward 5'-gggaagttggaccaccacat-3' and reverse 5'-ttctccacccggaaagtgaa-3' for IL-17, forward 5'-gaaagacaaccaggccatcag-3' and reverse 5'-tcatgaatgcatccttttttg-3' for IFN-γ, and forward 5'-cgggagagtttctcaagcac-3' and reverse 5'-ggagctcttgtccactgagg-3' for FoxP3.

The efficiencies for primers used in the study varied between 95% and 105%. Primer pairs were validated to ensure a correct size of PCR product and absence of primer dimers. To confirm the specificity of the primers, melting curve analysis and agarose gel electrophoresis were performed. Thermocycler conditions included an initial step at 95°C for 10 minutes followed by a two-step PCR program at 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. The β-actin gene was chosen as an internal control against which mRNA expression of the target gene was normalized. The resultant gene expression level was presented as $2^{-\Delta Ct}$, in which ΔCt was the difference between Ct values of target gene and β-actin.[24]

Statistical Analysis

Data are shown as means±SEM and statistical analysis was performed using the GraphPad Prism statistical package by ANOVA or Kruskal-Wallis test, as appropriate. In all cases, *p* values less than 0.05 were considered statistically significant. Experiments were performed in triplicate.

RESULTS

The EAE male Lewis rat model was set up in our laboratory. The susceptibility to EAE was 100% and the day that the first clinical signs were observed was 7 days after the disease induction. However, uneasiness was appeared in some animals 5 days after the immunization. Intra-CSF injection of chymotrypsin at the concentration of 0.2mg/ml led to a marked attenuation of clinical sign and weight loss, compared with saline or 0.1mg/ml chymotrypsin treatment (Figure 1). Since EAE is a T cell-mediated disease, we investigated the changes in expression of IFN-γ, IL-17, 1L-4, and FoxP3 as representative of Th1, Th17, Th2, and natural Treg, respectively.

The changes in expression of IFN-γ, IL-4, IL-17,

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

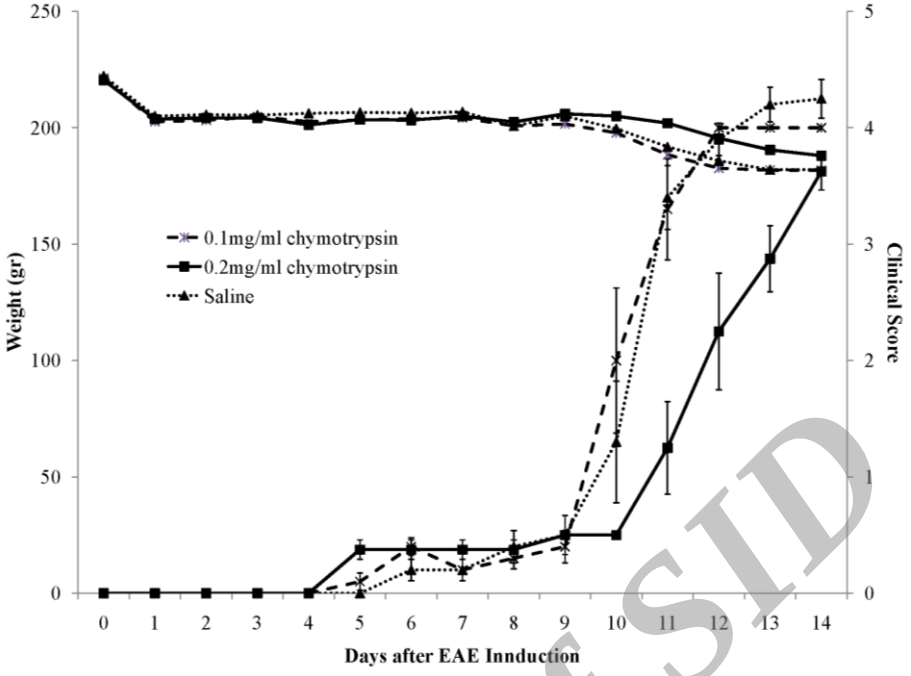

Figure 1. Treatment with saline (..▲..), 0.1mg/ml chymotrypsin (--*--), 0.2mg/ml chymotrypsin (_■_) affects clinical score and weight loss in male Lewis rats model of EAE. The rats were immunized via subcutaneous route with a suspension of guinea pig spinal cord and complete Freund's adjuvant (CFA). Intra-CSF injection was performed on day 7 at the onset of EAE. Clinical score was measured daily from day of disease induction. Data are presented as mean±SEM.

and FoxP3 were investigated in the brain, spinal cord, and DCLN of animals treated with saline, 0.1mg.ml, or 0.2mg/ml chymotrypsin during the peak of the disease (day 14 post EAE immunization).

