Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Helminthic therapy of experimental autoimmune encephalomyelitis by Dicrocoelium ova

Zahra Navi
Mashhad University of Medical Sciences
Mozhdeh Jafari Rad
Mashhad University of Medical Sciences
Abdolmajid Fata
Mashhad University of Medical Sciences
Elham Moghaddas (moghaddase@mums.ac.ir)
Mashhad University of Medical Sciences https://orcid.org/0000-0002-7080-2768
Mahmoud Mahmoudi
Mashhad University of Medical Sciences
Maryam Rastin
Mashhad University of Medical Sciences
Mojtaba Mousavi Bazaz
Mashhad University of Medical Sciences
Fahimeh Lavi Arab
Mashhad University of Medical Sciences
Sajad Sahab Negah
Mashhad University of Medical Sciences
Amir Reza Heidari
Mashhad University of Medical Sciences
Naser Morgan Azghadi
Ferdowsi University of Mashhad Faculty of Veterinary Medicine

Research article

Keywords: Encephalomyelitis, Autoimmune, Experimental, Dicrocoelium, Th1-Th2 Balance, Multiple Sclerosis

Posted Date: March 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-17288/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background Experimental Autoimmune Encephalomyelitis (EAE) is an animal model of Multiple Sclerosis (MS). This study was conducted to evaluate the efficacy of Dicrocoelium ova on Experimental Autoimmune Encephalomyelitis (EAE) treatment in C57BL6 mice.

Methods Twenty-eight C57BL/6 mice were assigned in four groups as control(C), prophylaxis (P), treatment1 (T1), and treatment2 (T2). Prior to induction of EAE in prophylaxis group and on days 7 and 18 in T1 and T2 groups, respectively, Dicrocoelium eggs were injected to each mouse. Clinical score, weight changes, and incidence time of EAE were recorded. IFN- γ and IL-4 assay and histopathological study by (H&E) and Toluidine-Blue (TB), and Luxol Fast Blue (LFB) were done. Data were analyzed using SPSS software version 21.

Results The disease score was significantly lower in P and T1 groups than the control group (p=0.01). IFN- γ was lower in P and T1 groups than the control group. The highest level of IL-4 was observed in the T1 group. The total number of Neuroglia cells of corpus callosum was similar in all groups, but density increased in T1 group compared to the control group (P = 0.03).

Conclusions : The results of the study showed that, Dicrocoelium eggs have the great potential efficacy to stimulate immunomodulation toward treatment of EAE during the initial phase.

Background

A dramatic increase has been observed in the prevalence of autoimmune diseases in populations who have born in developed countries during1980-1999 [1, 2]. Expansion of inflammatory diseases has been coincided with the improvement of vaccination and antibiotics consumption over such a short time [3]. These mentioned factors prevent the natural contact of individual's immunity with infectious agents ,which contributed to the development of immune system disorders suggesting the popular hygiene hypothesis [4]. Multiple Sclerosis(MS) disease is mediated with autoimmune $CD4^+$ Th_1 and Th_{17} responses [5, 6]. There are documented evidences showing IFN-γ (Th_1) is abundant in CNS of patients suffering from MS. Moreover, it is responsible for aggravation of their signs and symptoms [7],[8]. Experimental Autoimmune Encephalomyelitis (EAE) is an animal model of demyelinating disease so called MS in human. EAE extensively helps us to clarify the role of some specific cells in the pathology of MS. [9]. Helminths are known to be the dominant immune regulators with more than 20 different mechanisms for induction of immune regulation [10, 11]. Infections with helminth can shift the immune system toward Th_2 responses and suppress the Th_1, Th_{17} immune responses and induce M_2 macrophage differentiation [12, 13].Especially, IL4 (Th_2) is mostly effective in suppression of Th_1 pro inflammatory cells and diminishes the production of TNFα and IL_1 [14, 15]. Helminths also develop the activity of Tregs consequently suppressing the increase in Th_1 inflammatory productions in autoimmune diseases. In spite of extensive researches, treatment of MS has been remained unsolved. In addition, long time drug use is ineffective, which might be followed by severe toxic side effects.

There are controversial notions about helminth therapy of MS due to the existence of some limitations in treatment of patients with live worms, and it might be harmful for humans. Immunoregulation by helminths components could be more safe and reliable [13]. In this study, the effect of Dicrocoelium ova administration as crud antigens in treatment of EAE was investigated. This study was conducted because of high overall prevalence of MS (about 11.4%) in Iran [16] .Also, Dicrocoelium spp.is the most common liver fluke found in the ruminants in Iran [17].

Methods

Animals

Twenty-eight, 5-6 -week-old C57BL/6 female mice were purchased from Rouyan Institute of Iran (Tehran) and were housed in the pathogen-free animal lab, at 23±2^{o} C, with a relative humidity of 50±5 % and a 12-h light/dark cycle. Facilities regarding the access to food and water supply were provided for the paralyzed mice. This study was approved by the Committee for Animal Ethics of Mashhad University of Medical Sciences (Ethical code: IR – MUMS. FM. REC 1396. 203).

Dicrocoelium spp. Eggs Preparation

Livers of the slaughtered sheep infected with *Dicrocoelium* flukes were obtained from a slaughterhouse located in Mashhad (Iran). *Dicrocoelium* dendriticum adults were identified and were separated according to their microscopic characters, then were washed with sterile PBS. Eggs were washed with penicillin 500 U/mL- streptomycin 0.5 mg/mL (with a catalog number of XC-A4122/100). Then, the antibiotics were removed by10 times washing the eggs with sterile PBS. Finally, eggs were counted using Neobar slide, and were stored at -80 ^{o} C.

