

Oral tolerance reduces Th17 cells as well as the overall inflammation in the central nervous system of EAE mice

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammatory immune response directed against myelin antigens of the central nervous system. In its murine model, EAE, Th17 cells play an important role in disease pathogenesis. These cells can induce blood-brain barrier disruption and CNS immune cells activation, due to the capacity to secrete high levels of IL-17 and IL-22 in an IL-6 + TGF- β dependent manner. Thus, using the oral tolerance model, by which 200 μ g of MOG 35–55 is given orally to C57BL/6 mice prior to immunization, we showed that the percentage of Th17 cells as well as IL-17 secretion is reduced both in the periphery and also in the CNS of orally tolerated animals. Altogether, our data corroborates with the pathogenic role of IL-17 and IFN- γ in EAE, as its reduction after oral tolerance, leads to an overall reduction of pro-inflammatory cytokines, such as IL-1 α , IL-6, IL-9, IL-12p70 and the chemokines MIP-1 β , RANTES, Eotaxin and KC in the CNS. It is noteworthy that this was associated to an increase in IL-10 levels. Thus, our data clearly show that disease suppression after oral tolerance induction, correlates with reduction in target organ inflammation, that may be caused by a reduced Th1/Th17 response.

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1. Introduction

Multiple sclerosis (MS) and its murine model, EAE, are characterized by an autoimmune response against central nervous system (CNS) proteins, which culminates in inflammatory infiltrate, gliosis, damage of the myelin sheath and also neuronal death (Neumann, 2003; Rebenko-Moll et al., 2006; Rodriguez, 2007). Many studies have focused on the correlation between different cell types infiltrating the CNS during EAE and the clinical features of the disease. For instance, it has been shown that T CD4⁺ (Kroenke and Segal, 2007) and CD8⁺ (Goverman et al., 2005) cells, as well as macrophages and microglial cells are involved in EAE pathogenesis (Weiner, 2008).

In this context, a relatively new population of T cells has been described to play important role in EAE. Th17 cells are regulated by the expression of the ROR γ t transcription factor (Ivanov et al., 2006), and secrete IL-17 and IL-22 (Kreimberg et al., 2007) in an IL-6 + TGF- β

(Ivanov et al., 2006; Zhou et al., 2007) dependent manner. Moreover, we have previously shown that the TGF- β activation for Th17 commitment is thrombospondin-1 (TSP-1) dependent (Yang et al., 2009). Corroborating the relevance of IL-17 in EAE pathogenesis, it has been shown that IL-17 KO (Komiya et al., 2006) as well as IL-6 KO (Korn et al., 2007a,b) mice are resistant to EAE and the reciprocal development of Tregs or Th17 cells is related to IL-6 (Betelli et al., 2006). Furthermore, it has been shown that IL-23 is more important than IL-12 in inducing EAE (Cua et al., 2003) related to the fact that IL-23 is important in maintaining the viability of Th17 cells after their generation (Kreimberg et al., 2007). Human studies have reported that MS patients during active disease have higher levels of IL-6 and also IL-17 mRNA both in blood and spinal fluid (Matusevicius et al., 1999).

Oral tolerance has been classically defined as the specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the same antigen by the oral route, as extensively reviewed (Weiner, 2001; Peron et al., 2009). Oral tolerance has been shown to suppress pathology in different animal models, including EAE (Miller et al., 1992), EAU (Suh et al., 1993; Rizzo et al., 1994), asthma (Keller et al., 2006; Torseth and Gregerson, 1998) and also arthritis (Mins et al., 2004). Oral tolerance is orchestrated by distinct mechanisms. Depending on the amount fed, oral antigen can

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induce T cell deletion/anergy, or induce regulatory T cells (Benson and Whitacre, 1997; Faria and Weiner, 1999; Weiner, 2001). Other important factors are molecules, such as CTLA-4 (Samoilova et al., 1998), and CD11b (Lider et al., 1989; Zhang et al., 2001; Coombes et al., 2007; Mucida et al., 2007; Ehrchiou et al., 2007). Furthermore, it has been recently reported that lamina propria CD103⁺ dendritic cells favor the generation of T regulatory cells and to reciprocally inhibit the development of Th17 cells due to their capacity to generate retinoic acid from retinal (Mucida et al., 2007), which is an important factor for the induction of oral tolerance (Coombes et al., 2007). Very interestingly, it has already been demonstrated that oral feeding is able to significantly reduce the secretion of IL-17 and the percentage of CD4⁺IL-17⁺ cells in the periphery. On the other hand, to our knowledge, there have been no studies to date on the effect of antigen-specific oral tolerance on IL-17 responses in animal models of autoimmunity.

Given this background, we decided to investigate the effect of oral tolerance over the IL-17 response as well as the cytokines secreted by CNS infiltrating mononuclear cells from C57BL/6 mice fed and immunized with the same MOG 35–55 antigen. Our data showed a reduction of Th1 and Th17 responses in the CNS, which correlates to a milder clinical disease in animals treated with oral MOG. Moreover, besides the reduced IL-17 secretion in the periphery, several pro-inflammatory cytokines are also reduced in the CNS. These results demonstrate that in addition to previously described immunologic effects, oral antigen induces suppression of autoimmunity by affecting the secretion of IL-17 and IFN- γ , which in turn culminates with a reduction of several other pro-inflammatory cytokines.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice of 6–8 weeks of age were used to perform all experiments. Mice were housed at 5 mice per cage with water and chow given *ad libitum*. All animals were bred at the animal facility of the Center for Neurologic Diseases, Harvard Medical School, Boston, MA, USA. Some experiments were performed with mice from the animal facility of the Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil. All experiments were performed in accordance with the guidelines of the Committee on Animal Research of Harvard Medical School and University of Sao Paulo.

