



## Different MOG<sub>35–55</sub> concentrations induce distinguishable inflammation through early regulatory response by IL-10 and TGF- $\beta$ in mice CNS despite unchanged clinical course



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### ABSTRACT

Multiple sclerosis (MS) shows distinct clinical courses. Experimental autoimmune encephalomyelitis (EAE), a model to study multiple sclerosis, can be induced by different protocols, which show distinct cytokine and antibody production. The factors involved in this heterogeneity remain unclear. The relevance of MOG concentration in triggering a regulatory response in the chronic model of EAE is imprecise. The aim of this study was to investigate if 100 or 300  $\mu$ g of MOG<sub>35–55</sub> could induce different EAE profiles. Modifications in the concentration of MOG were able to change the patterns of chemokines, cytokines, percentage of cells, inflammatory infiltrate and the development of a regulatory response. However, these changes were unable to modify the intensity of response, which explains the chronic progression of the disease in both concentrations. The results presented in this study contribute to understanding the intricate mechanisms that trigger EAE and provide insights into the pathogenesis of various forms of MS.

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### 1. Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system (CNS) [1,2]. MS is characterized by heterogeneous clinical, pathological and immunological phenotypes that might better be described as a syndrome rather than a single disease entity, a concept that has important implications with respect to the development of effective therapeutic strategies [1–3].

There are four distinct clinical courses observed in patients with multiple sclerosis: the relapsing–remitting, the most frequent form; the secondary–progressive; the primary–progressive and the progressive–relapsing courses. The relapsing–remitting (RR) course is characterized by relapses followed by complete recovery or with sequelae and residual deficit upon recovery [2,4,5]. The

secondary–progressive course is characterized by the RR in the initial phase, with consequent progression of the disease and relapses. The primary–progressive course is characterized by progression from onset with temporary amelioration. In the progressive–relapsing course, there is a constant progression of the disease, with clear acute relapses [4,5]. However, the factors involved in this heterogeneity of MS are not completely understood, highlighting the importance of the use of experimental models in the effort to elucidate the MS clinical courses [5].

Experimental autoimmune encephalomyelitis (EAE), a model to study MS, is characterized by infiltration of T-helper (Th) 1 and Th17 and cytokines relating to these cells, such as IFN- $\gamma$  and IL-17 [6]. EAE can follow different disease courses, with diverse inflammatory process, which could mimic the forms of MS, but the mechanisms of these alterations are still unclear. Several research groups have tested different EAE induction protocols to determine the role of antigens and adjuvants in the progression of MS [5,7–9]. A previous study induced EAE, with different concentrations of myelin oligodendrocyte glycoprotein (MOG) and adjuvants, which modified the clinical course of the disease, with

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changes in the inflammatory process [5]. Thus, EAE progression in mice is determined by both genetic factors and the immunogenic/adjuvant used to induce disease.

In the EAE models, progression and remitting are controlled by the regulatory response [10]. The progression followed by complete remitting results from an established regulatory response able to promote recovery and stop relapsing. On the other hand, chronic and RR forms have inefficient regulatory response, which prevents the complete remitting of the disease [10].

MOG is a smaller constituent of the myelin, and is a well-characterized target antigen to study autoimmune responses in the CNS [11–14]. MOG-deficient mice have mild EAE, compared to the wild type mice, which shows the importance of this peptide in the generation of encephalomyelitis [14]. However, the relevance of MOG concentration in triggering a regulatory response in the chronic model of EAE remains unclear. The study of these gaps could favor the understanding of the EAE model.

With the aim to shed new light on these issues, the kinetics of regulatory and inflammatory mediators in the development of EAE with different MOG concentrations was evaluated. The results of this study show that different MOG<sub>35–55</sub> concentrations were unable to modify the clinical course of EAE, even though the lowest concentration induced a regulatory response in the CNS.

## 2. Materials and methods

### 2.1. Animals

Female C57BL/6 mice, 4–6 weeks old, were obtained from the animal care facilities of the Federal University of Juiz de Fora (UFJF) and maintained in micro isolator cages. All procedures were in accordance with the principles of the Brazilian Code for the Use of Laboratory Animals. All protocols involving mice handling were approved by the Committee on the use of laboratory animals from UFJF (Protocol 07/2011).

