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

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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

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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-mediated disease. However, it was later demonstrated that Th1 response-deficient mice which lack molecules, such as IFN- γ , IFN- γ R, 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- β 1 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 reported regarding 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 50 μ g guinea pig spinal cord and 400 μ g *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 tonic 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

Neuroimmunomodulation of Chymotrypsin by Upregulation of IL-4 & IL-17

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 saline and 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 5 μ l master mix, 100 nM primers for β -actin, IFN- γ , IL-4, IL-17, FoxP3, and 1 μ l template cDNA.

The sequences of primers were forward 5'-

aggccaaccgtgaaaagatg-3', and 5'-accagaggcatacagggacaa-3' for β -actin and forward 5'-ccacggagaacagactcatc-3' and reverse 5'-gagaaccccagactgtttctca-3' for IL-4, forward 5'-gggaagtggaccaccacat-3' and reverse 5'-ttctccaccggaaagtga-3' for IL-17, forward 5'-gaaagacaaccaggccatcag-3' and reverse 5'-tcataatgcatcctttttg-3' for IFN- γ , and forward 5'-cgggagagtttctcaagcac-3' and reverse 5'-ggagctctgtccactgagg-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 \pm 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, IL-4, and FoxP3 as representative of Th1, Th17, Th2, and natural Treg, respectively.

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

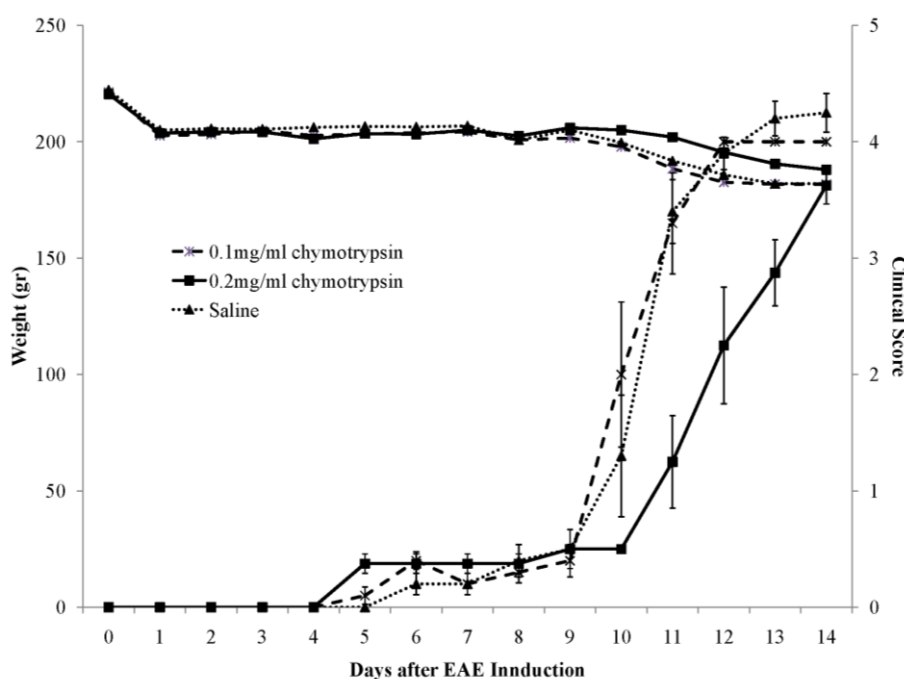


Figure 1. Treatment with saline (●▲●), 0.1mg/ml chymotrypsin (---*---), 0.2mg/ml chymotrypsin (—■—) affects clinical score and weight loss in male Lewis rats model of EAE. The rats were immunized via subcutaneous route with a suspension of guinea pig spinal cord and complete Freund's adjuvant (CFA). 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 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-