Gene Expression in the Brain

As illustrated in Figure 2, treatment with 0.2mg/ml chymotrypsin induced significant decreased expression of IFN-γ compared with saline-treated animals. In addition, the expression of IFN-γ was significantly decreased in animals treated with 0.1mg/ml chymotrypsin compared with saline-treated animals. IFN-γ expression was not markedly different between 0.1mg/ml chymotrypsin- and 0.2mg/ml chymotrypsin-treated animals. Inversely, animals injected with 0.2mg/ml chymotrypsin significantly up-regulated IL-17 expression compared with saline-treated animals. Accordingly, IL-17 expression in 0.1mg/ml chymotrypsin-treated animals was markedly increased compared with saline-treated animals. There was no significant difference between 0.1mg/ml chymotrypsin- and 0.2mg/ml chymotrypsin-treated animals. To further explore the cause of decreased score of clinical sign upon 0.2mg/ml chymotrypsin treatment, the expression of IL-4 and FoxP3 was investigated. FoxP3 is a representative of naturally occurring regulatory T cells, which have anti-inflammatory effect. IL-4 is also is a representative of Th2 response with ameliorating effect on EAE.[11,36] There was a significant up-regulation of IL-4 expression in the 0.2mg/ml chymotrypsin -treated animals compared with 0.1mg/ml chymotrypsin- and saline-treated animals, suggesting an up-regulation of Th2 upon 0.2mg/ml chymotrypsin treatment. As for FoxP3, there was no significant difference in FoxP3 expression among the groups treated with saline, 0.1mg/ml chymotrypsin, or 0.2mg/ml chymotrypsin.

Gene Expression in the Spinal Cord

As shown in Figure 3, administration of 0.1mg/ml chymotrypsin led to a significant suppression in IFN-γ expression, as compared to saline- and 0.2mg/ml chymotrypsin treated animals. However, IFN-γ expression in 0.2mg/ml chymotrypsin -treated animals did not show significant change compared with saline-

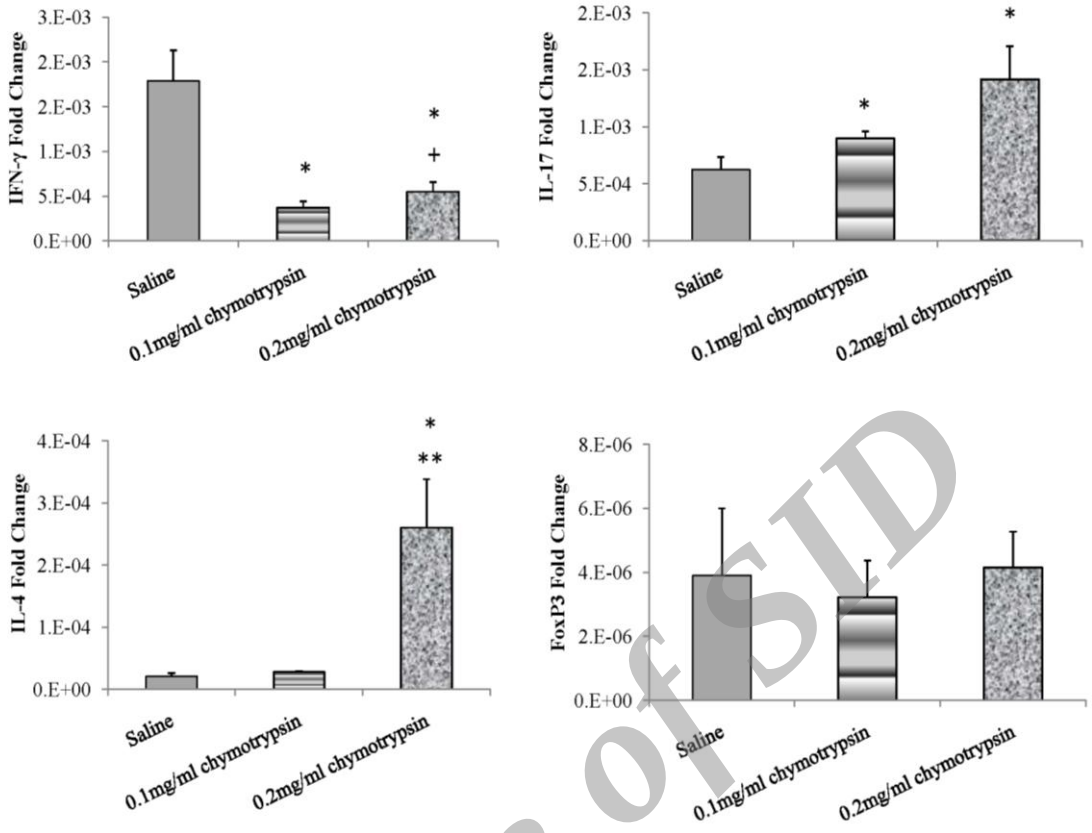

Figure 2. Comparison of IFN-γ, IL-17, IL-4, and FoxP3 expression in the brain. The animals were treated via intra-CSF route with saline, 0.1mg/ml chymotrypsin, or 0.2mg/ml chymotrypsin on day 7 after the disease induction. The animals were sacrificed on day 14 post EAE induction at the peak of the disease. Quantitative PCR was performed after RNA extraction and cDNA synthesis, when beta-actin was used as the reference gene for normalization. Data are presented as mean±SEM. (*): significant difference with saline-treated animals (p<0.05). (**): significant difference with 0.1mg/ml chymotrypsin-treated animals (p<0.05). (+): partial significant difference (p=0.1).

treated animals.