2.2. Induction of EAE and oral tolerance

MOG_{35–55} peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Dr. Teplow (Biopolymer Facility, Center for Neurologic Diseases, Boston, MA) or as kind gift from Prof. Niels Olsen Saraiva Camara (Immunology Department – University of Sao Paulo, Brazil). For the induction of oral tolerance, mice were orally treated by gavage with PBS or 200 μ g of MOG 35–55 every other day for 10 days with a total of 1 mg. At day 10 mice were immunized subcutaneously with 150 μ g of MOG 35–55 emulsified in CFA (v/v) containing 400 μ g of BCG. Mice also received 2 doses, 0 and 48 h after immunization of 200 ng of *Bordetella pertussis* toxin intraperitoneally. All animals were followed daily and scores were given as followed: 0 – no disease, 1 – limp tail, 2 – weak/partially paralyzed hind legs, 3 – completely paralyzed hind legs, 4 – complete hind and partial front leg paralysis, 5 – complete paralysis/death.

2.3. CNS infiltrating cell separation

All mice were sacrificed in CO₂ chambers and perfused with 10 mL of cold PBS. Brain and spinal cords were excised, macerated and maintained in 4 mL of HBSS supplemented with 2.5% collagenase D (Roche®) at 37 °C, 5% CO₂ incubator. 45 min later suspensions were

washed in HBSS and centrifuged at 450 $\times$ g for 5 min at 4 °C. After that, cells were resuspended in percoll 37% and gently laid over percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950 $\times$ g for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in HBSS and centrifuged at 450 $\times$ g for 5 min. Cellular suspensions were then resuspended in complete DMEM medium, counted and analyzed.

2.4. Flow cytometry analysis

Splenocytes and CNS mononuclear infiltrating cells were resuspended in HBSS containing 2% FBS. Cells were blocked with anti-CD16/anti-CD32 at a 1:100 dilution on ice for 20 min to prevent non-specific binding via Fc receptor. After Fc blocking, cells were stained with PE-, PercP-conjugated antibodies according to manufacturer's specifications for 30 min at 4 °C. The following antibodies were obtained from BD Biosciences® (San Diego, CA): purified anti-mouse CD16/CD32 antibodies; PercP-conjugated anti-mouse CD4, PE conjugated anti-mouse IL-17, APC conjugated anti-mouse IFN- γ . For intracellular staining, CNS-extracted cells were plated individually at 5 $\times$ 10⁵ cells/well and stimulated with 50 ng/mL of PMA and 1 μ g/mL of Ionomycin in the presence of brefeldin A for 3 h. After the incubation period, cells were washed and submitted to flow cytometry staining protocol for surface staining of CD4. Later, cells were fixed and permeabilized with Cytofix/Cytoperm kit (e-biosciences®) according to manufacturer's protocol. Cells were then washed and incubated with anti-IL-17 and anti-mouse IFN- γ for 30 min at 4 °C. After incubation cells were washed twice in Perm wash (e-bioscience), fixed in 1% paraformaldehyde and taken to flow cytometer (FacsCanto-BD Biosciences®).

2.5. Cytokine assay

Cells from lymph nodes or CNS were obtained as described and plated at 5.10⁵ cells/well for lymph nodes and 2 $\times$ 10⁵ cells/well for CNS-extracted cells in 96 well round-bottom plates. Cultures were stimulated or not with MOG 35–55 at 10, 50 or 100 μ g/mL for 72 h in 10% CO₂ incubator at 37 °C. Supernatants were collected and submitted to ELISA or Multiplex (Millipore®) assays according to manufacturer's protocol. The cytokine IL-17 was detected with the ELISA kit from e-biosciences (San Diego, CA). IL-1 α , IL-6, IL-9, IL-10, IL-12p70, MIP-1 β , RANTES, Eotaxin and KC were detected by multiplex (millipore®). The assays were performed according to manufacturer's specifications.

2.6. Lymphoproliferative response

Cells from draining lymph nodes were obtained as described and plated at 5 $\times$ 10⁵ cells/well in 96 well round-bottom plates. Cultures were stimulated with MOG 35–55 at 0, 10 and 100 μ g/mL in 10% CO₂ incubator at 37 °C. After the first 48 h 1 μ Ci/well was added and plates stored in the incubator for another 16 h. Cells were then collected in cell harvester (Cambridge technology Inc., Watertown, MA, USA) and radioactivity was measured in β -counter (Beckman LS 100C). Data are represented by counts per minute (c.p.m.).

