

## Beneficial Effect of Tolerogenic Dendritic Cells Pulsed with MOG Autoantigen in Experimental Autoimmune Encephalomyelitis

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### Keywords

Dendritic cells; Experimental autoimmune encephalomyelitis; Regulatory T cells; Tolerance; Vitamin D<sub>3</sub>.

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### SUMMARY

**Background:** Treatment with tolerogenic dendritic cells (TolDC) is a promising, cell-based strategy to regulate autoimmune diseases such as multiple sclerosis (MS) in an antigen-specific way. This technique involves the use of TolDC from MS patients cultured in the presence of vitamin D<sub>3</sub> (VitD3) and pulsed with myelin peptides to induce a stable hyporesponsiveness in myelin-specific autologous T cells. **Aim:** The purpose of this study was to analyze the *in vivo* effect of VitD3-TolDC treatment on experimental autoimmune encephalomyelitis, an animal model of MS. **Methods:** Bone marrow-derived TolDC cultured in the presence of VitD3 and pulsed with peptide 40–55 of the myelin oligodendrocyte glycoprotein (MOG<sub>40–55</sub>) were administered preventively, preclinically, and therapeutically to EAE-induced mice. **Results:** We found that VitD3-TolDC-MOG treatment showed a beneficial effect, not only decreasing the incidence of the disease but also reducing the severity of the clinical signs mediated by induction of regulatory T cells (Treg), as well as IL-10 production and reduction of Ag-specific lymphoproliferation. Our results support VitD3-TolDC-peptide(s) treatment as a potential strategy to restore tolerance in autoimmune diseases such as MS.

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## Introduction

Multiple sclerosis (MS) is a chronic demyelinating autoimmune-mediated disease affecting the central nervous system (CNS). Although the etiology of MS remains unknown, evidence obtained from animal models and patient studies indicate that myelin-specific, autoreactive T cells play an important role in MS pathology [1–3].

Current approved therapies for MS patients consist of nonspecific immunomodulatory or immunosuppressive treatments that result in a decrease in the frequency of relapses, but have a limited effect on disease progression. In addition, as a consequence of their nonspecific mechanism of action, these therapies have various adverse effects. Thus, more effective and specific strategies to block the autoimmune reaction are needed [4]. Toward this end, cell-based therapies such as regulatory T cells (Treg), regulatory macrophages (Mreg), myeloid suppressor cells, and tolerogenic dendritic cells (DC) are novel, emerging strategies for inducing antigen-specific tolerance [4,5].

Dendritic cells are professional antigen-presenting cells (APC), and as they are able to induce both effector responses and toler-

ance, they function as key regulators of the immune system [6]. The tolerogenic response induced by DC is promoted by T-cell antigen recognition under suboptimal costimulatory signaling and biased cytokine production. The resulting antigen-specific tolerogenic mechanisms include induction of T-cell anergy, promotion of Treg differentiation, and/or induction of T-cell death [4,7,8]. The promising therapeutic application of tolerogenic DC (TolDC) for autoimmune diseases and allograft rejection has triggered the generation of different strategies to produce TolDC including genetic manipulation or differentiation in the presence of biological or pharmacological agents [4,9,10]. Our group has recently established an *in vitro* strategy to differentiate TolDC from peripheral blood monocytes of relapsing–remitting MS patients using the active form of vitamin D, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (VitD3), as the tolerogenic agent. We have found that *in vitro*, VitD3-TolDCs primed with myelin peptides are able to induce a stable hyporesponsiveness in both allogeneic and myelin-specific autologous T cells [11].

With the aim to eventually translate this therapeutic approach to MS patients and initiate a phase I/IIa clinical trial, we investigated the *in vivo* effect of VitD3-generated TolDC loaded with

MOG<sub>40–55</sub> peptide in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Our results demonstrate a beneficial effect of VitD3-TolDC-MOG in EAE by reestablishing specific tolerance to MOG, supporting their potential as tolerogenic therapy to restore tolerance in MS patients.

## Material and Methods

### Mice

Female C57BL/6J mice, 8–10 weeks old, were purchased from Harlan Laboratories (Italy). The mice were housed under standard light- and climate-controlled conditions, with standard chow and water provided *ad libitum*. All experiments were performed in strict accordance with EU and governmental regulations (Generalitat de Catalunya, Decret 214/97 30th July). The Ethics Committee on Animal Experimentation of the “Germans Trias i Pujol” Research Institute approved all procedures described in this study (protocol number: 5315). Endpoint criteria were established to minimize suffering and ensure animal welfare.

### Induction of EAE

Mice were anesthetized and then immunized subcutaneously with 100 µg of MOG<sub>40–55</sub> (YRSPFSRVVHLYRNGK) (Institut de Recerca Biomèdica de Barcelona, IRBB, Barcelona, Spain), emulsified (1:1) in Freund’s complete adjuvant containing 4 mg/mL of *Mycobacterium tuberculosis* (strain H37RA, Difco, Detroit, MI). In addition, 250 ng of pertussis toxin (Sigma Chemical, St. Louis, MO, USA) was injected intravenously at day 0 and 2.

### Clinical Evaluation

All animals were weighed and examined daily for welfare and clinical signs according to the following criteria: 0, asymptomatic; 0.5, lost of distal half of tail tone; 1, lost of entire tail tone; 1.5, hindlimb weakness; 2, hindlimb paralysis; 2.5, hindlimb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia; and 6, death. Clinical evaluation was performed in a blinded manner by two different observers.