Experimental Design

28 mice were randomly assigned in four different groups including prophylaxis (P), treatment 1 (T1), and treatment 2 (T2), each group had 8 mice and the control (C) group had 4 mice. 2 weeks before EAE induction, 10,000 *Dicrocoelium* eggs were injected intraperitoneally (IP) to the prophylaxis group. 4 days prior to EAE, a second dose of eggs was injected to prophylaxis group; induction was performed using 5000 eggs via IP and 5000 via SC injections. EAE induction was initiated, when all mice were 8-9 weeks old. Both T_1 & T_2 groups received 20000 *D. Dendriticum* ova on 7th day after EAE induction and 18th day after appearance of paralysis, respectively. The control group remained untreated. Mice in the control group received PBS. According to an approved protocol[18] , on 40th day ,all mice were sacrificed ,and the spleen and brain of mice were removed for cytokine and histopathological assays, respectively.

EAE Induction

To perform the EAE induction, all mice were immunized with synthetic peptide of Myelin Oligodendrocyte Glycoprotein (MOG) amino acid residues 35–55 (MEVGWYRSPFSRVVHLYRNGK) (SBS Genetech Co. Ltd.,

Beijing, China). Induction was performed by preparing an emulsion of MOGp 35–55 (300 mg) in complete Freund's adjuvant containing 5mg/ ml of heat- killed *Mycobacterium tuberculosis* (H37Ra strain; Sigma, St. Louis, MO); yielding a solution containing1mg of MOG/ml. Each mouse received 200 ml of this emulsion through SC injection into two sites on its flank (100 ml/ site). Each mouse also received 250ng of pertussis toxin (Sigma, Germany) through IP injection on the day of immunization and 48h later [19]. Animals were assessed clinically according to the standard criteria(0: normal; 1: loss of tail tone; 2: hind limb weakness; 3: hind limb paralysis; 4: hind limb and forelimb paralysis; and 5: moribund or dead [18]. To evaluate the pre -immunization and efficacy of *Dicrocoelium* eggs on EAE, clinical score, weight changes, and incidence time of EAE were recorded in all groups each day.

Methods of Euthanasia

CO2 narcosis is done for euthanasia procedure, a method approved by the University of Connecticut Health Center Animal Care Committee (IACUC).

Histologic Analysis

Histopathologic evaluation was performed on 20 mice brains (6 P, 6 T1, 4 T2, and 4 C). Haematoxylin-Eosin (H&E) and Toluidine-Blue (TB) staining techniques were used to evaluate the neuroglia cells number in corpus callosum area. Also, brain sections were stained by Luxol Fast Blue (LFB) to evaluate the density. A total of 9 sections per mouse in each group were investigated and observed as blind. To perform the statistical analysis, Kruskal- Wallis test was performed. The p –value of < 0.05 was considered as statistically significant.

RNA Extraction

To compare the expression of $Th_{1/}$ Th_2 related cytokines between different groups, the mRNA gene expression levels of IFN- γ and IL-4 in mice splenocytes were investigated by Real -Time Polymerase Chain Reaction (RT- PCR) (Rotor Gene Q - CIAGEN). About 2 million cells were separated from the spleens and were stored at -2$\mathring{0}$ C with 750 µl of TRIZOL (Invitrogen – Germany). Total RNA was isolated using TRIZOL (Invitrogen – Germany), and then the reverse transcription was performed according to easy cDNA synthesis kit (Pars-Tous, Mashhad, Iran). All the procedures were conducted according to the manufacturer's instructions. The primer sequences are listed in Table 1. The relative expression of each gene was calculated as the ratio of gene expression to the housekeeping gene (B2 macroglobulin).

Table1. Sequence of real-time primer sequences implemented in this study

Primer	Sequence
IFN-gamma	5′-CCAAGTTT GAGGTCAACA-3′5′-CTGGCAGAATTATTCTTATTGG-3′
B2mG	5′- CCTGTATGCTATCCAGAA -3′ 5′- GTAGCAGTTCAGTATGTTC -3′
IL4	5′CTGGATTCATCGATAAGC-3′ 5′-GATGCTCTTTAGGCTTTC-3′

Statistical Analysis

Data were analyzed using SPSS software version 21. 95% confidence interval and 5% significance level were considered in all tests. Normality of the data was evaluated by Kolmogorov-Smirnov test. Levenes test was used to assess the homogeneity of variances. To describe the data, frequency, mean, standard deviation, or mean tables were used. ANOVA test was used to compare the means. Tukeys post hoc test was used for multiple comparisons. Repeated -measures ANOVA was used to evaluate the differences in weight over time in each of four groups. Normality of residuals was evaluated by Shapiro-Wilk test. Mauchly's test was applied to test the sphericity.

Results

There were statistically significant differences between group mean scores as determined by one-way ANOVA ($F(3,210) = 10.08$, $p = .001$). The results showed that, disease score was significantly lower in P and T_1 groups than the control group ($p = 0.01$). Mice in P and T_1 had trivial sign of paralysis which developed slowly, however, the scores over than 3 were not observed. No significant difference was found in clinical scores between T_2 and control groups ($p > 0.05$) (Fig. 1). Peaks of EAE were shorter and fewer in P and T1 groups than the control group. Otherwise, EAE developed faster and reached to score 5 in T_2 group, and we missed one mouse same as the control group. After injection of eggs on day 18 in T2 group, remission was observed compared to the control group, but signs and symptoms were presented very quickly after 1 week.

