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Diazepam Inhibits Proliferation of Lymph Node Cells Isolated from Rats with Experimental Autoimmune Encephalomyelitis

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Key Words
Autoimmunity · Experimental autoimmune encephalomyelitis · Myelin basic protein · Benzodiazepines · T lymphocytes

Abstract
Objective: Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease with similarities to human multiple sclerosis involving peripheral activation of autoreactive T cells which infiltrate the central nervous system and react to self antigens leading to damage. In previous studies, we have demonstrated that treatment with diazepam decreases the incidence and histological signs associated with the disease and diminishes immunological responses. The aim of the present work was to evaluate direct effects of diazepam on isolated T cells involved in immune responses during the development of EAE. ***Methods:*** Animals were sensitized with whole myelin to induce EAE and sacrificed during the acute phase of the disease. In mononuclear cells isolated from popliteal lymph nodes, cell viability, apoptosis induction, proliferation and cytokine production were evaluated. ***Results:*** Diazepam did not have a toxic or proapoptotic effect on the cells, at least up to the concentration of 25 μM, but proliferation, CD8+ T-cell activation and proinflammatory cytokine production were dose-dependently decreased. ***Conclusions:*** Diazepam has a direct inhibitory effect on the proliferation and activation of T lymphocytes isolated from the main lymphoid organ involved in disease onset and this could be one of the mechanisms that contribute to the beneficial effect previously observed with diazepam in vivo during EAE development.

Introduction

Benzodiazepines are classically known to be psychoactive drugs that share the pharmacological properties of tranquilizers, such as anxiolytic, sedative, hypnotic, skeletal muscle relaxant and anticonvulsant effects [1]. This action is mediated by the central benzodiazepine receptor (CBR). The CBR forms part of the γ-aminobutyric acid $(GABA)_A$ receptor complex, a heteropentameric channel permeable to chloride ions and responsible for the clinically exploited drug actions [2]. Binding of benzodiazepines to this receptor complex does not substitute GABA but promotes binding of GABA, which in turn increases the conduction of chloride ions across the neuronal cell membrane. As a result, the difference between the resting potential and the threshold potential is increased and firing is less likely [3]. Benzodiazepines have an alternative site of binding named peripheral benzodiazepine receptor (PBR), which may produce different pharmacological effects. Although the term PBR is widely accepted, multiple other names have been used to refer to this protein, including mitochondrial benzodiazepine receptor, mitochondrial diazepam-binding inhibitor receptor complex, PK11195-binding sites,

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isoquinoline-binding protein, pk18 and v3 receptor. TSPO or translocator protein (18 kDa) has also been proposed to reflect more accurately its subcellular role(s) and putative tissue-specific function(s), regardless of its subcellular localization [4]. These receptors are expressed in abundance in endocrine organs, skin, heart, lung epithelium, bone marrow, liver and spleen, and to a lesser extent in the central nervous system (CNS), where it is associated with glial cells [5]. PBR are neither structurally related nor coupled to $GABA_A$ receptors. Several functions have been attributed to PBR, among them the regulation of mitochondrial cholesterol delivery, the rate-determining step in steroidogenesis [4]. As a constituent of the mitochondrial permeability transition pore, PBR is thought to play a role in mitochondrial respiration, cell proliferation, differentiation and protein and ion transport [6]. Also, it is known that benzodiazepines could modulate immune responses via interaction with PBR on immune cells [7–10]. Diazepam, which is a mixed-type benzodiazepine, can act on both CBR and PBR, and exert an inhibitory effect on human T-cell function [10]. It was also reported that treatment of pregnant rats with diazepam from gestational days 14 to 20 resulted in severe and long-lasting depression of cellular immune responses in male and female offspring [11]. A recent work shows that acute and long-term diazepam treatment decreased the number of apoptotic cells, increased the percentage of T-cytotoxic cells and decreased the percentage of B cells, and increased corticosterone serum levels [12]. In this respect, we described that administration of low doses (0.1 mg/kg/day) of diazepam after active induction of experimental autoimmune encephalomyelitis (EAE) led to a marked decrease in the incidence and clinical signs of the disease, and diminished delayed-type hypersensitivity (DTH) responses, proliferation following stimulation with the encephalitogenic myelin basic protein (MBP), anti-MBP antibody production and spinal cord histological lesions associated with the disease [13]. Interestingly, alprazolam, another benzodiazepine derivative, also decreased the severity of clinical, histological and neuroendocrine manifestations of EAE in Lewis rats but only when the animals were exposed to stressful conditions [14]. In the context of EAE, it was also observed that phenobarbitone sodium and sodium valproate inhibited the clinical signs of EAE; however, high doses of diazepam failed to control EAE signs in this experiment [15].

