Modulation by Anisakis simplex antigen of inflammatory response generated in experimental autoimmune encephalomyelitis

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ABSTRACT

The impact of immunization with *Anisakis simplex* larval antigen on the occurrence and progression of experimental autoimmune encephalomyelitis (EAE) induced in mice was studied. C57BL/6J mice were immunized with the MOG35–55 peptide and one batch was treated with *A. simplex* total larval antigen on days 1, 8, 10 and 12 after EAE induction. Significantly higher values were obtained in the EAE clinical parameters of the antigen treated group. Likewise, there was a significant decrease in the weights of the animals. *Anisakis*-treatment produced a significant decrease in anti-MOG35–55 specific IgG1 on day 21. On day 14 there was an increase in serum IL-2, IL-6, IL-10, IL-17A, and TGF-β in the treated group. On day 21, a decrease in IL-4, IL-6, TNF-α, TGF-β was observed. All brain determinations were made on day 21. The treatment decreased values of IL-6, IL-10, IL- 17A and TNF-α. *A. simplex* antigen caused a significantly higher incidence of EAE and an advance in the appearance of the disease manifestations. However, treatment with the antigen was able to cause a decrease in proinflammatory cytokines (IL-6, IL-17A, and TNF-α) in nervous tissue that could establish a future preventive scenario for myelin damage.

Keywords: *Anisakis* antigen, Experimental autoimmune encephalomyelitis, C57BL/6J mice, MOG35–55 peptide, Antibodies, Cytokines

1. Introduction

Multiple sclerosis (MS) is considered a disease of the white matter of the central nervous system (CNS) in which there is an autoimmune attack, usually initiated by T lymphocytes. There are different cells involved in the inflammatory reaction that damages the myelin sheaths, including Th1, Th17, Tc, B, and regulatory T (Tregs) cells [1]. There is a negative correlation between the incidence of MS and naturally acquired helminth infections [2].

Experimental autoimmune encephalomyelitis (EAE) is a murine model that resembles MS mostly induced by the administration of the MOG35–55 peptide. Regulatory effects in C57BL/6J mice and using various helminths have been observed [3–8].

Anisakis larvae have developed mechanisms to modulate the dichotomy of the host immune response [9–12]. Both excretory-secretory products and total larval antigen of *Anisakis simplex* produce tolerogenic dendritic cells that induce the expansion of functional Tregs in vitro. At the same time, they promote the production of Th1 cytokines, such as IFN-γ [13].

Previous contact with the antigens released by *A. simplex* living larvae is associated with an increase of the regulatory cytokines IL-10 and TGF-β in anisakiosis patients [14,15].

Anisakiosis is an important pathology in Japan due to the nutritional habits of the general population [16]. MS is characterized by a low prevalence in the Asian population and has been very rare in Japan [17].

The aim of this work was the evaluation of the impact of immunization with *A. simplex* larval antigen on the appearance and progression of induced EAE in mice.

2. Materials and methods

2.1. Preparation of antigens

A. simplex larval extracts were prepared by the method previously described [18]. Antigens were filtered through 0.22 μm membranes. The possible contamination by LPS was determined in all samples used by the test with Limulus Amoebocyte Lysate (LAL) (Pierce). Antigen samples were purified by using appropriate columns (Detoxi-Gel Endotoxin Removing Gel, Thermo Scientific).

2.2. Animals

Twenty-four C57BL/6J female mice that are susceptible to EAE [19] of 8–12 weeks of age obtained from Harlan Laboratories (Barcelona, Spain) were used. The authors have involved the minimum number of animals to produce statistically reproducible results. All procedures were carried out in accordance with Royal Decree 53/2013, of 1 February, which establishes the basic rules applicable for the protection of animals used in experimentation and other scientific purposes. The project was approved by the Ethics Committee of the UCM and evaluated and approved by an authorized body authorized by the Community of Madrid for the evaluation of Projects. The paralyzed animals were given access to water and food.

2.3. Induction of EAE

Animals were anesthetized with intraperitoneal injection of atropine (0.6 mg/kg of weight) and 2.5% of 2,2,2-tribromoethanol (avertin) (Sigma-Aldrich, Darmstadt, Germany) (0.01 ml/g of weight). Animals were immunized subcutaneously (s.c.) on both sides of the tail base with 100 μg of peptide MOG35–55 (Sigma-Aldrich, Darmstadt, Germany) (MEVGWYRSPFSRVVHLYRNGK) emulsified vol/vol in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, Darmstadt, Germany). On the day of the immunization, 300 ng/animal of pertussis toxin (Sigma-Aldrich, Darmstadt, Germany) was injected intraperitoneally (i.p.) with the injection repeated 48 h later.

2.4. Clinical evaluation

The mice were weighed and their clinical signs of EAE were observed daily (by the same investigators) until day 21 postimmunization (p.i.) (acute phase) in the control group of

EAE and in the group subjected to the therapeutic protocol with the antigen. The change in average body weight was calculated by the percentage of the initial body weight (at day 1). Clinical status was assessed by a score assigned to certain body parts of each mouse as discussed below.

Grade 0, normal; Grade 1, flaccid tail; Grade 2, mild weakness of the hind limbs; Grade 3, severe weakness of the hind limbs; Grade 4, paralysis of the hind limbs; Grade 5, paralysis of the hind limbs and partial weakness of the forelimbs. The final clinical score was obtained by adding all the scores of all the individuals [20].

2.5. Treatment with Anisakis simplex antigen

A group of 12 mice were treated with 100 μg of *A. simplex* antigen (s.c.) on days 1, 8, 10 and 12 after the induction of the EAE. The mice were sacrificed on day 21 of the study.