In the control group, EAE was not seen in only one mouse. In T1 and P groups, just 2 mice from 8 mice in each group afflicted to the EAE. 50% decrease of cumulative incidence in the two mentioned groups was attributed to protective effect of Dicrocoelium eggs immunization and treatment. Dicrocoelium eggs immunization and treatment significantly reduced cumulative incidence rate in prophylaxis and treatment 1 groups ($p = 0.0$), and caused a delay of about 5 days in the onset of EAE in the mentioned groups. (Control n = 4), (Prophylaxis n = 8), (Treatment 1 n = 8), and (Treatment 2 n = 8). EAE developed in most mice in two groups (the control and treatment 2 groups) on day 10 [mean day of onset] but in prophylaxis and treatment1 groups, EAE developed just in 25% of mice on day 15 after induction (Fig. 2).

There were no statistically significant differences between group mean weight changes at baseline as determined by one-way ANOVA ($F(3,20) = 1.995$, $p = 0.147$). Mauchly's test indicated the violation in the

assumption of sphericity, χ 2(35) = 93.578 p < .001, therefore degrees of freedom were corrected using the Greenhouse-Geisser estimates of sphericity (ε = 0.429). The result showed that, there was no significant difference in weight changes between the groups in four times of measurement (weight * groups), F (10.28, 65.1), p = .0.115. But, helminthic therapy with Dicrocoelium eggs could inhibit severe weight loss in prophylaxis and treatment1 groups. In the control group, after induction of EAE, mean weight of mice reduced dramatically from 19 gr on day 10 to 17.5 gr on day 20. During 10 days, the mean weight loss was about 1.5 gr. In treatment group 2, mean weight loss from 20.5 gr on day 5 reached to 17.8 gr on day 15. Approximately, a 2.8 -gr weight loss was observed in this group. However, the maximum weight loss was estimated lower than 0.5 gr after induction of EAE in other two groups (Prophylaxis, treatment1groups) (Fig. 3).

Evaluation of Neuroglia Cell Number and Density in Corpus Callosum

To evaluate the effect of Dicrocoelium egg administration on the number of neuroglia cells in corpus callosum, H&E and TB staining were investigated by light microscope. The neuroglia cells were counted in a Scale bar of 200 µm.

The results demonstrated that, the quantity of neuroglia cells in corpus callosum is similar in 4 groups (P > 0.05; Fig. 4). The LFB stained sections showed more density in treatment 1 group compared to the control group (P = 0.03). No significant difference was observed between prophylaxis, treatment 2, and control groups (P > 0.05, Fig. 5).

Brains from each mouse (collected on day 40 post-immunization) were fixed, and were embedded in paraffin, sections (5-µm) were prepared, and then the tissues were stained with H&E (Fig. 4A) and TLB (Fig. 4B) to count the number of neuroglia cells (as presented in the left side of Fig. 4A and 4B). The quantification of neuroglia cells is shown in the right side of Fig A and B. (Control n = 4), (Prophylaxis n = 6), (Treatment 1 n = 6), and (Treatment 2 n = 4). No significant difference was found between 4groups (P > 0.05).

Brains from each mouse tissues were stained with LFB to assess the extent of density of brain tissue. Histologic features were scored semi quantitatively as presented in the right side of Fig. 5 (control, n = 4), (Prophylaxis, n = 6), (Treatment 1, n = 6), and (Treatment 2, n = 8). The quantification of brain tissue density is shown in the left side of Fig. 5. No significant difference was found between prophylaxis, treatment 2, and control groups (P > 0.05). There was a significant difference in EAE incidence between treatment1 and control groups (P = 0.03, *p < 0.05).

Evaluation of IFN- γ and IL-4 m RNA Expression

There were no statistically significant differences between group means of IFN- γ and IL-4 as determined by one-way ANOVA (F (3, 19) =.90, p = .459) (F (3,19) =1.210, p = .333). In prophylaxis and treatment 1 groups, the treatment induced a great up-regulation of IL-4, compared to the control group. The highest level of IL-4 was observed in the treatment 1 group (RQ=2.65). The IL-4 mRNA gene expression level was

lowest in the treatment 2group (RQ= 0.92), also, different levels of mRNA gene expression were observed in the IFN- γ. The mRNA gene expression level of IFN- γ was at highest copies in the control (RQ=8.09) and T2 (RQ= 5.91) groups, whereas the mRNA gene expression of IFN- γ was lower in the prophylaxis (RQ=4.03) and treatment 1 groups (RQ=0.37) than the control group (RQ=8.09) (Figure 6).

Relative mRNA gene expression of genes was determined compared to the housekeeping gene, β2 microglobulin.

Discussion

Immunotherapy by helminth is considered as one of the effective therapeutic methods for treatment of multiple sclerosis and other autoimmune diseases. The evidence indicates that helminthic infections lead to the reduction of autoimmune paroxysm in animal models [20, 21]. In most studies, the prevalence of helminthic infections before deterioration of EAE not only plummeted EAE scores but also decreased the incidence rate in the infected animal groups [22–24]. Many studies have been conducted so far to investigate the immune therapy of EAE (animal model of Multiple Sclerosis) by different helminth species such as Schistosoma mansoni, Trichinella spiralis, Fasciola hepatica, Trichinella psudospiralis, Taenia crassiceps, Strongyloides venezuelensis, Schistosoma japonicum, and Trichuris suis [18, 20, 21, 23, 25–30]. Among all of these studies, F. chiuso-minicucci et al. (2011) showed that, the infection with Strongyloides venezuelensis could not be effective in treatment of EAE. Yet, they emphasized that, their study alone cannot reject the hygiene hypothesis, and they recommended the conduction of more studies to determine the immune regulation role of other species of helminth [25].

In this study, the effect of Dicrocoelium ova immunization in EAE was evaluated for the first time. Clinical scores, IL4, and IFN-γ genes expression along with neuroglia cell counting and demyelination of brain cells in corpus callosum were evaluated and analyzed between the groups. The results illustrated that, pre-immunization of EAE in prophylaxis and treatment1 groups attenuated the scores, delayed onset day of EAE and reduced its incidence in the mentioned groups dramatically, as confirmed in the study by Swell et al. (2003) who investigated the treatment of EAE by Shistosoma mansoni so that, EAE helminthic therapy just could be effective in initial phase of EAE not in effective phase[18].