### 2.2. EAE induction

The mice were divided in three experimental groups: non immunized group, immunized group with 100 µg of MOG<sub>35–55</sub> and immunized group with 300 µg of MOG<sub>35–55</sub>. The groups were evaluated at days 7, 10, 14, 21 and 58 postimmunization. The mice of the immunized groups were sub-cutaneous injected at the tail base with 100 or 300 µg of myelin oligodendrocyte glycoprotein peptide MOG<sub>35–55</sub> emulsified v/v in complete Freund's adjuvant (Sigma Chemical Co., Saint Louis, USA) supplemented with 400 µg of attenuated *Mycobacterium tuberculosis* H37RA (Difco, Detroit, USA), the MOG<sub>35–55</sub> was synthesized in the laboratory of Biophysics of the Federal University of São Paulo. Pertussis toxin 300 ng/animal (Sigma Chemical Co., Saint Louis, USA) was injected intraperitoneally (i.p.) on the day and after 2 days of immunization. Animals were monitored daily and neurological impairment was quantified.

### 2.3. Clinical assessment

Mice were weighed and the clinical signs of EAE observed daily for up to 58 days. The clinical status was assessed individually according to previous study [15,16]. The final clinical score was obtained adding all individual scores assessed.

### 2.4. Histological staining

To evaluate brain and spinal cord tissue histology, groups of mice ( $n = 6$ ) were euthanized under anesthesia, perfused by intracardiac puncture with 5 ml of 4% buffered formalin at days 7, 10,

14, 21 and 58 postimmunization. The brains or spinal cords were fixed in 4% buffered formalin and embedded in paraffin. 5-µm-thick sections were stained with hematoxylin and eosin (H&E) to assess tissue damage and inflammation. The mean score of inflammation (MSI) in the brain and spinal cord of each animal were evaluated blindly, in each slide, using a semi quantitative system as previously described [17]. 10-µm-thick sections of spinal cord of 58 day postimmunization were stained with Luxol fast blue (LFB) – cresyl violet, to access the demyelination.

### 2.5. Cytokines and chemokines production

At days 7, 10, 14, 21 and 58 postimmunization mice were euthanized under anesthesia (i.p.) and perfused through the left ventricle with phosphate buffered saline (PBS). Brains, spinal cords and lymph nodes of 6 mice per group were removed and homogenized (100 mg/ml) in the extraction solution containing 0.4 M NaCl, 0.05% tween 20 (Merck & Co., Inc., Whitehouse Station, USA), 0.5% bovine serum albumin (BSA), 0.1 M phenylmethylsulfonyl fluoride (PMSF), 0.1 M benzethonium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA) and 20 kIU/ml aprotinin (Sigma Chemical Co., Saint Louis, USA). The homogenate was centrifuged at 2000×g for 15 min at 4 °C and supernatants were collected to determine the concentration of IFN-γ, IL-17A, IL-6, TGF-β and IL-10. CCL5 and CCL20 were determined only in the brain. Cytokines and chemokines production were assayed by ELISA using commercially available antibodies according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, USA).

### 2.6. Isolation of brain mononuclear cells

At days 7 and 10 after induction, eight mice of each group were euthanized under anesthesia (i.p.) and perfused through the left ventricle with PBS. Brains were removed, macerated and filtered using 70 µm cell strainer (BD Biosciences, Bedford, USA) in RPMI 1640 medium (Gibco, Grand Island, USA) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, USA). Dispersed brain cells were incubated in RPMI containing 2 mg of collagenase D (Roche, Mannheim, Germany) at 37 °C for 45 min. Brain mononuclear cells were collected after Percoll density gradient centrifugation and washed with staining buffer (PBS, 1% FBS, 0.09% sodium azide) [18]. The cell pellet was suspended in ACK solution, centrifuged at 350×g for 5 min, and suspended in staining buffer for flow cytometry analysis.

### 2.7. Flow cytometry analysis

Isolated mononuclear brain cells were incubated with anti-mouse CD3-APC and anti-mouse CD4-PerCP (BD Biosciences Pharmingen, San Diego, USA). After 30 min at 4 °C, cells were washed in staining buffer and permeabilized with BD Pharmingen Fix/Perm solution, washed in BD Pharmingen Perm/Wash Buffer, and then stained with anti-mouse IL-17-Alexa Fluor 488 and anti-mouse IFN-γ- PE (BD Biosciences Pharmingen, San Diego, USA). After 30 min at 4 °C, cells were acquired and analyzed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, San Diego, USA).

### 2.8. Statistics

The present results are representative of three independent experiments, shown as the mean ± SEM. The significance of differences between groups was analyzed using a Student's *t*-test, Mann-Whitney test, or two-way ANOVA (GraphPad Prism 5.00) when appropriate. The differences were considered significant when  $p < 0.05$ .