Administration of 0.2mg/ml chymotrypsin markedly induced IL-17 expression compared with 0.1mg/ml chymotrypsin- and saline-treated animals.

However, there was no significant difference between saline- and 0.1mg/ml chymotrypsin-treated animals with respect to IL-17 expression. The Th2 cytokine, IL-4 level was only significantly elevated in animals treated with 0.2mg/ml chymotrypsin compared with saline- and 0.1mg/ml chymotrypsin-treated animals. FoxP3 expression in 0.2mg/ml chymotrypsin- and 0.1mg/ml chymotrypsin-treated animals was statistically similar to that of saline-treated group.

Gene Expression in Deep Cervical Lymph Node (DCLNs)

Since DCLNs are regional lymph nodes for brain in the rat and they appear to play a role in T cell mediated immunity in the brain,[37] we investigated the expression of IFN-γ, IL-17, IL-4, and FoxP3 in DCLNs of the three groups (Figure 4). IFN-γ expression in DCLN was not markedly different among the three groups. In contrast, mRNA level of IL-17 was significantly stimulated in DCLNs of 0.1mg/ml or 0.2mg/ml chymotrypsin-treated animals relative to saline-treated animals. However, IL-4 expression showed a significant increase upon treatment with 0.1mg/ml or 0.2mg/ml chymotrypsin relative to treatment with saline. However, treatment with 0.2mg/ml chymotrypsin did not result to a significant stimulation of IL-4 expression relative to treatment with 0.1mg/ml chymotrypsin. There was not a significant difference in FoxP3 mRNA level among the three groups.

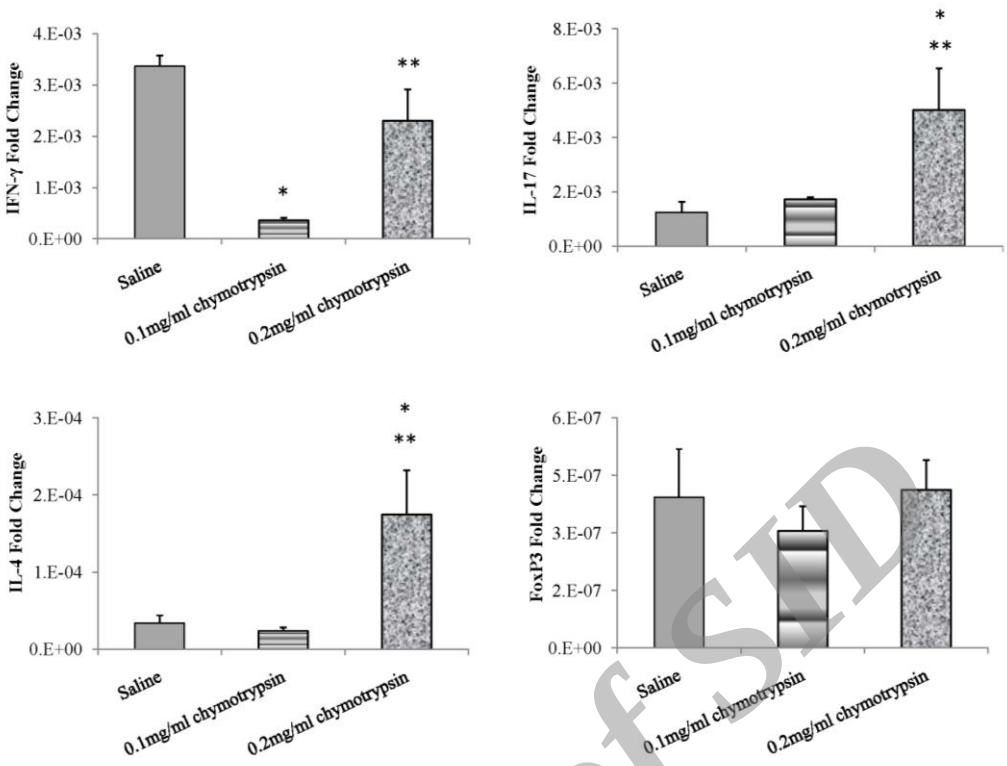

Figure 3. Comparison of IFN-γ, IL-17, IL-4, and FoxP3 expression levels in the spinal cord, as explained in previous legend.

