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ORIGINAL INVESTIGATION

Application of dendritic cells stimulated with *Trichinella spiralis* excretory–secretory antigens alleviates experimental autoimmune encephalomyelitis

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Abstract The parasitic nematode, *Trichinella spiralis* (*T. spiralis*), exerts an immunomodulatory effect on the host immune response through excretory–secretory products (ES L1) released from encysted muscle larvae. Our model of combined *T. spiralis* infection and experimental autoimmune encephalomyelitis (EAE) in Dark Agouti (DA) rats demonstrated a significant reduction in EAE severity in infected animals. Recently, we have created an immune status characteristic for the live infection by in vivo application of dendritic cells (DCs) stimulated with ES L1 products of *T. spiralis* muscle larvae. Moreover, these cells were able to ameliorate EAE when applied 7 days before EAE induction. ES L1-stimulated DCs increased production of IL-4, IL-10 and TGF-β, and decreased production of IFN-γ and IL-17, both at the systemic level and in target organs. A significant increase in the proportion of CD4+CD25+Foxp3+ T cells was found among spleen cells, and CNS infiltrates from DA rats treated with ES L1-stimulated DCs before EAE induction, compared to controls injected with unstimulated DCs. Regulatory T cells, together with elevated levels of IL-10 and TGF-β, are most likely involved in restraining the production of Th1 and Th17 cytokines responsible for autoimmunity and thus are responsible for the beneficial effect of ES L1-educated DCs on the course of EAE. Our results show that ES L1 antigen-stimulated DCs are able not only to provoke, but also to sustain anti-inflammatory and regulatory responses regardless of EAE induction, with subsequent amelioration of EAE, or even protection from the disease.

Keywords Trichinella spiralis · Dendritic cells · Experimental autoimmune encephalomyelitis

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Introduction

The immune system evolved under constant pressure from a wide spectrum of infectious agents, ranging from harmless environmental saprophytes to pathogens like helminths. These "old friends" have influenced the development of immunoregulatory mechanisms necessary for the correct functioning of the immune system [1]. The absence of such stimuli, due to clean sanitary conditions, application of vaccines or antibiotics, may result in dysregulation of the immune system, which leads to the development of chronic inflammatory disorders, such as allergies and autoimmunity [2]. Indeed, in the last 70 years, there has been a remarkable increase in the incidence of autoimmune diseases, like multiple sclerosis or type 1 diabetes, in developed countries [3]. This gave rise to the "hygiene hypothesis" [4] or, as recently reformulated, "Old Friends Hypothesis" [1] postulated to explain the alarming increase in allergic and autoimmune diseases in urban environments. According to this hypothesis, the decreased incidence of infections with parasites and bacterial pathogens may be the underlying reason for immunoregulatory problems that lead to development of allergic and autoimmune diseases [5]. Data from epidemiological studies [2, 6, 7] have been upgraded by results obtained from animal model systems [8] and clinical trials [9, 10] and most of them favor this hypothesis.

Our model of joint *Trichinella spiralis* (*T. spiralis*) infection and experimental autoimmune encephalomyelitis (EAE) has shown that helminth infection can dampen, or even prevent autoimmune disease [11]. Previously established *T. spiralis* infection creates an environment unsuitable for the development of EAE. Proposed immunomodulatory mechanisms were parasite-driven inhibition of IL-17 and lower IFN-γ production, together with simultaneous activation of Th2 and/or regulatory T cells. Acting through cytokines IL-4, IL-10 and TGF-β, they participate in the alleviation of EAE [12]. Many investigations have pointed to correlations between defects in immune regulation and the development of autoimmunity. There is substantial evidence that both adaptive and naturally occurring Tregs are important in the control of autoimmune disease [13]. Besides the beneficial effect of helminth administration in animal models of autoimmune diseases [14], and promising results from clinical trials [15, 16], the use of live worms as therapy, in addition to causing strong aversion and disgust, carries risks for the host organism [17]. However, this model of joint infection and autoimmunity could provide insight into mechanisms and molecules utilized by parasites to modulate the immune response not only to themselves but to unrelated, foreign or self-antigens as well. Like other helminths, *T. spiralis* is considered a master of immunomodulation. It persists in the host organism for a considerably long time, inhabiting muscles by transforming the cells into encapsulated "nurse" cells [18]. From this immunoprivileged place, *T. spiralis* communicates with the host organism and affects cells of the immune system through excretory–secretory products (ES L1) released from the muscle larvae [19]. In a way not fully understood yet, *T. spiralis* brings the host organism to a new level of homeostasis, by induction of regulatory mechanisms and rebalancing the status of tolerance in the host. We investigated the impact of ES L1 antigens on dendritic cells (DCs), antigen-presenting cells crucial for both initiation and regulation of immune responses [20, 21]. Under the influence of ES L1 products, DCs acquired a semi-mature phenotype [22] and were able to polarize naïve T cells in vitro and in vivo [23]. Application of ES L1-stimulated DCs into naïve Dark Agouti (DA) rats in vivo created a cytokine milieu that resembled the situation in live infection. This encouraged us to design a new approach in EAE treatment, that is, to use ES L1-stimulated DCs to pre-treat animals undergoing EAE induction. In this study, we analyzed "educated" DCs for their capacity to ameliorate EAE. ES L1-stimulated DCs were able to reduce the clinical signs and duration of the disease. Investigation into underlying mechanisms revealed that ES L1-stimulated DCs altered the immune response responsible for the development of EAE via decreased production of IFN-γ and IL-17 and increased production of IL-4, IL-10 and TGF-β, as well as through activation of regulatory T cells.

Materials and methods

Animals and antigen preparation

DA rats were purchased from the animal facility of the Military Medical Academy (MMA, Belgrade) and given free access to food and water. *T. spiralis* strain (ISS 161) was maintained by passage in Wistar rats. Muscle larvae were recovered by digestion of the carcasses in pre-warmed gastric juice [24] and kept under controlled conditions (37 °C, 5 % CO2) in complete Dulbecco's modified Eagle medium (DMEM) (Sigma), for 18 h [25]. Excretory–secretory products of the muscle larvae (ES L1) were obtained by dialysis and concentration of culture supernatants. The quality of ES L1 products was checked by *Trichinella* ELISA test (INEP, Serbia).

