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ASSOCIATION OF MYELIN PEPTIDE WITH VITAMIN D PREVENTS AUTOIMMUNE ENCEPHALOMYELITIS DEVELOPMENT

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Abstract—Multiple sclerosis is a chronic, inflammatory and demyelinating disease of the central nervous system (CNS). As there is no cure for this disease, new therapeutic strategies and prophylactic measures are necessary. We recently described the therapeutic activity of the association between myelin oligodendrocyte glycoprotein peptide (MOG) and active vitamin D3 (VitD) against experimental autoimmune encephalomyelitis (EAE). The objective of this work was to evaluate the prophylactic potential of this association in EAE. C57BL/6 mice were vaccinated with MOG in the presence of VitD and then subjected to EAE induction. Animals were euthanized 7 and 19 days after disease induction and the following parameters were evaluated: body weight, clinical score, inflammatory process in the CNS, amount of dendritic cells (DCs) and regulatory T cells in the spleen and cytokine production by spleen and CNS cell cultures. Vaccination with MOG associated with VitD determined a drastic reduction in clinical score, body weight loss, CNS inflammation, DCs maturation and also in the production of cytokines by CNS and spleen cell cultures. Collectively, our data indicate that this association prevents EAE development. A similar effect from specific self-antigens associated with VitD is expected in other autoimmune conditions and deserves to be experimentally appraised.

Key words: multiple sclerosis, experimental autoimmune encephalomyelitis, myelin oligodendrocyte glycoprotein, vitamin D3, tolerogenic adjuvant.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) affecting mainly young people (Ellwardt and Zipp, 2014). According to a recent update, the estimated number of people with MS is 2.3 million worldwide (Browne et al., 2014). Although etiology and immunopathogenesis of this disease are still not entirely elucidated, it is believed that this pathology is mainly mediated by Th1 and Th17 subsets (Sospedra and Martin, 2005; Luchtman et al., 2014). Cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin (IL)-17, produced by these T cell subsets, mediate inflammation and subsequent axonal degeneration, oligodendrocyte death and neuronal dysfunction (Lucchinetti et al., 2000; Sospedra and Martin, 2005; Furuzawa-Carballeda et al., 2007). Defects in the functional activity of regulatory T cells (Tregs) (CD4 + CD25 +) have been described in MS patients (Viglietta et al., 2004). Genetic predisposition and environmental factors also contribute to MS initiation and development (Lin et al., 2012; Krementsov and Teuscher, 2013).

Experimental autoimmune encephalomyelitis (EAE) has been extensively used to elucidate the pathophysiology and the potential therapeutic measures applied to MS (França et al., 2014; Zorzella-Pezavento et al., 2014; Rahimi et al., 2015). Current therapeutic approaches to control the destructive immune response in autoimmune disease are mainly based on non-specific drugs that systemically suppress the function of many immune effector cells. This extensive immunosuppression often causes serious and sometimes life-threatening side effects (Damal et al., 2013). Therefore, the need for more specific treatments resulting in lower toxicity and long-term effectiveness is highly desirable. Tolerogenic vaccines comprise a new class of vaccines, designed to re-establish immunological tolerance and thereby theoretically able to reverse autoimmune diseases. Substantial advances have been made in the generation of these vaccines that inhibit EAE in a preclinical setting. Some of the most relevant and recent findings in this context include dendritic cells (DCs) vaccines (van Brussel et al., 2014), myelin oligodendrocyte glycoprotein (MOG)-DNA constructions (Fissolo et al., 2012), cytokine-neuroantigen fusion proteins (Mannie et al., 2012) and polymeric biodegradable lactic-glycolic acid particles loaded with MOG plus IL-10 (Cappellano et al., 2014).

An alternative and very straightforward approach could be based on the concept of tolerogenic adjuvants.

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Abbreviations: CFA, complete Freund's adjuvant; CNS, central nervous system; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; Foxp3, forkhead box P3; IFN-γ, interferon gamma; IL, interleukin; MHC-II, major histocompatibility complex class II; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RPMI, Roswell Park Memorial Institute; TNF-α, tumor necrosis factor alpha; Tregs, regulatory T cells; VitD, 1α,25-dihydroxyvitamin D3.

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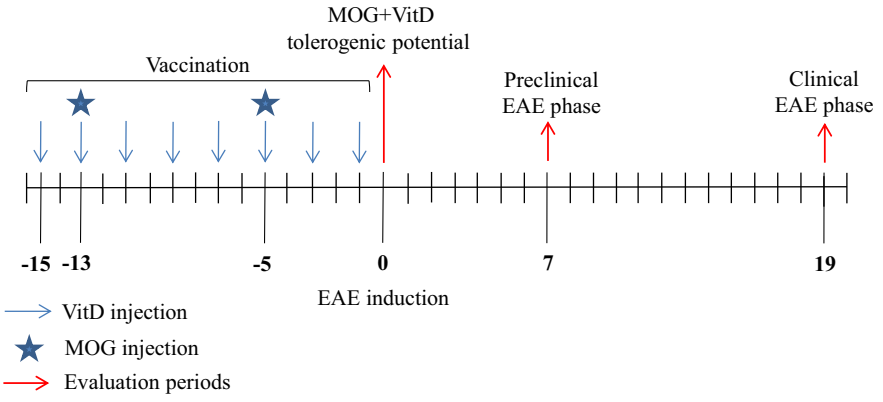
In contrast to the conventional immunogenic adjuvants that intensify the immune response, the so-called tolerogenic adjuvants have the ability to suppress or modify the specific immune response when associated with specific antigens. This concept and its functional application to induce T cell tolerance in autoimmune diseases was conceived by Kang et al. (2008). These authors demonstrated that dexamethasone combined with an insulin peptide and FK506 associated with MOG were prophylactic in diabetes and encephalomyelitis, respectively (Kang et al., 2008, 2009). In this context, we hypothesized that active vitamin D3 (VitD) could also behave as a tolerogenic adjuvant if associated with a specific antigen. This possibility was raised by strong evidences that VitD is able to modulate the immune response at both, innate and adaptive levels. It is capable, for example, to promote antimicrobial response by macrophages through the induction of antibacterial proteins (Wang et al., 2004). On the other hand, it suppresses inflammation and promotes immune tolerance by affecting antigen presentation and T cell proliferation and differentiation (Chun et al., 2014).