### Generation of Bone Marrow-Derived Dendritic Cells

Progenitor bone marrow cells were flushed from femur and tibias of C57BL/6 donor mice and were cultured in RPMI 1640 (PAA Laboratories) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA), 2 mM glutamine (Sigma Chemical), 100 IU/mL penicillin and streptomycin (Normon SA, Madrid, Spain and Laboratorio Reig Jofre, Sant Joan Despi, Spain, respectively), 1 mM sodium pyruvate (Biowest SAS, Nuaille, France), and 50 µM β-mercaptoethanol (Sigma, St. Louis, MO, USA). Bone marrow cells were derived from DC after 8 days in culture medium containing 1000 IU/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF; Prospec, Rehovot, Israel). TolDC were generated by adding 1 nM

1α,25-dihydroxyvitamin D<sub>3</sub> (Calcijex, Abbott Laboratories, IL, USA) to the culture medium. Cultures were refreshed on days 2, 4, and 6 by replenishment of half the volume of fresh medium supplemented with GM-CSF and VitD3 for TolDC. On day 7, the maturation stimulus, 0.1 µg/mL lipopolysaccharide (LPS; Sigma), was added to the culture medium of mature DC (mDC) and TolDC, but not to immature DC (iDC). After 22–24 h, DC were pulsed with 10-µM MOG<sub>40–55</sub> (TolDC-MOG) for 18 h or cultured with only medium (unpulsed TolDC).

### In vivo Administration of Tolerogenic DC

Tolerogenic DC and TolDC-MOG were washed twice with PBS after which cell viability was assessed by Annexin V and 7-aminocoumarin D (7-AAD) staining. Mice received 2 or 4 doses of 10<sup>6</sup> viable TolDC-MOG, TolDC, or PBS (vehicle) intravenously. Preventive treatment with TolDC-MOG was administered on days 2 and 5 postimmunization (pi); preclinical treatment was performed on days 5 and 9 pi and therapeutic on days 15, 19, 23, and 33 pi.

### Cellular Tracking

Tolerogenic DC-MOG were labeled with CellVue NIR815 dye (MTTI, PA, USA) following the manufacturer’s instructions. Briefly, washed TolDC-MOG were incubated with NIR815 for 5 min at room temperature and blocked by adding culture medium containing 20% FBS (1:1 v/v). After washing cells twice with PBS to eliminate unbound dye and FBS, cell viability was assessed by Annexin V and 7-AAD staining. A total of 10<sup>6</sup> viable TolDC-MOG-NIR815 were administered iv in EAE-induced mice (n = 9) at day 5 pi. Imaging was performed *in vivo* at 0 and 24 h and *ex vivo* at 24 h, 48 h, 96 h, 7 days, and 14 days postadministration using a Pearl™ Impulse Imager (Li-COR Biosciences, Lincoln, NE, USA). Signal from mice treated with PBS (vehicle, n = 8) was used as negative control.

### Flow Cytometry

On day 8 of culture, iDC, mDC, and TolDC were stained for 25 min at room temperature with specific monoclonal antibodies (mAb) to determine the expression of the surface markers I-A<sup>b</sup> (MHC class II), CD86 (both from e-Bioscience, San Diego, CA, USA), and CD40 on CD11c<sup>+</sup>-gated cells (Becton Dickinson [BD] Pharmingen, San Diego, CA, USA).

For analysis of Treg, 0.5 × 10<sup>6</sup> splenocytes were stained with anti-CD3, CD4, CD25, and FoxP3 (all from BD Pharmingen) mAb following manufacturer’s instructions. Cell viability and counting were calculated using Perfect-Count Microspheres (Cytognos SL), Annexin V–allophycocyanin (APC) (Immunotools, Friesoythe, Germany), and 7-AAD (BD Pharmingen) staining.

### Allogeneic Proliferative Response

To determine the stimulatory capacity of DC, either 2500 or 5000 iDC, mDC, or TolDC pulsed with MOG<sub>40–55</sub>, were incubated for 5 days with 10<sup>5</sup> allogeneic splenocytes (six replicas per each condition) from Balb/C mice (ratio 1/40 and 1/20, respectively),

adding 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] methylthymidine (PerkinElmer, Waltham, MA, USA) for the last 8 hrs. Cells were harvested onto glass fiber filters, and [ $^3\text{H}$ ] methylthymidine incorporation was measured in a  $\beta$ -scintillation counter (Trilux, Wallac).

### Antigen-Specific Proliferation and Cytokine Production

Mice from the preventive experiment ( $n = 8$ : TolDC-MOG and  $n = 5$ : PBS [sham]) as well as a subgroup of 15 EAE-induced mice treated preclinically ( $n = 5$  for TolDC-MOG, TolDC, and PBS), and therapeutically (TolDC-MOG:  $n = 5$ , and  $n = 3$  for TolDC and PBS) were euthanized on day 36, 12, and 46 pi for functional studies. Briefly, splenocytes were cultured in a 96-well plate at  $2 \times 10^5$  cells/well in 200  $\mu\text{L}$  of supplemented RPMI containing 5  $\mu\text{M}$  MOG<sub>40–55</sub> and either 5  $\mu\text{M}$  of pokeweed mitogen (positive control) or culture medium (negative control). After 48 h of culture, 50  $\mu\text{L}$ /well of supernatant was collected and stored for cytokine detection and 1  $\mu\text{Ci}$ /well of [ $^3\text{H}$ ]-thymidine was added to each well for the last 18 h of culture. The stimulation index (SI) for each stimulus was calculated as the mean counts per minute (cpm) of antigen-stimulated cultures divided by the mean cpm of the nonstimulated cultures.