2.6. Serum samples

To obtain the sera on days 0 and 14 p.i., the mice were bled from the retroorbital venous sinus. The blood samples of the day 21 p.i. were collected by intracardiac puncture. The mice were anesthetized with intraperitoneal administration of pentobarbital (Euta-Lender, Normon Laboratories, Madrid, Spain).

2.7. Preparation of brain tissue extracts

Brain tissue extracts were obtained from both control and *Anisakis* treated mice. Brains were removed and hemispheres were kept on ice. Hemispheres were homogenized in RPMI 1640 (Sigma-Aldrich, Darmstadt, Germany) (100 mg of tissue per 750 μl) by means of a tissue homogenizer. Brain homogenates were centrifuged 10 min at 10,000g at 4◦C and the supernatants were collected.

2.8. Detection of antibodies by ELISA

For detection of anti-MOG35–55 antibodies, ELISA plates (Costar, Corning, NY) were treated with a 2% glutaraldehyde solution in PBS pH 5 (200 μl/well, overnight at 4 ◦C). After washing (PBS pH 5, 3 times) plates were coated overnight at 4 ◦C with 100 μl/well

of murine MOG35–55 peptide at 10 μg/ml in carbonate buffer pH 9.6. Plates were washed (PBS-Tween) and blocked with 0.1% BSA in PBS pH 7. Sera were then incubated in duplicate at the 1/200 dilution (PBS-Tween-BSA) for 2 h at 37 ◦C and after washing incubated with a Goat anti-Mouse IgG1 Secondary Antibody, HRP (Invitrogen, Thermo Fisher Scientific, Waltham, MA). For detection of anti-*Anisakis* antibodies plates were overlaid overnight at 4 ◦C with 100 μl/well of antigen at 10 μg/ml in carbonate buffer. The following steps were carried out as in the previous case.

For the detection of antibodies in the CNS we used Nunc™ MaxiSorp ™ flat-bottom 96 well plates (Thermo Fisher Scientific, Waltham, MA). Brain tissue extracts were tested at dilution 1/2.

2.9. Quantification of cytokines

ELISAs for the detection of murine (IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-17A, IFN-γ, TNF-α and TGF-β were carried out using Mouse IL-10 ELISA Ready-SET-Go! (2nd Generation) (Invitrogen, Thermo Fisher Scientific, Affymetrix, Inc., San Diego, CA) following the manufacturer's instructions.

2.10. Statistical analysis

All statistical analyses were performed using the program GraphPad Prism 6. Differences of clinical scores and weights between control and treated groups were analysed by multiple t tests. Variations in antibody and cytokine levels from sera were analysed by the repeated measured two-way ANOVA adjusted for Tukey's multiple comparisons test until day 21 p.i. Differences between the means of two groups in serum and brain antibody and cytokine levels were analyzed by multiple t tests, with correction for multiple comparisons using the Holm-Sidak method, with alpha = 0.05. Differences were considered to be statistically significant at a level of $P < 0.05$.

3. Results

3.1. Clinical characteristics of EAE

EAE control and *Anisakis*-treated animals developed clinical signs of disease between day 8 and day 21p.i and day 6 and 21 p.i., respectively. EAE treated animals presented with more severe clinical symptoms than the EAE control group. Significantly higher values of Clinical Scores were obtained from day 14 p.i. in the group treated with the antigen until the end of the experiment. The Anisakis EAE group had a significant increase of Clinical Scores on day 14 p.i. in comparison with the EAE control group ($P < 0.05$; $P < 0.01$) and this persisted throughout the rest of the study. Clinical evidence of disease in the *Anisakis*-treated group (first noticed at day 6 p.i.) peaked at day 18 p.i. while in the untreated control group it peaked at day 21 p.i. according to the Clinical Score assessment (Fig. 1A).

Final Clinical Scores were obtained by adding all the scores of all the individuals. Significantly higher values were observed in the *Anisakis* treated group (days 6–10 and days 12–21, $P < 0.0001$). The range of the values of the EAE treated group was twice that of the EAE control group from day 14 p.i. (Fig. 1B).

The percentages of mice with any disease symptoms were higher in the *Anisakis*-treated group from day 6 to 15 p.i. ($P < 0.0001$). After day 16 p.i. the disease incidence was 100% in both control and treated groups (Fig. 1C).

3.2. Weight of mice

From day 15 p.i. a significant reduction in body weight was observed in the *Anisakis*-treated group ($P < 0.05$; $P < 0.01$) compared to the control group. On the other hand, while the mean weight for the treated mice remained lower, control mice increased in weight at the endpoint (Fig. 2A).

There was a dramatic weight lost in the *Anisakis*-treated group from day 15 to 21 p.i. (Fig. 2B and C).

3.3. Antibody levels against Anisakis larval antigen and MOG35–55

The immunization with *Anisakis* antigen produced specific IgG1 antibodies. We observed a significant increase of anti-*Anisakis* IgG1 levels from day 14 p.i. when compared to day 0 ($P < 0.001$). On day 21 p.i. the increase turned out to be much higher ($P < 0.0001$ and $P < 0.01$ versus day 0 and day 14 p.i., respectively) (Fig. 3A).

When comparing both groups, control and treated, we observed significant differences in the anti-*Anisakis* IgG1 mean O.D. values ($P < 0.01$ and $P < 0.001$ on days 14 and 21 p.i., respectively). In the case of the brain extracts, all determinations were made on day 21 p.i. A very significant response of IgG1 anti-*Anisakis* was observed with respect to the control in the EAE group treated with the antigen ($P < 0.01$) (Fig. 3B).

Immunization with MOG35–55 triggered activation of autoreactive MOG-specific B cells. *Anisakis*-treated EAE mice have decreased levels of autoantibodies.