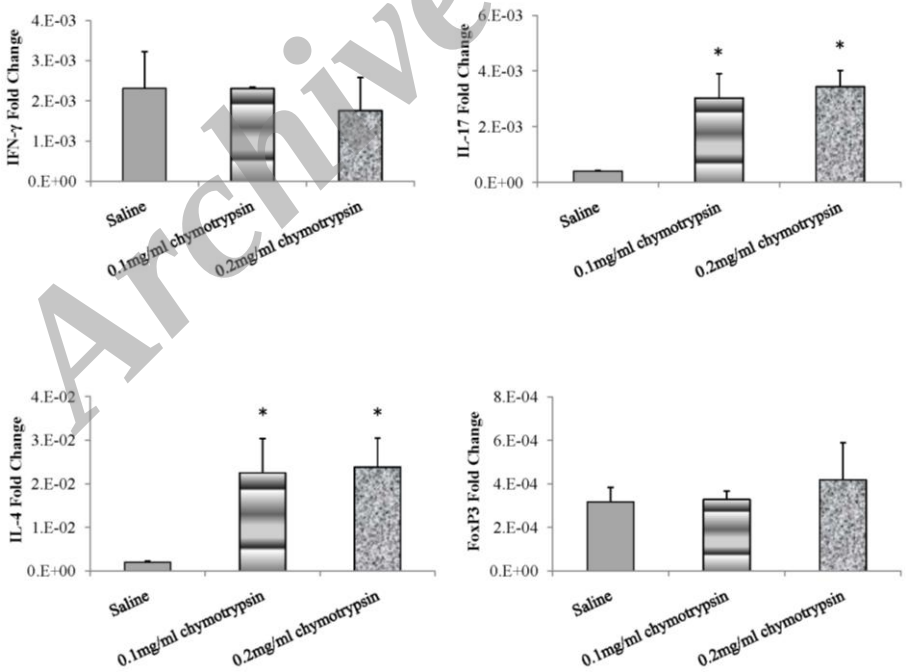

Figure 4. Comparison of IFN-γ, IL-17, IL-4, and FoxP3 levels in the deep cervical lymph node. Treatment of the animals with saline-, 0.1mg/ml chymotrypsin-, or 0.2mg/ml chymotrypsin and quantitative PCR were performed as explained in Figure 2 legend. Data are presented as mean±SEM. (*): significant difference with saline-treated animals (p<0.05).

Comparison of Gene Expression between Brain and Spinal Cord

To determine the differences between brain and spinal cord, we compared IFN-γ, IL-17, 1L-4, and FoxP3 expression level in brain and spinal cord in each of saline-, 0.1mg/ml chymotrypsin-, or 0.2mg/ml chymotrypsin-treated animals, as shown in Figure 5.