2.7. Real-time PCR

Lymph node cells were obtained and submitted to protocols of mRNA extraction according to Qiagen® protocols. Briefly, samples containing 10⁶ cells were maintained in RNase DNase free eppendorf® tubes. Cells were lysed with 600 μ L of RLT buffer (kit supplied). All samples were then centrifuged at 10000 $\times$ g for 2 min on silic matrix columns. The flow through was discarded and the columns received 600 μ L of 70% ethanol. All columns were homogenated and centrifuged again at 10000 $\times$ g for 10 s. The flow through was discarded again and 600 μ L of RW1 buffer (kit supplied) was added to the columns which were centrifuged again at 10000 $\times$ g for 2 min. All columns were

transferred to another collector tube and received 40 μ L of DNase RNase free water and centrifuged at 10000 $\times$ g for 1 min. The flow through containing the RNA was collected. For cDNA synthesis, 1 μ g of total RNA was used. For real-time PCR, we used the Taqman Universal Master Mix also from Applied Biosystem® according to manufacturer's protocol. All plates were then taken to the Applied Biosystems 7900HT Real-Time PCR System®. All curves were normalized against the housekeeping gene GAPDH.

2.8. Statistical analysis

The two-way ANOVA followed by Bonferroni post-test were used for clinical score and lymphoproliferative responses. Real-time PCR were analyzed by parametric Student t-test. Other assays were analyzed by one-way ANOVA. All analyses were performed using the software graph pad prism 5 (San Diego, CA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Oral MOG suppresses EAE and lymphoproliferative responses

As already described, oral feeding prior to immunization is able to suppress disease score in several different models (Keller et al., 2006; Miller et al., 1992; Rizzo et al., 1994). Thus, we first evaluated the clinical scores of EAE in C57BL/6 mice orally administered MOG 35–55. One day after the last feeding dose, animals were immunized according to Materials and methods. Mice were followed daily and disease scores monitored (Lider et al., 1989). Thus, as shown in Fig. 1A,

MOG 35–55 fed animals had milder disease scores when compared to the control group, particularly from days 12 to 24 after immunization ($p < 0.01$). Moreover, hematoxylin and eosine staining of spinal cords at the peak of disease clearly demonstrates a reduction in the cellular infiltrate of orally tolerated animals (MOG-Score 1.5) compared to control (PBS-Score 3.5) (Fig. 1B).

Often, the disease suppression observed after oral tolerance is associated with a reduction of the antigen-specific adaptive immune responses. In this context, spleen and lymph node cells from PBS and MOG 35–55 fed mice were harvested on days 10 post-immunization and their proliferative responses were measured after re-stimulation *in vitro*. In fact, reduced T cell proliferation of draining lymph node cells was observed for both conditions, i.e., 10 and 100 μ g/mL of MOG 35–55 (Fig. 1C left panel), whereas for splenocytes, the reduction was observed only after 100 μ g/mL of MOG 35–55 re-stimulation (Fig. 1C right panel).

3.2. Oral MOG suppresses IL-17 and upregulates IL-10 transcription and secretion by lymph node and spleen cells respectively

The primary goal of our study was to evaluate whether oral tolerance would modulate the immune response related to secretion of the inflammatory cytokines IL-17 and IL-6 that were recently described to play a fundamental role in the pathogenesis of MS and also in the EAE model (Ivanov et al., 2006; Komiyama et al., 2006). Besides, due to the regulatory role of IL-10 observed during oral tolerance (Rizzo et al., 1999), we also decided to evaluate its transcriptional level. Thus, utilizing real-time PCR, we evaluated the expression of the *il-6*, *il-10* and *il-17* genes from draining lymph node cells obtained at day 10 post-immunization. This time point was chosen based on our observation