Neuroimmunomodulation of Chymotrypsin by Upregulation of IL-4 & IL-17

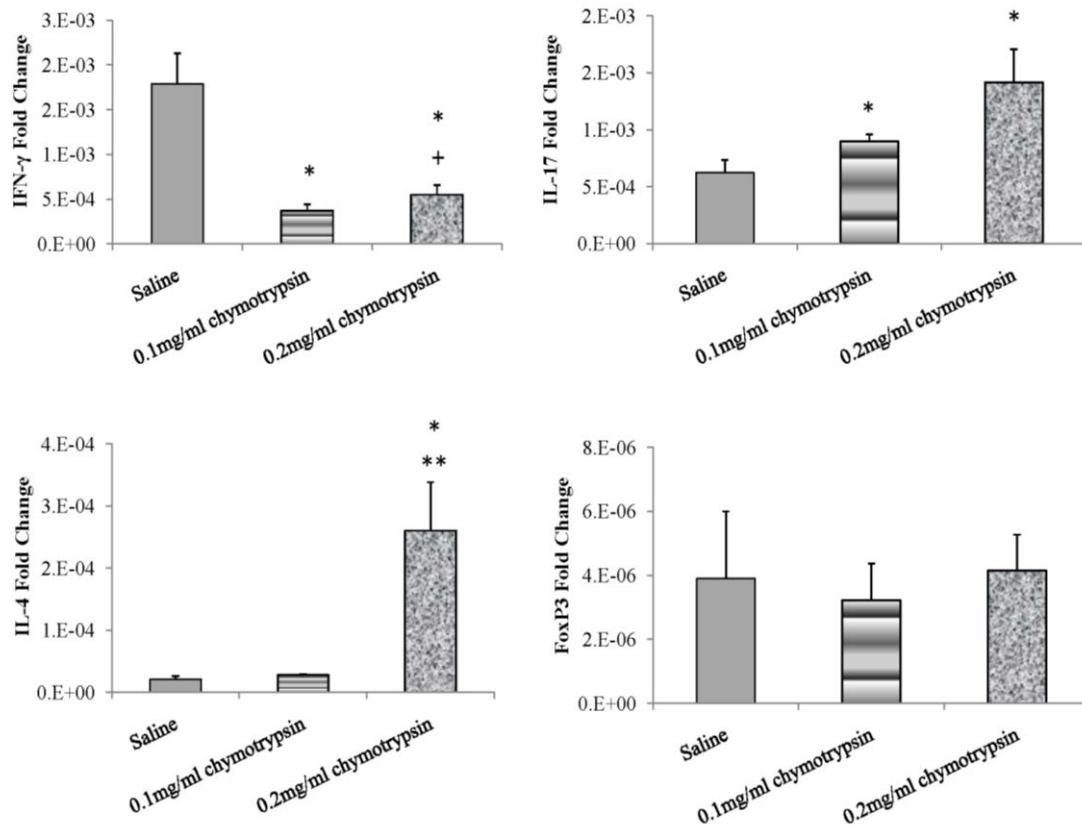


Figure 2. Comparison of IFN- γ , IL-17, IL-4, and FoxP3 expression in the brain. The animals were treated via intra-CSF route with saline, 0.1mg/ml chymotrypsin, or 0.2mg/ml chymotrypsin on day 7 after the disease induction. The animals were sacrificed on day 14 post EAE induction at the peak of the 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 \pm 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.