### 3. Results

#### 3.1. EAE induced with 100 or 300 $\mu\text{g}$ of $\text{MOG}_{35-55}$ showed the same clinical course

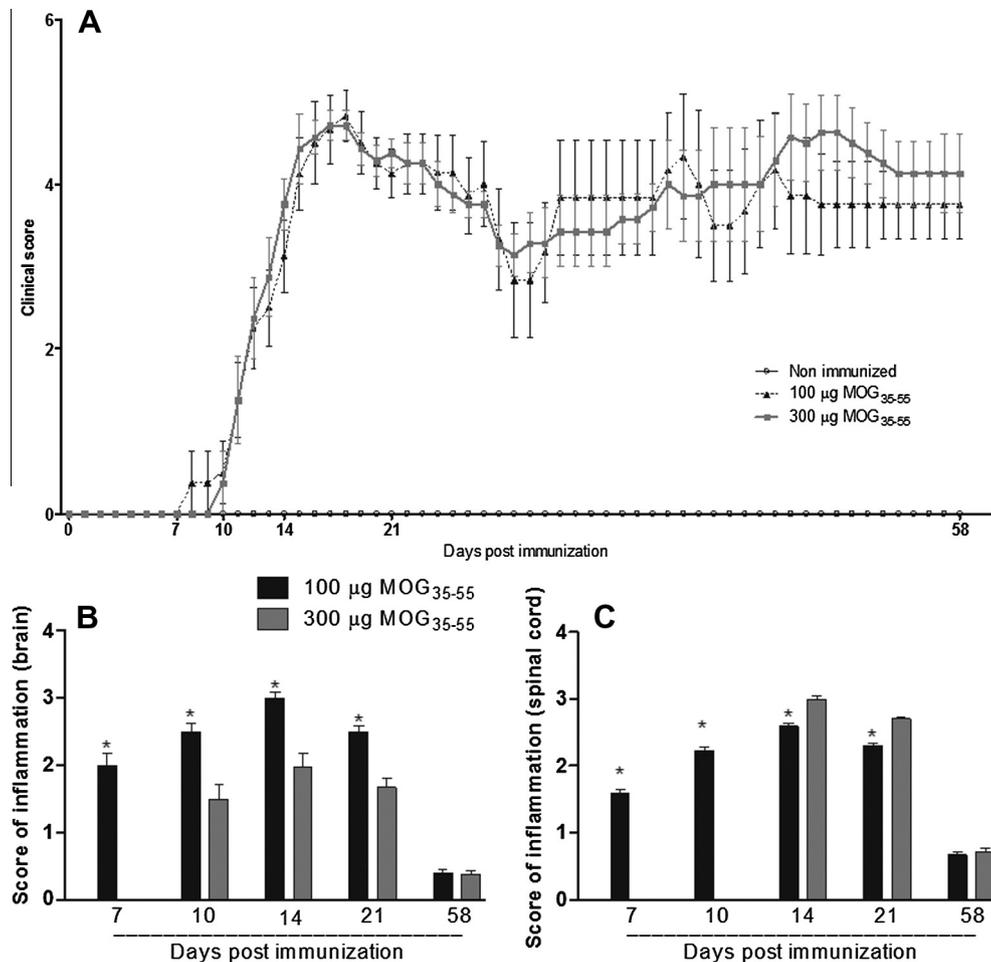
All immunized animals developed clinical signs with no difference between groups in the peak of clinical score ( $4.8 \pm 0.3$  versus  $4.7 \pm 0.2$ ) and the end-stage mean score (day 58;  $3.8 \pm 0.4$  versus  $4.1 \pm 0.5$ ) (Fig. 1A). Tail paralysis and hind limb weakness were the major clinical features observed. Concomitantly with the clinical symptoms above, there was significant weight loss between days 11 and 21 after immunization in both immunized groups, when compared to the non-immunized group (Supplementary 1).

#### 3.2. The 100 $\mu\text{g}$ $\text{MOG}_{35-55}$ group had earlier inflammatory infiltrate in the CNS

The presence of inflammatory infiltrate in the brain and spinal cord of EAE-induced mice on days 7, 10, 14, 21 and 58 after immunization was assessed through histological analysis. The non-immunized group showed standard CNS histology (Supplementary 2).