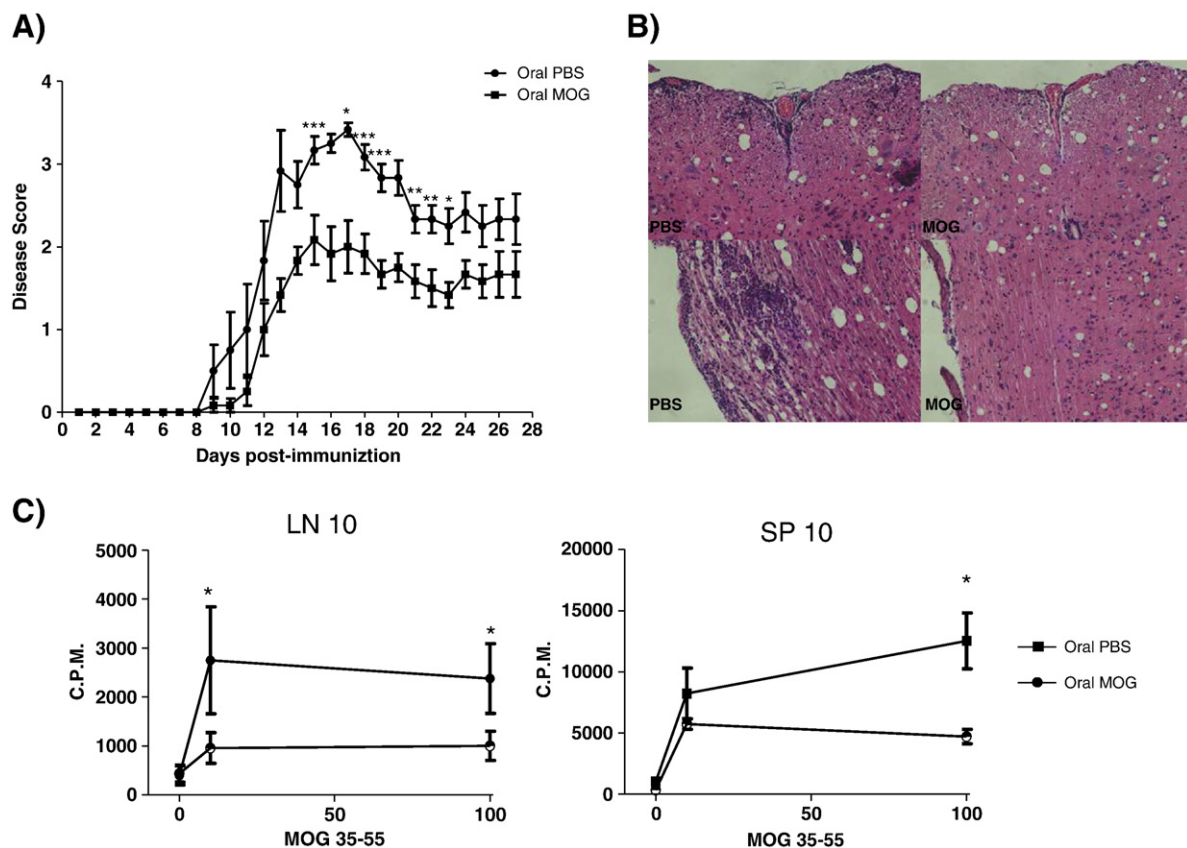


Fig. 1. Oral tolerance reduces clinical scores and lymphoproliferative response of EAE mice. Animals were orally treated with PBS or 200 μ g of MOG 35–55 every other day for 10 days and then immunized subcutaneously with 150 μ g of MOG 35–55 emulsified in CFA (v/v). All animals were followed daily. A) Daily disease EAE score of PBS and MOG 35–55 treated animals. Data shown as mean $\pm$ SE. B) Histological analysis of spinal cord from PBS (Score 3.5) and MOG 35–55 (Score 1.5) fed animals at day 16th post-immunization. C) At day 10 post-immunization, splenocytes and lymph node cells were obtained, plated and re-stimulated or not with 10 and 100 μ g/mL of MOG 35–55. Cellular proliferation was measured by thymidine- H^3 incorporation according to Materials and methods. Data shown as mean $\pm$ SD. $n = 5$ mice per group. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

that lymphoproliferative responses were significantly suppressed 10 days after immunization, demonstrating that tolerance was taking place. Moreover, this is when animals start to display clinical symptoms. Thus, as shown in Fig. 2A, MOG 35–55 fed animals had significantly less *il-17* transcripts compared to the PBS-treated group. On the other hand, we did not detect differences either in IL-6 or in IL-10 expression. As expected, spleen cells from tolerated animals had significantly higher level of IL-10 transcription when compared to its control (Fig. 2A). However, no differences for IL-6 nor IL-17 were observed.

Due to possible post-transcriptional changes of gene expression, we also evaluated the secretion of IL-17 *in vitro* after specific re-stimulation with 50 µg/mL of MOG 35–55. Consistent with the real-time PCR data, ELISA assay revealed that lymph node cells from MOG-fed mice secreted less IL-17 after re-stimulation when compared to those from PBS-treated animals (Fig. 2B). Thus, oral MOG suppresses the secretion of the cytokine IL-17 in the periphery, which is central in the pathogenesis of EAE.