In addition, EAE suppression was accompanied with a dramatic increase in the density in brain of mice in T1 group. Considering similar count of neuroglia cells in 4 groups, a dramatic increase in the density observed in treatment1 group is not associated with the number of myelin-producing cells. Merrill JE. et al (1992) found that, the demyelination reduction in spinal cords was related to the reduction of inflammatory leukocytes and cytokines [22]. Zhiliang Wu et al (2010) showed that, the administration of 200 larvae of Trichinella psudospiralis (15 days before induction) attenuated EAE score and delayed the incidence of EAE. Also, demyelination in spinal cord decreased ,and inflammatory brain cells reduced [28]. La Flamma et al (2003) indicated that, the reduction of demyelination in spinal cord of EAE mice was rooted in the plummeted inflammatory macrophage [21].

In our study, the assessment of cytokines with Real Time PCR showed that, IFN- γ gene expression was lower in prophylaxis and treatment1 groups than the control and treatment2 groups. In contrast, IL-4 gene expression was more in prophylaxis and treatment1 groups than the control and treatment2 groups. So, slight down- regulation of IFN- γ along with up- regulation of IL-4 in T1 and P groups could not exclusively interpret significant preventable effect of Dicrocoelium eggs immunization. Previous studies showed that, Th1 lymphocytes are the critical cells causing the EAE. However, recent studies have demonstrated that, Th17 cells are more responsible for autoimmune diseases especially in case of multiple sclerosis. Briefly, remission of EAE relatively but not completely depends on the existence of balance between Th1/ Th2 [31, 32]. Cross immunomodulation of Th1/Th2 in helminthic therapy is disputable yet [33]. Due to variable functions of cytokines, it is difficult to obtain a definite conclusion and interpretation of the condition [34]. The suppression of EAE has been demonstrated to result from a decrease in IL12 in helminth therapy with Shistosoma mansoni. Also, the reduction of IFN- γ and increase of IL-4 production was not significant in the treated groups ,indicating that EAE attenuation was related to the suppressed IL12 ,and shift of immune response toward Th2 cytokines was not responsible for EAE amelioration [21]. However, Swell et al proved that, EAE improvement is merely related to predominant Th2 cytokines[18].In addition, the role of innate immune system [35] such as macrophages, B lymphocytes, regulator cells of immune system CD4 CD8 Tregs is effective regarding mediation of most of the protective mechanisms against EAE and MS. Besides, [36] Th17 is the most effective element in EAE induction explaining that, why the mechanisms of autoimmune diseases such as MS and EAE cannot be exclusively interpreted via Th1/Th2 responses [37, 38]. Therefore, it is suggested to evaluate Th17 and Tregs cytokine and inflammation as well as assessing the infiltration of immune cells in spinal cord in future studies, which could be considered as a limitation in the present study.

Conclusion

The results of the current study showed that, the treatment with Dicrocoelium ova immunization attenuated EAE score and delayed the incidence of EAE especially in initial phase of diseases with down - regulation of IFN- γ and up -regulation of IL-4. Dicrocoelium egg did not have any effect on neuroglia cells number, so EAE amelioration alongside increase of density in the brain cells might be related to another factors. Hence, it can be concluded that, Dicrocoelium eggs have the potential to be considered as useful therapy for treatment of MS in initial phase in future studies especially in human clinical trial studies.

Declarations

Acknowledgments

We thank the Animal lab employees for feeding and watering of the mouse and checking the room temperature daily.

Authors' contributions

Designed and performed experiments: E.M and M.M. Analyzed data and co-wrote the paper: A.F and N.M.A. Performed experiments: Z.N and M.J.R. Performed transporter experiments: M.R. Performed Statistical Analysis: M.M.B. Supervised the research: A.R.H and F.L.A. Histologic Analysis: S.S.N.

Funding

The authors gratefully acknowledge the support provided from the Student Research Committee (grant # 970556) and the Grants-in-Aid for Scientific Research (Grant # 951683) from Mashhad University of Medical Sciences (MUMS), Mashhad, Iran.

Competing Interests

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Anandan, C., et al., *Is the prevalence of asthma declining? Systematic review of epidemiological studies.* Allergy, 2010. **65**(2): p. 152-167.
2. Okada, H., et al., *The 'hygiene hypothesis' for autoimmune and allergic diseases: an update.* Clin. Exp. Immunol, 2010. **160**(1): p. 1-9.
3. Brooks, C., N. Pearce, and J. Douwes, *The hygiene hypothesis in allergy and asthma: an update.* Current opinion in allergy and clinical immunology, 2013. **13**(1): p. 70-77.
4. Greenwood, B., E.M. Herrick, and A. Voller, *Suppression of autoimmune disease in NZB and (NZB× NZW) F1 hybrid mice by infection with malaria.* Nature, 1970. **226**(5242): p. 266.
5. Joller, N., et al., *Immune checkpoints in central nervous system autoimmunity.* Immunological reviews, 2012. **248**(1): p. 122-139.
6. Zozulya, A.L. and H. Wiendl, *The role of regulatory T cells in multiple sclerosis.* Nat Rev Neurol, 2008. **4**(7): p. 384.
7. Goverman, J., *Autoimmune T cell responses in the central nervous system.* NAT REV IMMUNOL, 2009. **9**(6): p. 393.
8. Schoenborn, J.R. and C.B. Wilson, *Regulation of interferon-γ during innate and adaptive immune responses.* ADV IMMUNOL, 2007. **96**: p. 41-101.
9. O'Connor, R., L. Taams, and S. Anderton, *Translational Mini-Review Series on Th17 Cells: CD4+ T helper cells: functional plasticity and differential sensitivity to regulatory T cell-mediated regulation.* Clin. Exp. Immunol, 2010. **159**(2): p. 137-147.
10. Fleming, J., et al., *Probiotic helminth administration in relapsing–remitting multiple sclerosis: a phase 1 study.* Mult. Scler. J, 2011. **17**(6): p. 743-754.
11. Maizels, R.M., et al., *Helminth parasites–masters of regulation.* Immunol. Rev, 2004. **201**(1): p. 89-116.