All animal experiments were performed according to institutional guidelines and were approved by the local Institutional Animal Care and Use Committee.

Generation of bone-marrow-derived DCs

Male DA rats, 16–18 weeks old, were used as the source of bone marrow. DCs were generated from rat bone marrow as described previously [22, 26]. Briefly, DCs were obtained by culturing bone marrow cells from DA rats in RPMI 1640 (PAA Laboratories Gmbh) supplemented with 10 % FCS (PAA Laboratories Gmbh), 2 mM L-glutamine, 1 mM Na-pyruvate, 10 mM Hepes, 50 μM 2-ME (Sigma), 50 U/ml gentamycin and growth factors: 25 ng/ml GM-CSF, 25 ng/ml IL-4 and 25 ng/ml Flt-3 ligand (all from Biosource Invitrogen, USA). Cells were plated on 6-well plates and maintained at 37 °C in 5 % CO_2/95 % air. Fresh medium was added on days 3 and 6. On day 8 of cultivation, cells were pulsed with 50 μg/ml of ES L1 (DC/ES L1) for 48 h. Non-adherent DCs were collected on day 10, washed to remove excess antigen and resuspended in serum-free medium. DCs were analyzed for the expression of surface markers (CD80, CD86 and ICAM1). DC culture supernatants were collected for IL-10 and IL-12p70 determination. DCs cultivated in medium alone (DC/medium) or in the presence of *T. spiralis* muscle larvae excretory–secretory antigens (DC/ES L1) were injected intraperitoneally (i.p.) into healthy female DA rats (12–14 weeks of age) in a volume of 1 ml.

EAE induction

To evaluate the effect of ES L1 antigen-educated DCs on the development of EAE, DA rats were divided into groups (5 animals/group) receiving different doses of DC/ES L1 (0.5, 1 and 5 × 10^6 DCs). The control group received 5 × 10^6 unstimulated DCs, that is, DCs cultivated in

medium only. Seven days after i.p. application of DCs, DA rats were induced to develop EAE by subcutaneous injection of rat spinal cord tissue homogenate (50 % w/v in saline) emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA) containing 5 mg/ml *Mycobacterium tuberculosis* (Difco) in the right hind footpad. Disease development was monitored over 25 days post-immunization. Animals were evaluated daily for the presence of clinical symptoms, using the following clinical score: 0 = no clinical signs; 1 = flaccid tail; 2 = hind limb paresis; 3 = complete bilateral hind limb paralysis often associated with urine incontinence; and 4 = moribund state or death. For the analyses of cytokine production and the presence of regulatory T cells at different phases of EAE development, EAE-induced animals given 5×10^6 stimulated or unstimulated DCs were killed during the inductive (day 8 p.i.), peak (day 15 p.i.) and recovery phase (day 25 p.i.) and cells were harvested from spleens and CNS.

Cells and cell cultures

Single-cell suspensions from spleens and CNS were isolated from DA rats treated with DC/ES L1 or DC/medium at the indicated time points. CNS inflammatory cells were obtained from the spinal cords (SC) of rats perfused with sterile PBS. Spinal cords were homogenized, adjusted to 30 % Percoll (Sigma, MO) and overlaid on a 40 %/70 % Percoll gradient. Following centrifugation at $700 \times g$ for 20 min, mononuclear cells were recovered from the 40 %/70 % Percoll interface and washed in RPMI medium (Sigma). Spleen cells and SC-infiltrating cells were grown at 5 % CO_2 and 37 °C in RPMI-1640 (PAA Laboratories, Pasching, Austria) supplemented with antibiotics and 5 % fetal calf serum (PAA Laboratories, Pasching, Austria) for spleen cells or 2 % rat serum for SC-infiltrating cells. Spleen cells were seeded in 24-well plates (2×10^6/ml) and cultivated in the presence of ConA (2.5 μg/ml) (INEP, Serbia) or in medium alone for 48 h, while SC-infiltrating cells were seeded in 96-well plates (5×10^5/ml) without stimulation for 72 h. Culture supernatants were collected and analyzed for cytokines. The obtained cells from spleens and CNS were also stained for markers of T regulatory cells: CD4, CD25 and Foxp3. Dead cells that appear in SC-infiltrating cell isolates were removed by Dead Cell Removal Kit on MS MACS column (Miltenyi Biotec, USA) before staining for T regulatory cell markers.

Cytokine ELISAs

Sandwich ELISAs were used to measure IL-12p70 (Biosource, USA), IL-4, IL-10, IFN-γ (BD Biosciences), IL-17 (eBioscience) and TGF-β (Diaclone, A Tapnel Company, France) in culture supernatants according to the manufacturers' instructions.

Flow cytometry

Expression of surface molecules on DCs was quantified using FITC-conjugated anti-MHC class II, anti-CD86 and anti-ICAM1 antibodies (Abcam PLC, Cambridge, UK). Before staining for Treg markers, spleen and CNS cells were incubated with FcR Ab (FcR block, BD Biosciences). Afterward, they were phenotyped according to the expression of surface markers (CD4, CD25) and intracellular staining (Foxp3). Cells (1×10^6) were incubated with FITC-conjugated mouse anti-rat CD4 Ab and PE-conjugated mouse anti-rat CD25 Ab (BD Pharmingen). For Foxp3 detection, cells were permeabilized and stained with anti-Foxp3-PE-Cy5 according to the manufacturer's instructions (eBioscience). FACS data were collected on EPICS XL-MCL (Coulter, Krefeld, Germany) using SYSTEM II Software (Coulter). Data were analyzed using FlowJo software.

Statistical analysis

The Mann–Whitney test was used for statistical analyses of clinical parameters, and Student's t tests were used to determine statistical significance in all other assays. In all cases, a p value less than 0.05 was considered statistically significant.