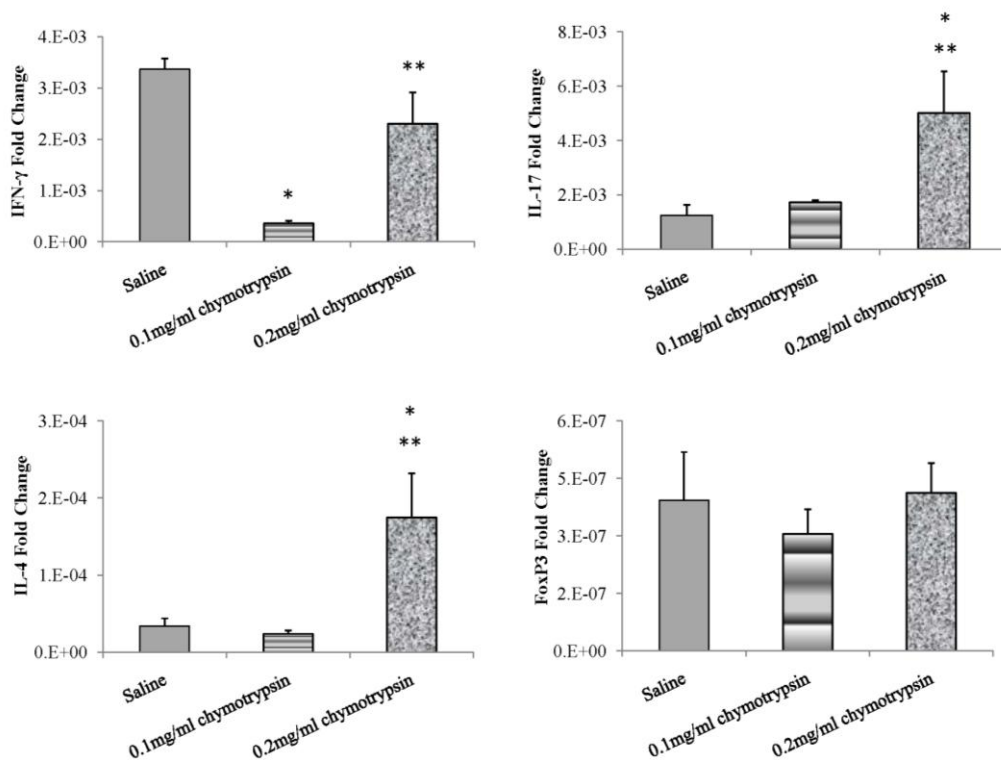


Figure 3. Comparison of IFN- γ , IL-17, IL-4, and FoxP3 expression levels in the spinal cord, as explained in previous legend.

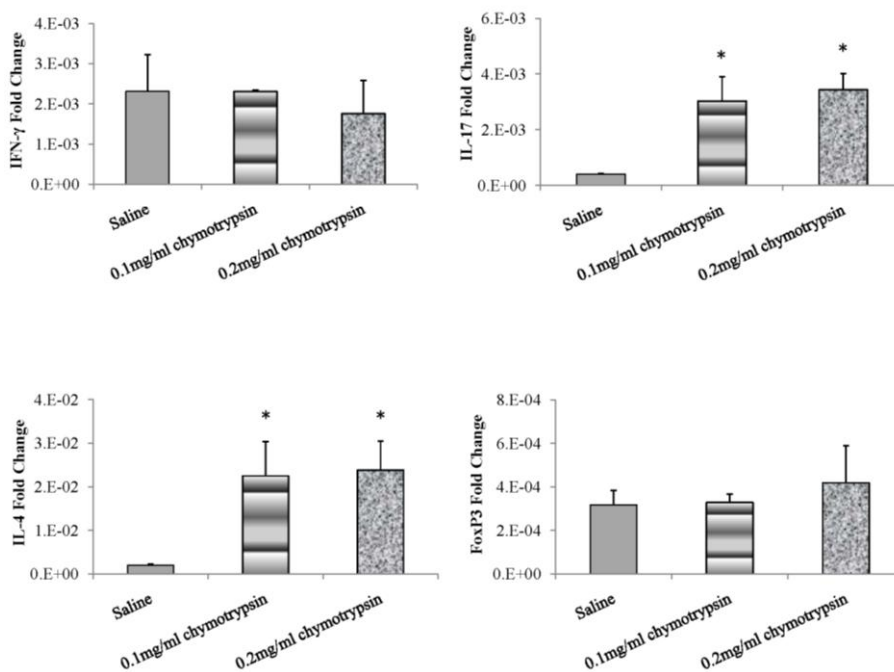


Figure 4. Comparison of IFN- γ , IL-17, IL-4, and FoxP3 levels in the deep cervical lymph node. Treatment of the animals with saline-, 0.1mg/ml chymotrypsin-, or 0.2mg/ml chymotrypsin and quantitative PCR were performed as explained in Figure 2 legend. Data are presented as mean \pm SEM. (*): significant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