12. Lowther, D.E. and D.A. Hafler, *Regulatory T cells in the central nervous system.* Immunol. Rev, 2012. **248**(1): p. 156-169.
13. Smallwood, T.B., et al., *Helminth immunomodulation in autoimmune disease.* FRONT IMMUNOL, 2017. **8**: p. 453.
14. Imitola, J., T. Chitnis, and S.J. Khoury, *Cytokines in multiple sclerosis: from bench to bedside.* PHARMACOL THERAPEUT, 2005. **106**(2): p. 163-177.
15. Urcelay, E., et al., *Role of interleukin 4 in Spanish multiple sclerosis patients.* J NEUROIMMUNOL, 2005. **168**(1-2): p. 164-167.
16. Moosazadeh, M., et al., *Prevalence of familial multiple sclerosis in Iran: A systematic review and meta-analysis.* Iran. J. Neurol, 2017. **16**(2): p. 90.
17. Arbabi, M., et al., *Prevalence and intensity of Dicrocoelium dendriticum in sheep and goats of Iran.* Res J Parasitol, 2011. **6**(5): p. 160-167.
18. Sewell, D., et al., *Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization.* Int. Immunol, 2003. **15**(1): p. 59-69.
19. Nosratabadi, R., et al., *St. John's wort and its component hyperforin alleviate experimental autoimmune encephalomyelitis through expansion of regulatory T-cells.* J IMMUNOTOXICOL, 2016. **13**(3): p. 364-374.
20. Gruden-Movsesijan, A., et al., *Mechanisms of modulation of experimental autoimmune encephalomyelitis by chronic Trichinella spiralis infection in Dark Agouti rats.* Parasite Immunol, 2010. **32**(6): p. 450-459.
21. La Flamme, A.C., K. Ruddenklau, and B.T. Bäckström, *Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis.* Infect Immun, 2003. **71**(9): p. 4996-5004.
22. Merrill, J.E., et al., *Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10. PL mice.* PNAS, 1992. **89**(2): p. 574-578.
23. Walsh, K.P., et al., *Infection with a helminth parasite attenuates autoimmunity through TGF-β-mediated suppression of Th17 and Th1 responses.* J. Immunol, 2009. **183**(3): p. 1577-1586.
24. Wilson, M.S., et al., *Helminth-induced CD19+ CD23hi B cells modulate experimental allergic and autoimmune inflammation.* Eur. J. Immunol, 2010. **40**(6): p. 1682-1696.
25. Chiuso-Minicucci, F., et al., *Experimental autoimmune encephalomyelitis evolution was not modified by multiple infections with Strongyloides venezuelensis.* Parasite Immuno, 2011. **33**(5): p. 303-308.
26. Kuijk, L.M., et al., *Soluble helminth products suppress clinical signs in murine experimental autoimmune encephalomyelitis and differentially modulate human dendritic cell activation.* Mol Immunol, 2012. **51**(2): p. 210-218.
27. Reyes, J.L., et al., *Taenia crassiceps infection abrogates experimental autoimmune encephalomyelitis.* Cell Mol Immunol 2011. **267**(2): p. 77-87.

28. Wu, Z., et al., *Infection of non-encapsulated species of Trichinella ameliorates experimental autoimmune encephalomyelitis involving suppression of Th17 and Th1 response.* PARASITOL RES, 2010. **107**(5): p. 1173-1188.
29. Zheng, X., et al., *Soluble egg antigen from Schistosoma japonicum modulates the progression of chronic progressive experimental autoimmune encephalomyelitis via Th2-shift response.* J NEUROIMMUNOL, 2008. **194**(1-2): p. 107-114.
30. Zhu, B., et al., *Immune modulation by Lacto-N-fucopentaose III in experimental autoimmune encephalomyelitis.* Clin. Immunol, 2012. **142**(3): p. 351-361.
31. Hosken, N.A., et al., *The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model.* J Exp Integr Med, 1995. **182**(5): p. 1579-1584.
32. Lehmann, P.V., O.S. Targoni, and T.G. Forsthuber, *Shifting T-cell activation thresholds in autoimmunity and determinant spreading.* Immunol. Rev, 1998. **164**(1): p. 53-61.
33. Marshall, A.J., et al., *Toxoplasma gondii and Schistosoma mansoni synergize to promote hepatocyte dysfunction associated with high levels of plasma TNF-α and early death in C57BL/6 mice.* J Immunol, 1999. **163**(4): p. 2089-2097.
34. Steinman, L., *Some misconceptions about understanding autoimmunity through experiments with knockouts.* J. Exp. Med, 1997. **185**(12): p. 2039.
35. Zaccone, P., et al., *Schistosoma mansoni antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes.* Eur. J. Immunol, 2003. **33**(5): p. 1439-1449.
36. Correale, J. and M. Farez, *Association between parasite infection and immune responses in multiple sclerosis.* Ann Neurol, 2007. **61**(2): p. 97-108.
37. Hofstetter, H.H., et al., *Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis.* Cell Mol Immunol 2005. **237**(2): p. 123-130.
38. Komiyama, Y., et al., *IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis.* J. Immunol, 2006. **177**(1): p. 566-573.