The secretion of IL-2, IL-4, IL-6, IFN- $\gamma$ , TNF- $\alpha$ , IL-17, and IL-10 cytokines was quantified in the culture supernatants using the BD cytometric bead array mouse Th1/Th2/Th17 cytokine kit (BD) and Fortessa cytometer (BD) according to the manufacturer's instructions.

### Statistical Analysis

Data were expressed as the mean  $\pm$  standard deviation (SD) values unless otherwise stated. Statistical analyses were performed using Graphpad Prism version 6.00 for Windows (La Jolla, CA, USA). A Mann–Whitney or one-way ANOVA with Dunnett's multiple comparisons test were applied to compare the mean values between 2 or  $\geq 3$  groups, respectively. For very small samples ( $n \leq 3$ ), an unpaired  $t$ -test was performed [12]. Fisher's exact test was used to compare qualitative variables. Differences were considered statistically significant when  $P < 0.05$ .

## Results

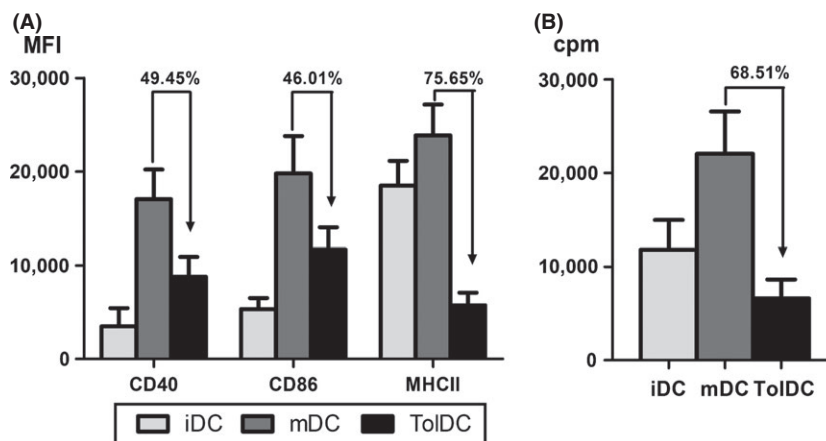
### Characterization of VitD3-TolDC from C57BL/6

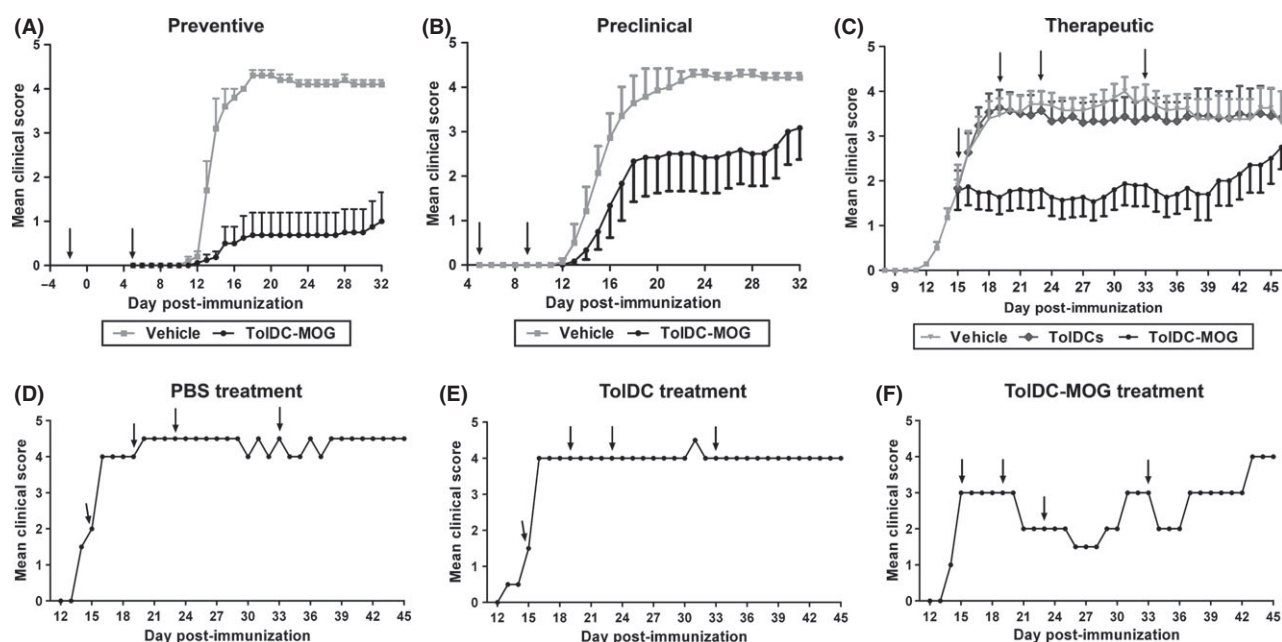
VitD3 treatment during DC differentiation generated TolDC showing a semimature phenotype with decreased expression of MHC class II, CD40, and CD86 molecules compared to mDC (MHC II:  $75.65 \pm 10.41\%$ , CD40:  $49.45 \pm 8.07\%$ , and CD86:  $46.01 \pm 9.74\%$  of reduction compared to mDC expression levels; Figure 1A). In addition, functional analysis of VitD3-TolDC revealed their poor stimulatory ability, exhibiting  $68.51 \pm 15.06\%$  reduction of allogeneic proliferative response compared to mDC (Figure 1B). Data were obtained from five representative experiments.