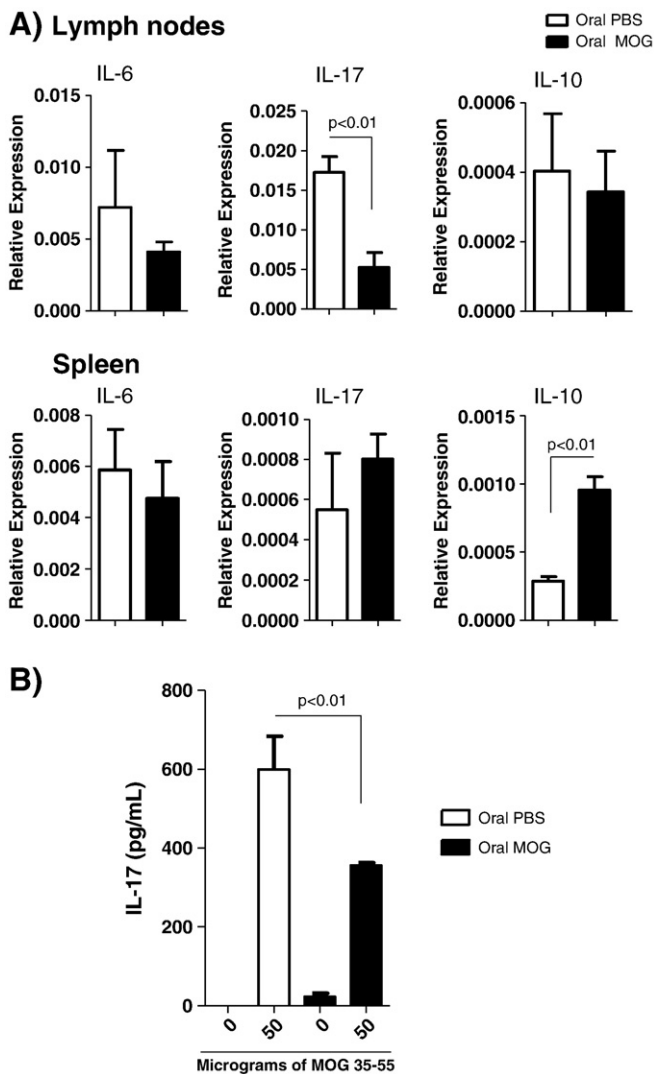


Fig. 2. Oral tolerance suppresses *il-17* transcription as well as IL-17 secretion by draining lymph node cells. C57BL/6 mice were orally treated with PBS or 200 µg of MOG 35–55 every other day for 10 days and then subcutaneously immunized with 150 µg of MOG 35–55. At day 10 post-immunization animals were sacrificed and draining lymph node cells were obtained. A) Ex vivo real-time PCR for *il-6*, *il-10* and *il-17* from draining lymph node cells and splenocytes of EAE mice. Samples were normalized against GAPDH. B) IL-17 secretion was measured by ELISA from draining lymph node cells supernatants after *in vitro* re-stimulation with 50 µg of MOG 35–55 for 72 h. n = 5 mice per group. Data are shown as mean ± SD.

3.3. Oral MOG treatment suppresses Th1 and Th17 infiltration as well as IL-17 Secretion by CNS infiltrating mononuclear cells of EAE mice

As we observed a decreased capacity of IL-17 secretion by draining lymph node cells from mice orally treated with MOG 35–55, we evaluated the profile of cytokines found in the CNS during EAE. The goal was to correlate the suppression in EAE scores with a possible reduced *in situ* production of pro-inflammatory cytokines. We obtained CNS mononuclear infiltrating cells from brain and spinal cord of PBS or MOG 35–55 fed mice at day 10 post-immunization, the same time point in which we had previously detected differences in IL-17 secretion in the periphery. As demonstrated by Fig. 3A, mononuclear CNS infiltrating secrete less IL-17 after *in vitro* re-stimulation both with 10 and 100 µg/mL of MOG 35–55. Besides the fact that other cell types may also secrete IL-17, such as macrophages (Song et al., 2008) and microglia (Kawanokuchi et al., 2008), in fact, the percentage of Th17 cells infiltrating the CNS is significantly reduced in MOG-fed animals. Moreover, these data correlates with a significant reduction in Th1 cells in the CNS as well (Fig. 3B), as observed after intracellular staining of CNS mononuclear infiltrating cells re-stimulated *in vitro* with PMA + Ion. It is noteworthy that IL-4 and IL-5 were not detected, probably due to the Th1/Th17 type of response characteristics of EAE (data not shown).

3.4. Oral tolerance reduces the inflammatory cytokine profile in the CNS of EAE mice

Due to the essential role of IL-6 in generating Th17 cells, we decided to evaluate the levels of this cytokine in the CNS. Although more relevant in the periphery, it is possible that naive CD4 T cells are committed to the Th17 population inside the CNS due to characteristics of CNS resident antigen presenting cells (APCs) (Bailey et al., 2007). In this context, we observed a significantly decreased secretion of IL-6 by CNS mononuclear cells obtained at day 10th post-immunization from MOG 35–55 fed mice when compared to PBS-treated group (Fig. 4). Despite the fact that IL-6 is essentially an innate immunity derived cytokine, it has already been demonstrated that T CD4⁺ cells are able to do so in the CNS of EAE mice (Korn et al., 2007a,b).

Still in the context of pathogenic T cells in EAE, and associated with the fact that we observed decreased amounts of IL-6 and IL-17 secreted by CNS infiltrating mononuclear cells, we next evaluated the secretion of several other pro-inflammatory cytokines that may be involved in the CNS inflammation during EAE. Consistent with the previous data, we observed a significant reduction in the secretion of the pro-inflammatory cytokines IL-1α, IL-12p70 and IL-9 (Fig. 4). Moreover, chemokines were also reduced, as observed for MIP-1β, RANTES, eotaxin and KC (Fig. 4). Associated to the decreased level of inflammatory cytokines, we also observed an increase in IL-10 secretion by these same cells (Fig. 4). In summary, our data show that somehow oral tolerance reduces the secretion/infiltration of IL-17 and IFN-γ-secreting CD4⁺ cells in the CNS of MOG-fed animals. More interesting, this overall reduction correlates with a wide reduction in the inflammatory pattern of cytokines found inside the CNS.

4. Discussion

Oral tolerance is a well known phenomenon responsible for maintaining integrity of the intestinal tract, mainly due to the regulatory characteristics of the GALT (Vieira and O' Garra, 2007). One good example is that IL-10 KO mice develop severe colitis after several weeks of life. More interesting is the fact that oral tolerance can be used in order to suppress autoimmune diseases, as already shown for EAE (Santos et al., 1994), MS (Weiner et al., 1993), experimental autoimmune uveitis (EAU) (Rizzo et al., 1999), collagen induced arthritis (CIA) (Mins et al., 2004) and also the allergic model of asthma (Keller et al., 2006; Peron et al., 2009).