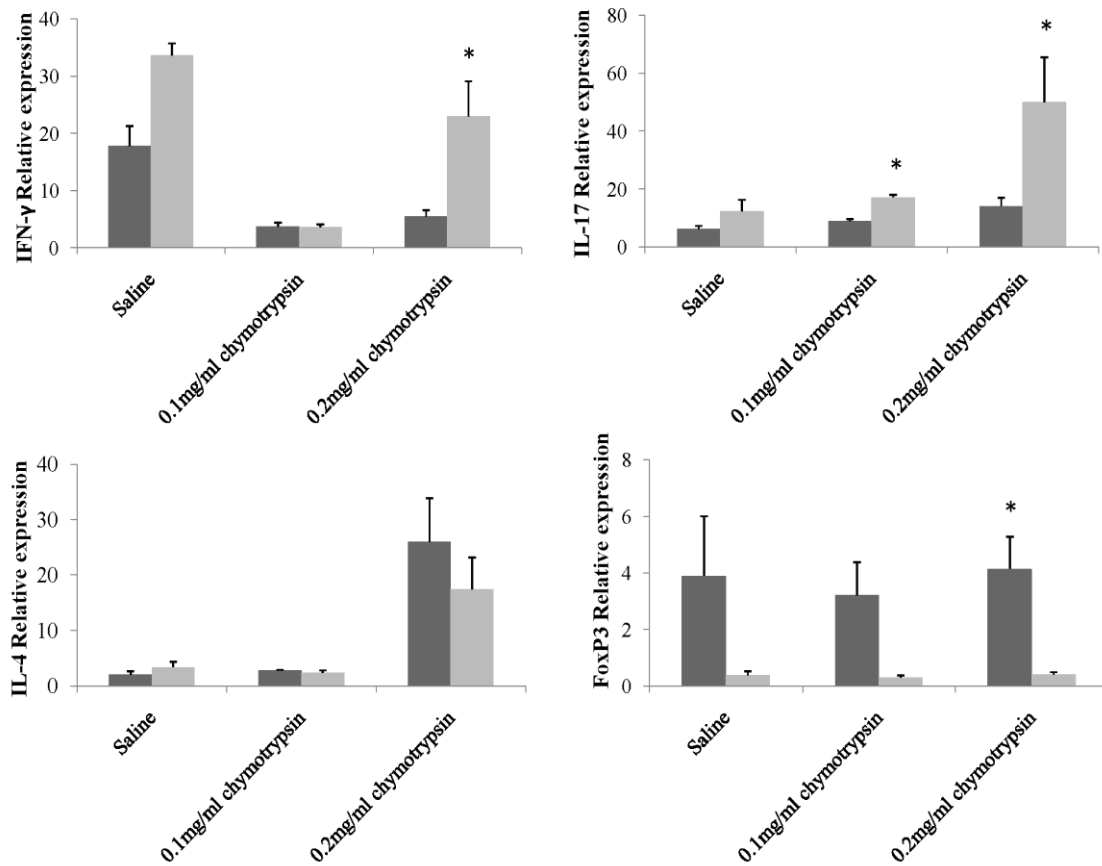


Figure 5. Comparison of brain and spinal cord in terms of IFN- γ , IL-17, IL-4, and FoxP3 levels in animals treated with saline, 0.1mg/ml chymotrypsin, or 0.2mg/ml chymotrypsin (■Brain & ■Spinal Cord). Data are presented as mean \pm SEM. (*): significant difference with brain ($p < 0.05$).

There was no significant difference in the expression of IFN- γ , IL-17, IL-4, and FoxP3 levels 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

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 cyclophosphamide⁵³ 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 investigated⁵⁵ 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-4¹¹ 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 Th17 cytokines 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.

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