Figures

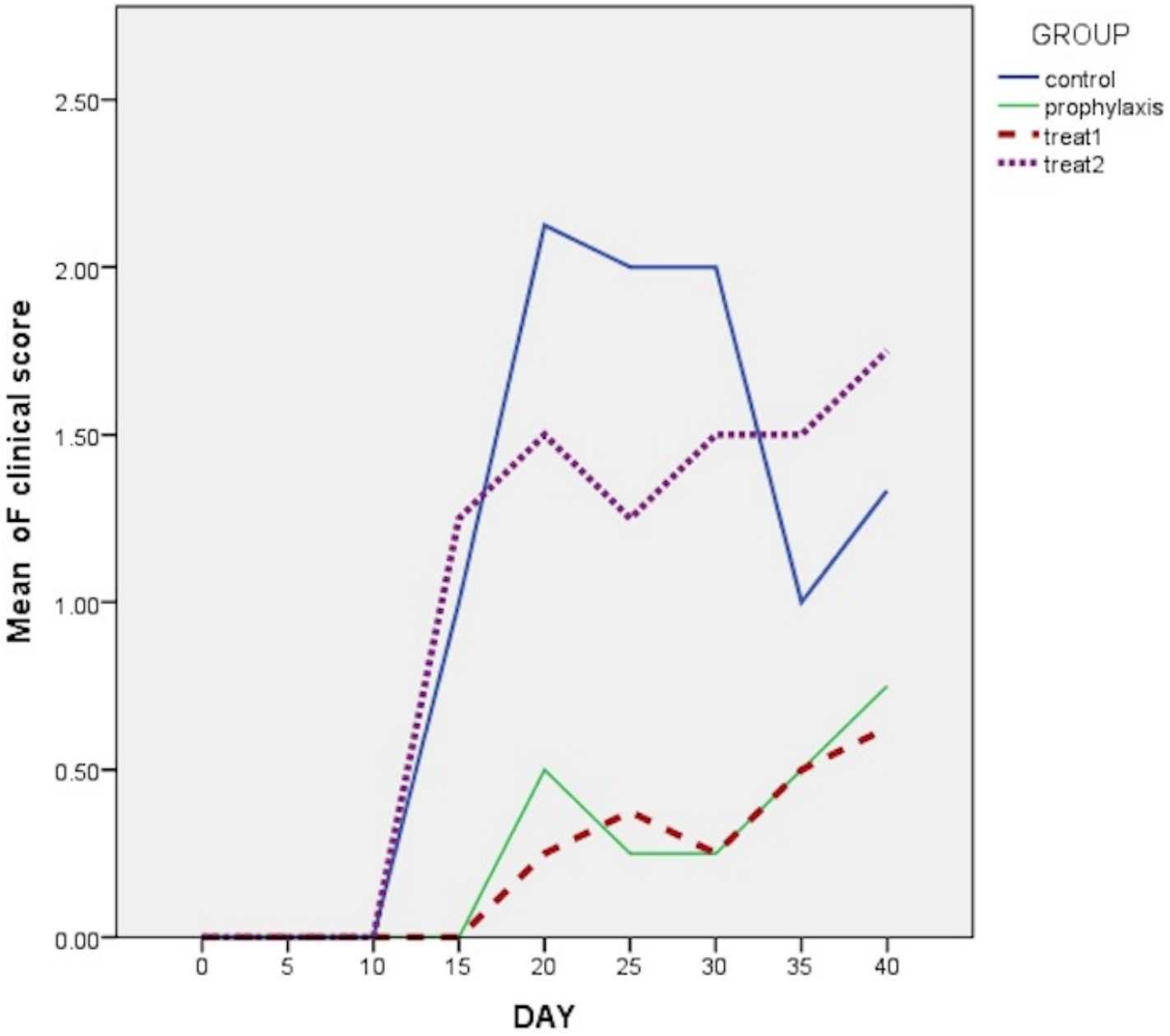

Figure 1

Mean of clinical score in helminthic therapy of EAE mice with Dicrocoelium eggs in four groups (control, prophylaxis, treatment 1, and treatment 2)