In the present study we evaluated if vaccination with MOG in the presence of VitD could be prophylactic and therefore prevent autoimmune encephalomyelitis development. This procedure prevented disease development and triggered reduction in DC maturation, number of Foxp3+ Tregs and proinflammatory cytokine production by spleen and CNS cell cultures. An increased production of TGF-β was observed in the spleen and CNS cell cultures.



EXPERIMENTAL PROCEDURES

Experimental design

Initially, to assess VitD tolerogenic potential, mice were allocated to four groups: 1. CTL group (negative control group) that received only saline by i.p. route; 2. MOG group that was injected only with 2 MOG doses (150 μg by i.p. route); 3. VitD group that was injected only with 8 VitD doses and 4. MOG + VitD group that was injected with both, i.e., 8 VitD doses and 2 MOG doses. VitD tolerogenic potential was tested one day after the last VitD dose and included the evaluation of cytokines, DCs and Tregs determinations. Body weight and serum calcium levels were also measured. To determine the effect of MOG + VitD vaccination strategy in EAE clinical development, mice were first vaccinated with MOG + VitD and then subjected to active induction of EAE (MOG35-55 + Complete Freund's Adjuvant (CFA)). In this case mice were allocated to the following four groups: 1. EAE group (subjected to EAE induction only); 2. MOG/EAE group; 3. VitD/EAE group; 4. M + V/EAE group. Groups 2, 3 and 4 were injected with MOG, VitD or MOG + VitD respectively before EAE induction. Evaluation was performed 19 days after EAE induction, i.e., during the acute encephalomyelitis phase. The following parameters were evaluated: clinical score, body weight and histopathological analysis of the CNS. The effect of vaccination on specific immunity was assessed 7 and 19 days after EAE induction, that is, at the pre-clinical and clinical disease phases, respectively. For this mice were allocated to two groups: 1. EAE group that was subjected to EAE induction and 2. M + V/EAE group that was vaccinated with MOG in the presence of VitD before being subjected to EAE induction. The effect of this vaccination strategy was assessed by cytokine production by spleen cells during pre-clinical and clinical disease phases and also by cytokine production by CNS cells during the clinical disease stage. This experimental design is illustrated below by a timeline scheme.

Animals

Female C57BL/6 mice 5–6 weeks old were purchased from University of São Paulo (USP, Ribeirão Preto, SP, Brazil). The animals were manipulated in accordance with the Ethics Committee for Animal Experimentation – Institute of Bioscience of Botucatu, Universidade Estadual Paulista (protocol number 571).

Vaccination with MOG in the presence of VitD

Mice were i.p. injected with 0.1 μg of VitD from Sigma (St. Louis, MO, USA), every other day during 15 days (on days −15, −13, −11, −9, −7, −5, −3 and −1). On days −15 and −5 the animals were also injected by i.p. route with 150 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) synthesized by Genemed Synthesis Inc. (San Antonio, TX, USA). Mice injected only with VitD or only with MOG were used as

controls. The rationale used to support this experimental procedure was based on our own experience (Chiuso-Minicucci et al., 2015) and also on other author's findings (Lemire and Archer, 1991).

EAE induction

Mice were subcutaneously injected with 150 μg of MOG emulsified with CFA containing 4 mg/mL of *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA). Mice also received two intraperitoneal doses, 0 and 48 h after immunization, of 250 ng of *Bordetella pertussis* toxin (Sigma). EAE clinical score was daily assessed according to the following criteria: 0 – no disease, 1 – limp tail, 2 – weak hind legs, 3 – partially paralyzed hind legs, 4 – complete hind leg paralysis, and 5 – complete paralysis/death.

CNS cell isolation

Nineteen days after EAE induction, mice were anesthetized with ketamine/xylazine and perfused with 10 mL of saline solution. To isolate CNS cells, brain and the whole spinal cord were collected and digested with collagenase D (2.5 mg/mL, Roche Applied Science, Indianapolis, IN, USA) in 4 mL of Roswell Park Memorial Institute (RPMI) (Sigma) at 37 °C for 45 min. Then, suspensions were washed in RPMI and centrifuged at 450×*g* for 15 min at 4 °C. Cells were resuspended in percoll (Sigma) 37% and gently laid over percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950×*g* for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI, and centrifuged at 450×*g* for 10 min. Cells were then resuspended in complete RPMI medium, counted, and analyzed.

Cell culture conditions and cytokine quantification

Spleen and CNS-isolated cells were collected and adjusted to 5×10^6 cells/mL, and 2.0×10^5 cells/mL, respectively. Cells were cultured in complete RPMI medium (RPMI supplemented with 10% of fetal calf serum and 2 mM of glutamine). Spleen cells were restimulated with Con A or MOG (20 μg/mL and 50 μg/mL, respectively) and CNS-isolated cells were restimulated MOG (50 μg/mL). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IFN-γ, TGF-β and IL-10 BD OptEIA Sets (Becton Dickinson) and IL-6, TNF-α and IL-17 Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions.

Flow cytometry

Spleen cells were collected and the red blood cells were lysed with buffer containing NH_4Cl. For Tregs analysis, cells were incubated with 0.5 μg of FITC labeled anti-mouse CD4 (clone GK1.5) and 0.25 μg of APC-labeled anti-mouse CD25 (clone PC61.5) (eBiosciences, San Diego, CA, USA) for 20 min at room temperature. Intracellular forkhead box P3 (Foxp3) transcription factor was detected using Foxp3 PE Staining Set (eBiosciences) according to manufacturer's instructions. For DCs analysis, splenic cells were incubated with 0.25 μg of FITC-labeled anti-mouse CD11c (clone N418), 0.03 μg of APC-labeled anti-mouse Major histocompatibility complex class II (MHC-II) (clone M5/114.15.2) and 0.125 μg of PE-labeled anti-mouse CD86 (clone GL1) (eBiosciences) for 30 min at 4 °C. After incubation, the cells were washed, resuspended in flow cytometry buffer and fixed in paraformaldehyde 1%. The cells were analyzed by flow cytometry using the FACSCanto II (Becton Dickinson, San Jose, CA, USA). Tregs and DCs analyses were performed by FlowJo software (TreeStar, Ashland, OR, USA) and Infinicyt software (Cytognos, Salamanca, Spain), respectively.