In the brain, only the group immunized with 100  $\mu\text{g}$   $\text{MOG}_{35-55}$  showed cellular infiltration on day 7. Both immunized groups showed the presence of inflammatory infiltrate on day 10. On days 14 and 21 after immunization, infiltrate levels were higher in the 100  $\mu\text{g}$   $\text{MOG}_{35-55}$  group, compared to the 300  $\mu\text{g}$   $\text{MOG}_{35-55}$  group. On the 58th day after immunization, a reduction in inflammatory infiltrate levels was observed in the brain (Supplementary 2). Only the 100  $\mu\text{g}$   $\text{MOG}_{35-55}$  group presented MSI (MSI =  $2.0 \pm 0.2$ ) on day 7. The peak of MSI for immunized groups was observed on day 14. Fifty-eight days after immunization, MSI was reduced, and no significant differences were observed between the immunized groups (Fig. 1B).

For the spinal cord, the cellular infiltrate was observed only in the 100  $\mu\text{g}$   $\text{MOG}_{35-55}$  group on days 7 and 10 after immunization. On days 14 and 21 after immunization, the infiltrate level was higher in the 300  $\mu\text{g}$   $\text{MOG}_{35-55}$  group (Supplementary 3). As in the histology analysis, only the 100  $\mu\text{g}$   $\text{MOG}_{35-55}$  group presented MSI on days 7 and 10, while on days 14 and 21, MSI was higher for the 300  $\mu\text{g}$   $\text{MOG}_{35-55}$  group (Fig. 1C). On day 58 after immunization, the degree of cellular infiltrate and MSI were reduced, though significant differences were not observed between the immunized groups (Fig. 1C and Supplementary 3); however, demyelination was present (Fig. 2). Demyelination was observed on day 58 after



**Fig. 1.** C57BL/6 immunized with 100 or 300  $\mu\text{g}$  of  $\text{MOG}_{35-55}$  showed the same clinical course and different inflammatory progress. Clinical score (A) and score of inflammation in the brain (B) and spinal cord (C) of C57BL/6 non immunized and immunized with 100 or 300  $\mu\text{g}$  of myelin oligodendrocyte ( $\text{MOG}_{35-55}$ ) peptide. Clinical score ( $n = 14$  per group) were registered from day 0 to day 58 postimmunization. Each point represents mean  $\pm$  SEM. The score of inflammation ( $n = 6$  per group) was evaluated at 7, 10, 14, 21 and 58 days postimmunization. Each bar represents the arithmetic mean  $\pm$  SEM. \* $p < 0.05$  versus 300  $\mu\text{g}$  of  $\text{MOG}_{35-55}$  group. Results are representative of three different experiments.

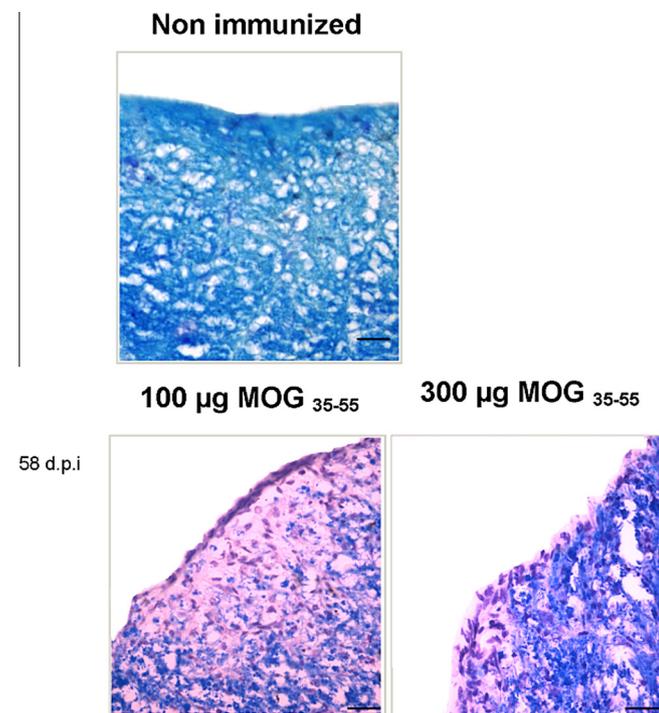
immunization in the spinal cords of both groups, but it was higher in the group immunized with 100  $\mu\text{g}$  MOG<sub>35–55</sub> than in the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group (Fig. 2).

### 3.3. Cytokines production

The levels of IFN- $\gamma$ , IL-17A, IL-6, TGF- $\beta$  and IL-10 were analyzed in supernatants of macerated brains, spinal cords and lymph nodes on days 7, 10, 14, 21 and 58. The concentrations of cytokines in the brain were detected earlier, with production peaks on day 7, in the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group. In the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group, cytokines were detected from day 10 on, with production peaks on day 10 (Supplementary 4). In the spinal cord, only IFN- $\gamma$  of the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group was not detected on day 7 (Supplementary 5). The production peak followed a similar pattern in the brain and in the spinal cord for the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group; however, in the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group, cytokines had a production peak in the spinal cord after the onset of the disease. On the other hand, oppositely to what was observed in the CNS, in lymph nodes all cytokines were detected from day 7 on in the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group, while in the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group, cytokines were detected from day 10 on, with production peak on day 21 (Supplementary 6). In relation to IL-10 and TGF- $\beta$ , the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group showed a high production of these cytokines in the CNS on day 7. The same finding was observed for the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group on day 10, but the opposite was observed in lymph nodes (Fig. 3).