On day 14 p.i. the control group showed a detectable response with respect to day 0 ($P < 0.05$), while in the group treated with *Anisakis* antigen, no statistically significant differences were detected against the MOG35–55 peptide when compared to day 0 (Fig. 3C). On day 21 p.i., high levels of MOG-specific IgG1 were detected, although they were much higher in the control group than in the *Anisakis*- treated group, where there were significant differences with respect to day 0 ($P < 0.01$). Comparing both control and treated groups, a significant decrease in MOG-specific IgG1 antibodies was observed in mice immunized with the antigen ($P < 0.01$).

In the case specific antibodies in brain extracts, the treatment with *Anisakis* antigen produced a significant decrease in IgG1 anti-MOG35–55 levels on day 21 p.i. ($P < 0.05$) (Fig. 3D).

3.4. Cytokine levels

On day 14 there was an increase in serum IL-2, IL-6, IL-10, IL-17A, and TGF-β in the EAE treated group (Fig. 4), as well as IL-17A/IL-10 ratio (Fig. 5). On day 21 p.i., a decrease in IL-4, IL-6, TNF-α, TGF-β (Fig. 4) and IFN-γ/IL-10, TNF-α/IL-17A and TGF-β/IL-17A ratios (Fig. 5) was observed.

In the case of the brain, the treatment decreased the values of IL-6, IL-10 and IL-17A on day 21 p.i. The TNF-α assessment revealed much lower levels in the *Anisakis*-treated mice compared to the untreated-control group (Fig. 6). IL-10/IL-4 and TNF-α/IL-4 ratios decreased after *Anisakis* treatment, while TGF-β/IL-17A ratio experienced an increase (Fig. 7).

4. Discussion

A large number of animal models have been generated for the study of pathology in MS, as well as to identify biomarkers and therapeutic targets [21].

In this work we studied the impact of immunization with *A. simplex* larval antigen on the occurrence and progression of EAE induced in mice. The MOG35–55 EAE mouse model has been established on a C57BL/6 genetic background. We characterized previously *Anisakis* infection/immunization in this mouse strain. In a murine experimental model orally inoculated with an *A. simplex* living L3 we observed that responses of the IgG1 subclass were mainly produced in the C57BL/10 strain [22]. Recently, by means of the study of DC stimulation, we observed that C57BL/6J mice developed a more discrete and resistant response against *A. simplex* antigens with lower expression of IL-12 and higher IL-10 than in BALB/c mice [13].

In this study, C57BL/6J mice were treated with 100 μg of *A. simplex* antigen (s.c.) on days 1, 8, 10 and 12 after the induction of the EAE.

Zheng et al. carried out a similar therapeutic protocol by the administration of 100 μg soluble egg antigen from *Schistosoma japonicum* on days 0, 7, 9 and 11 after EAE induction. They observed a Th2-polarizing response in both periphery and CNS [4]. Our results showed that several injections of *Anisakis* antigen before the peak phase of EAE exacerbated the disease severity. In EAE + *Anisakis* mice the clinical severity of EAE was greater in comparison to mice with EAE induction only. Multiple injections of *Anisakis* antigen after EAE induction not only were not able to ameliorate disease clinical

manifestations but also induced a worsening of EAE clinical signs during the acute phase of EAE.

Likewise, a significant reduction in body weight was observed in the EAE *Anisakis*-treated group.

When antibodies in serum and brain against MOG35–55 were measured we observed that *A. simplex* antigen treatment produced a significant decrease in anti-MOG35–55 specific IgG1 on day 21 p.i. Therefore, exposure to *Anisakis* larval antigen after EAE induction exacerbated the clinical symptoms but not MOG35–55 specific IgG1.

The importance of the presence of B cells, plasma cells and immunoglobulins in the pathology of the disease has become evident [23,24].

Such antibodies can be detected at very early stages of the disease [25,26]. We have been able to detect them from day 14 p.i. although there was a drastic decrease in both serum and brain from the 21 p.i. in the group of treated mice. These antibodies are directed towards the myelin sheaths and are responsible for the pathogenicity since they cause damage to them and as a consequence generate the characteristic symptoms of MS [27,28].

Therefore, the decrease in the levels of these antibodies could improve the pathology triggered by them. Although in our case the treated animals did not improve their symptoms, it is likely that this effect was not manifested since animals were slaughtered on day 21 p.i. Secondly, it could be due to the immunization regimen carried out, since Anisakis antigen was administered after immunization with MOG35–55, that is, after the appearance of autoantibodies, so it would be advisable to proceed to the evaluation of the antigen in early stages prior to the triggering of demyelinated inflammatory lesions in the CNS.

It should also be borne in mind that in 85% of cases, MS is characterized by the presence of outbreaks followed by total or partial remissions [29,30]. Therefore, it would be interesting to be able to determine if treatment with the *Anisakis* antigen causes a

remission of these outbreaks or a distancing in time. The presence of autoantibodies in brain samples shows that an alteration of the blood–brain barrier (BBB) has occurred, which very effectively regulates the entry of both antibodies and cells into the CNS [31,32].

In our experimental conditions, this alteration has been revealed with the detection of high levels of brain autoantibodies, although these levels have been lower than those detected in serum. On the other hand, treatment with Anisakis antigen caused a decrease of more than 70% in serum anti-MOG35–55 IgG1 levels, which shows not only a lower production of these but also a possible restoration of the permeability of the BBB since less than 30% of them managed to cross it.

On day 14 p.i. there was an increase in serum IL-2, IL-6, IL-10, IL-17A, and TGF-β in the *Anisakis*-treated group, as well as IL-17A/IL-10 ratio.