### Treatment with TolDC-MOG<sub>40–55</sub> Abrogates EAE Induction and Ameliorates Clinical Signs of the Disease

To determine the *in vivo* effect of TolDC loaded with MOG<sub>40–55</sub> peptide in EAE, three different approaches were used: preventive (before immunization, on days  $-2$  and  $5$  pi), preclinical (postimmunization, on days  $5$  and  $12$  pi), and therapeutic (after the onset of the disease; days  $15$ ,  $19$ ,  $23$ , and  $33$  pi). When  $10^6$  TolDC-MOG were administrated preventively, 75% of treated mice showed a lower incidence of EAE induction compared with the control group (25% vs. 100%;  $P = 0.021$ ; Figure 2A). On the other hand, when TolDC-MOG treatment was performed after EAE induction (preclinical and therapeutic approaches), the treated mice showed an ameliorated clinical course compared with both the control and unpulsed TolDC groups (Figure 2B,C). In accordance, cumulative score of TolDC-MOG-treated mice was significantly reduced preclinically ( $P = 0.051$ ) and therapeutically (vehicle vs. TolDC-MOG;  $P = 0.037$  and TolDC vs. TolDC-MOG;  $P = 0.037$ ; Table 1). In the same way, the severity of mice treated therapeutically with TolDC-MOG was only moderate compared with PBS- or unpulsed TolDC-treated mice (vehicle vs. TolDC-MOG;  $P = 0.027$  and TolDC vs. TolDC-MOG;  $P = 0.038$ ; Table 1). Interestingly, individual analysis of the clinical effect on mice with established EAE revealed that TolDC-MOG administration was able to either ameliorate clinical signs of EAE or abrogate EAE progression, as

**Figure 1** Characterization of VitD3-TolDC from C57BL/6. Expression of CD40, CD86, and MHC-II molecules on immature dendritic cells (DC) (iDC), mature DC (mDC), or tolerogenic DC (TolDC). Data are expressed as mean fluorescence intensity (MFI) (A). Allogeneic proliferation of splenocytes cocultured with iDC, mDC, or TolDC. Data are expressed as counts per minute (cpm) (B). Representative data from five independent experiments. Error bars correspond to SEM.





**Figure 2** TolDC-MOG treatment ameliorates experimental autoimmune encephalomyelitis (EAE). Clinical follow-up of mice treated with 106 TolDC-MOG, PBS (vehicle), or unpulsed TolDC preventively, on days  $-2$  and  $+5$ pi, (PBS:  $n = 5$  and TolDC-MOG:  $n = 8$ ) (A), pre-clinically, on days  $+5$  and  $+9$ pi, (PBS:  $n = 6$  and TolDC-MOG:  $n = 11$ ) (B), and therapeutically, on days  $+15$ ,  $+19$ ,  $+23$ ,  $+33$ pi, (PBS:  $n = 14$ , TolDC:  $n = 15$  and TolDC-MOG:  $n = 15$ ) (C). Representative graph of clinical follow-up from one individual mouse treated therapeutically (D), TolDC (E) and TolDC-MOG (F). Arrows indicate days of 106 TolDC-MOG, TolDC, or PBS administration. Error bars correspond to SEM.

**Table 1** Clinical data of experimental autoimmune encephalomyelitis (EAE) mice treated preclinically and therapeutically with tolerogenic DC (TolDC) and TolDC-MOG

	Preclinical approach		Therapeutic approach		
	Vehicle	TolDC-MOG	Vehicle	TolDC	TolDC-MOG
Incidence (%)	11/11	5/6	14/14	14/15	14/15
	100	83.33	100	93.33	93.33
Maximum score	$4.43 \pm 0.2$	$3.25 \pm 1.9$	$4.07 \pm 1.2$	$3.83 \pm 1.6$	$2.77 \pm 1.6^*$
Cumulative score <sup>a</sup>	$72.00 \pm 12.5$	$42.67 \pm 32.8$	$98.18 \pm 35.1$	$96.57 \pm 52.5$	$52.93 \pm 47.5^*$
Disease onset (day)	$14.71 \pm 2.6$	$15.80 \pm 2.6$	—	—	—

<sup>a</sup>Mean of the sum of daily scores for each mouse. Data are expressed as mean  $\pm$  SD. \* $P < 0.05$  (comparison between both Vehicle–TolDC-MOG and TolDC–TolDC-MOG).

opposed to animals treated with TolDC or PBS, which rapidly reached high clinical scores (Figure 2D–F). Nevertheless, the effect of TolDC-MOG was transient (5–6 days), with new doses of TolDC-MOG being required to reestablish the beneficial effect of the therapeutic vaccine (Figure 2C,F).