### 3.4. CCL5 and CCL20 were first produced in the brains of the 100 $\mu\text{g}$ MOG<sub>35–55</sub> group

In addition to the evaluation of inflammatory cell infiltrate, the levels of CCL5 and CCL20 were analyzed in supernatants of macerated



**Fig. 2.** Demyelination was evaluated in the spinal cords of C57BL/6 mice non immunized and immunized with 100 or 300  $\mu\text{g}$  of myelin oligodendrocyte (MOG<sub>35–55</sub>) peptide ( $n = 6$  per group) at 58th day postimmunization (d.p.i.). The figures are representative of the histological analysis of experimental group stained with Luxol fast blue and captured at a magnification of 40 $\times$ , bar scale = 20  $\mu\text{m}$ . Results are representative of three different experiments.

ated brains on days 7, 10, 14, 21 and 58. High levels of both chemokines were detected on day 7 only in the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group, and remained so until day 21. In the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group, chemokines were detected only on day 10 and 14, and returned to control levels from day 21 on (Fig. 4).

### 3.5. The frequency of CD4+IL-17+ T cells and CD4+IFN- $\gamma$ + T cells was elevated in the brains of immunized groups

The percentage of CD4+IL-17+ cells was higher in the group immunized with 100  $\mu\text{g}$  MOG<sub>35–55</sub> on day 7 after immunization (Fig. 5). The frequency of cells expressing CD4+IFN- $\gamma$ + was higher on day 10 after immunization, for both immunized groups (Fig. 5).

## 4. Discussion

The EAE model has been studied for decades as an animal model to understand the pathogenesis and progression of MS [19,20]. In this context, different EAE models can be developed with diverse clinical progression courses, such as complete remitting, relapsing–remitting or chronic progression. The incomplete remitting course in chronic and RR models could be explained in part by the absence of a regulatory response. The regulatory response in this model is well studied, and has been linked to the establishment of a tolerance to self-antigens; moreover, treatments that induce a regulatory response are related to the improvement of clinical signs [21–23].

In the present study, the different concentrations of MOG were able to trigger differentiated regulatory responses that, despite the influence in the inflammatory process, were unable to induce tolerance and improve clinical signs, suggesting that the availability of the antigen could be a crucial factor to determine regulatory response.