Evaluation of inflammatory infiltrates in the CNS

The histological analysis was performed during EAE acute phase (19 days after EAE induction). After euthanasia, brain and lumbar spinal cord samples were fixed in 10% formaldehyde and then embedded in paraffin. Paraffin slides (5 μm) were stained with Hematoxylin & Eosin and analyzed with a Nikon microscope.

Calcium level

The serum calcium level was analyzed using Cálcio Arsenazo III commercial kit (Bioclin, Minas Gerais, Brazil), according to the manufacturer's instruction.

Statistical analysis

Results were expressed as mean ± standard deviation or as median and interquartile (25–75%) ranges. Comparisons between two samples were made by *t* test and more than two samples were made by a one-way analysis of variance (ANOVA) followed by Tukey's test for parametric variables and by Kruskal–Wallis followed by Dunn's test for non-parametric variables. Chi-square was performed for EAE incidence. Statistical analysis was accomplished with SigmaPlot software version 12.0 (Jandel Corporation, USA) and $p < 0.05$ was considered significant.

RESULTS

Immunomodulatory effect of VitD

Eight doses of VitD, injected every other day, statistically down-modulated TNF-α, IL-6 and IL-17 production by splenic cells stimulated with Con A as demonstrated in Fig. 1A, B, D, respectively. A downmodulatory effect in the amount of CD11c + CD86 + MHCII + DCs in the spleen was also elicited by VitD injection (Fig. 1E). On the other hand, mice injected with VitD presented a slightly higher number of CD4 + CD25 + Foxp3 + T cells (Fig. 1F) in the spleen. Body weight (Fig. 1G) and calcium levels (Fig. 1H) were also affected by VitD injection. These animals presented significant hypercalcemia and accentuated body weight loss. The presence of MOG did not affect the VitD downmodulatory effect over IL-6, IFN-γ and IL-17.