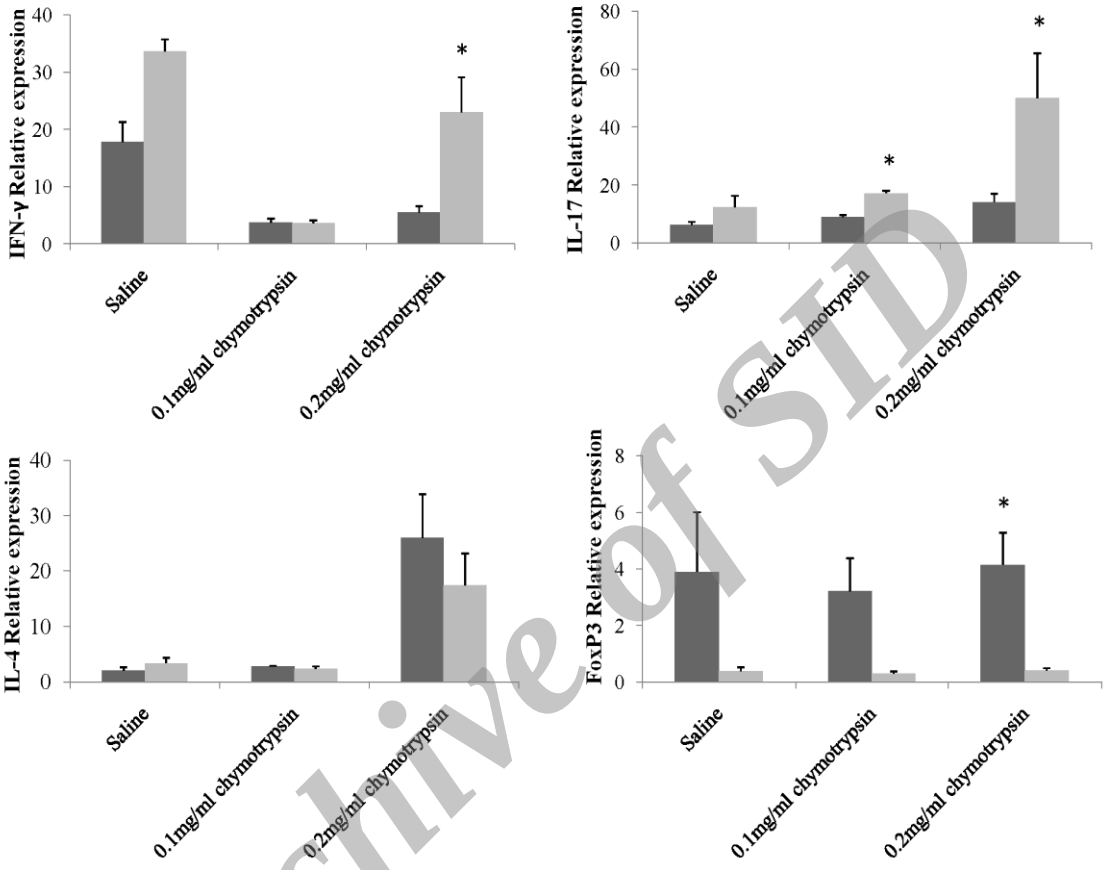

Figure 5. Comparison of brain and spinal cord in terms of IFN-γ, IL-17, IL-4, and FoxP3 levels in animals treated with saline, 0.1mg/ml chymotrypsin, or 0.2mg/ml chymotrypsin (■Brain & ■Spinal Cord). Data are presented as mean±SEM. (*): significant difference with brain (p<0.05).

There was no significant difference in the expression of IFN-γ, IL-17, IL-4, and FoxP3 leves between brain and spinal cord of saline-treated animals. The expression of IL-17 was significantly higher in the spinal cord comparing with the brain of the animals treated with 0.1mg/ml chymotrypsin. However, there was no significant difference in IFN- γ, IL4, and FoxP3 expression between brain and spinal cord of 0.1mg/ml chymotrypsin-treated animals.

In the animals treated with 0.2mg/ml chymotrypsin, the expression of IFN-γ, IL-17 levels showed significant induction in the spinal cord as compared with the brain. Inversely, FoxP3 expression in the brain was more than that of spinal cord. IL-4 expression in the brain and spinal cord were not statistically different.

DISCUSSION

MS is considered as a chronic inflammatory autoimmune disorder of the CNS. Inflammatory reaction of the disease causes de-myelination, axonal degeneration, and gliosis.[38] A number of anti-inflammatory agents have been used to ameliorate of MS and/or EAE. Corticosteroids are widely used for the suppression of chronic inflammatory and

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

autoimmune diseases.[39-42] Corticosteroids specifically target the immune response and cause a shift from Th1 to Th2 immune responses, leading to directly inhibition of pro-inflammatory cytokines production.[43] Treatment with an H1R antagonists or H2R agonist reduces the clinical signs in MS and EAE. H1R and H2R are believed to stimulate pro- and anti-inflammatory effect of histamine, respectively.[44-48] IFN-β reduces the production of pro-inflammatory cytokines and induces the production of anti-inflammatory cytokines.[49,50] Glatiramer acetate,[51] laquinimod.[52] and cyclophosphamide[53] increase Th2 response. Importantly, sex hormones including androgens attenuate MS and EAE severity through activation of Th2 response.[54]

The role of chymotrypsin in inflammation has been vastly investigated[55] some of which indicated an anti-inflammatory role for chymotrypsin in the peripheral organs.[13,16-20] We previously demonstrated that chymotrypsin could ameliorate EAE in female Lewis rats.[24] Because of unknown reason(s) EAE is more severe in males than females. In this study, anti-inflammatory effect of chymotrypsin on CNS in a male model of EAE was investigated. Based on previous reports showing the effectiveness of intra-CSF delivery of enzyme in reducing the range of neuropathological effects in the CNS,[56] chymotrypsin was administered via intra-CSF route. There is an unexplained preferential targeting of inflammation to the spinal cord in the EAE model used in this study.[57,58] Administration of 0.1mg/ml or 0.2 mg/ml chymotrypsin significantly downregulated IFN-γ and upregulated IL-4 level at the spinal cord, compared to saline treatment. However, IFN-γ expression in 0.2mg/ml chymotrypsin-treated animals was more than that of 0.1mg/ml chymotrypsin-treated animals. This is consistent with the ameliorating effect of IFN-γ[59] and IL-4[11] in EAE. In addition, IL-17 level was increased in 0.2mg/ml chymotrypsin-treated animals compared to 0.1mg/ml chymotrypsin- and saline-treated animals, consistent with anti-inflammatory role of IL-17 at higher doses.[9] Co-expression of IL-4 and IL-17 has been previously explained and reviewed.[60] Presumably, chymotrypsin manipulates the cytokine network in a dose-dependent manner. This is consistent with the previous results indicating that absolute level of IL-17 is not the only determinant of inflammation. Indeed, the balance of Th1, Th2 and Th17cytokines determines the fate of immune events.[61,62] Although, Th1/Th17 double-positive cells have been frequently reported in humans,[10,63,64] but any Th2/Th17 commonality has been rarely evidenced.[65] This study is the second report indicating anti-inflammatory property for IL-17 at higher concentration. It seems that chymotrypsin does not affect FoxP3-expressing Treg population and exerts its modulatory effect via activation of different effector T cells.