### TolDC-MOG Migrate to the Spleen

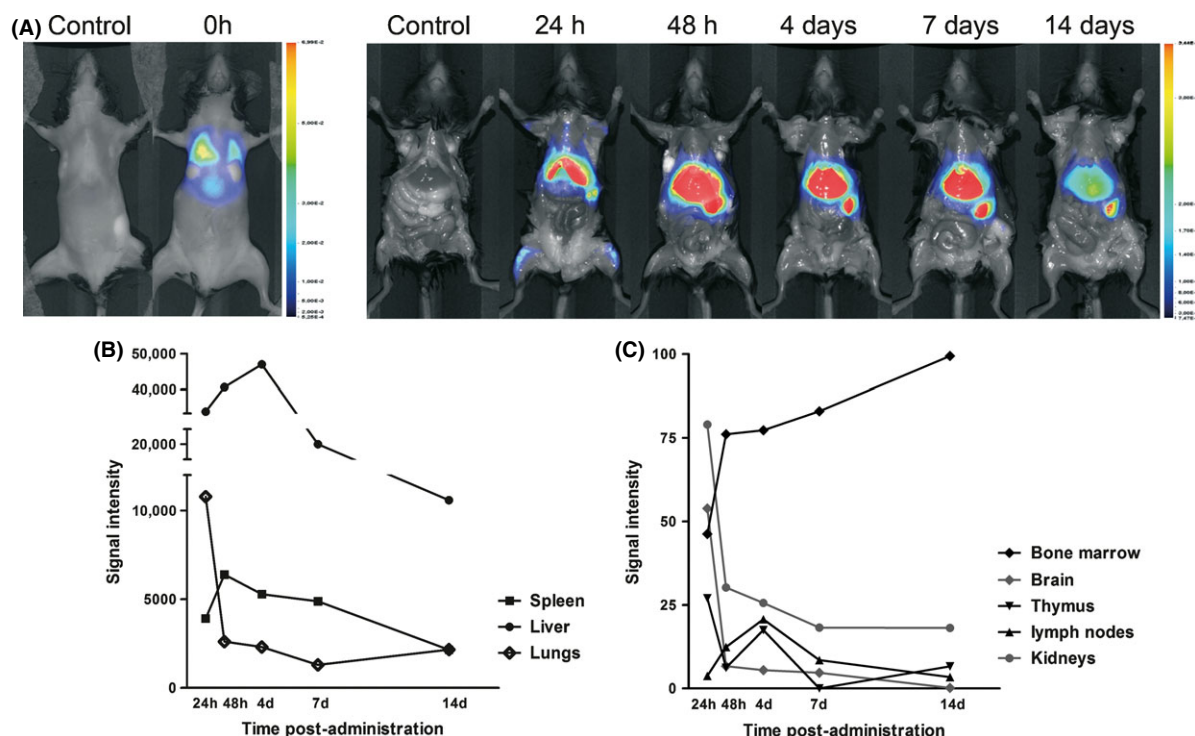
Image data obtained from mice receiving TolDC-MOG labeled with NIR815 ( $n = 9$ ) showed that following iv administration, the cells reached the lungs immediately after administration (Figure 3A). At 24 h postadministration, cells were found at a high concentration in the liver, with a moderate signal becoming

visible in the spleen, where it increases after 48 h and remained elevated until day 7 postadministration (Figure 3A,B). A low level signal from TolDC-MOG-NIR815 cells was also detected in other irrigated organs and tissues such as thymus, lymph nodes, brain, kidneys, and bone marrow (Figure 3C).

### Reduced Antigen-Specific Reactivity and Increased IL-10 Secretion by Splenocytes of Mice Treated with TolDC-MOG

Splenocytes were obtained on day 36 and 46 pi for preventive and therapeutic approaches, respectively, or on day 12 pi (during the inflammatory phase of the disease) in the preclinical





**Figure 3** Migration of TolDC-MOG after iv injection in mice with experimental autoimmune encephalomyelitis (EAE). Cellular tracking images from mice treated with TolDC-MOG labeled with NIR815 dye ( $n = 9$ ). Image acquisition is performed *in vivo* at 0 h and *ex vivo* at 24 h, 48 h, 4 days, 7 days, and 14 days postadministration (A). Signal intensity from spleen, lungs, liver, brain, thymus, lymph nodes, bone marrow, and kidneys are obtained *ex vivo* at different time points (B, C). Signal from mice treated with PBS ( $n = 8$ ) is used as negative control. The images are representative results from at least two independent experiments.