We have observed that on day 14 p.i. there was a significant increase in serum IL-6 and IL-10 in the group of treated mice, which shows that there has been an increase in the Th2 population.

Anisakis antigen is known to cause a polarization of the immune response to a Th2 phenotype [33,14]. Th2 profile related cytokines have been associated with reduced inflammation and improved symptoms in MS patients [34]. *Anisakis* antigen has led to increased IL-10 levels on day 14 p.i. which caused a decrease in both serum IL-17A/IL10 and IFN-γ/IL-10 ratios. The ratio of IL-17A/IL-10 may act as a predicted marker of inflammation and immune imbalance [35].

Likewise, on day 21, a decrease in IL-4, IL-6, TNF-α, TGF-β and IFN-γ/IL-10, TNF-α/IL-17A and TGF-β/IL-17A ratios were observed. In the case of the brain, the *A. simplex* treatment decreased IL-6, IL-10, IL-17A and TNF-α, as well as IL-10/IL-4, TNF-α/IL-4 ratios, while TGF-β/IL-17A ratio experienced an increase.

We detected on day 14p.i an increase in TGF-β levels which could be responsible for the increase in the Th2 cytokine profile. TGF-β is a negative regulator against Th1 differentiation and is able to block IL-12 increasing the Th2 profile [36,37].

Indeed increasing Th2 response in the onset of the EAE demonstrated by both cytokines and antibodies could achieve suppression of the inflammatory response and have a beneficial effect on the clinical course of MS. The decrease in IL-10/IL-4 and TNF-α/IL-4 ratios in the brain shows an increase in the Th2 response.

Likewise, increases in IL-17A and IL-2 levels were also observed. The increase in these cytokines could be related to the increase in TGF-β since TGF-β + IL-6 has been shown to drive differentiation of Th17 cells in vitro.

It has been observed that the polarization of cells towards a Th17 profile requires both the presence of IL-6 and TGF-β. The absence of IL-6 and the presence of TGF-β generate a stimulation of FoxP3 + Tregs which will help maintain self-tolerance and prevent reaction against self-antigens [38].

We have observed a decrease in IL-6 levels on day 21p.i in both serum and brain. Likewise, although IL-17A levels were elevated in sera from *Anisakis*-treated animals on day 14 p.i., this proinflammatory cytokine was reduced in brains on day 21 p.i. This could indicate the activation by *Anisakis* antigen of Tregs, which could exert their suppressive effects by releasing IL-10. This caused a decrease in IL-17A/IL-10 and IFN-γ/IL-10 ratios. Importantly, an impressive suppression not only of TNF-α but also of TNF-α/IL-4 ratio was observed in sera and brains of *Anisakis*-treated mice in comparison to untreated animals on day 21 p.i.

It has been observed that MS patients have elevated levels of TNF-α at the site of active lesions as well as in serum and that their presence is related to the severity of the lesions [39]. Astrocytes present in the basal lamina of the BBB have been shown to be capable of releasing cytokines such as IL-6 and TNF-α during the inflammatory process. These cytokines increase the permeability of the BBB, while the production of TGF-β induces

the opposite effect [40]. We have observed that the mice treated with the *Anisakis* antigen present in the brain a decrease in both IL-6 and TNF-α levels as well as an increase in TGF-β/IL-17A ratio. This fact could be causing a decrease in the permeability of the BBB that could cause a decrease in the passage of both antibodies and proinflammatory cytokines to the CNS.

Interestingly, IL-17A levels rose in serum on day 14 p.i. in the *Anisakis*-treated group with respect to the control, and no differences were observed on day 21 p.i. between both groups. In contrast, TGF-β levels were significantly higher in the *Anisakis*-treated group on day 14 p.i. but were significantly lower in this group on day 21 p.i. Elevated TGF- β levels together with high IL-6 rates promote IL-17A production [41]. IL-17A levels were significantly reduced in the brain on day 21 p.i. despite being significantly elevated in serum on day 14 p.i. in the *Anisakis* treated group. Likewise, IL-6 levels were elevated in serum on day 14 p.i. along with TGF- β and IL-17A levels. The production of the latter was probably promoted by both previous cytokines. As a consequence of the decrease in IL-6 and TGF- β on day 21 p.i., the decrease in IL-17A will have occurred. This decrease was not detectable in serum but in brain extracts on day 21 p.i. It is very important to note that, despite the fact that in both groups a significant increase in serum IL-6 was observed on day 14 p.i., the drastic decrease observed on day 21 p.i. only occurred in the *Anisakis*-treated group. Our results indicate that in the absence of IL-6, TGF- β /IL-17A ratio increased.

The concentration of IL-17A in the serum of mice with EAE was significantly higher after *Anisakis* immunization at 14 days p.i. compared to EAE control mice on the same day. The IL-17A level in the serum at 21 days p.i. was lower compared to day 14 p.i. Likewise, the IL-17A level in the brain of *Anisakis*-treated mice at 21 days p.i. was significantly lower compared to the EAE control mice. On the other hand, the mean serum TGF-β concentration was higher in *Anisakis* treated animals on day 14 p.i.

Astrocytes are considered as non-professional antigen presenting cells and TGF-β has been shown to inhibit the expression of MHC II molecules [42]. The decreased serum IL-

17A and dramatic increase in TGF-β/IL-17A ratio in *Anisakis*-treated animals further indicate that dysregulation of the Treg/Th17 axis of T effector cells occurred in EAE mice has been balanced by the higher values of TGF-β observe on day 14 p.i.