Results

Stimulation with *T. spiralis* ES L1 antigens induces partial maturation of DCs

Rat bone-marrow-derived DCs (BMDCs) cultivated in medium alone (DC/medium) exhibited the typical morphological characteristics of immature DCs [22]. As in our previous studies, stimulation with ES L1 resulted in moderate upregulation of CD 86, significant upregulation of ICAM 1 and no upregulation of MHC II, compared to DCs cultivated in medium alone (Table 1). Culture supernatants were analyzed for IL-12p70 and IL-10. ES L1-stimulated DCs (DC/ES L1) produced significantly less IL-12p70, a cytokine essential for the Th1 response, than control DCs (DC/medium). At the same time, IL-10 production was highly elevated due to ES L1 stimulation, which could represent the potential of these cells to induce tolerance (Table 1).

Table 1 DC activation status under the influence of ES L1

	DC/medium	DC/ES L1
% Expression of surface markers		
MHC II	55 ± 3.8	56 ± 2.2
CD86	17.5 ± 3.4	31.4 ± 1.9
ICAM 1	51 ± 2.5	82 ± 7.1*
Cytokine production		
IL-12p70	149.1 ± 29.3	85.1 ± 14.3**
IL-10	39.8 ± 7.4.	536.2 ± 98.5***

Data represent the mean ± SD from four independent DC preparations

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$

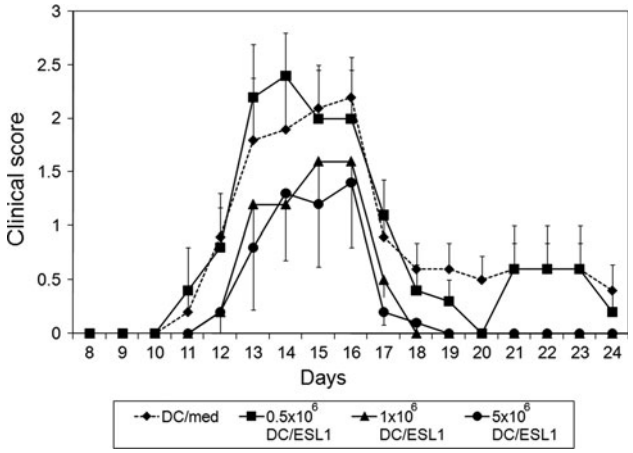

Fig. 1 Effect of a single injection of ES L1-stimulated DCs on the course of EAE. DCs were cultivated in the presence of *T. spiralis* ES L1 antigens, and different numbers of cells were applied to naïve DA rats 7 days before EAE induction. Control EAE rats received 5×10^6 DCs cultivated in medium only 7 days before EAE induction. Rats were observed for neurological signs. Data are shown as the daily mean clinical scores of disease (±SEM) for five rats per group and are representative of two independent experiments with similar results

ES L1-stimulated DCs ameliorate EAE

It was previously shown that ES L1-stimulated DCs possess the capacity to polarize naïve T cells in vitro and in vivo, provoking release of Th2 and regulatory-type cytokines [23]. Therefore, we wished to investigate whether DC/ES L1 applied to naïve DA rats could ameliorate EAE. ES L1-stimulated DCs were injected i.p. 7 days before EAE induction in three different doses (0.5×10^6, 1×10^6 and 5×10^6), while control animals received 5×10^6 DCs cultivated in medium only. The course of EAE was not affected by injection of 0.5×10^6 DC/ES L1 (Fig. 1; Table 2). However, the higher doses of DC/ES L1 ameliorated the disease in a dose-dependent manner, as evaluated from the disease parameters indicating the severity of EAE (Table 2; Fig. 1).

The incidence of EAE was lower in rats given 5×10^6 DC/ES L1 when compared to the other groups, so only this dose gave protection to a number of animals. Animals that received 1×10^6 and 5×10^6 DC/ES L1 and developed disease exhibited reduction in disease severity (Fig. 1), together with a lower mean maximal clinical score and cumulative index compared to the controls (Table 2). Onset of the disease was unchanged regardless of the treatment, but its duration was significantly shorter in these two groups (Table 2).

To assess immunomodulatory mechanisms underlying EAE amelioration seen in rats that received ES L1-stimulated DCs, we used the dose showing the best results in the above model. Rats injected with 5×10^6 DC/ES L1 or 5×10^6 DC/medium were subjected to EAE induction, and the T-cell response was analyzed at different time points during the course of EAE, that is, at induction (day 8 p.i.), effector (day 15 p.i.) and recovery phases (day 25 p.i.) of the disease (Fig. 2). EAE was reduced in severity in animals treated with ES L1-stimulated DCs prior to EAE induction. The cumulative index for DC/ES L1 group, which was observed till day 24, was 0.16 ± 0.1, while for the control group (DC/medium recipients), it was 0.79 ± 0.2. The difference clearly shows that ES L1-stimulated DCs had a significant impact on disease development.

Immune response in the spinal cord during the course of EAE

Inflammatory cells accumulate within the central nervous system from the onset till the recovery phase of EAE in the DA rat model. Here, we examined the effect of DC/ES L1 on the degree of inflammatory cell infiltration within the spinal cord of EAE-induced DA rats. Spinal cord-infiltrating cells were isolated in the inductive, effector and recovery phases of EAE from DC/ES L1-treated and control animals. The number of SC-infiltrating cells in control EAE rats increased throughout the disease, reaching a maximum in the recovery phase, which is in line with findings of other authors [27]. DC/ES L1-treated EAE rats showed a decline in the number of infiltrating cells to one-third of the control value at day 25 p.i. (Fig. 3). The results suggest that in DC/ES L1-treated EAE rats, cell infiltration into the spinal cord is significantly reduced compared to control EAE rats.