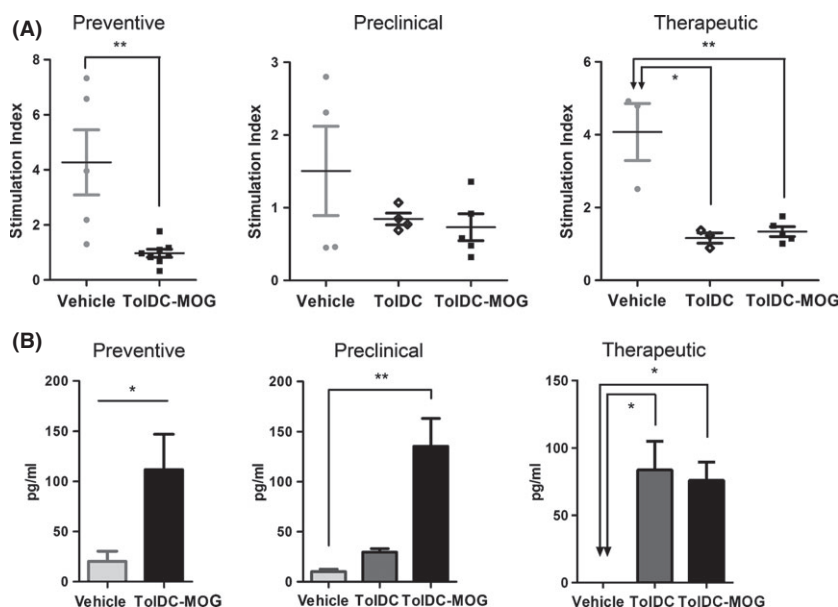
approach. Results revealed that splenocytes of mice treated either with TolDC-MOG or unpulsed TolDC showed a reduction in MOG-specific proliferation (expressed as stimulation index, SI) compared with control mice, when treatment was administrated preventively (vehicle:  $4.27 \pm 2.65$  vs. TolDC-MOG:  $0.97 \pm 0.42$ ,  $P = 0.003$ ;  $n = 5$  and  $n = 8$ , respectively), preclinically (vehicle:  $1.51 \pm 1.23$  vs. TolDC:  $0.73 \pm 0.41$  and TolDC-MOG:  $0.85 \pm 0.16$ , ns;  $n = 4$ ,  $n = 4$  and  $n = 5$ , respectively), or after the onset of the disease (therapeutic approach) (vehicle:  $4.07 \pm 1.36$  vs. TolDC:  $1.16 \pm 0.25$ ,  $P = 0.022$ ,  $n = 3$  in each group; and TolDC-MOG:  $1.34 \pm 0.30$ ,  $P = 0.004$ ,  $n = 5$ ; Figure 4A).

In addition, cytokine levels were analyzed in the supernatant of MOG-stimulated splenocytes. Among all the cytokines analyzed, the most relevant result was exhibited by IL-10 secretion. We found a significant increase in IL-10 production related to TolDC-MOG administration in all the treatment approaches, preventive ( $P = 0.019$ ), preclinical ( $P = 0.008$ ), and therapeutic ( $P = 0.036$ ; Figure 4B). In the same way, splenocytes from mice treated therapeutically with unpulsed TolDC also showed higher levels of IL-10 secretion compared to PBS-treated mice (vehicle;  $P = 0.017$ ), although no differences were found in the preclinical approach (Figure 4B). Furthermore, there were increased levels of secretion of the antiinflammatory cytokine IL-4 for splenocytes derived from mice treated preventively or preclinically with TolDC-MOG compared with both vehicle and TolDC groups (preventive, PBS vs. TolDC-MOG:  $P = 0.045$ ; and preclinical, PBS vs. TolDC-MOG:

$P = 0.056$  and TolDC vs. TolDC-MOG:  $P = 0.032$ ). When cytokine analysis was performed using restimulated splenocytes obtained on day 12 pi (preclinical approach), high and variable levels of cytokines related to the initiation of the inflammatory phase of the disease were detected. Consequently, we not only detected high levels of IL-4 and IL-10, but also IL-6 in TolDC-MOG-treated mice when compared to the vehicle group ( $P = 0.056$ ). Similarly, we found an increase IL-2 in the supernatant of splenocytes from mice receiving TolDC-MOG therapeutically as compared to PBS-treated mice ( $P = 0.036$ ), but there was no difference when compared with the unpulsed TolDC group.

### Treg induction Following Antigen-Specific TolDC Administration

The percentage of Treg (% of CD25<sup>+</sup> FoxP3<sup>+</sup> cells from the CD3<sup>+</sup> CD4<sup>+</sup> population) in splenocytes from mice of the different groups was analyzed *ex vivo* (prior to *in vitro* stimulation with MOG<sub>40–55</sub>; Figure 5A). Results revealed that mice receiving TolDC-MOG treatment had increased the % of Treg cells as compared to control or TolDC-treated mice in all the treatment approaches: preventive (day 32 pi, vehicle:  $9.78 \pm 3.6$  vs. TolDC-MOG:  $38.1 \pm 10.9$ ,  $P = 0.002$ ); preclinical (day 12 pi, vehicle:  $11.86 \pm 2.7$ , and TolDC:  $12.18 \pm 2.9$  vs. TolDC-MOG:  $16.72 \pm 2.2$ ,  $P = 0.016$ , and  $P = 0.032$ , respectively); and therapeutic (day 46 pi, vehicle:  $16.23 \pm 5.4$  and TolDC:  $13.90 \pm 1.2$  vs. TolDC-MOG:



**Figure 4** Reduced antigen-specific reactivity and increased IL-10 secretion of splenocytes from mice treated with TolDC-MOG. **(A)** MOG-induced proliferative response from mice treated preventively, preclinically, or therapeutically with PBS (vehicle), unpulsed TolDC, or TolDC-MOG. The data are expressed as stimulation index (SI). **(B)** Analysis of the antiinflammatory cytokine IL-10 secretion in supernatants of MOG-stimulated splenocytes from mice treated preventively, preclinically, or therapeutically with PBS (vehicle), unpulsed TolDC, or TolDC-MOG. Error bars correspond to SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

$20.38 \pm 4.2$ ,  $P = 0.393$  and  $P = 0.036$ , respectively; Figure 5B–D). In relation to the analysis of the absolute numbers, we observed some variability in the number of CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes among mice. Nevertheless, similar results were observed compared to percentages: preventive (day 32pi, vehicle:  $489.20 \pm 489.10$  vs. TolDC-MOG:  $565.60 \pm 337.80$ ,  $P = 0.524$ ); preclinical (vehicle:  $1151.0 \pm 314.20$ , and TolDC:  $874.0 \pm 249.0$  vs. TolDC-MOG:  $1490.0 \pm 254.30$ ,  $P = 0.393$ , and  $P = 0.036$ , respectively); and therapeutic (day 46 pi, vehicle:  $409.0 \pm 147.20$  and TolDC:  $405.0 \pm 122.70$  vs. TolDC-MOG:  $551.40 \pm 137.70$ ,  $P = 0.151$ ,  $P = 0.056$ , and  $P = 0.036$ , respectively).