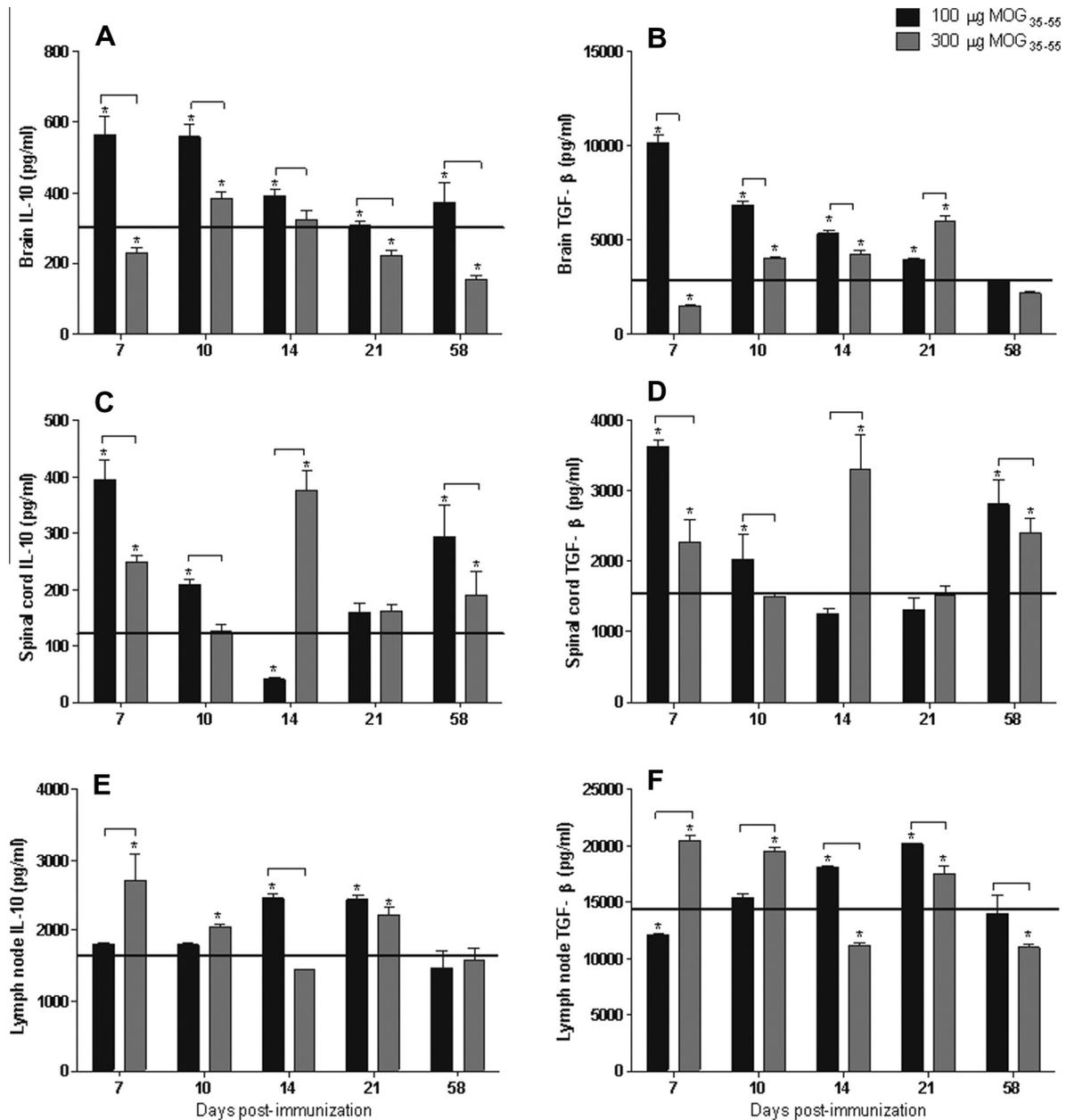
The increase of IL-10 and TGF- $\beta$  in the lymph nodes of the 300  $\mu\text{g}$  MOG<sub>35–55</sub> group on the 7th day suggests an immunosuppressive environment that probably could contribute to the development of Tregs, which culminates in the delayed onset and inflammation in the CNS and the lower demyelination on day 58 after immunization, when compared to the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group.

A previous investigation reported on a similar mechanism observed when the difference between susceptible and resistant rat strains was studied [24]. After immunization of an Albino Oxford resistant rat strain, a marked up-regulation of TGF- $\beta$  occurred in several hepatic structures. This developed an immunosuppressive environment in the liver, which contributed to the lower susceptibility to the induction of EAE observed [24].

There is growing evidence that the generation of regulatory T cells is necessary to maintain self-tolerance and prevent the onset of autoimmune disease. It has been shown that such suppression could act through the secretion of suppressor cytokines, such as TGF- $\beta$  or IL-10, and via a cell–cell contact mechanism [25–27].

Another study showed that the interplay between TGF- $\beta$  and dendritic cells is a key event in the control of EAE [28]. The lack of TGF- $\beta$  signaling in the innate system was sufficient to induce severe and spontaneous autoimmune encephalomyelitis, demonstrated by early infiltration of T cells into CNS, activation of microglia cells, inflammation in the CNS, locomotion dysfunction, and premature death [28].

The present study observed an earlier increase of the inflammatory infiltrate in the CNS in the 100  $\mu\text{g}$  MOG<sub>35–55</sub> group (7th day after immunization). The results were consistent with previous data that showed detectable number of leukocyte rolling and adhesion in the cerebral microvasculature on days 7, 14 and 21 after disease induction with 100  $\mu\text{g}$  of MOG<sub>35–55</sub> [29].