Proteases are involved in the inflammatory autoimmune response and leukocyte extravasation due to the myelinolytic process and demyelination. Myelin basic proteins are vulnerable to digestion by proteases.[66] Cross-regulation of T cell responses by chymotrypsin could be due to altered digestion of myelin basic protein, a component of myelin,[67,68] similar to "altered peptide ligand" phenomenon of glatiramer acetate.[52] Alternatively, chymotrypsin may have role in the shedding of specific cell surface receptors.[19] In conclusion, our results demonstrated that the potent neuroimmunomodulatory effect of chymotrypsin is sex-independent. This study also provided more evidence for the anti-inflammatory effect of IL-17 at higher doses along with decreased IFN-γ and increased IL-4 expression in male Lewis rat model of EAE. Our previous study on female Lewis rat model of EAE demonstrated amelioration of the disease by decreased IFN-γ/IL-17 and increase in IL-4/Foxp3 expression. However, when comparing this study with the previous one it is notable that this study was performed using a two-step real-time PCR and the previous one performed using a one-step kit. More research is required to elucidate and compare the underlying anti-inflammatory role of chymotrypsin and how to increase its beneficial effect.

REFERENCES

1. Hartley MD, Altowaijri G, Bourdette D. Remyelination and multiple sclerosis: therapeutic approaches and challenges. Curr Neurol Neurosci Rep 2014; 14(10):485.
2. Becher B, Durell BG, Noelle RJ. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. J Clin Invest 2002; 110(4):493-7.
3. Ferber IA, Brocke S, Taylor-Edwards C, Ridgway W, Dinisco C, Steinman L, et al. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J Immunol 1996; 156(1):5-7.

4. Aranami T, Yamamura T. Th17 cells and autoimmune encephalomyelitis (EAE/MS). Allergol Int 2008; 57(2):115-20.
5. Chen Y, Langrish CL, McKenzie B, Joyce-Shaikh B, Stumhofer JS, McClanahan T, et al. Anti-IL-23 therapy inhibits multiple inflammatory patheways and ameliorates autoimmune encephalomyelitis. J Clin Invest 2006; 116(5):1317-26.
6. Hartung HP, Kieseier BC, Hemmer B. Purely systemically active anti-inflammatory treatments are adequate to control multiple sclerosis. J Neurol 2005; 252 Suppl 5:v30-7.
7. Hofstetter H, Gold R, Hartung HP. Th17 Cells in MS and Experimental Autoimmune Encephalomyelitis. Int MS J 2009; 16(1):12-8.
8. Touil T, Fitzgerald D, Zhang GX, Rostami AM, Gran B. Pathophysiology of interleukin-23 in experimental autoimmune encephalomyelitis. Drug News Perspect 2006; 19(2):77-83.
9. Ke Y, Liu K, Huang GQ, Cui Y, Kaplan HJ, Shao H, et al. Anti-inflammatory role of IL-17 in experimental autoimmune uveitis. J Immunol 2009; 182(5):3183-90.
10. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nat Med 2008; 14(3):337-42.
11. Bitan M, Weiss L, Reibstein I, Zeira M, Fellig Y, Slavin S, et al. Heparanase upregulates Th2 cytokines, ameliorating experimental autoimmune encephalitis. Mol Immunol 2010; 47(10):1890-8.
12. Blaber SI, Ciric B, Christophi GP, Bernett MJ, Blaber M, Rodriguez M, et al. Targeting kallikrein 6 proteolysis attenuates CNS inflammatory. FASEB J 2004; 18(7):920-2.
13. Müller AM, Jun E, Conlon H, Sadiq SA. Inhibition of SLPI ameliorates disease activity in experimental autoimmune encephalomyelitis. BMC Neurosci 2012; 13:30.
14. Nelissen S, Vangansewinkel T, Geurts N, Geboes L, Lemmens E, Vidal PM, et al. Mast cells protect from post-traumatic spinal cord damage in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4. Neurobiol Dis 2014; 62:260-72.
15. Safavi F, Rostami A. Role of serine proteases in inflammation: Bowman-Birk protease inhibitor (BBI) as a potential therapy for autoimmune diseases. Exp Mol Pathol 2012; 93(3):428-33.
16. Frenkel K, Chrzan K, Ryan CA, Wiesner R, Troll W. Chymotrypsin-specific protease inhibitors decrease H2O2 formation by activated human polymorphonuclear leukocytes. Carcinogenesis 1987; 8(9):1207-12.
17. Jutila MA, Kishimoto TK, Finken M. Low-dose chymotrypsin treatment inhibits neutrophil migration into sites of inflammation in vivo: effects on Mac-1 and MEL-14 adhesion protein expression and function. Cell Immunol 1991; 132(1):201-14.
18. Sarkar N, Foskick LS. Mode of action of chymotrypsin on pleural inflammation. J Pharmacol Exp Ther 1964; 146:258-64.
19. Pham CT. Neutrophil serine proteases fine-tune the inflammatory response. Int J Biochem Cell Biol 2008; 40(6-7):1317-33.
20. Viswanatha Swamy AH, Patil PA. Effect of some clinically used proteolytic enzymes on inflammation in rats. Indian J Pharm Sci 2008; 70(1):114-7.
21. Kitano H, Saito T, Kanayama N. Substrate monolayers as electrochemical sensing elements for α-Chymotrypsin. J Colloid Interface Sci 2002; 250(1):134-41.
22. Kostetskii PV. The volume and structure of the Chymotrypsin active site. Biofizika 2005; 50(6):993-7.
23. Martin GJ, Brendal R, Beiler JM. Absorption of enzymes from the intestinal tract. Am J Pharmacol 1957; 129(6):194-7.
24. Ghaffarinia A, Jalili C, Riazi-Rad F, Mostafaie A, Parvaneh S, Pakravan N. Anti-inflammatory effect of chymotrypsin to autoimmune response against CNS is dose-dependent. Cell Immunol 2014; 292(1-2):102-8.
25. Soldin OP, Chung SH, Mattison DR. Sex differences in drug disposition. J Biomed Biotechnol 2011; 2011:187103.
26. Donovan MD. Sex and racial differences in pharmacological response: effect of route of administration and drug delivery system on pharmacokinetics. J Womens Health (Larchmt) 2005; 14(1):30-7.
27. Ruigrok AN, Salimi-Khorshidi G, Lai MC, Baron-Cohen S, Lombardo MV, Tait RJ, et al. Meta-analysis of sex differences in human brain structure. Neurosci Biobehav Rev 2014; 39:34-50.
28. Allen JS, Damasio H, Grabowski TJ, Bruss J, Zhang W. Sexual dimorphism and asymmetries in the gray-white composition of the human cerebrum. Neuroimage 2003; 18(4):880-94.
29. Bayless DW, Daniel JM. Sex differences in myelin-associated protein levels within and density of projections between the orbital frontal cortex and dorsal striatum of adult rats: implications for inhibitory control. Neuroscience 2015; 300:286-96.