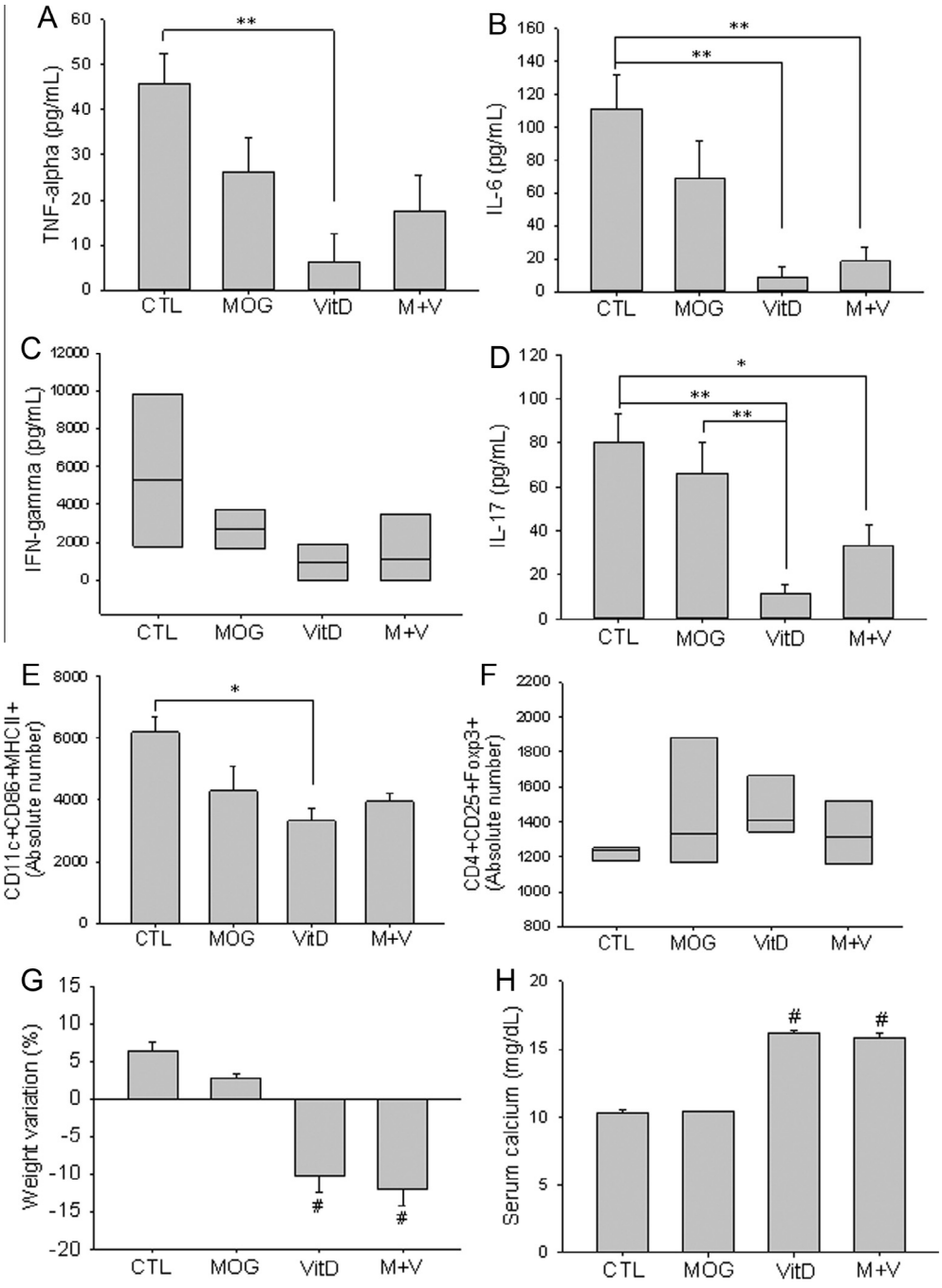


Fig. 1. Immunomodulatory effect of MOG + VitD association in C57BL/6 mice. Mice were injected with MOG + VitD and one day after the last VitD dose the levels of TNF-α (A), IL-6 (B), IFN-γ (C) and IL-17 (D) were measured in spleen cell cultures stimulated with Con A. Absolute number of CD11c + CD86 + MHCII + DCs (E) in total CD11c + cell in 500,000 acquired events and CD4 + CD25 + Foxp3 + Tregs in total CD4 + cell (F) in 100,000 acquired events. Mice were daily weighted (G) and serum calcium levels were measured in the end of vaccination procedure (H). $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{\#}p < 0.001$ represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables and by Kruskal–Wallis followed by Dunn's test for non-parametric variables. Results were expressed as mean ± SEM or medians (25–75%, box) of five to six animals per group.

Association of MOG with VitD was highly prophylactic

To test the prophylactic potential of MOG + VitD association, C57BL/6 mice were vaccinated with MOG in the presence of VitD and then subjected to EAE induction. Disease incidence is shown in Table 1. The incidence in the EAE group reached 85.71%. These animals presented the expected clinical signals as paralysis and accentuated body weight loss, as shown in Fig. 2. Previous vaccination with the MOG + VitD association triggered a clear protection. This group presented no signs of disease as paralysis or body weight loss, as illustrated in Fig. 2B, C, respectively. Differently from this protective effect, VitD or MOG alone slightly delayed clinical disease manifestations (Fig. 2A)

Table 1. EAE incidence in mice previously immunized with MOG + VitD

Groups	Incidence (%)	*p* value
EAE	85.71	0.007
MOG/EAE	60	
VitD/EAE	83.33	
M + V/EAE	0	

and did not affect the maximum clinical score (Fig. 2B) nor prevented the body weight loss (Fig. 2C).

These findings were reinforced by the histopathological analysis. As illustrated in Fig. 3, lumbar spinal cord samples from EAE group (Fig. 3B) presented the expected inflammatory foci concentrated in the tissue periphery. Similar inflammatory infiltrates were observed in samples obtained from MOG/EAE (Fig. 3C) and VitD/EAE (Fig. 3D) groups. Otherwise, no inflammatory infiltrates were detected in spinal cord samples from mice previously vaccinated with MOG associated with VitD (Fig. 3E).

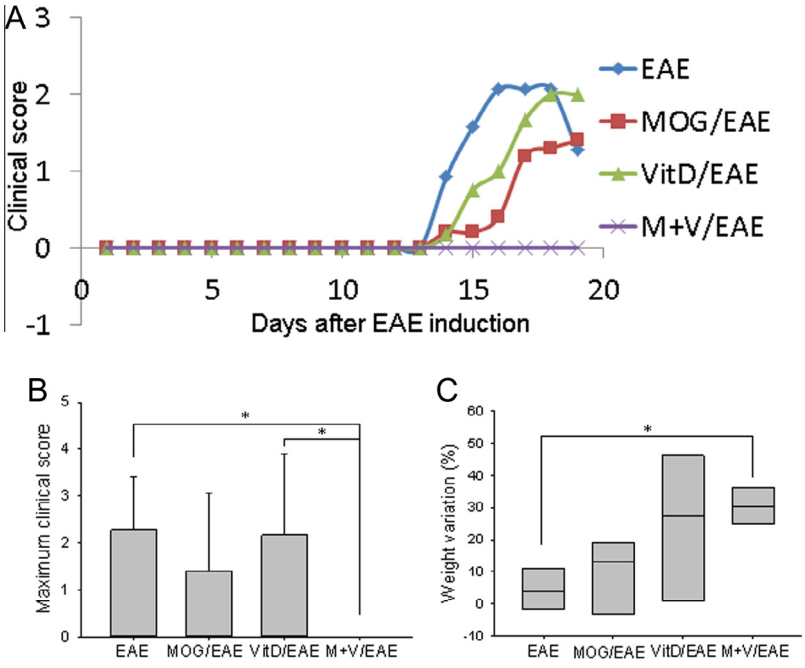


Fig. 2. Effect of MOG + VitD vaccination before EAE induction. C57BL/6 mice were previously vaccinated with MOG, VitD or MOG + VitD and then submitted to EAE induction. Clinical scores (A), maximum clinical score (B) and body weight variation (C). $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables and by Kruskal–Wallis followed by Dunn's test for non-parametric variables. Results were expressed as mean ± SEM or medians (25–75%, box) of five to six animals per group. Data are representative of two independent experiments.

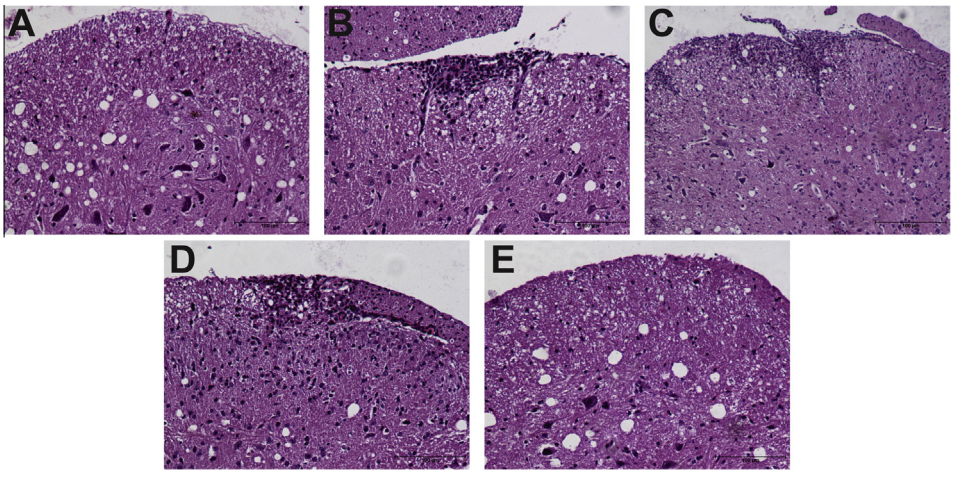


Fig. 3. Inflammatory process in the lumbar spinal cord of C57BL/6 mice vaccinated with MOG + VitD before EAE induction. C57BL/6 mice were previously vaccinated with MOG, VitD or MOG + VitD and then submitted to EAE induction. Euthanasia was performed at the clinical phase to evaluate the presence of inflammatory infiltrates in the lumbar spinal cord. Control (A), EAE (B), MOG/EAE (C), VitD/EAE (D) and M + V/EAE (E) samples. Scale bar = 100 μm. Micrographs are representative of five animals per group.