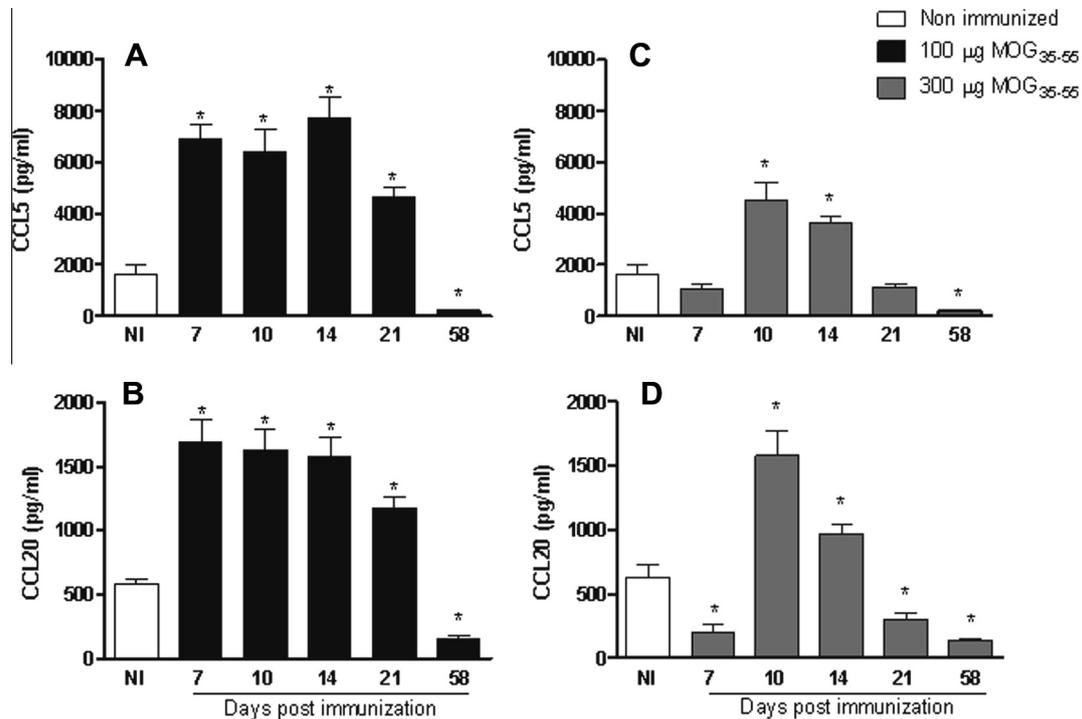


**Fig. 3.** Anti-inflammatory cytokine levels in the brain, spinal cord and lymph nodes. Cytokines levels of IL-10 and TGF- $\beta$  in the brain (A and B), spinal cord (C and D) and lymph nodes (E and F) of C57BL/6 mice non immunized and immunized with 100 or 300  $\mu$ g of myelin oligodendrocyte (MOG<sub>35-55</sub>) peptide ( $n = 6$  per group) were evaluated at 7, 10, 14, 21 and 58 days postimmunization. Brain, spinal cord and inguinal lymph nodes were removed and homogenized in extraction solution (100 mg of tissue per 1 ml). Supernatants were collected to determine the concentration of IL-10 (A, C and E) and TGF- $\beta$  (B, D and F), measured by ELISA. Each bar represents the arithmetic mean  $\pm$  SEM. \* $p < 0.05$  versus non immunized group.  $\rightarrow p < 0.05$  between the groups. Line, cytokine level of the non immunized group. Results are representative of three different experiments.

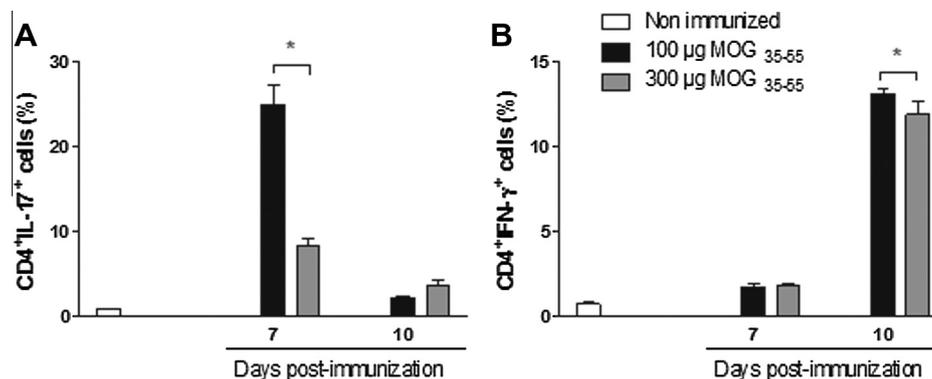
Previous evidence showed that chemokines expressed in the inflamed CNS had a time-based relationship with disease activity and with the site of CNS lesions [18,30]. A time correlation between chemokines release and cellular infiltration in the brain was observed in the present study, when the production of CCL5 and CCL20 was evaluated for both groups. In addition, the peak of chemokines overlaps the onset of clinical signs of the disease. However, in the 300  $\mu$ g MOG<sub>35-55</sub> group, the lower cellular infiltration could be a consequence of the decrease in the chemokines observed after the 10th day. The reduction of CCL5 and CCL20 has been suggested to be one of the factors responsible for the improvement of clinical signs of EAE, when subjects are treated

with an anthraquinone derivative [31]. A similar improvement, due to reduced numbers of cells in CNS, was observed when CCR5 T cells were depleted [32].