Spinal cord-infiltrating cells were analyzed for the production of cytokines and the presence of CD4CD25Foxp3-positive cells. The production of cytokines by SC-infiltrating cells was determined in culture supernatants of unstimulated cells (basic production). The release of IFN-γ by SC-infiltrating cells from DC/ES L1-treated animals was significantly lower than in control

Table 2 Effects of DC/ES L1 on EAE

Groups	Incidence	Onset	Duration	Mean max. clinical score	Cumulative index[a]
5×10^6 DC/medium + EAE	10/10	12.4 ± 1.1	9.4 ± 3.7	2.4 ± 0.6	0.6 ± 0.2
0.5×10^6 DC/ES L1 + EAE	10/10	12.2 ± 0.8	7.2 ± 2.5	2.4 ± 0.9	0.5 ± 0.3
1×10^6 DC/ES L1 + EAE	10/10	12.8 ± 0.5	4.2 ± 2.4*	1.8 ± 1.3	0.3 ± 0.2*
5×10^6 DC/ES L1 + EAE	6/10	13 ± 1.0	3.0 ± 3.1**	1.6 ± 1.5	0.2 ± 0.2*

Data represent the mean ± SD from two independent experiments

* $p < 0.05$; ** $p < 0.01$

[a] Cumulative disease index is the sum of the daily mean clinical scores for a group of rats, divided by the number of days in the experiment, that is, 25 days

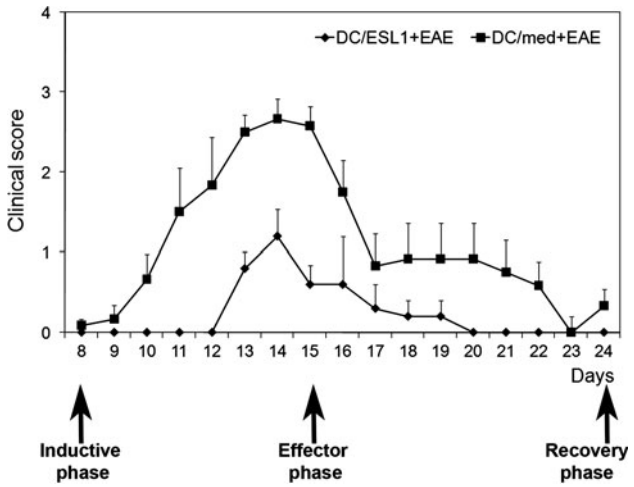

Fig. 2 EAE course in control DA rats and rats treated with ES L1-stimulated DCs. Rats receiving 5×10^6 DC/ES L1 or 5×10^6 DC/medium (control rats) were divided into groups (5 animals/group) and killed at the indicated time points. The presented results concern groups analyzed at the end of the observation period (day 24 p.i., recovery phase). Data are shown as the daily mean clinical scores of disease (±SEM) for five rats per group and are representative of two independent experiments

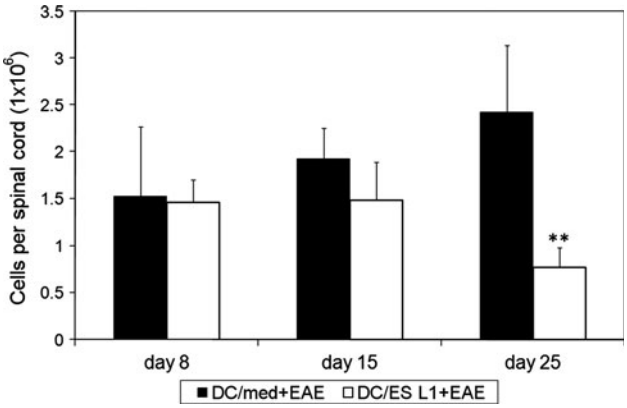

Fig. 3 Cellularity of spinal cord infiltrates during the course of EAE. SC-infiltrating cells were isolated from DA rats that received DC/medium ($n = 5$) and DC/ES L1 ($n = 5$), at the indicated time points after EAE induction and the number determined. Data represent the mean ± SD from two independent experiments

animals at day 8 p.i. (Fig. 4a). This trend was maintained in the effector phase, but without statistical significance. The production of IFN-γ in treated EAE animals reached the level of untreated EAE animals in the recovery phase of the disease. SC-infiltrating cells from DC/ES L1-treated EAE rats produced significantly less IL-17 than control EAE rats (Fig. 4b) throughout the disease. The capacity of these cells to produce IL-17 was drastically reduced in the inductive and effector phases of EAE. At the same time, the production of Th2 and regulatory cytokines by SC-infiltrating cells from DC/ES-L1 treated EAE rats was elevated during the period of observation (Fig. 4c, d, e). Basic IL-4 release was significantly increased in DC/ES L1 EAE rats (Fig. 4c) compared with control EAE animals. Measured levels of IL-10 release increased from day 8 onwards, but reached statistical significance only at the end of the observation period, in the recovery phase (Fig. 4d). On the other hand, production of TGF-β was greatly elevated throughout the disease in SC-infiltrating cells from DC/ES L1-treated EAE rats, when compared to controls (Fig. 4e).

SC-infiltrating cells were analyzed for the presence of CD4CD25Foxp3-positive cells. Flow cytometric analysis revealed that the percentage of CD4+CD25+ T cells expressing Foxp3 was greater in SC infiltrates from DC/ES L1 recipients subjected to EAE induction than in SC infiltrates from control EAE animals, at each indicated time point (Fig. 5).

It was obvious that there is a relation between the presence of Foxp3-positive T cells and the severity of EAE. To examine the assumed correlation, we compared the cumulative index from animals killed 25 days after EAE induction and the proportion of CD4CD25Foxp3-positive cells in their spinal cords. The cumulative index is a parameter that indicates disease severity and represents the sum of the daily mean clinical scores for a group over a given number of days. The relative expression of Foxp3 on CD4+CD25+ cells showed a close inverse relationship