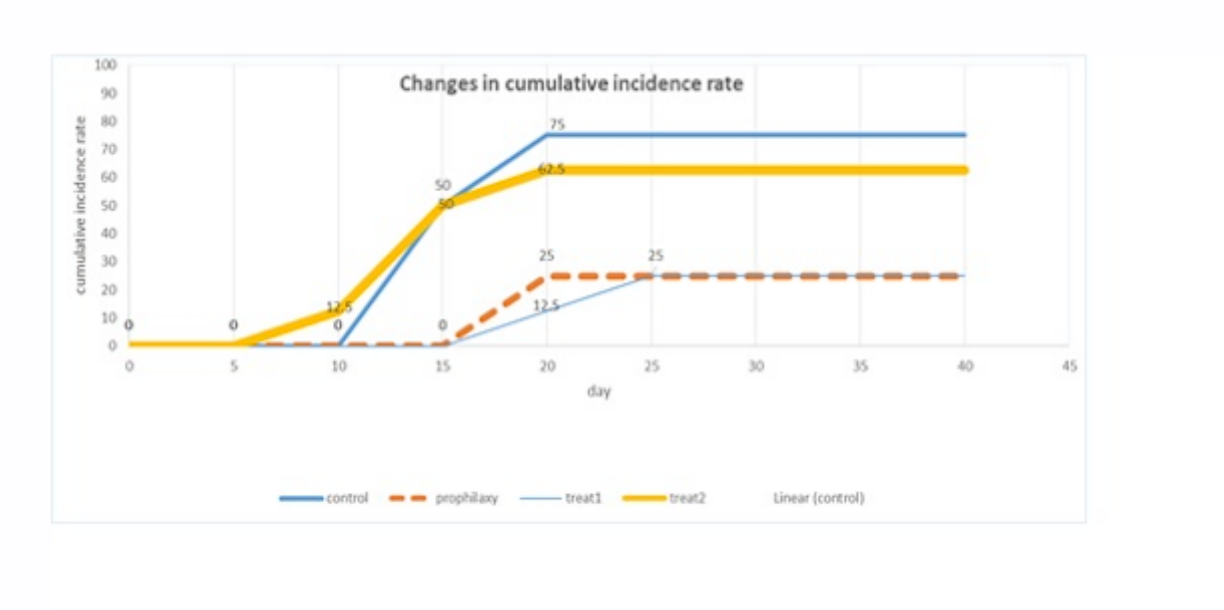

Figure 2

Cumulative incidence in control, prophylaxis, treatment 1, and treatment 2 groups obtained as 75, 25, 25, and 62.5%, respectively.

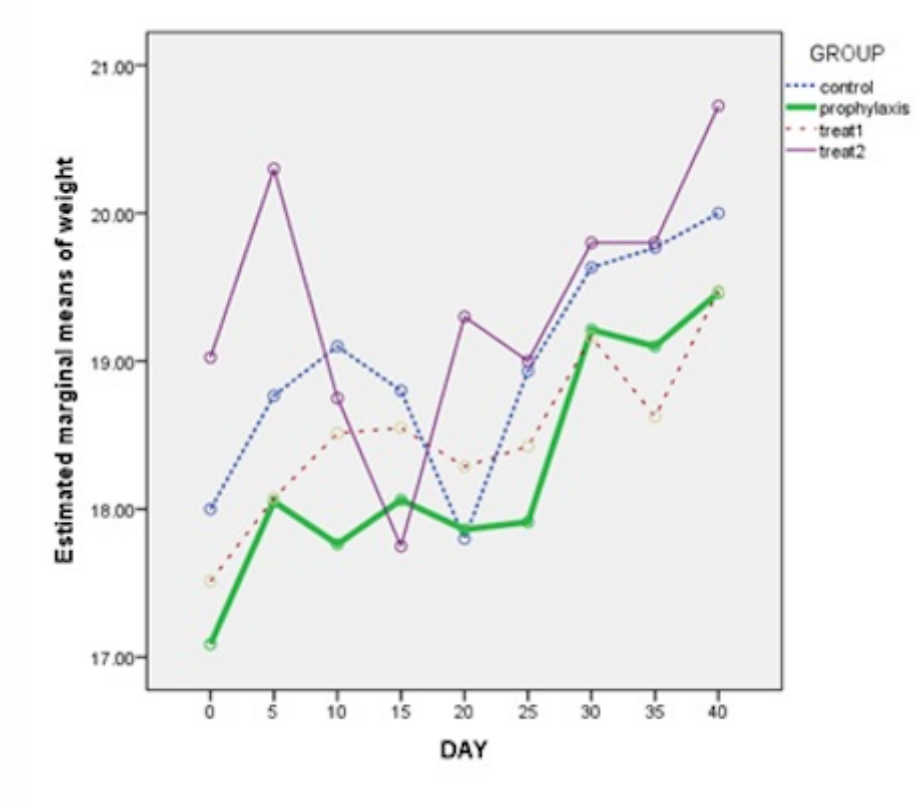

Figure 3

Mean weight in immunotherapy of EAE mice with Dicrocoelium eggs in four groups (control, prophylaxis, treatment 1, and treatment 2)

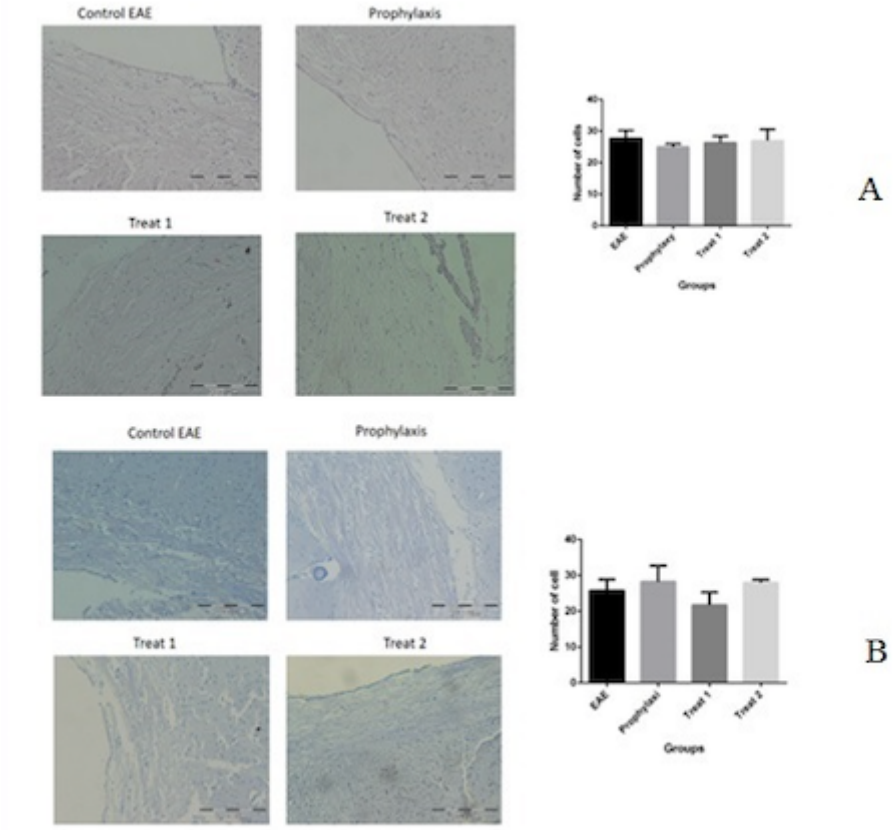

Figure 4

Effects of Dicrocoelium egg administration on the pathologies associated with EAE by H&E (Fig 4A) and TLB (Fig 4B) to count the number of neuroglia cells (as presented in the left side of Fig 4A and 4B).