Consequently, if the ratio of TGF-β/IL-17A may act as a predicted marker of future anti-inflammation and immune balance the results of the study could suggest that a scene conducive to recovery of EAE progression is being prepared. IFN-γ/IL-10 ratio was significantly decreased in the brain extracts on day 21 p.i. due to the *Anisakis* treatment compared to control group. Definitely, change in IFN-γ/IL-10 cytokines ratio pattern (Th1/Treg balance) toward to IL-10 (Treg) results in prevention of autoimmune-disease such as the EAE model of MS [43].

In comparison to untreated group, *Anisakis* antigen led to an anti-inflammatory condition with an increase in Treg/Th17 balance through Treg cytokine (TGF-β) predominantly. Furthermore, *Anisakis* antigen also disturbed the balance of Th1/Th2 (TNF-α/IL-4 ratio) toward to Th2 through more decrease in the value of TNF-α. According with our results, *Anisakis* antigen leads to an imbalance among four subtypes of T cells (Th1/Th2/Th17/Treg) towards Th2/Treg cells along with their cytokines. *Anisakis* larval antigen impress Th2 (IL-4) and Treg (IL-10, TGF-β) more than Th1 (IFN-γ, TNF-α) and Th17 (IL-17A) cells which leads a significant reduction in Th1/Th2 (TNF-α/IL-4 ratio), Th1/Treg (IFN-γ/IL-10 ratio) and Th17/Treg (IL-17A/TGF-β ratio) balance. Investigating cytokine levels in sera from patients diagnosed with anisakiosis, it was shown that prior contact with antigens released by living *Anisakis* larvae is associated with an increase in regulatory cytokines, IL-10 and TGF- β [14]. In this context *Anisakis* treatment could be a therapeutic approach for manipulating improper response in therapy. The results of the present study indicated that *Anisakis* larval antigen might alter the release of cytokines in murine EAE, in particular a decrease of Th1 and Th17 cytokines, and an increase of Th2 and Treg cytokines, leading to an immune balance and an anti-inflammatory response.

In conclusion, the *A. simplex* larval antigen caused a significantly higher incidence of EAE. Our results showed that the presence of specific IgG1 generated in the brain against MOG35–55 was not related to the severity of the EAE. However, treatment with the *A. simplex* antigen was able to cause a decrease in proinflammatory cytokines (IL-6, IL-17A, and TNF-α) in nervous tissue that could establish a future preventive scenario for myelin damage. Similarly, in our present study, *Anisakis* antigen strongly decreased the Th1 and Th17 cytokines and IL-17A/TGF-β ratio, while increased Th2 and Treg cytokines, revealing that the antigen exposure induced robust anti-inflammatory responses and promoted a bias towards a Th2/Treg immune response, which ultimately might control inflammatory cell recruitment, focal inflammation and tissue destruction. The results obtained show a possible prior immunomodulatory role of the antigen on the immune responses induced in murine EAE although injections of *Anisakis* larval antigen before the peak phase of EAE exacerbated the disease severity. For this reason, it is necessary to study the effect of a treatment on the response already induced. Likewise, it would be interesting to study the effect of preventive treatments before induction of EAE.

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CRediT authorship contribution statement

Marta Rodero: Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. Carmen Cuéllar: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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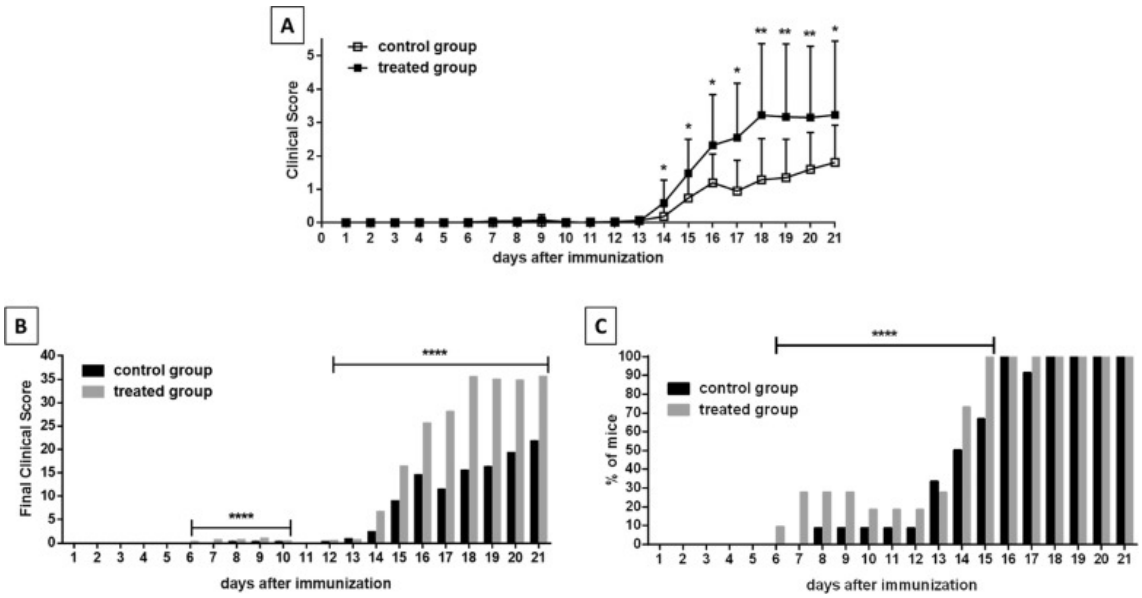

Fig. 1. (A) Clinical characteristics of EAE in female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). The values of the Clinical Score are represented throughout the 21 days p.i. of the experiment. Data are presented as mean ± standard deviation of 12 mice from two independent experiments. (B) Final clinical scores were obtained by adding all the scores of all the individuals. (C) Percentage of mice with any disease symptoms. (*P < 0.05; **P < 0.01; ****P < 0.0001).