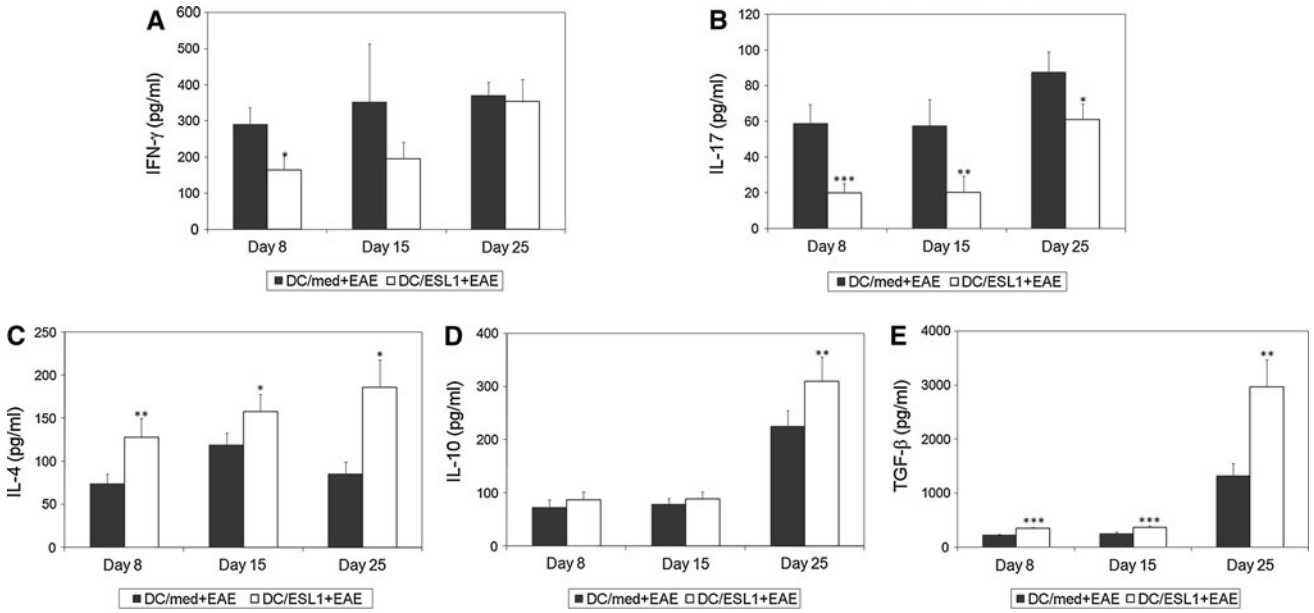

Fig. 4 Cytokine production by SC-infiltrating cells isolated from DA rats injected with DC/ES L1 before EAE induction. SC-infiltrating cells were isolated at the indicated time points and cultivated in medium only. Culture supernatants were collected after 72 h and assayed for IFN-γ (**a**), IL-17 (**b**), IL-4 (**c**), IL-10 (**d**) and TGF-β (**e**) using cytokine-specific ELISA. The results shown represent the mean ± SD of data from two independent experiments, five animals per group, done in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$

with disease severity ($r = -0.829$). The obtained data indicated that the proportion of Foxp3-positive T cells within CNS infiltrates was associated with EAE mitigation.

Immune response in the spleen along the course of EAE

To investigate the immune response in the periphery outside the CNS, we isolated spleen cells from DC/ES L1-treated and DC/ES L1-untreated DA rats at the indicated time points during the observation period. Spleen cells were cultivated in medium alone, and the culture supernatants were analyzed for cytokine content, which means that the basic production was measured. Cytokine analyses revealed that splenocytes from recipients of DC/ESL1 subjected to EAE induction produced half the amount of IFN-γ as EAE controls in the inductive phase. This trend of reduced IFN-γ production was maintained throughout the disease, but in the recovery phase, the difference was not statistically significant (Fig. 6a). The production of IL-17 gradually declined from the beginning of the disease till the end of the experiment in DC/ES L1-treated EAE-induced animals, compared to continuously high production in splenocytes from control EAE rats (Fig. 6b). ES L1-stimulated DCs markedly suppressed the release of IL-17, despite EAE induction. Culture supernatants were analyzed for the presence of Th2 and regulatory cytokines. Splenocytes from DC/ES L1-treated rats produced significantly more of the Th2 cytokine—IL-4, compared to cells from control EAE rats in the inductive and effector phases (Fig. 6c). Moreover, production of the regulatory cytokines, IL-10 and TGF-β, was greatly elevated in DC/ES L1 recipients, during the course of EAE (Fig. 6d, e). The potential of isolated spleen cells to produce cytokines was investigated by re-stimulation with non-specific stimulus—ConA. Cytokine production by ConA-stimulated cells reached much higher values compared to those presented in Fig. 6, but the overall pattern resembled basic production, which means that spleen cells retained the mode of releasing cytokines regardless of stimulation (data not shown).

High levels of IL-10 and TGF-β indicated activation of the regulatory network. To check this assumption, spleen cells from DC/ES L1-treated EAE-induced rats and EAE controls were analyzed for the presence of Treg cells by determining the number of CD4CD25Foxp3-positive cells (Fig. 7). Injection of DC/ES L1 7 days before EAE induction provoked a significant increase in the proportion of CD4CD25Foxp3-positive cells among spleen cells from recipient animals, compared to values obtained for splenocytes from control EAE animals. Thus, DC/ESL1 elicited Th2 and regulatory responses, as measured by cytokine production and Foxp3 cell determination.

Discussion

We have shown that amelioration of EAE can be induced by bone-marrow-derived dendritic cells (BMDC) that had