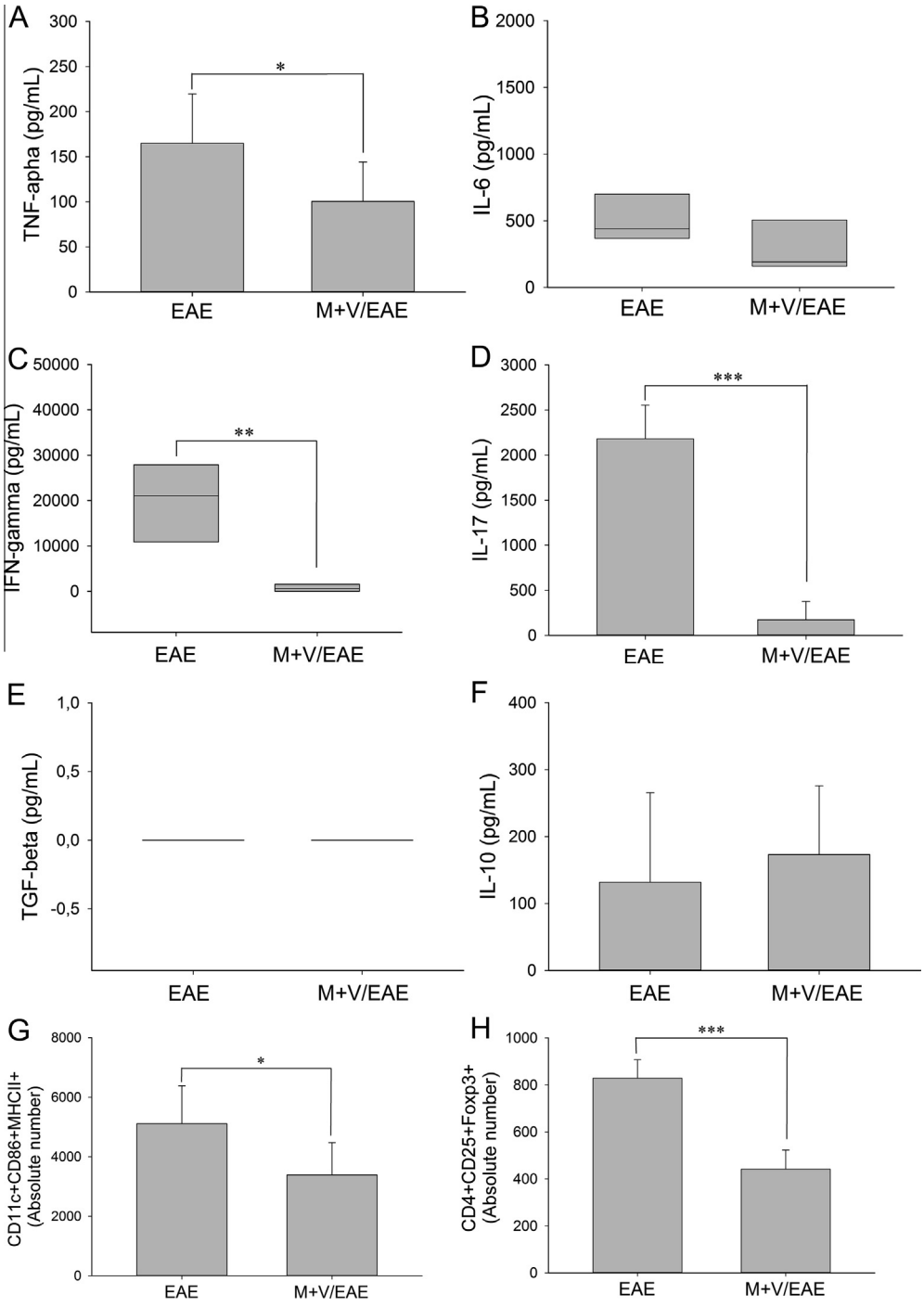


Fig. 4. Effect of MOG + VitD vaccination before EAE induction in the immune response analyzed during the preclinical disease phase. C57BL/6 mice were previously vaccinated with MOG + VitD and then submitted to EAE induction. After 7 days, TNF-α (A), IL-6 (B), IFN-γ (C), IL-17 (D), TGF-β (E) and IL-10 (F) levels were measured in spleen cell culture stimulated with MOG. Absolute number of CD11c + CD86 + MHCII + DCs (G) in total CD11c + cell in 500,000 acquired events and CD4 + CD25 + Foxp3 + Tregs in total CD4 + cell (H) in 100,000 acquired events. Comparisons between groups were performed by *t*-test. Data were presented by mean ± SE or medians (25–75% ranges) of six animals per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ represent comparisons between groups performed by *t*-test. Results were expressed as mean ± SEM or medians (25–75%, box) of five to six animals per group.