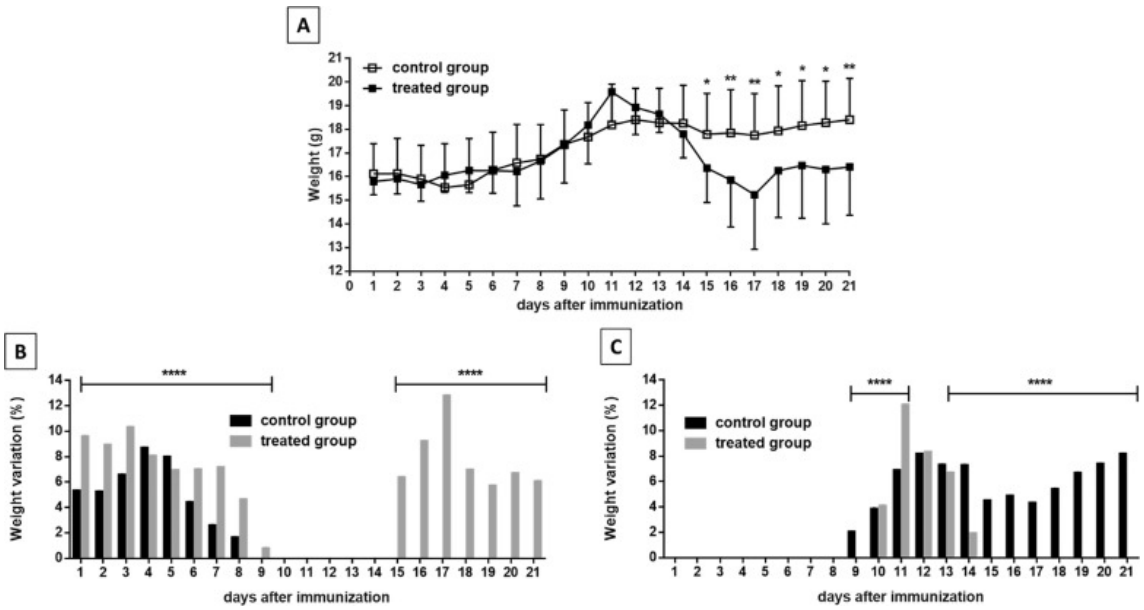

Fig. 2. (A) Mean weight in female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). Data are presented as mean ± standard deviation of 12 mice from two independent experiments. (B, C) Weight variation. (B) lost weight, (C) gained weight. (*P < 0.05; **P < 0.01; ****P < 0.0001).

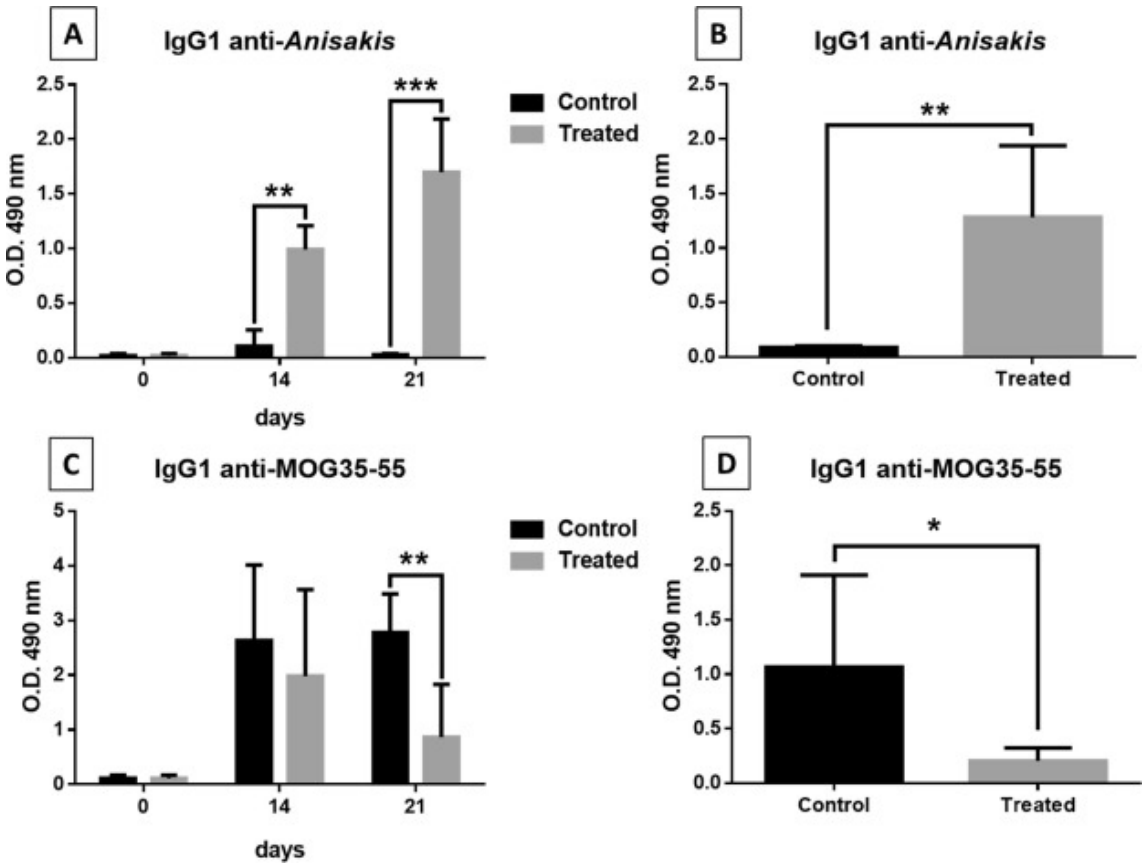

Fig. 3. Antibody responses (IgG1) against Anisakis larval antigen (A, B) or MOG35-55 (C, D) in sera (A, C) and brains (B, D) from female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). The average values of the O.D. of the sera are represented throughout the 21 days p.i. of the experiment (A, C). In the case of brain tests were carried out on day 21 p.i. (B, D). Data are presented as mean ± standard deviation of eight mice from two independent experiments. (*P < 0.05; **P < 0.01; ***P < 0.001).