EAE is a well-accepted model that mimics many of the clinical and pathological features of multiple sclerosis (MS). This pathology can be induced in genetically susceptible animals by a single injection of CNS antigens homogenized in an adequate adjuvant [16]. Wistar rats develop a monophasic course of the disease (acute stage) 11–13 days post-induction (dpi) characterized by ataxia and hind limb paralysis associated with weight loss and fecal and urinary incontinence. Affected animals show spontaneous neurological improvement 2–4 days after disease onset and regain the full ability to walk by 17–18 dpi [17]. In the EAE model, calcium-dependent glutamate release decreased in isolated nerve terminals of the cerebral cortex from EAE animals, which was coincident with the onset of clinical signs [18]. Since GABA is the major inhibitory neurotransmitter balanced with glutamate in the CNS we also have demonstrated that, in contrast to controls, synaptosomes from EAE rats showed decreased inhibition of glutamate release mediated by GABA. Furthermore, we observed a decrease in flunitrazepam-sensitive $GABA_A$ receptor density in synaptosomes from symptomatic EAE rats [19]. These results indicate that both glutamatergic and GABAergic neurotransmission is impaired in symptomatic EAE animals, and could contribute to clinical symptoms and disease progression. Treatment with diazepam inhibited this reduction in glutamate release in frontal cortex synaptosomes from EAE animals [13]. However, the modulatory effect of benzodiazepines on T cells is limited and the underlying mechanism is poorly defined. Therefore, the aim of this work is to ascertain the in vitro action of diazepam on encephalitogenic lymphocytes in order to understand the effect of this drug on the clinical, pathological and neuroendocrine manifestations of acute EAE in Wistar rats.

Materials and Methods

Chemicals

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3H)-one, Valium; 5 mg/ml) was from Roche International Limited (Montevideo, Uruguay). Myelin and MBP were purified from bovine spinal cords as previously described [20]. Complete Freund's adjuvant (CFA), bovine serum albumin, concanavalin A (Con A) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich Co. (St. Louis, Mo., USA). RPMI 1640 medium was purchased from Life Technologies (Grand Island, N.Y., USA). The fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) apoptosis detection kit was from BD Biosciences (San Diego, Calif., USA). Fetal bovine serum (FBS) was from NATOCOR (Córdoba, Argentina). [Metyl-^{3}H]thymidine ([^{3}H]TdR) was from NEN Life Science Products Inc. (Boston, Mass., USA). Peridinin chlorophyll protein complex (PerCp)-conjugated mouse anti-rat CD8a (clone OX-8), FITC-conjugated mouse anti-rat CD4 (clone OX-35), R-phycoerythrin-conjugated mouse anti-rat CD11b/c (clone OX-42) antibodies and the interferon (IFN)-γ ELISA kit were purchased from BD Pharmingen (San Jose, Calif., USA). Biotin-labeled mouse monoclonal anti-rat CD45RA (clone OX-33) antibody was from Invitrogen (Freder-

ick, Md., USA). Allophycocyanin-streptavidin and allophycocyanin-conjugated anti-rat CD25 (IL-2Ra, clone OX-39) antibody and the interleukin (IL)-17 rat IL17A ELISA kit were from eBioscience (San Diego, Calif., USA). The rest of the chemicals were analytical grade reagents of the highest purity available.

EAE Induction

Forty-five-day-old albino rats from a Wistar strain inbred in our laboratory for 40 years were used. All experiments were performed in accordance with international and institutional guidelines for animal care, and the protocol was approved by the local institutional review committee for animal studies (Exp. No. 15-99-40426). Every effort was made to minimize both the number of animals used and their suffering. Animals were anesthetized with a mixture of xylazine and ketamine (10 and 65 mg/kg i.p., respectively), and active disease was induced by intradermal inoculation with 8 mg whole myelin purified from bovine spinal cords in 0.5 ml of an emulsion consisting of 0.25 ml saline solution and 0.25 ml CFA (EAE group) in both hind feet. Animals were weighed, assessed daily for clinical signs of the disease and sacrificed at 12–14 dpi when the rats from the EAE group showed maximum clinical signs (acute period) [20].