30. Yang S, Li C, Zhang W, Wang W, Tang Y. Sex differences in the white matter and myelinated nerve fibers of Long-Evans rats. Brain Res 2008; 1216:16-23.
31. Ghaffarinia A, Jalili C, Parvaneh S, Mir-Aghaee S, Pakravan N. Damage of urinary/respiratory system and survival rate is affected by gender in EAE model of Lewis rat. Acta Scientiae Veterinariae 2015; 43:1269-1277.
32. Papenfuss TL, Rogers CJ, Gienapp I, Yurrita M, McClain M, Damico N, et al. Sex differences in experimental autoimmune encephalomyelitis in multiple murine strains. J Neuroimmunol 2004; 150(1-2):59-69.
33. Tremlett H, Zhao Y, Rieckmann P, Hutchinson M. New perspectives in the natural history of multiple sclerosis. Neurology 2010; 74(24):2004-15.
34. Dunn SE, Lee H, Pavri FR, Zhang MA. Sex-Based Differences in Multiple Sclerosis (Part I): Biology of Disease Incidence. Curr Top Behav Neurosci 2015; 26:29-56.
35. Dunn SE, Gunde E, Lee H. Sex-Based Differences in Multiple Sclerosis (MS): Part II: Rising Incidence of Multiple Sclerosis in Women and the Vulnerability of Men to Progression of this Disease. Curr Top Behav Neurosci 2015; 26:57-86.
36. Buc M. Role of regulatory T cells in pathogenesis and biological therapy of multiple sclerosis. Mediators Inflamm 2013; 2013:963748.
37. Goldmann J, Kwidzinski E, Brandt C, Mahlo J, Richter D, Bechmann I. T cells traffic from brain to cervical lymph nodes via the cribroid plate and the nasal mucosa. J Leukoc Biol 2006; 80(4):797-801.
38. Sospedra M, Martin R. Immunology of multiple sclerosis. Ann Rev Immunol 2005; 23:683–747.
39. Ciccone A, Beretta S, Brusaferri F, Galea I, Protti A, Spreafico C. Corticosteroids for the long-term treatment in multiple sclerosis. Cochrane Database Syst Rev 2008; 23(1):CD006264.
40. Frequin STFM, Barkhof F, Lamers KJB, Hommes OR. The effects of high-dose methylprednisolone on gadolinium-enhanced magnetic resonance imaging and cerebrospinal luidmeasurements inmultiple sclerosis. J Neuroimmunol 1992; 40(2-3):265–72.
41. Sloka JS, Stefanelli M. The mechanism of action of methylprednisolone in the treatment of multiple sclerosis. Mult Scler 2005; 11(4):425-32.
42. Then Bergh F, Kümpfel T, Schumann E, Held U, Schwan M, Blazevic M, et al. Monthly intravenous methylprednisolone in relapsing-remitting multiple sclerosis - reduction of enhancing lesions, T2 lesion volume and plasma prolactin concentrations. BMC Neurol 2006; 23:6-19.
43. Barnes PJ. Anti-inlammatory actions of glucocorticoids: molecular mechanisms. Clin Sci 1998; 94(6):557–72.
44. Alonso A, Jick SS, Hernán MA. Allergy, histamine 1 receptor blockers, and the risk of multiple sclerosis. Neurology 2006; 66(4):572-5.
45. Emerson MR, Orentas DM, Lynch SG, LeVine SM. Activation of histamine H2 receptors ameliorates experimental allergic encephalomyelitis. Neuroreport 2002; 13(11):1407-10.
46. Lapilla M, Gallo B, Martinello M, Procaccini C, Costanza M, Musio S, et al. Histamine regulates autoreactive T cell activation and adhesiveness in inflamed brain microcirculation. J Leukoc Biol 2011; 89(2):259-67.
47. Logothetis L, Mylonas IA, Baloyannis S, Pashalidou M, Orologas A, Zafeiropoulos A, et al. A pilot, open label, clinical trial using hydroxyzine in multiple sclerosis. Int J Immunopathol Pharmacol 2005; 18(4):771-8.
48. Passani MB, Ballerini C. Histamine and neuroinflammation: insights from murine experimental autoimmune encephalomyelitis. Front Syst Neurosci 2012; 6:32.
49. Vollmer T, Stewart T, Baxter N. Mitoxantrone and cytotoxic drugs' mechanisms of action. Neurol 2010; (74 Suppl 1):S41-6.
50. Kay M, Hojati Z, Dehghanian F. The molecular study of IFNβ pleiotropic roles in MS treatment. Iran J Neurol 2013; 12(4):149-56.
51. Minagar A. Current and future therapies for multiple sclerosis. Scientifica (Cairo) 2013; 2013:249101.
52. Ruggieri M, Avolio C, Livrea P, Trojano M. Glatiramer acetate in multiple sclerosis: a review. CNS Drug Rev 2007; 13(2):178-91.
53. Preiningerova J. Oral laquinimod therapy in relapsing multiple sclerosis. Expert Opin Investig Drug 2009; 18(7):985-9.
54. Spence RD, Voskuhl RR. Neuroprotective effects of estrogens and androgens in CNS inflammation and neurodegeneration. Front Neuroendocrinol 2012; 33(1):105-15.
55. Pham CT. Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol 2006; 6(7):541-50.
56. Hemsley KM, Beard H, King BM, Hopwood JJ. Effect of high dose, repeated intra-CSF injection of sulphamidase on neuropathology in MPS IIIA mice. Genes Brain Behav 2008; 7(7):740–53.
57. Mix E, Meyer-Rienecker H, Hartung HP, Zettl UK. Animal models of multiple sclerosis-potentials and