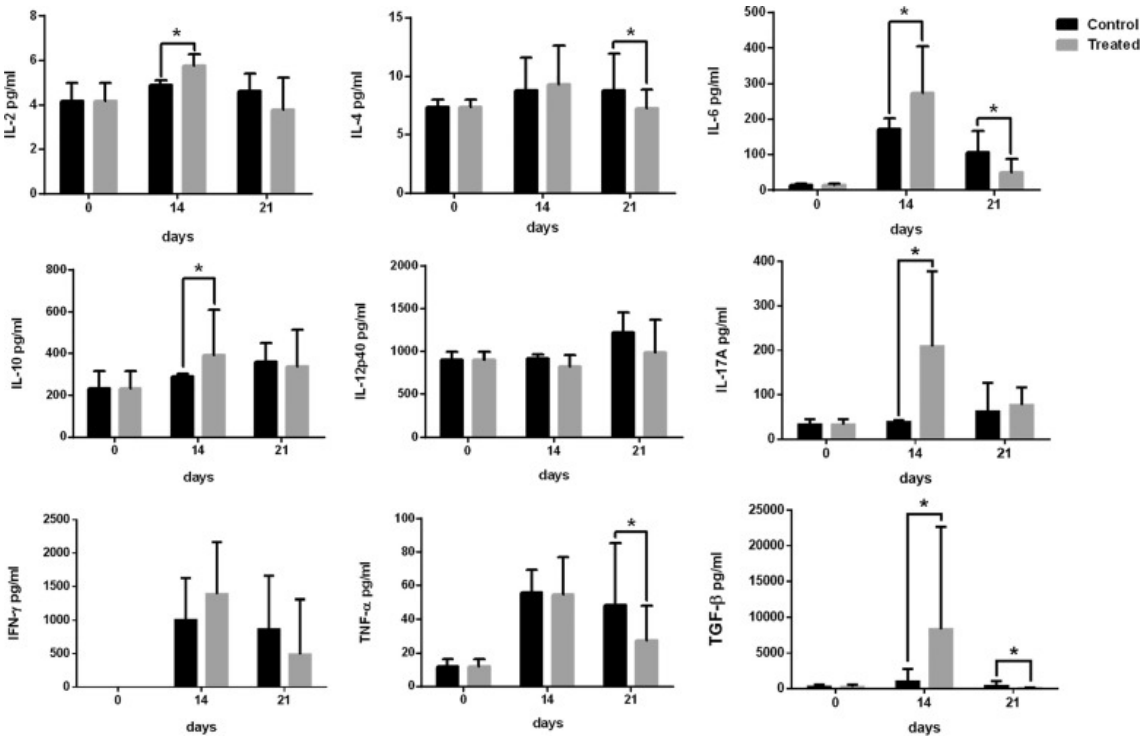

Fig. 4. Levels of IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-17A, IFN-γ, TNF-α and TGF-β in sera from female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). The average values of the pg/ml are represented throughout the 21 days p.i. of the experiment. Data are presented as mean ± standard deviation of eight mice from two independent experiments. $P < 0.05$).

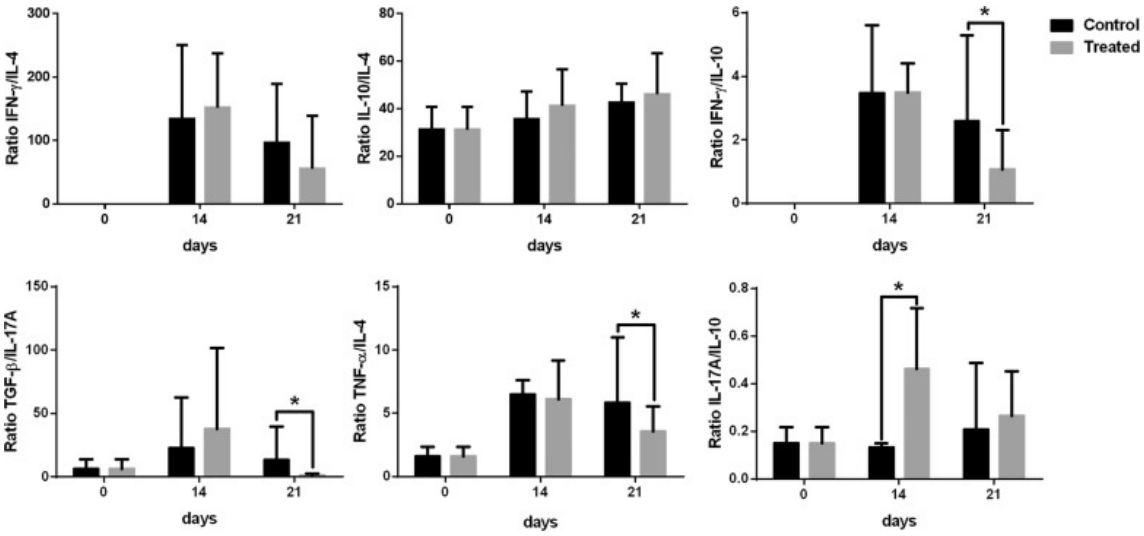

Fig. 5. Ratio IFN-γ/IL-4, IL-10/IL-4, IFN-γ/IL-10, TGF-β/IL-17A, TNF-α/IL-4 and IL-17A/IL-10, in sera from female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). The average values are represented throughout the 21 days p.i. of the experiment. Data are presented as mean ± standard deviation of eight mice from two independent experiments. (*P < 0.05).