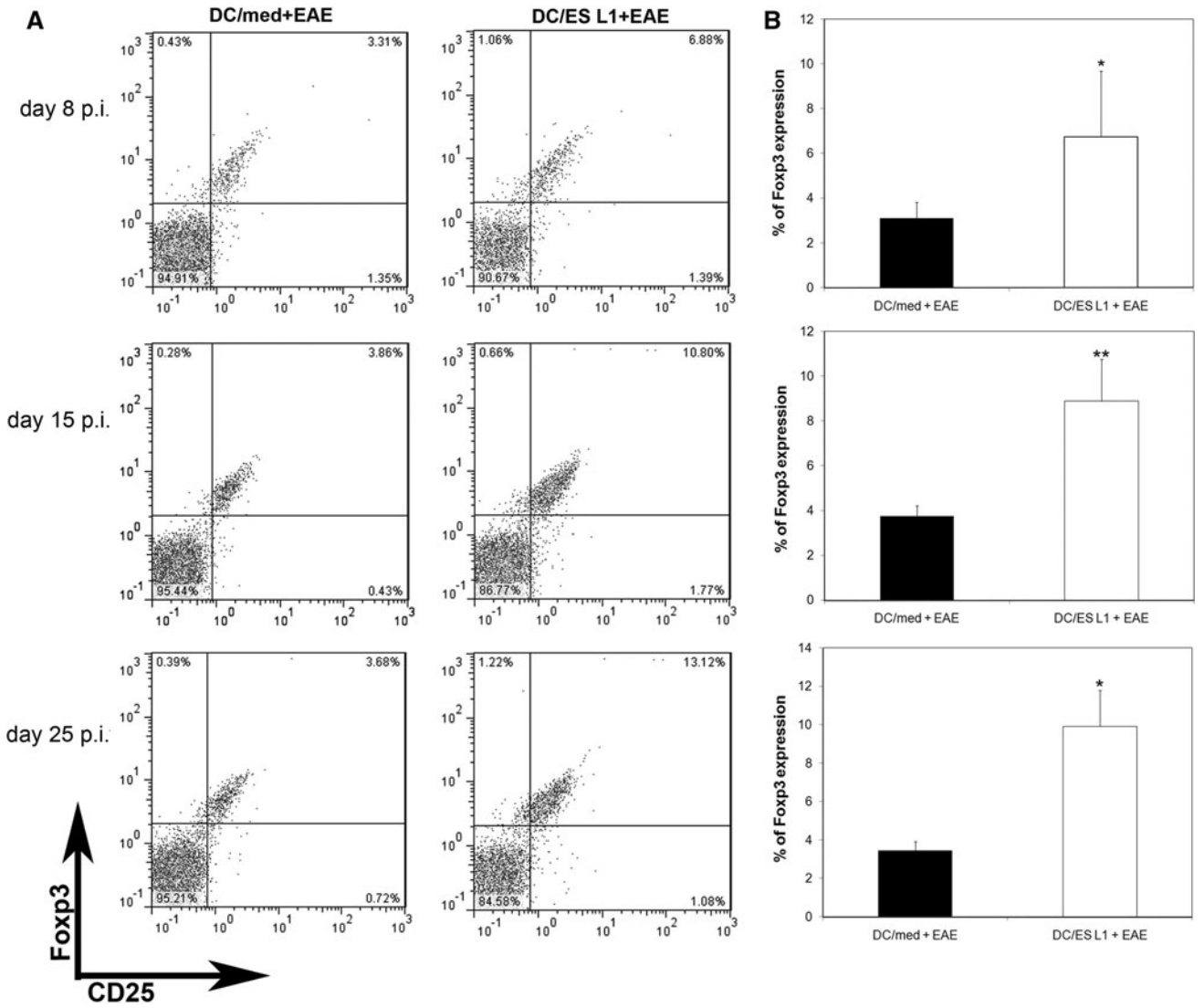

Fig. 5 Foxp3 expression in SC-infiltrating cells during the course of EAE. SC-infiltrating cells were isolated in the inductive (day 8 p.i.), effector (day 15 p.i.) and recovery (day 25 p.i.) phases and analyzed for Treg markers. **a** Representative plots for each time point, from one of two experiments present CD25 versus Foxp3 expression, gated on CD4+ T cells. **b** The percentage of Foxp3+ cells within the CD4+ T-cell population in SC-infiltrating cells from DC/ES L1-treated animals compared to control, DC/med-treated rats, at the indicated time points. Data represent the mean ± SD from two independent experiments, five animals per group. $*p < 0.05$; $***p < 0.005$

been pulsed with *T. spiralis* muscle larvae excretory–secretory antigens and injected intraperitoneally into healthy recipient DA rats 7 days prior to EAE induction. The reduction in disease severity was dose dependent, that is, it correlated with the number of injected DCs/ES L1. This modulation of an autoimmune disease could be a consequence of a restored state of tolerance induced by parasite antigens. These antigens induce a tolerogenic status in DCs and consequently shift T-cell polarization toward the regulatory type. Activated Tregs are able to suppress not only the specific response against the parasite but also toward autoantigens.

At first, it was thought that immature DCs are solely responsible for the induction of tolerance [28]. However, a growing amount of evidence supports the notion that semi-mature DCs can also induce tolerance [29, 30]. This type of maturation was found to be characteristic for a number of parasites like *Schistosoma mansoni* [31], *Nipostrongilus brasiliensis* [32] and *Ascaris lumbricoides* [33]. Our results have shown that in vitro stimulation of DCs with *T. spiralis* ES L1 antigens caused partial maturation of these cells, as indicated by expression of the determined surface markers. Stimulation of DCs from DA rats with *T. spiralis* ES L1 antigens also resulted in significant reduction in IL-12p70 production and increased production of IL-10. IL-12 is held responsible for provoking a Th1 response, while IL-10 participates in the induction of Th2 and the regulatory type of response [34, 35] and at the same time maintains the