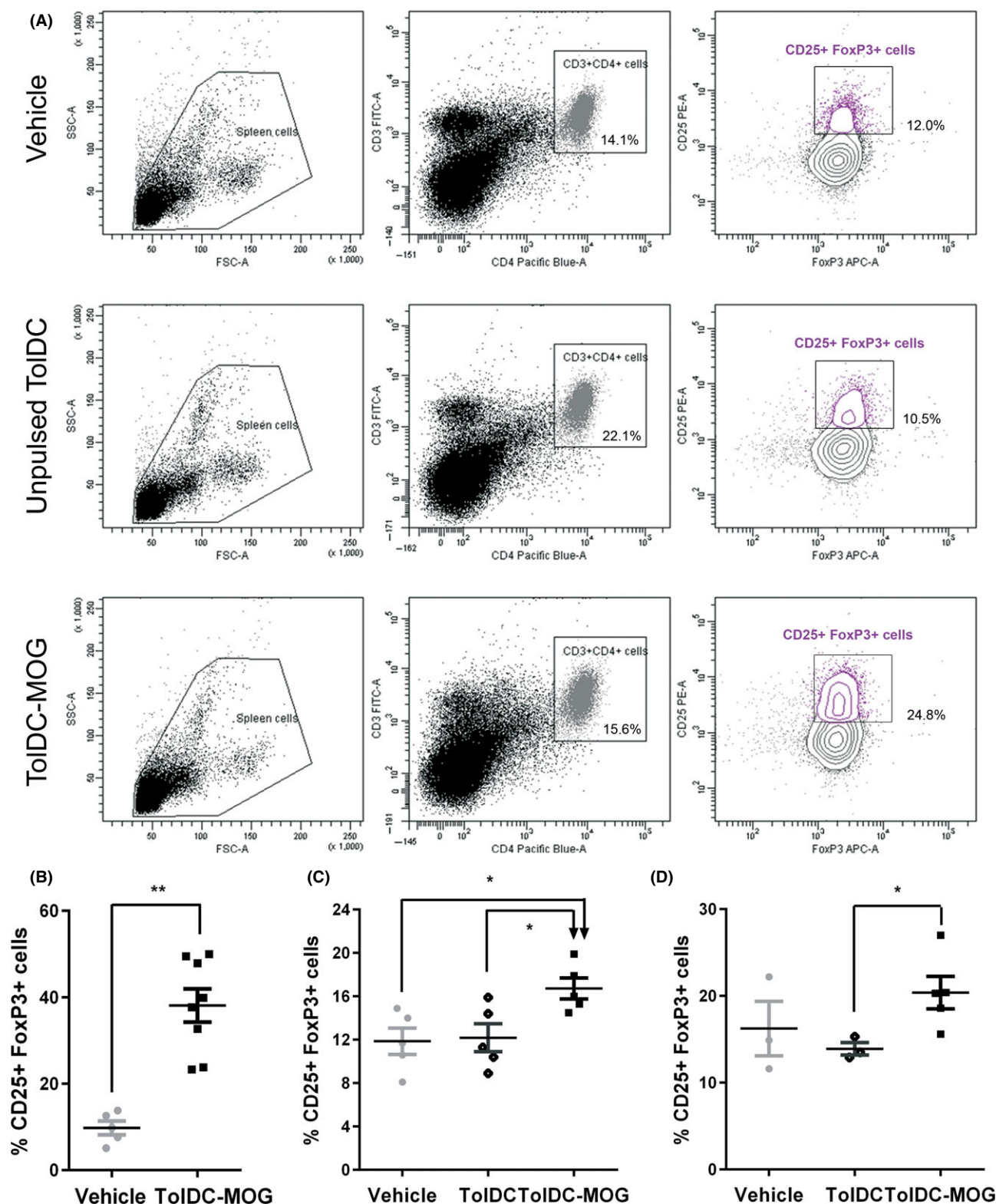
## Discussion

Tolerogenic DC are a promising immunosuppressive strategy to control pathogenic-reactive T-cell response in autoimmune diseases such as rheumatoid arthritis, type 1 diabetes, or MS [4,10]. Previous studies reported that bone marrow-derived iDC pulsed with MOG<sub>35–55</sub> are able to suppress EAE in an antigen-dependent way [13,14]. However, although iDC might be a powerful immunotherapy strategy to treat MS and other autoimmune diseases, they are potentially pathogenic, as they could mature in a proinflammatory environment and exacerbate autoimmune reactions. Consequently, important efforts have been devoted in the last years to generate mature DC with tolerogenic functions (TolDC). To date, several strategies have been developed to generate potent and stable TolDC *in vitro* from murine bone marrow or human peripheral blood mononuclear cells. These protocols consist in treating DC with immunosuppressive drugs or biological agents (such as antiinflammatory cytokines, dexamethasone, VitD3 and rapamycin) or modifying DC function by genetic engineering (transducing DC with viral vectors expressing immunoregulatory molecules or silencing immunostimulatory molecules) (reviewed in [4,5,8,10,15]).