MOG + VitD specific modulatory effect

The modulatory effect of MOG + VitD previous immunization was evaluated 7 and 19 days after EAE induction, that is, during the preclinical and clinical disease phases, respectively. A very clear downmodulatory activity was observed in the preclinical phase. Splenic cell cultures, derived from vaccinated mice and stimulated *in vitro* with MOG produced significantly lower amounts of the encephalitogenic cytokines TNF-α (Fig. 4A), IFN-γ (Fig. 4C) and IL-17 (Fig. 4D). At this early phase no differences were

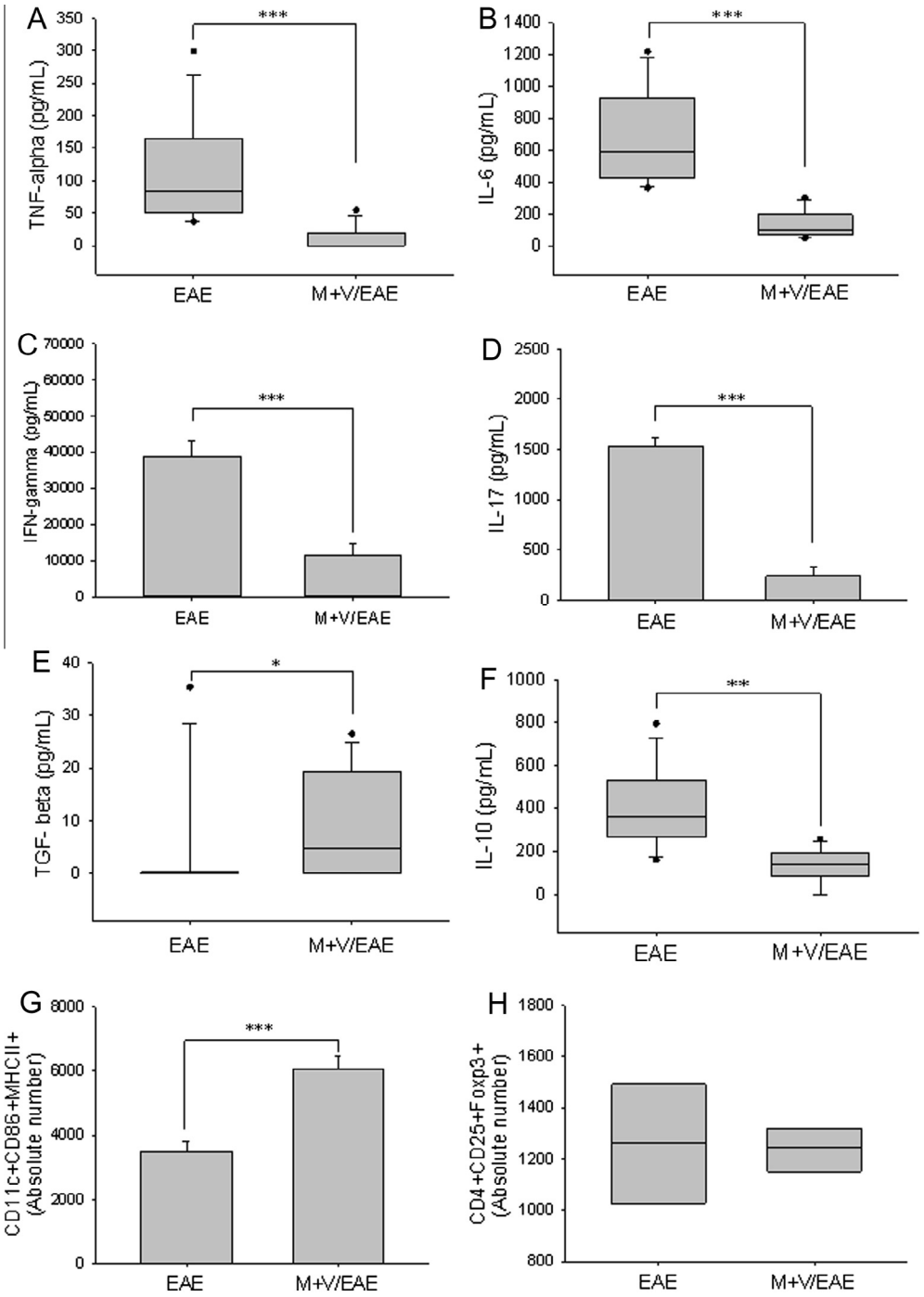


Fig. 5. Effect of MOG + VitD vaccination before EAE induction in the immune response analyzed during the clinical disease phase. C57BL/6 mice were previously vaccinated with MOG + VitD and then submitted to EAE induction. After 19 days, TNF-α (A), IL-6 (B), IFN-γ (C), IL-17 (D), TGF-β (E) and IL-10 (F) levels were measured in spleen cell culture stimulated with MOG. Absolute number of CD11c + CD86 + MHCII + DCs (G) in total CD11c + cell in 500,000 acquired events and CD4 + CD25 + Foxp3 + Tregs in total CD4 + cell (H) in 100,000 acquired events. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ represent comparisons between groups performed by t-test. Results were expressed as mean ± SEM or medians (25–75%, box) of 12 animals per group. Data are representative of two independent experiments.

observed in the production of TGF-β and IL-10 by EAE and M + V/EAE groups, as illustrated in Fig. 4E, F, respectively. Vaccinated animals also presented significantly lower numbers of CD11 + CD86 + MHCII + DCs and CD4 + CD25 + Foxp3 + Tregs in the spleen as depicted in Fig. 4G, H, respectively.

The production of TNF-α (Fig. 5A), IL-6 (Fig. 5B), IFN-γ (Fig. 5C) and IL-17 (Fig. 5D) remained significantly lower in the MOG + VitD vaccinated group during the clinical disease phase. However, other parameters were clearly distinct from the ones observed in the previous phase. This was the case of TGF-β (Fig. 5E) whose production

was significantly higher in the previously vaccinated group and IL-10 (Fig. 5F) whose production was decreased in the vaccinated group. Interestingly, there was an increased number of mature DCs in this group even though the number of CD4 + CD25 + Foxp3 + cells was similar in both groups, as shown in Fig. 5G, H, respectively.