Isolation of Mononuclear Cells from Lymph Nodes

EAE rats were sacrificed by CO_2 euthanasia; popliteal lymph nodes were aseptically removed and placed in ice-cold RPMI 1640 medium. Then the tissue was mechanically disrupted and single-cell suspensions filtered through a 70-μm cell strainer (Nylon membrane BD; Becton Dickinson, Buenos Aires, Argentina). After washing with 50 ml of Dulbecco's phosphate-buffered saline (PBS) containing 2% FBS, mononuclear cells (MNC) were preserved in complete medium (RPMI 1640, 10% FBS and 40 mg/ml gentamicin). Then, 1.25×10^6 cells/ml (250,000 cells/well) were cultured in triplicate in a volume of 200 μl in 96-well flat-bottom plates with different stimuli for 24–48 h. For the characterization of popliteal lymph node cell populations, cells were stained with surface antigen antibodies such as FITC-conjugated anti-CD4, PerCp-conjugated anti-CD8, biotin-conjugated anti-CD45Ra (B cells) coupled with allophycocyanin-streptavidin and phycoerythrin-conjugated anti-CD11b/c analyzed on a FACSCanto II flow cytometer (Becton Dickinson). FlowJo software was used to identify and quantify distinct cell populations by means of fluorescent intensity. A minimum of 50,000 cells per sample were counted. The effects of diazepam on cell viability, apoptosis induction, proliferation, cytokine production and activation were evaluated. Therefore, cells were treated with 2.5–500 μM diazepam and stimulated for 24 or 48 h with 1 μg/ml Con A or 100 μg/ml MBP.

Cell Viability and Cytotoxicity Assay

Viability was measured with MTT, a yellow tetrazole that is reduced to purple formazan in living cells. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials. Different concentrations of diazepam were added to MNC cultured without any stimulus. After 20 or 44 h, 20 μl of MTT (5 mg/ml) was added to each well and the cells were further incubated for 4 h at 37°C to allow MTT metabolization. The plates were centrifuged (350 *g*, 10 min), supernatants were discarded and 200 μl dimethyl sulfoxide were added per well. Absorbance was recorded at 570 nm on a microplate reader [21].

Apoptosis and Cell Death Detection

Staining with FITC-annexin V/PI was used to identify apoptotic and death cells. Briefly, after a 24-hour culture, MNC incubated with different amounts of diazepam were washed twice in PBS and resuspended in 100 μl of annexin V binding buffer before being incubated with FITC-conjugated annexin V ($0.5\ \mu g/7.5 \times 10^5$ cells) and PI solution ($0.1\ \mu g/7.5 \times 10^5$ cells). Cells were then analyzed by flow cytometry after a 10-min incubation in the dark at room temperature. Early apoptotic cells were stained with annexin V alone, whereas necrotic and late apoptotic cells were stained with both annexin V and PI [22, 23].

Proliferation of MNC

MNC were cultured in triplicate in 96-well flat-bottom plates essentially as described previously [19]. Briefly, 1.25×10^6 cells/ml were cultured in a total volume of 200 μl of RPMI 1640 per well with 1 μg/ml Con A or 100 μg/ml of MBP, for 24 or 48 h, respectively. When indicated, different concentrations of diazepam (μM) were also added to the cultures. Each well was pulsed with 20 μl of medium containing 1 μCi of [^{3}H]TdR during the last 18 h of culture; subsequently cells were harvested onto fiberglass filters and the radioactivity incorporated counted using standard liquid scintillation techniques. Each treatment was performed in triplicate, and results are expressed as stimulation index (SI), which was defined as the ratio between mean counts per minute of antigen-stimulated culture/mean counts per minute of the unstimulated culture; an SI ≥2 was considered as a positive response.