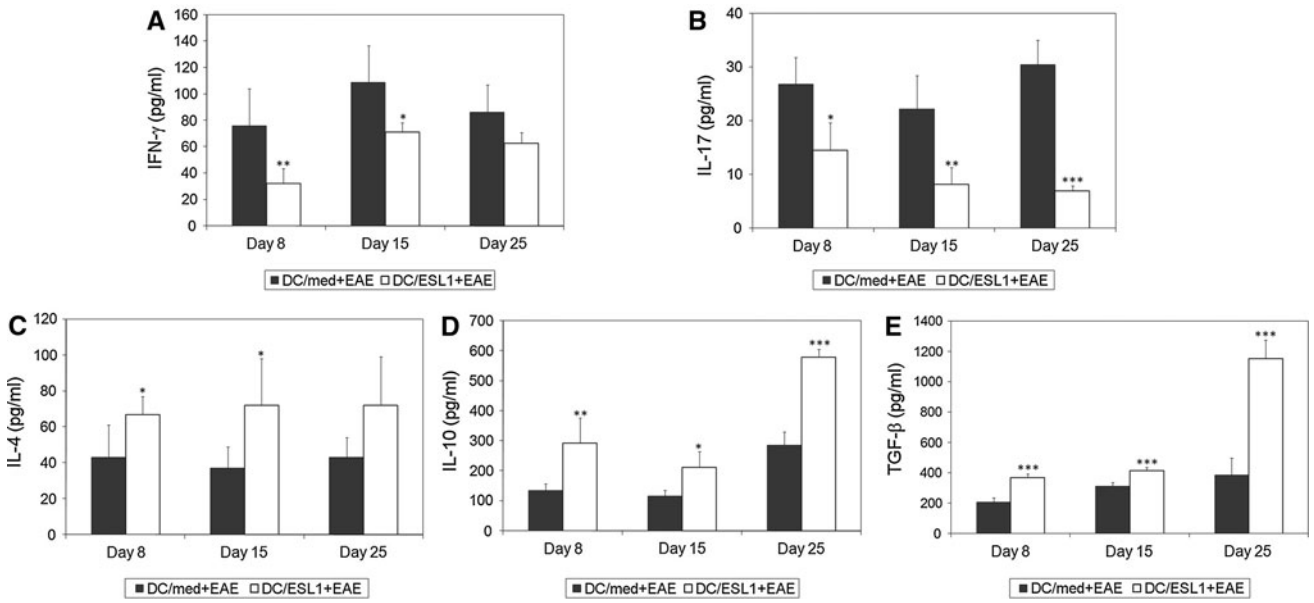

Fig. 6 Cytokine production by splenocytes isolated from DA rats injected with DC/ES L1 before EAE induction. Splenocytes were isolated at the indicated time points and cultivated in medium only (basic production). Culture supernatants were collected after 48 h and assayed for IFN-γ (**a**), IL-17 (**b**), IL-4 (**c**), IL-10 (**d**) and TGF-β (**e**) using cytokine-specific ELISA. The results shown represent the mean ± SD of the data from two independent experiments, five rats per group, done in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$

tolerogenic status of DCs in an autocrine way [36]. Our results concerning the activation status of ES L1-stimulated DCs point to the tolerogenic nature of these cells. Tolerogenic DCs are characterized by low expression of co-stimulatory molecules, low production of pro-inflammatory cytokines and high production of immunosuppressive cytokines [37]. A similar DC phenotype as in our experiments was obtained by treatment of DCs with immunosuppressive agents, such as the anti-inflammatory cytokines IL-10 and TGF-β. DCs treated with IL-10 and/or TGF-β are characterized by low expression of co-stimulatory molecules, low production of IL-12 and high production of IL-10. These cells induced T-cell tolerance and suppressed diseases in experimental models of autoimmunity [38]. Our previous investigations demonstrated that ES L1-stimulated DCs possess the potential to polarize the immune response toward Th2 and the regulatory type and to induce expansion of Foxp3-expressing regulatory T cells, when injected in vivo [23]. When applied to healthy DA rats 7 days before EAE immunization, these cells reduced the severity of the disease. It became apparent that in DC/ES L1 recipients, the number of infiltrating cells within CNS in the recovery phase was much lower than in controls, suggesting that ES L1-pulsed DCs were able effectively to suppress inflammatory process in the target organ. This was consistent with our previous findings concerning the degree of CNS infiltration in *T. spiralis*-infected, EAE-induced animals [11]. The amelioration of EAE was accompanied by abundant production of IL-10 and particularly TGF-β, both in the CNS and at the periphery, and significantly reduced production of the pro-inflammatory cytokines IFN-γ and IL-17, which are crucial for the induction and development of EAE [39]. DC/ES L1 also facilitated induction/expansion of CD4CD25Foxp3-positive T cells, which persisted in increased proportions in both the CNS and spleen throughout the disease. The increased presence of these cells in the spinal cord of DC/ES L1 recipients correlated with significant reduction in EAE severity.

Induction of Foxp3-positive Tregs is one of the proposed mechanisms by which tolerogenic DCs keep autoreactive T cells and hence immune response under control [40]. Regulatory T cells induced in *T. spiralis* infection [12] or by application of ES L1-stimulated DCs, together with elevated levels of IL-10 and TGF-β, cytokines closely related with Treg function, could be involved in restraining both the Th2 response toward the parasite and the Th1 and Th17 responses responsible for the development of EAE. IL-10 is a key cytokine for the recovery of EAE [41], while TGF-β is necessary for the survival of Foxp3+ Tregs and is recognized as a central component of Treg suppressive activity [42].

The role that DCs play in inducing tolerance nominated these cells for immunotherapy of autoimmune diseases [37]. Increasing knowledge about the strategies for tolerogenic DC generation and potentials of such DCs to maintain tolerance to self-antigens has mostly been gained from animal models. In our model, ES L1-stimulated DCs,