Changes in the CNS

Brain histological analyses confirmed the spinal cord findings, that is, typical inflammatory foci, with a mononuclear predominance, were detected in the EAE control group (Fig. 6A), whereas no inflammatory infiltrates were found in previously vaccinated mice (Fig. 6B). However, enough cells were recovered from the CNS of previously vaccinated mice to allow their *in vitro* restimulation with MOG. Comparison of cell numbers eluted from the CNS of both experimental groups is shown in Fig. 6C and clearly indicates that the absolute number of cells was significantly smaller in vaccinated mice. TNF-α (Fig. 6D), IL-6 (Fig. 6E), IFN-γ (Fig. 6F), IL-17 (Fig. 6G) and IL-10 (Fig. 6H) production was significantly down-regulated in the previously vaccinated group. On the other hand, a high level of TGF-β was observed in the vaccinated group (Fig. 6I).

DISCUSSION

The main objective of this work was to evaluate if vaccination with MOG in the presence of VitD could be prophylactic in the EAE model. We initially evaluated the immunomodulatory effect of MOG + VitD association in C57BL/6 mice that is a widely accepted strain to develop EAE. This procedure did not affect the number of Tregs but slightly decreased the amount of mature DCs. It also decreased the production of encephalitogenic cytokines, this effect being highly significant in the case of IL-6 and IL-17. Similar immunomodulatory effects have been attributed to VitD alone in C57BL/6 and other mice strains (Chang et al., 2010; Joshi et al., 2011). As most of VitD effects were preserved when MOG was included in the procedure, we tested if MOG + VitD was able to prevent EAE. Previous immunization with MOG + VitD association was highly protective and this experimental group presented no clinical disease manifestations, that is, no body weight loss nor paralysis signs. MOG triggered a discrete protective effect characterized by a lower disease incidence and also a delayed paralysis appearance. VitD alone was not protective. The histopathological analysis of the CNS confirmed these findings showing no inflammatory infiltrates in the previously vaccinated group. To the best

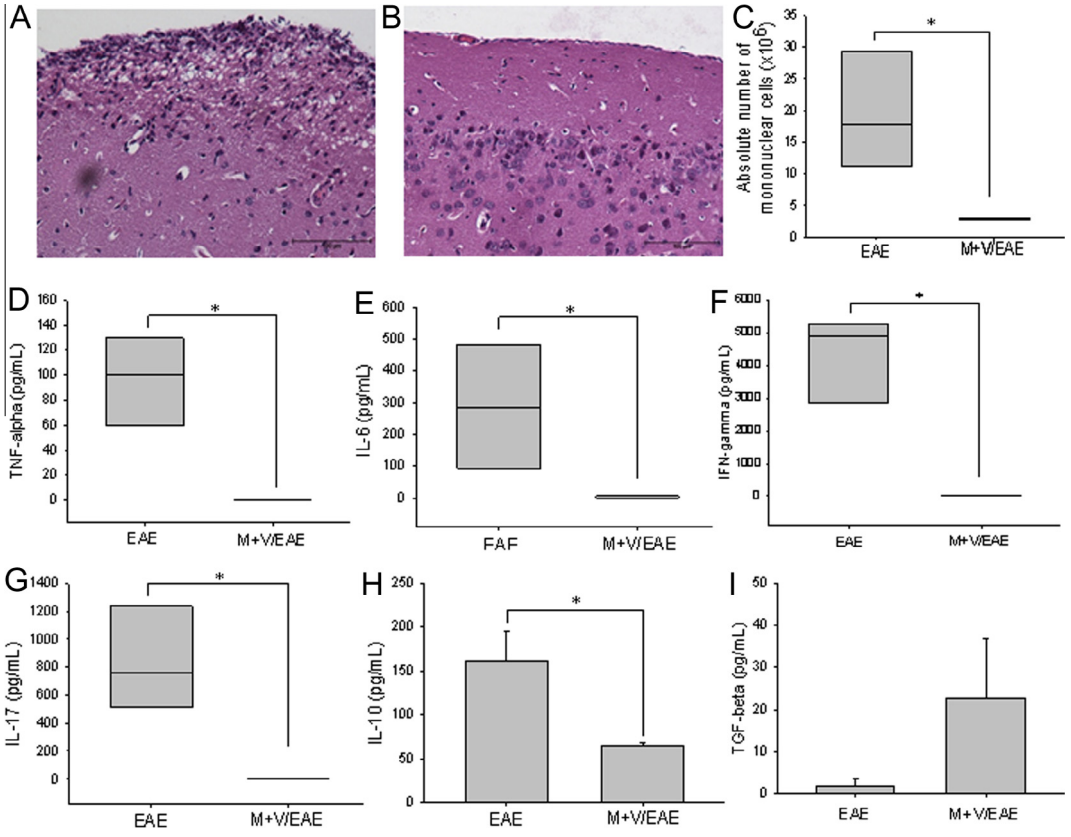


Fig. 6. Effect of MOG + VitD vaccination before EAE induction in the CNS. C57BL/6 mice were previously vaccinated with MOG + VitD and then submitted to EAE induction. 19 days after EAE induction, brains were collected for histopathological analyses: EAE (A) and M + V/EAE samples (B). Scale bar = 100 μm. Number of mononuclear cells eluted from the CNS (C). TNF-α (D), IL-6 (E), IFN-γ (F), IL-17 (G) and IL-10 (H), TGF-β (I) levels were measured in CNS cell cultures stimulated with MOG. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ represent comparisons between groups performed by *t*-test. Results were expressed as mean ± SEM or medians (25–75%, box) of four pools (each pool contains brains and spinal cords from three animals) per group in CNS cultures. Data are representative of two independent experiments.

of our knowledge, this is the first demonstration that MOG + VitD association can determine such a strong prophylactic activity in a MS experimental model. The knowledge that purified self-antigens are able to induce specific tolerance in this or other autoimmune diseases is not new. A plethora of strategies employing MOG have been devised and tested and showed promising results. Levy Barazany et al. (2014) described reduction in both, clinical score and CNS inflammation in non-obese diabetic mice after nasal administration of MOG. MOG was also effective when delivered as a genetic vaccine (Fissolo et al., 2012), as a portion of a fusion protein (Divekar et al., 2011) and as mannan-conjugated myelin peptides (Tseveleki et al., 2015). Nonetheless, the use of VitD as a tolerogenic adjuvant when associated with a specific antigen is a new proposition in the autoimmunity field that was recently suggested by us in a therapeutical context (Chiuso-Minicucci et al., 2015) and that is now being also proposed in a prophylactic scenario.