To evaluate if the incubation of MNC with diazepam induced permanent cell anergy, cells were incubated for 24 h in the presence of diazepam (0, 10 or 25 μM) without any stimuli. Then, cells were harvested, washed with PBS to remove diazepam, resuspended in new complete medium and plated for 24 h in the presence of Con A or anti-CD3 antibody. Each well was pulsed with 20 μl of medium containing 1 μCi of [^{3}H]TdR during the last 18 h of culture; subsequently, cells were harvested onto fiberglass filters and the radioactivity incorporated counted using standard liquid scintillation techniques.

Determination of Cytokine Levels in vitro

Cytokines in conditioned medium were determined using an enzyme-linked immunosorbent assay (ELISA). MNC were prepared and cultured as described above with the correspondent stimuli and different concentrations of diazepam. After 24–48 h, plates were centrifuged at 350 *g* for 10 min, and culture supernatants were collected to assess cytokine levels. IFN-γ and IL-17 concentrations were determined using matching antibodies in a sandwich ELISA according to the manufacturer' instructions. Absorbance was read at 450 nm and cytokine levels were compared with the linear portion of the standard curve.

CD25 Expression

To determine the functional response of T cells, expression of the surface activation marker CD25 was measured in cultured cells in the presence or absence of Con A. MNC were cultured in 96-well flat-bottom plates at a density of 1.25×10^6 cells/ml in a total volume of 200 μl of RPMI 1640 per well for 24 h. When indicated, different doses of diazepam were added. At 24 h, cells were harvested, 3 wells were pooled and stained for surface antigens with FITC-conjugated anti-CD4, PerCp-conjugated anti-CD8 and allophycocyanin-conjugated anti-CD25. The populations were analyzed by flow cytometry.

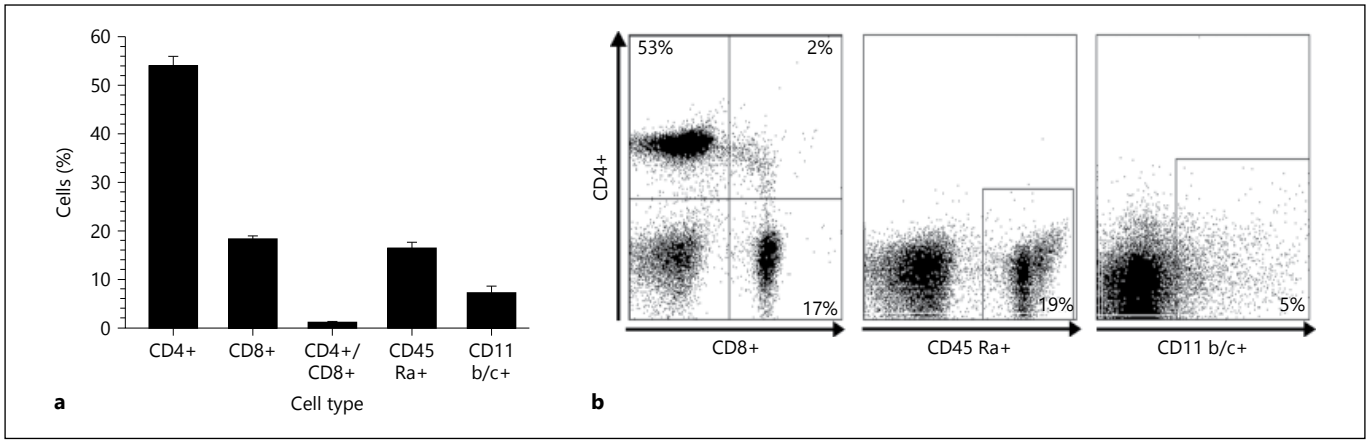

Fig. 1. Analysis of cell frequency in popliteal lymph nodes from EAE rats. Anti-CD4 and anti-CD8 antibodies identified subsets of T lymphocytes, anti-CD45 Ra antibody-stained B lymphocytes and anti-CD11 b/c monocytes, granulocytes, macrophages and dendritic cells. **a** Mean percentages of each population from data accumulated using flow cytometry of 20 individual samples. **b** Two-dimensional dot plots are representative of one individual determination.