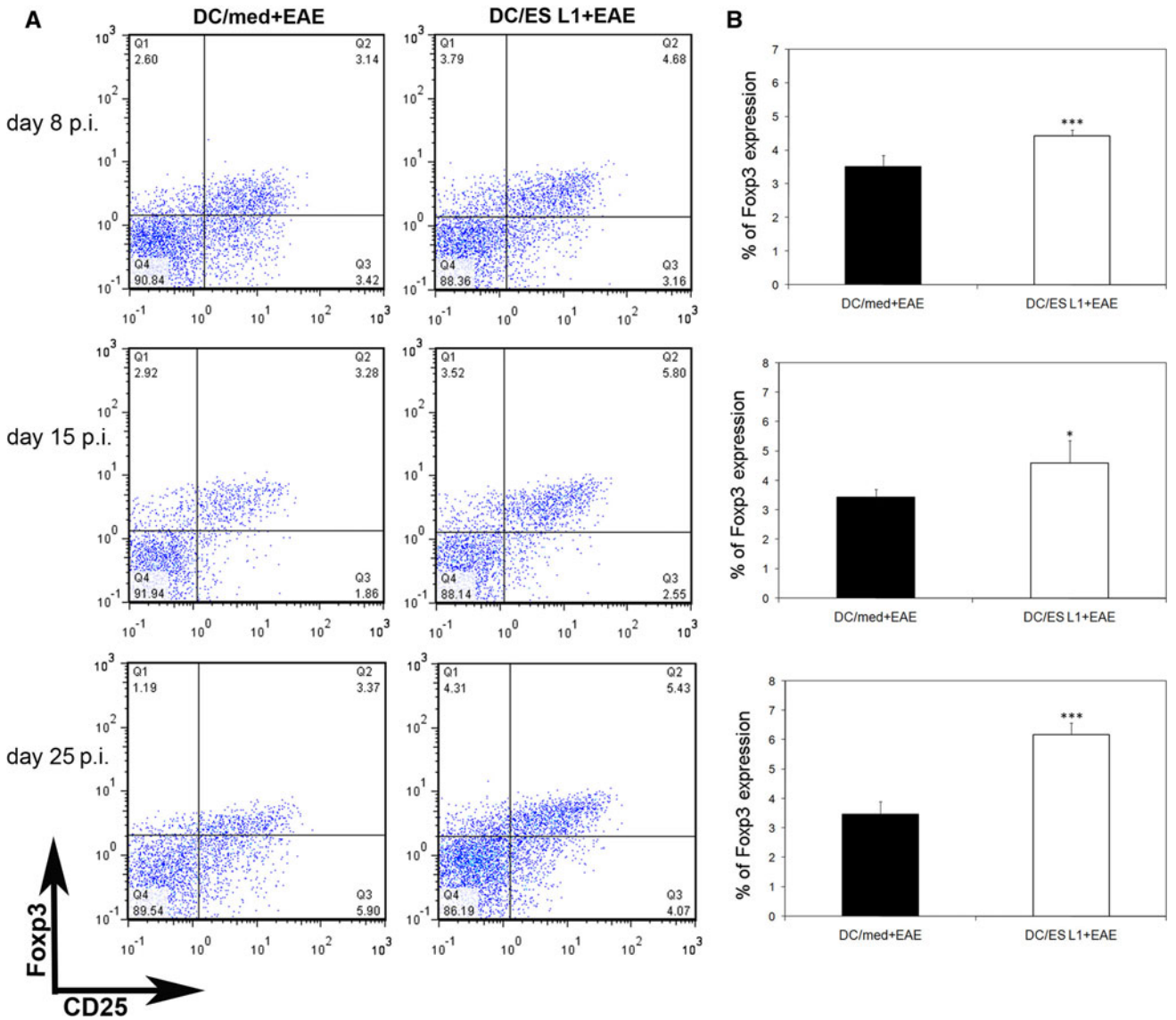

Fig. 7 Foxp3 expression in splenocytes during the course of EAE. Spleen cells were isolated in inductive (day 8 p.i.), effector (day 15 p.i.) and recovery (day 25 p.i.) phases and analyzed for Treg markers. **a** Representative plots for each time point, from one of two experiments, presence of CD25 versus Foxp3 expression, gated on CD4+ T cells. **b** The percentage of Foxp3+ cells within the CD4+ T-cell population in spleen cells from DC/ES L1-treated animals compared to the control, DC/med-treated rats, at the indicated time points. Data represent the mean ± SD from two independent experiments, five animals per experimental group. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$

applied as a single dose, achieved suppression of EAE through activation of regulatory mechanisms. The applied DCs managed to hold elevated levels of anti-inflammatory and regulatory cytokines (IL-10 and TGF-β), low levels of disease-associated IFN-γ and IL-17, and an increased proportion of Foxp3+ Tregs throughout the disease, without any alteration. Huang and colleagues [43] succeeded in protecting Lewis rats from EAE by injecting encephalitogenic myelin basic protein peptide 68–86-pulsed DCs. Cells that were stimulated with auto-antigen represented the adherent fraction of cultivated BMDCs that expressed high levels of IL-10 mRNA and low levels of IL-12 mRNA, which resembles cytokine production in DCs/ES L1 in our experiment. The authors speculate that these cells could be tolerogenic DCs. DCs matured with TNF-α and pulsed with auto-antigen MOG peptide were able to reduce the severity of EAE [44]. Apparently, treatment with TNF-α-induced incomplete DC maturation characterized with low/moderate expression of MHC II and costimulatory molecules and low cytokine production. These cells induced generation and proliferation of IL-10-producing T cells. A single injection of TNF-α-treated DCs ameliorated EAE, while three consecutive injections enabled full protection, which could indicate the

importance of repeated antigen stimulation. However, the authors discuss the need for three doses in the context of Treg generation, since some findings indicate the effectiveness of a single dose in the expansion of regulatory T cells in vivo. Our findings revealed the expansion of Treg in DA rats treated with a single injection of DC/ES L1. Hu and Wan [45] gave a nice review on different approaches for generating tolerogenic DCs and challenges to their potential clinical application. The main issue concerning therapeutic application of tolerogenic DCs is their stability in vivo. It is always a question whether these cells, once in the recipient's organism, would succumb to various influences and reverse phenotype into immunogenic. We cannot tell for sure the destiny of DC/ES L1 after injection, but considering that they suppress the disease and maintain an anti-inflammatory environment from EAE induction till the end of the observation period, we can speculate that they are stable.

The above-presented examples refer to antigen-specific induction of suppressive activities of regulatory T cells. However, these cells can also act through bystander suppression [46], which is most likely the case in our model. Regulatory T cells, once activated by their specific antigen, are able to inhibit effector function of T cells, regardless of their antigen specificity [47]. In experimental models of spontaneous development of EAE, transfer of CD4+ T cells from healthy animals prevented the onset of the disease [48], which indicated that preexisting Tregs acted in an antigen nonspecific manner. In many autoimmune diseases, target antigens still escape our knowledge. Therefore, the kind of approach where we can create bystander suppression without the need for recognition of the target antigen could be very promising.

In conclusion, we have shown that ES L1 antigen pulsed DCs gain immunomodulatory capacities, which are represented by their ability to modulate the outcome of EAE by prophylactic administration. DCs treated this way activate and maintain anti-inflammatory and regulatory mechanisms in recipient DA rats, which remain stable even after a robust challenge such as immunization with encephalitogenic emulsion. The therapeutic potential of tolerogenic DCs in treatment for various autoimmune diseases was suggested by a number of authors [37]. However, it is yet to be seen whether, in our model system, tolerogenic DCs could be used to suppress ongoing immunopathological reactions.

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Conflict of interest The authors confirm that there are no conflicts of interest.

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