The migration of auto reactive T cells to the inflamed CNS is promoted by the expression of the CCL20 receptor, while the CC-chemokine receptor 6 (CCR6) and the adhesion of leucocytes are mediated by the production of CCL5 [29,31,33]. Additionally, an amelioration of clinical signs, with reduction of adhered leucocytes in the microcirculation of the CNS, was observed when CCL5 antagonists were used to treatment of EAE [34,35]. Furthermore, CCR6-deficient mice developed a normal Th17 response in peripheral lymphoid organs, but failed to develop EAE [36].



**Fig. 4.** Chemokine levels in the brain. Chemokines levels in the brain of C57BL/6 in mice non immunized and immunized with 100 µg (A and B) or 300 µg (C and D) of myelin oligodendrocyte (MOG<sub>35-55</sub>) peptide ( $n = 6$  per group) were evaluated at 7, 10, 14, 21 and 58 days postimmunization. Brains were removed and homogenized in extraction solution (100 mg of tissue per 1 ml). Supernatants were collected to determine the concentration of CCL5 (A and C) and CCL20 (B and D) and measured by ELISA. Each bar represents the arithmetic mean  $\pm$  SEM. \* $p < 0.05$  versus non immunized group. Results are representative of three different experiments.



**Fig. 5.** Intracellular expression of IL-17 and IFN- $\gamma$ . Expression of intracellular IL-17 (A) and IFN- $\gamma$  (B) in brain of C57BL/6 mice non immunized and immunized with 100 or 300 µg of myelin oligodendrocyte (MOG<sub>35-55</sub>) peptide were determined by flow cytometry ( $n = 8$  mice per group) at 7 and 10 days postimmunization. Cells were stained with fluorescence-conjugated antibodies to IL-17A (Alexa Fluor 488), CD4 (PerCP), IFN- $\gamma$  (PE-Cy7) and CD3 (PerCP). Bars represent mean  $\pm$  SEM. \* $p < 0.05$  versus non immunized group.  $\rightarrow p < 0.05$  between the groups. Results are representative of three different experiments.

Related to the inflammatory infiltrate, an increase in the release of IFN- $\gamma$  and IL-17 on day 7 for the 100 µg MOG<sub>35-55</sub> group and on day 10 for the 300 µg MOG<sub>35-55</sub> group was observed in the CNS. The severity of EAE is related to the increased release of IFN- $\gamma$  [4,37,38]. IL-17 has an important role as inflammatory mediator in the development of EAE. As shown in previous studies, the use of IL-17 blockers, or mice deficient in IL-17, resulted in improvement of the clinical signs of EAE [39,40]. Moreover, the transfer of cells secreting IL-17 can induce EAE, but not the transfer of T cells that secrete IFN- $\gamma$  [41].

The release of IL-6 and TGF- $\beta$  could act through activation of microglia, astrocytes, and antigen presentation in the CNS, or by

promoting the differentiation and expansion of Th17 cells, as downstream inflammatory mediators of Th17 cells [42–45].

An inflammatory process determines the damage to myelin and destruction of oligodendrocytes, and the injury and loss of axons. Accumulating axonal and neuronal damage due to chronic neuroinflammation and neurodegeneration leads to increasing and permanent neurological dysfunction [46,47]. The earlier and more intense inflammatory infiltrate observed in the CNS of the 100 µg MOG<sub>35-55</sub> group could be responsible for the more severe demyelination observed on day 58 after immunization.

The T cells producing IL-17, but not the T cells producing IFN- $\gamma$ , are implicated in the initiation of inflammatory infiltrates in the

brain, and IFN- $\gamma$  has been shown to be more strongly associated with the onset and the peak of disease [48–50]. In the present study, the percentage of CD4+ T cells secreting IL-17 was more pronounced on the 7th day after immunization, mainly in the 100  $\mu$ g MOG<sub>35–55</sub> group. On the other hand, the expression of IFN- $\gamma$  was higher on day 10, which was closest to the onset of disease in both groups.

In conclusion, modifications only in the concentration of MOG were able to change the time a regulatory response was triggered; however, these changes were unable to modify the intensity of the response, which explains the chronic progression of the disease in both groups of mice.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2014.12.009>.

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