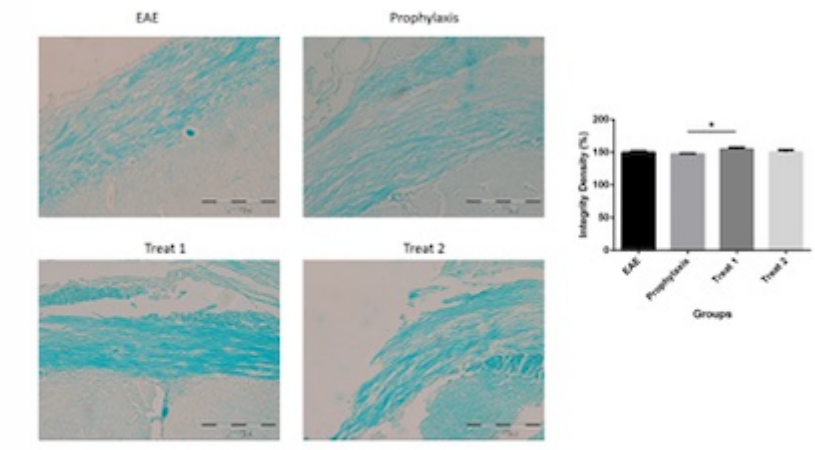

Figure 5

Effects of Dicrocoelium eggs administration on the pathologies associated with EAE

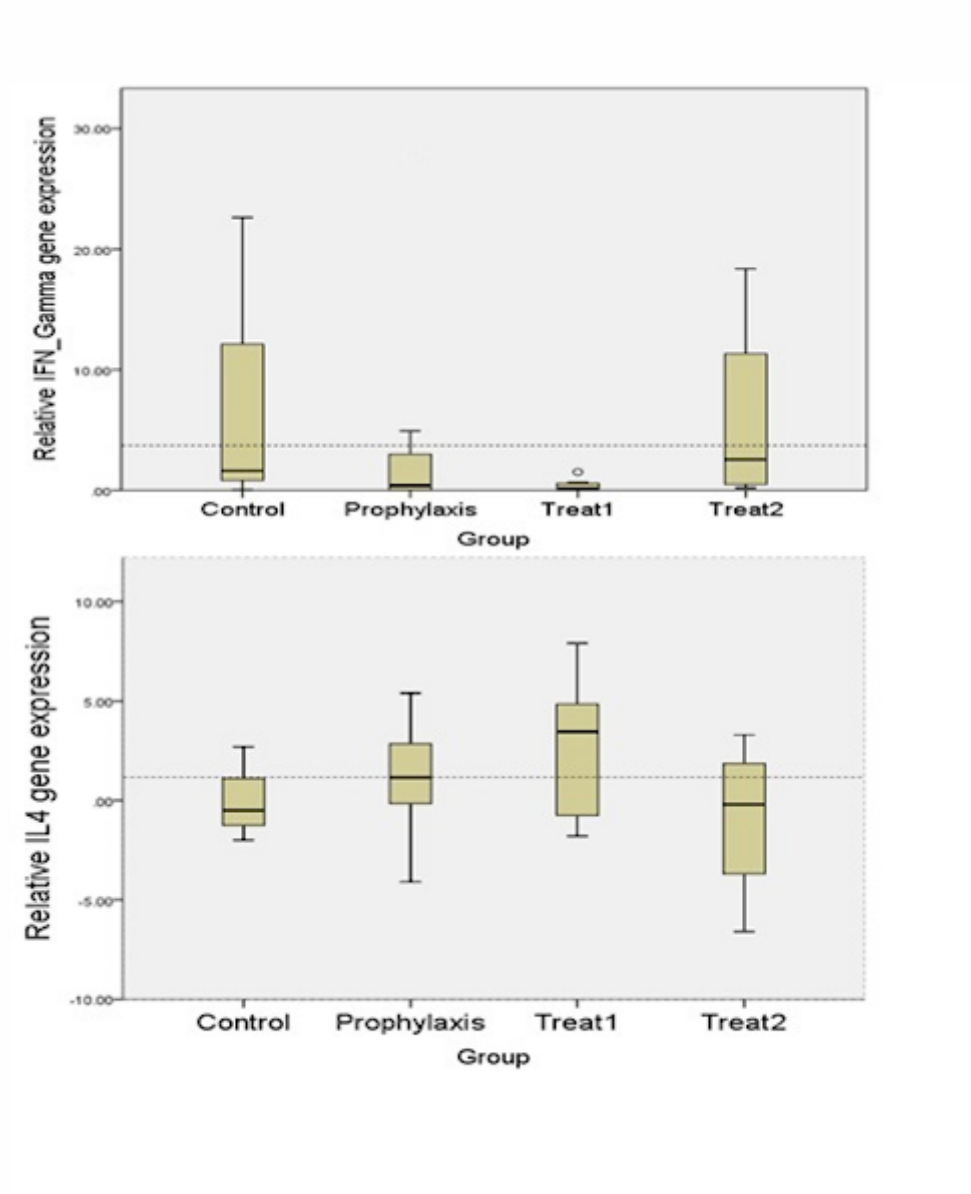

Figure 6

mRNA expression of IL4 and IFN- γ in EAE mice treated with Dicrocoelium eggs in four groups (control, prophylaxis, treatment 1, and treatment 2)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelines2013.docx