limitations. Prog Neurobiol 2010; 92(3):386-404.
58. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br J Pharmacol 2011; 164(4):1079-106.
59. Krakowski M. Owens T. Interferon-gamma confers resistance to experimental allergic encephalomyelitis. Eur J Immunol 1996; 26(7):1641-6.
60. Cooney LA, Fox DA. Regulation of Th17 maturation by interleukin 4. Crit Rev Immunol 2013; 33(5):379-87.
61. Cooney LA, Towery K, Endres J, Fox DA. Sensitivity and resistance to regulation by IL-4 during Th17 maturation. J Immunol 2011; 187(9):4440-50.
62. Sarkar S, Cooney LA, White P, Dunlop DB, Endres J, Jorns JM, et al. Regulation of pathogenic IL-17 responses in collagen-induced arthritis: roles of endogenous interferon-gamma and IL-4. Arthritis Res Ther 2009; 11(5):R158.
63. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. J Exp Med 2007; 204(8):1849-61.
64. Boniface K, Blumenschein WM, Brovont-Porth K, McGeachy MJ, Basham B, Desai B, et al. Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage. J Immunol 2010; 185(1):679-87.
65. Raymond M, Van VQ, Wakahara K, Rubio M, Sarfati M. Lung dendritic cells induce T(H)17 cells that produce T(H)2 cytokines, express GATA-3, and promote airway inflammation. J Allergy Clin Immunol 2011; 128(1):192-201.
66. Lajtha A, Banik NL. Role of Proteases in the Pathophysiology of Neurodegenerative Diseases. Amsterdam:Kluwer Academic Publishers, 2002; 5-24.
67. Katsara M, Yuriev E, Ramsland PA, Tselios T, Deraos G, Lourbopoulos A, et al. Altered peptide ligands of myelin basic protein (MBP87_99) conjugated to reduced mannan modulate immune responses in mice. Immunology 2009; 128(4):521-33.
68. Lees MB and Brostoff SW. Proteins of myelin, in: Myelin. P. Morell, ed., New York: Plenum, 1984: 197-224.