To get some insights into the immunological mechanism involved in this prophylactic effect, parameters usually affected by tolerogenic strategies as cytokines, DCs and Tregs were evaluated. These analyses were done during preclinical and clinical EAE phases, that is, at 7 and 19 days after EAE induction, respectively. A protective effect was already detected at the preclinical phase. By this time vaccinated animals were already producing smaller amounts of encephalitogenic cytokines upon *in vitro* stimulation with MOG. The similar production of IL-10 and the absence of TGF-β production by both groups, suggest that the lower levels of the encephalitogenic cytokines in the protected group were not due to an increased production of anti-inflammatory cytokines at this period. Interestingly, a smaller number of mature (CD11c + CD86 + MHCII +) DCs was detected in the vaccinated group in comparison to the non-vaccinated one. As it is well established that mature DCs are fundamental to promote a strong and effective immune response (Guermonprez et al., 2002), the smaller amount of mature DCs in the spleen of previously vaccinated mice could explain, at least partially, the weak immune response against MOG. This finding is especially relevant because DCs have been pointed as a critical and promising target in the immunomodulatory strategies devised to control autoimmune-mediated diseases (Kavousanaki et al., 2010; Segovia-Gamboa et al., 2014).

The concept that CD4 + CD25 + Foxp3 + Tregs are deeply involved in the control of autoimmune inflammatory processes is strongly supported by the literature (Smigiel et al., 2014). In addition, most of the therapeutical procedures involving the application of VitD, promoted an increased frequency of this type of Treg (Jeffery et al., 2009; Takeda et al., 2010). For these reasons an increased number of CD4 + CD25 + Foxp3 + T cells was expected in the group previously vaccinated with MOG + VitD. However, the number of these cells was significantly lower in this experimental group during the preclinical disease phase. Even though initially not expected, this finding is compatible with the observation that expansion of specific Treg depends upon the presence of proinflammatory cytokines (Korn et al., 2007). Also, a reduced expansion of encephalitogenic T cells without a concomitant increase in the number of Treg cells was associated with the therapeutical effect of atorvastatin in an MS experimental model (Weber et al., 2014). Also supporting our findings, human Treg express VitD receptor and their proliferation in response to IL-2 is inhibited by this vitamin (Khoo et al., 2011). In this scenario, it is possible that this prophylactic effect has been, at least partially, mediated by the presence of immature DCs. Several studies revealed that vitamin D has strong immunomodulatory potential including the capacity to induce tolerogenic/immature DCs. A plethora of *in vitro* findings indicate the possible benefit of VitD in autoimmune diseases, specially through APCs profile modulation (Farias et al., 2013; Ferreira et al., 2014; He et al., 2014). Our findings suggest that similar effects can be triggered by *in vivo* injection of VitD.

The lower production of pro-inflammatory cytokines in vaccinated mice was also observed later, during the clinical disease phase. Besides, a clear change was detected in the profile of TGF-β and IL-10 production. Previously vaccinated mice produced less IL-10 but higher TGF-β levels than the EAE control group. The number of cells eluted from the CNS of previously vaccinated mice was strikingly smaller, confirming, therefore, the histopathological analysis that showed no inflammatory infiltrates in the CNS of this experimental group. In addition, these cells produced TGF-β and IL-10 but not proinflammatory cytokines in response to MOG. This finding suggests that the MOG + VitD prophylactic effect could be associated with the generation and subsequent infiltration of anti-inflammatory cytokine producer T cells, mainly IL-10 and TGF-β, to the CNS. A similar beneficial role has been attributed to these two cytokines in other therapeutical scenarios. For example, Taher et al. (2008) demonstrated that these cytokines played a critical role in the protective effects of combined immunotherapy with allergen plus VitD in a mouse model of allergic asthma. Spach et al. (2006) described that IL-10 signaling is essential for VitD-mediated inhibition of EAE. Also, patients with MS that received VitD showed significantly higher levels of these two cytokines compared with the control group (Mosayebi et al., 2011).

The protective effect of this vaccination strategy depended upon the concomitant presence of MOG and VitD because these substances, isolatedly, were not able to induce a significant prophylactic activity. These results also suggest that VitD is working as a tolerogenic adjuvant. The possible role of immunosuppressive drugs as tolerogenic adjuvants was only recently reported. Kang et al. (2008), for example, demonstrated that ovalbumin peptide (323–339) sensitized BALB/c mice could be desensitized by a concomitant treatment with this ovalbumin peptide plus dexametasone. This tolerogenic activity was associated with a blockage of DC maturation. These authors also showed that dexametasone could be tolerogenic and prophylactic in non-obese diabetic mice when associated with an insulin peptide. Kang et al. (2009) demonstrated that this concept also worked in EAE.

Immunization with MOG-DNA vaccine in the presence of FK506 prevented disease development in mice.

The most promising aspect raised by this work is to reveal that combinations of specific antigens plus tolerogenic adjuvants can be highly prophylactic and possibly therapeutical for autoimmune diseases. We recently described that the early treatment with MOG + VitD of mice submitted to EAE induction was able to control disease development (Chiuso-Minicucci et al., 2015). Further preclinical investigations including tests with VitD analogs devoid of side effects (Leyssens et al., 2014) and more acceptable routes for self-antigen administration, as transdermal or oral ones (Majewska et al., 2007) are mandatory. The persistence of the therapeutic or prophylactic effect of this association and the efficacy of other CNS peptides also requires detailed investigation.

CONCLUSION

Association of MOG with VitD significantly down-modulated the specific immune response and prevented EAE development suggesting that VitD can be used as a tolerogenic adjuvant.

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LANM, FCM and AS were the main investigators of this study. TFCFS contributed to the experiments and processed the CNS samples for histological analyses. SFZP, TGDF and LLWI contributed with the immunological experiments. MP and MRVI performed cytometric analyses.

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