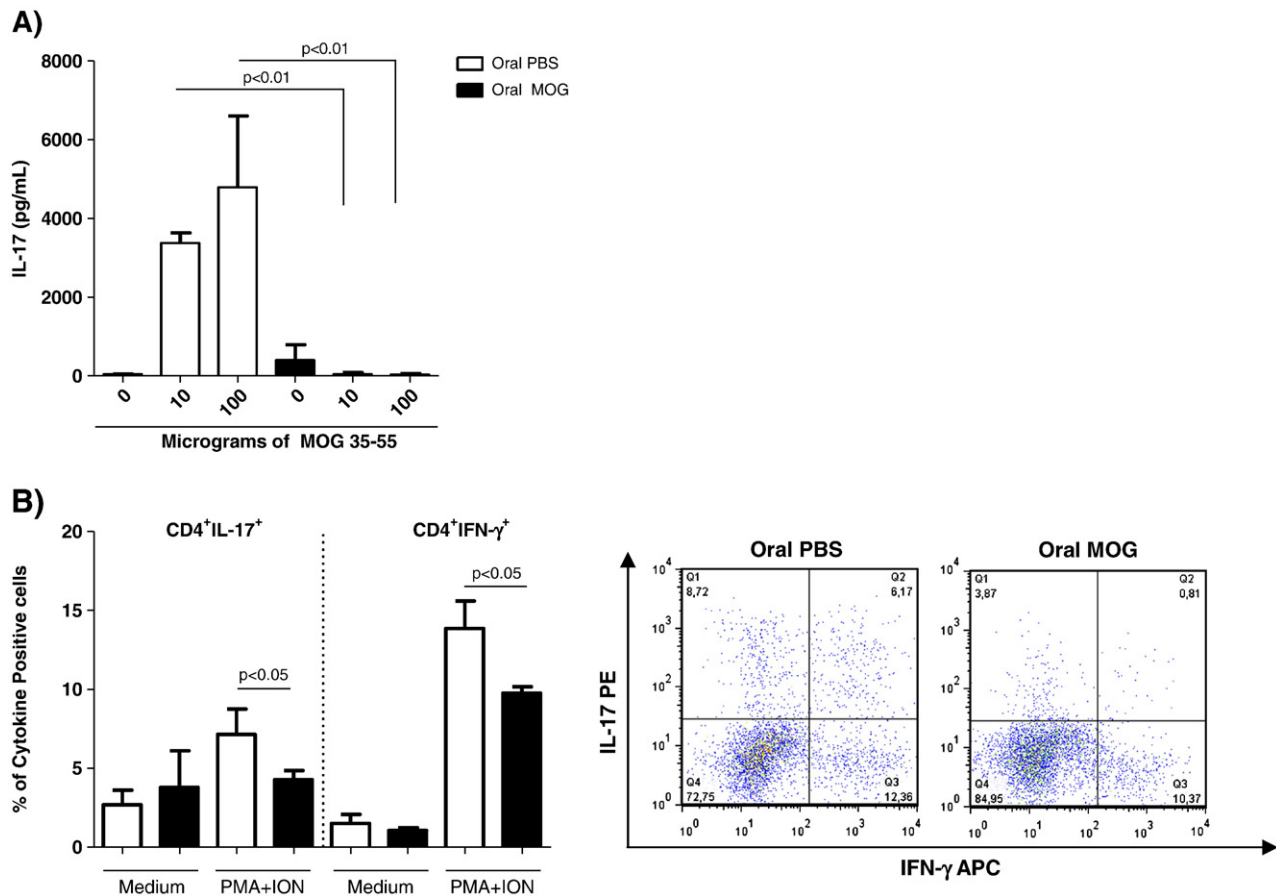


Fig. 3. Oral tolerance suppresses Th1 and Th17 response in the CNS of EAE mice. IL-17 secretion, CD4⁺IFN-γ⁺ and CD4⁺IL-17⁺ T cells percentage was assayed after *in vitro* re-stimulation of CNS mononuclear infiltrating cells. C57BL/6 mice were orally treated with PBS or 200 μg of MOG 35–55 every other day for 10 days and then subcutaneously immunized with 150 μg of MOG 35–55. At day 10 post-immunization animals were sacrificed and CNS infiltrating mononuclear cells were obtained. In A) ELISA for IL-17 secretion from CNS infiltrating cells re-stimulated with 0, 10 and 100 μg/mL of MOG 35–55. In B) Graph shows the percentage of CD4⁺IFN-γ⁺ and CD4⁺IL-17⁺ T cells in the CNS of EAE mice after PMA + ION *in vitro* re-stimulation. Right panel illustrates the quadrants used. Cells were CD4 gated. n = 5 mice per group. Data are shown as mean ± SD.

Due to the relevance of a newly described population of T cells, so-called Th17 cells (Ivanov et al., 2006; Iwakura and Ishigame, 2006; Yang et al., 2009), which secrete high amounts of IL-17A, IL-17F and also IL-22 (Kreimberg et al., 2007), we decided to study the relationship between the suppression taking place after oral tolerance and this pathogenic cytokine an autoimmunity model. In fact, our data clearly showed suppression of IL-17 responses after oral MOG administration, both in the periphery and in the CNS. This was observed not only at transcriptional level of the *il-17* gene in draining lymph nodes, but also for its secretion after *in vitro* antigen-specific re-stimulation. This suggests that Th17 generation/expansion may be somehow impaired in orally tolerated animals. It is noteworthy to mention that our data is corroborated by the literature in a sense that it has already been shown that oral tolerance reduced IL-17 level in the periphery, by a CD11b-dependent phenomenon (Ehrichiou et al., 2007). However, the study conducted by this group used the Balb/c OVA-immunized approach, whereas we used the both peripheral cells as well as CNS infiltrating cells in the experimental model of MS.