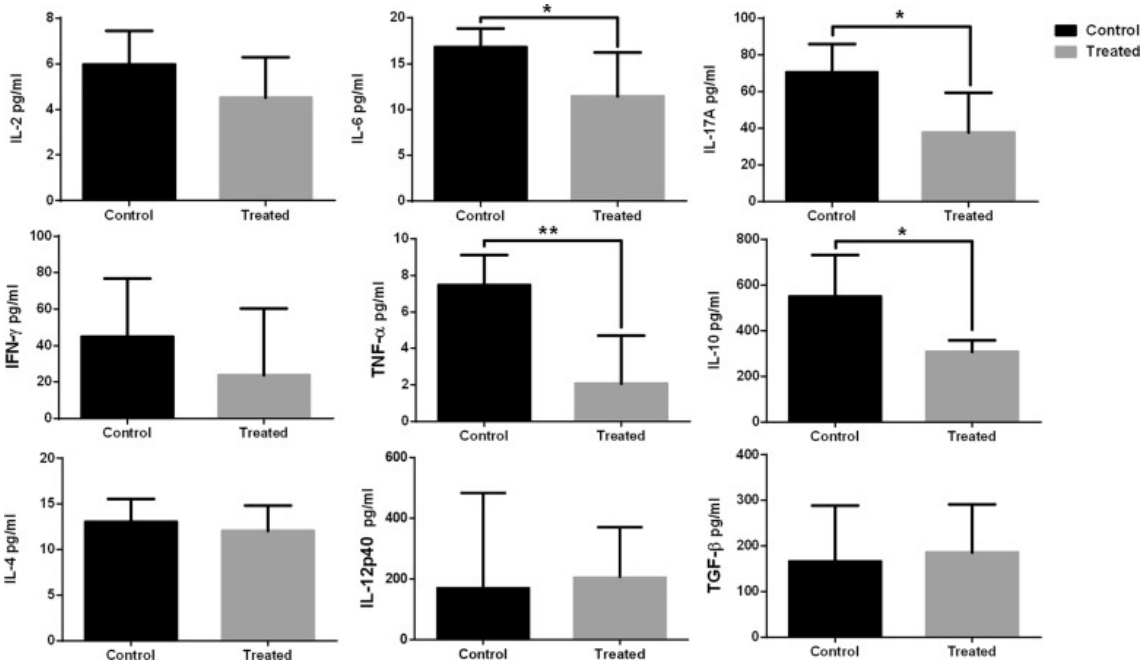

Fig. 6. Levels of IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-17A, IFN-γ, TNF-α and TGF-β in brains from female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). Tests were carried out on day 21 p.i. Data are presented as mean ± standard deviation of eight mice from two independent experiments. (*P < 0.05; **P < 0.01).

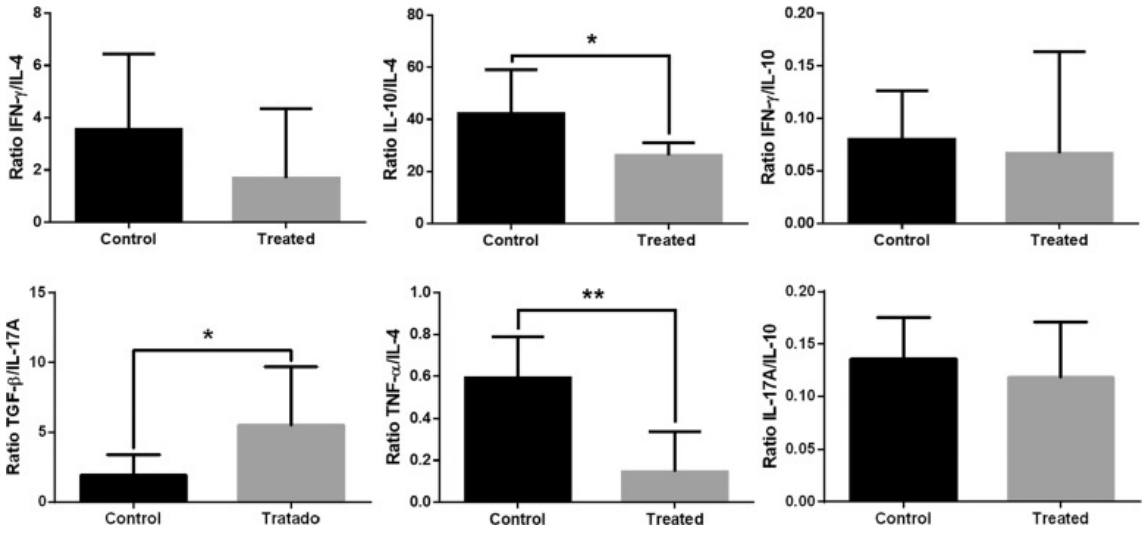

Fig. 7. Ratio IFN-γ/IL-4, IL-10/IL-4, IFN-γ/IL-10, TGF-β/IL-17A, TNF-α/IL-4 and IL-17A/IL-10, in brains from female C57BL/6J mice immunized with MOG35–55 (EAE control group) or immunized with MOG35–55 and Anisakis larval antigen (EAE treated group). Tests were carried out on day 21 p.i. Data are presented as mean ± standard deviation of eight mice from two independent experiments. (*P < 0.05; **P < 0.01).