The generation of TolDC using VitD3 alters the phenotype and functionality of monocyte and bone marrow-derived DC [11,16,17]. In this study, we show that VitD3-treated bone marrow-derived DC induced a decrease in the levels of costimulatory molecules (CD80, CD40, and MHC II) and low T-cell alloreactivity proliferation compared to mDC. This is in accordance with previous studies that have reported the efficacy of preventive or preclinical administration of a wide range of TolDC types in EAE [14,18–20]. Here, we demonstrated that iv administration of VitD3-TolDC loaded with MOG<sub>40–55</sub> peptide are capable of not only preventing, but also ameliorating the transient clinical signs of EAE when administrated to mice with clinical manifestations of the disease.

We performed cellular tracking experiments to determine the migratory capacity of TolDC after intravenous injection, the expected route of administration in a future clinical trial for MS patients. Results revealed that following iv injection, TolDC-MOG migrate mainly to the lungs, liver, and spleen. Although the cell signal was also detected transiently in the brain, these results support that DC do not need to cross the blood-brain barrier to perform their function. Furthermore, the *ex vivo* analysis of splenocytes from treated mice showed that antigen-specific reactivity was reduced in both TolDC- and TolDC-MOG-treated mice. However, in contrast to vehicle- or TolDC-treated mice, only mice receiving TolDC-MOG demonstrated an increase in the Treg population, which is in line with the amelioration of EAE clinical signs. These results suggest that one of the mechanisms of tolerance induction of TolDC-MOG is the generation of Treg.

Our results are in agreement with previous studies reporting that the tolerogenic effect of iDC and TolDC in EAE- and collagen-induced arthritis (CIA), respectively, require pulsing cells with auto-antigens [13,21]. In contrast, using the acute EAE model, Farias et al. [22] found that preventive treatment with unpulsed VitD3-TolDC was able to reduce antigen-specific reactivity



**Figure 5** Increase Treg population in ex vivo splenocytes from mice receiving ToIDC-MOG. The percentage of CD25+ FoxP3+ cells (Treg) is calculated from the CD3+ CD4+ T-cell population. Representative analysis of the % Treg in ex vivo splenocytes of PBS, ToIDC, and ToIDC-MOG therapeutically treated mice (A). Percentage of Treg from ex vivo splenocytes of mice treated preventively (B), preclinically (C), or therapeutically (D) with PBS, unpulsed ToIDC, or ToIDC-MOG. The error bars correspond to SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

and EAE severity. Our data confirm that unpulsed VitD3-TolDC have a tolerogenic effect, as we found that splenocytes from TolDC-treated mice restimulated with MOG<sub>40–55</sub> were able to reduce antigen-specific reactivity and occasionally, also increase secretion of the antiinflammatory cytokine IL-10. However, the *in vitro* tolerogenic effects of unpulsed TolDC were not sufficient to ameliorate the clinical signs of the chronic EAE model, which is characterized by an aggressive disease course. In contrast, treatment with VitD3-TolDC-MOG was found to be more potent than unpulsed VitD3-TolDC, as it was able to improve the clinical course of EAE *in vivo*. Furthermore, when studied *in vitro*, reduced MOG reactivity and increased IL-10 secretion were always found following antigen restimulation of splenocytes, while in *ex vivo* experiments, a high percentage of CD25<sup>+</sup> FoxP3<sup>+</sup> cells was detected in spleen cells. Although FoxP3 can be expressed by activated cells, functional results following antigen splenocytes restimulation revealed that animals treated with TolDC-MOG had reduced proliferation against MOG, thus supporting a regulatory function of those cells. This results are in agreement with other studies that have also demonstrated that EAE suppression is related to Treg expansion and is mediated by TolDC in an antigen-specific manner [23,24]. Moreover, clinical data has shown that the therapeutic effect of TolDC-MOG is transient, meaning that repetitive administrations are required to obtain a stable clinical effect. Although we were not able to analyze if the Treg induction in TolDC-MOG-treated mice was also transient, the clinical score did not correlate with the percentage of Treg of each mice (data not shown); Therefore, we consider that Treg response plays an important role in controlling the autoreactive T-cell response, but other mechanism or factors could be contributing to the regulatory effect triggered by TolDC-MOG treatment.

Altogether, these results suggest that antigen-specific DC-based therapy using VitD3 could represent an advantageous strategy for MS patients. So far, phase I clinical trials have shown that the

treatment with autologous TolDC is safe and well tolerated [25,26]. In the near future, studies establishing effective cell-dosing (number of antigen-specific TolDC and readministration timing) and identifying biomarkers to monitor tolerance induction will be crucial the steps to guarantee success of the therapy.

## Conclusions

We have demonstrated that TolDC-MOG (differentiated in the presence of VitD3) show a tolerogenic profile, demonstrated both by phenotype and *in vitro* functionality. When VitD3-TolDC were administrated in mice with clinical signs of EAE, only antigen-specific TolDC exhibited a beneficial effect *in vivo* by reducing disease incidence and ameliorating clinical symptoms. Furthermore, TolDC loaded with MOG peptide were able to increase the percentage of Treg cells, induce IL-10 secretion, and reduce EAE severity. Taken together, these results can be considered a proof of concept that VitD3-monocyte-derived DC could be a potential specific cell-based immunotherapy for MS patients.

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## Conflict of Interest

The authors declare no conflict of interest.

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