Data Analysis

Results are expressed as means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA). Whenever ANOVA indicated significant effects ($p < 0.05$), a pair-wise comparison of means of all groups with respect to the control group was carried out (Dunnett's multiple comparison test). In all cases, the assumptions of ANOVA (homogeneity of variance and normal distribution) were attained. When indicated, significant differences were performed using Student's t test for paired or grouped samples. In all statistic analyses, $p < 0.05$ was considered to represent a significant difference between groups.

Results

Cell Populations of Popliteal Lymph Nodes

Isolated MNC from draining popliteal lymph nodes of EAE rats were stained with surface antigen antibodies and analyzed by flow cytometry (fig. 1). The MNC population was composed of about 72% T lymphocytes (54% CD4+ and 18% CD8+) and 18% B lymphocytes. The number of immature T cells (double-positive T cells) was very low, around 0–1%. About 7% of the isolated cells were stained with anti-CD11b/c antibody corresponding to monocytes, granulocytes, macrophages and dendritic cells. These cell populations did not vary when MNC were placed in culture, both with and without Con A stimulation for 24 h (data not shown).

Effect of Diazepam on Cell Viability

MNC from EAE animals were cultured in the presence of different concentrations of diazepam added to the cell culture medium and the effect on cell viability was evaluated by MTT assay (fig. 2). Post hoc comparisons demonstrated that diazepam did not affect the viability of unstimulated cells at concentrations of up to 25 μM either at 24- or 48-hour exposure. However, at concentrations >50 μM, the percentage of viable cells was decreased and at the highest dose (500 μM) there was marked cytotoxicity. These data correlate with the results obtained in the apoptosis and cellular death assay using PI (fig. 3).

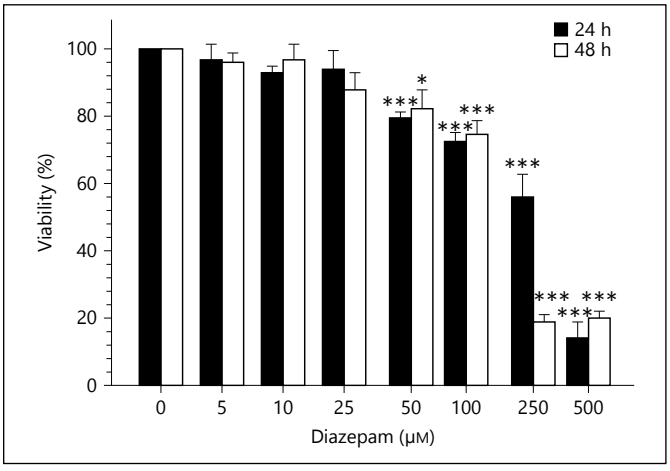

Fig. 2. Effect of diazepam on cell viability. Isolated cells from popliteal lymph nodes of EAE rats were cultured for 24 or 48 h without any stimulus and treated with increasing concentrations of diazepam. Cells were incubated with MTT for 4 h and their viability was measured as absorbance at 570 nm of the formazan-formed product of mitochondrial metabolism. Data indicate the percentage of viable cells and are presented as means ± SEM of 5 independent experiments. * $p < 0.05$, *** $p < 0.001$ (one-way ANOVA).