Despite the fact that Th17 cells had taken great relevance in autoimmunity, in fact, IL-17 may also be secreted by other cellular types. To name a few, macrophages (Song et al., 2008) microglia (Kawanokuchi et al., 2008) and astrocytes (Das Sarma et al., 2009) are also able to secrete biological levels of IL-17. In fact, besides secreting this cytokine, microglia and astrocytes constitutively express IL-17RA (IL-17 receptor) *in vivo*, exerting several biological effects after its activation. For instance, *in vitro* stimulation of these cells with rIL-17 induces upregulation of several inflammatory molecules, such as MIP-1, MIP-2,

MCP-1, MCP-5 and KC (Das Sarma et al., 2009). This is consistent with our data in a sense that orally tolerated animals had less IL-17 protein in the CNS, which may be responsible for the reduction in MIP-1β, RANTES and KC, important as macrophage-recruiting chemokines. Moreover, it has been shown that altered peptide ligands (APLs) treatment during EAE is able to widely reduce disease score, which correlates to lower levels of chemokines, such as MIP-1β, MCP-1 and RANTES (Fischer et al., 2000). Moreover, it has been recently demonstrated that microglial cells are able to convert naive T CD4 cells into Th17 cells. Interestingly, IL-17 secreted by Th17 cells upregulates costimulatory molecules, antigen-presentation and inflammatory cytokines secretion by microglial cells (Murphy et al., 2010). Thus, the overall reduction in pro-inflammatory cytokines in the CNS of tolerated mice also correlates with our observation that Th1 cells are also found in lower percentage in the CNS. As IFN-γ has a well known pro-inflammatory capacity, even over macrophages and microglial cells (Murphy et al., 2010), it is reasonable to believe that the reduction in IL-17 and IFN-γ may surely account for the overall reduced inflammatory cytokine pattern observed. The reduction of Th1 cells may also be corroborated by the lower levels of IL-12p70 observed. It is also worthy to mention that it has been recently described that IFN-γ acts synergistically with IL-6 to increase IL-12 secretion and CD40 expression by microglial cells (Lin and Levinson, 2009). This data corroborates our findings, as we detected less IL-6 and IFN-γ from MOG-fed animals, which correlates with less IL-12p70 and the decreased overall inflammation. Still in this concern, it has been previously demonstrated that, due its plasticity, Th17 cells may convert to IFN-γ secreting cells, as reviewed (Locksley, 2009; Peck and Mellins

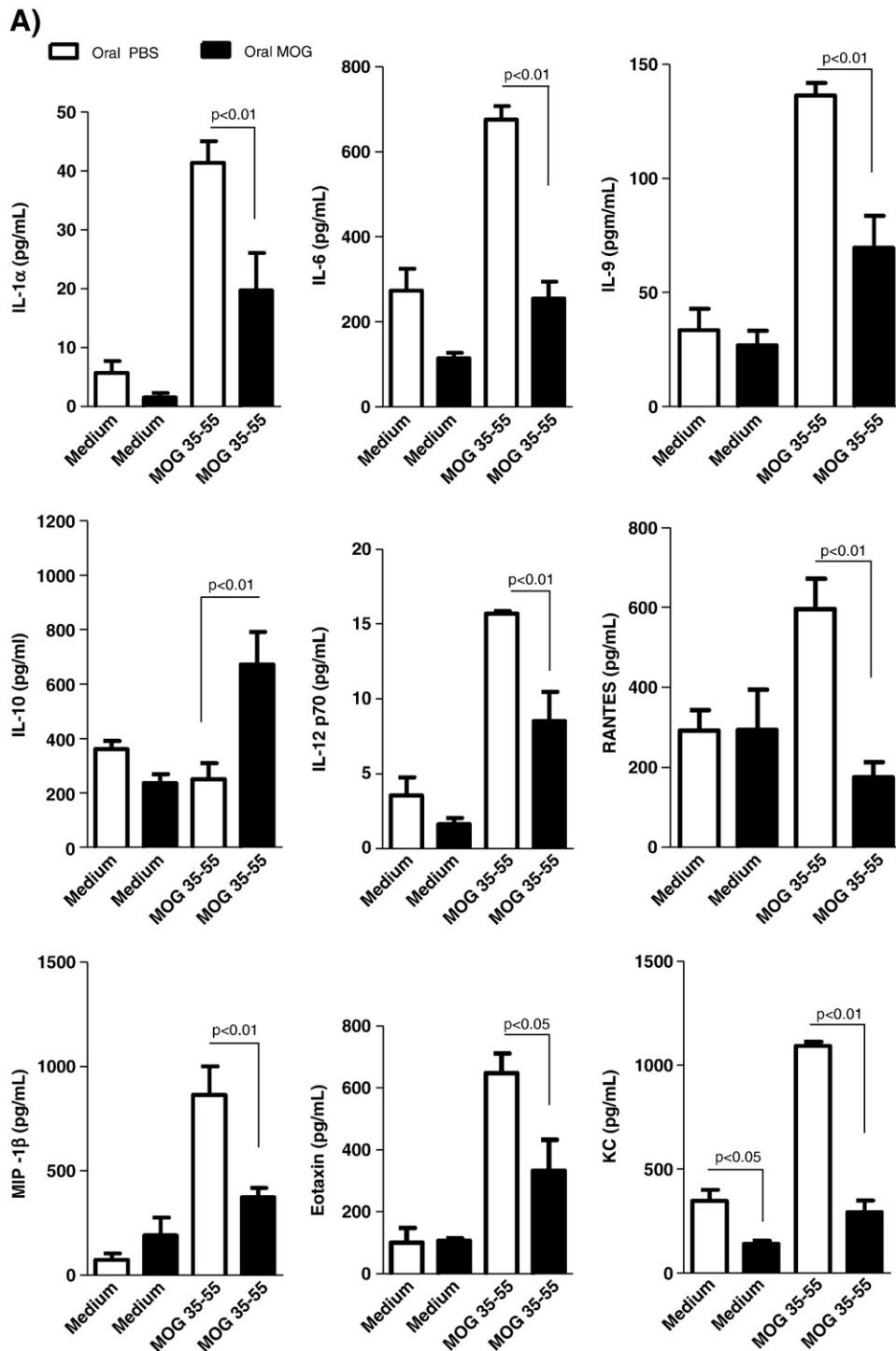


Fig. 4. Oral tolerance reduces inflammatory cytokines and chemokines in the CNS of EAE mice. CNS infiltrating mononuclear cells were obtained at day 10 post-immunization, plated and *in vitro* re-stimulated with 50 μ g/mL of MOG 35–55. A) Graphs show the spontaneous and MOG 35–55 re-stimulated secretion of IL-1 α , IL-6, IL-9, IL-12p70, MIP-1 β , RANTES, Eotaxin, KC and IL-10. $n = 5$ mice per group. Data are shown as mean $\pm$ SD.

2009). Thus, it is possible that in our model the reduction in Th17 cells directly correlates with the reduction in Th1 cells.

It has already been shown the relevance of CNS-derived APCs in generating Th17 cells, mainly CNS resident dendritic cells or microglia (Bailey et al., 2007). Thus, besides the reduced secretion of IL-17 in the draining lymph nodes, which suggests impaired Th17 expansion, we also detected less IL-6 and IL-17 secretion in the CNS of orally tolerated mice at day 10 post-immunization. This may also impair local conversion of infiltrating Th0 naive T cells to the Th17 pathogenic phenotype. Besides, despite that IL-6 is mainly secreted by cells from the innate immune

system, it has already been demonstrated that Th17 cells may also secrete IL-6 in the inflamed nervous system (Korn et al., 2007a,b). This is also consistent with our findings of reduced percentage of Th17 infiltrating cells in our experimental group. Moreover, after flow cytometric analysis of CNS infiltrating cells, we also observed that orally tolerated mice had less macrophages (CD11b⁺CD45^{high}) found in the CNS at day 7 post-immunization (data not shown). This may correlate to the reduced secretion of IL-1 α , MIP-1 β and also IL-6 in the CNS of MOG-fed animals. Moreover, it may also be possible that in MOG tolerated mice the cellular infiltrate may be constituted of alternatively- or less-activated

macrophages/microglia, with a reduced capacity to secrete pro-inflammatory cytokines, such as those already mentioned as IL-1, IL-6, IL-12p70, and a higher capacity to secrete IL-10, as observed by us. Moreover, due to the great relevance of IL-10 during oral tolerance, it may also seem suitable that IL-27-driven Tr1 cells may be involved, and be more present within the CNS (Awasthi et al., 2007).

Our study shows that oral tolerance is able to suppress the secretion of IL-17 both from draining lymph nodes of the immunization site, as well as from CNS infiltrating mononuclear cells. In addition, there were less macrophages as well as CD4⁺IL-17⁺ T cells in the CNS of MOG 35–55 treated animals, which is associated to the decreased secretion of both IL-1 α , IL-6, IL-9, IL-12p70, MIP-1 β , RANTES, eotaxin and KC. Moreover, concerning pathogenic T cells, we also observed reduced secretion of IL-9 by CNS infiltrating cells after MOG 35–55 re-stimulation *in vitro*. This may suggest that, besides Th17 cells, Th9 cells, which induce pathology in EAE (Jäger et al., 2009), may also be reduced in orally tolerated mice. Thus, it is possible that this reduction is responsible for the milder activation of innate immunity resident cells, such as microglia, astrocytes and also infiltrating macrophages. Moreover, this milder activation correlates also to the reduced inflammatory pattern of cytokines and more importantly with the reduced clinical scores observed.

On the other hand, whether cytokines that are important in maintaining Th17 cells, such as IL-21 and 23 (Cua et al., 2003; Korn et al., 2007a,b; Zhou et al., 2007), or those that are counter-regulatory, such as IL-13 and IL-25 (Kleinschek et al., 2007) and IL-27 (Fitzgerald et al., 2007) are involved in this phenomenon after oral feeding, still remains to be determined and is our further goal in the lab. It would be plausible for instance that somehow after oral feeding, IL-27 may be found in high amounts, and thus blocking Th17 generation and also leading to the Tr1 cells commitment, as already shown (Awasthi et al., 2007). Our results provide the basis to better understand the relationship between oral tolerance and the generation of regulatory mechanisms concerning EAE suppression through modulation of the Th1 and Th17 response, and to our knowledge, this is the first report to show that oral tolerance is able to suppress a pathogenic population of cells inside the target organ in the experimental model of MS. Altogether, we believe that our findings help to better understand the biological effect of IL-17 as well as IFN- γ over the inflammation of the CNS.

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