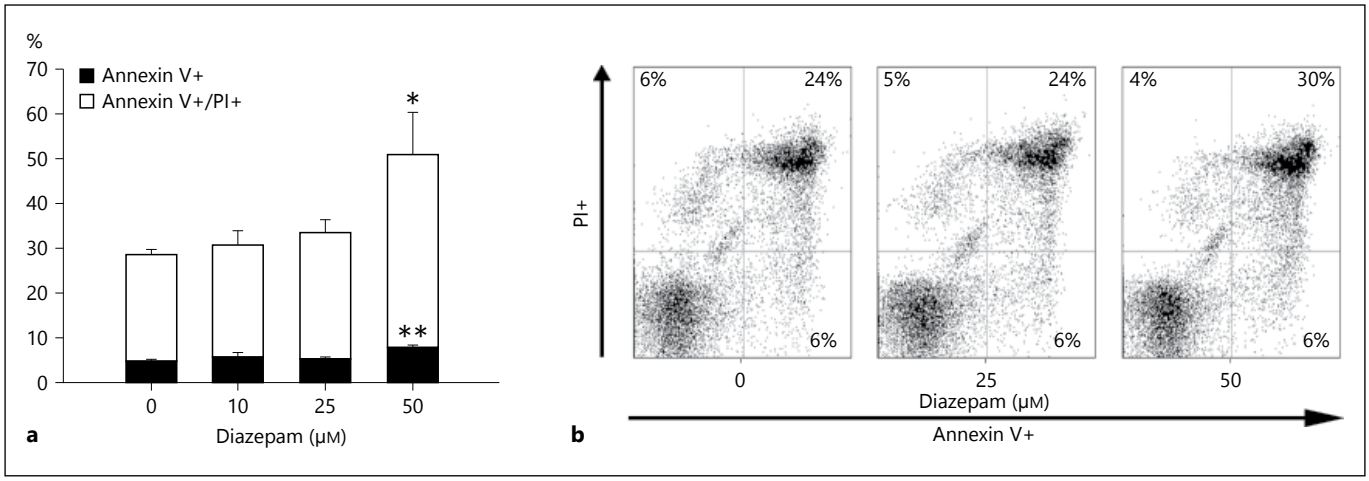

Fig. 3. Effect of diazepam on cell death. Isolated cells from popliteal lymph nodes of EAE animals during the acute state of the disease were incubated with different concentrations of diazepam without stimuli, and the induction of apoptosis after 24 h of culture was evaluated by flow cytometry. Annexin V+/PI– = Effect of diazepam on early apoptosis; annexin V+/PI+ = percentage of cells in late apoptosis or cell death. **a** Percentage of cells in each condition (means ± SEM of 5 independent experiments). **b** Two-dimensional dot plots are representative of one individual determination. * $p < 0.05$, ** $p < 0.01$, Student's t test for grouped samples.

Diazepam Does Not Induce Early Apoptosis

In order to analyze early apoptosis, isolated cells from popliteal lymph nodes of EAE animals during the acute state of the disease were incubated with diazepam in conditions that did not alter cell viability (fig. 2). Therefore, cells were incubated with 0–50 μM diazepam without stimuli, and the induction of apoptosis and cell death after 24 h of culture was evaluated by flow cytometry (fig. 3). In this experiment, the percentage of annexin V+, PI– cells did not change in the presence of diazepam, indicating no effect of the drug on early apoptosis. The double-positive population (annexin V+, PI+) did not change up to 25 μM, but there was a significant increase at 50 μM, indicating that this diazepam concentration increased the percentage of cells in late apoptosis or at cell death. This assay does not distinguish between these two conditions since both cell populations are annexin V+, PI+.

Diazepam Inhibits T-Cell Proliferation in a Dose-Dependent Manner

Activation and proliferation of encephalitogenic T cells in peripheral lymph nodes is a key event in the early development of EAE. Thus, the proliferation assay of isolated MNC from lymph nodes was tested in vitro at disease onset (11–14 dpi), and following unspecific stimulation with Con A or specific stimulation with MBP for 24 or 48 h, respectively, in the presence of different doses of diazepam. EAE cells without diazepam showed a positive proliferation response upon Con A and MBP stimulation (SI >2). The presence of diazepam reduced proliferation in a dose-dependent manner at nontoxic doses (2.5–25 μM; fig. 4). MNC from control CFA rats showed a similar response to diazepam when they were stimulated with Con A. When cells from CFA rats were cultivated with MBP (antigen-specific stimulation), there was neither proliferation with nor without the addition of diazepam (data not shown).

Preincubation with Diazepam Does Not Affect the Ability of T Cells to Respond to Later Stimulation

To evaluate whether the incubation of MNC with diazepam induced permanent cell anergy, cells were incubated for 24 h in the presence of 0–25 μM diazepam without stimuli. Then cells were washed to remove diazepam, resuspended in new complete medium and incubated again for 24 h in the presence of Con A or anti-CD3 antibody (fig. 5). This experiment showed that MNC respond similarly to Con A and anti-CD3 antibody stimulation after being previously being incubated for 24 h with different doses of diazepam, indicating that diazepam does not alter cell capacity to proliferate after future stimulations.

Diazepam Reduces the Production of Proinflammatory Cytokines

Taking into account that diazepam reduced the proliferation of MNC in vitro when stimulated with Con A and

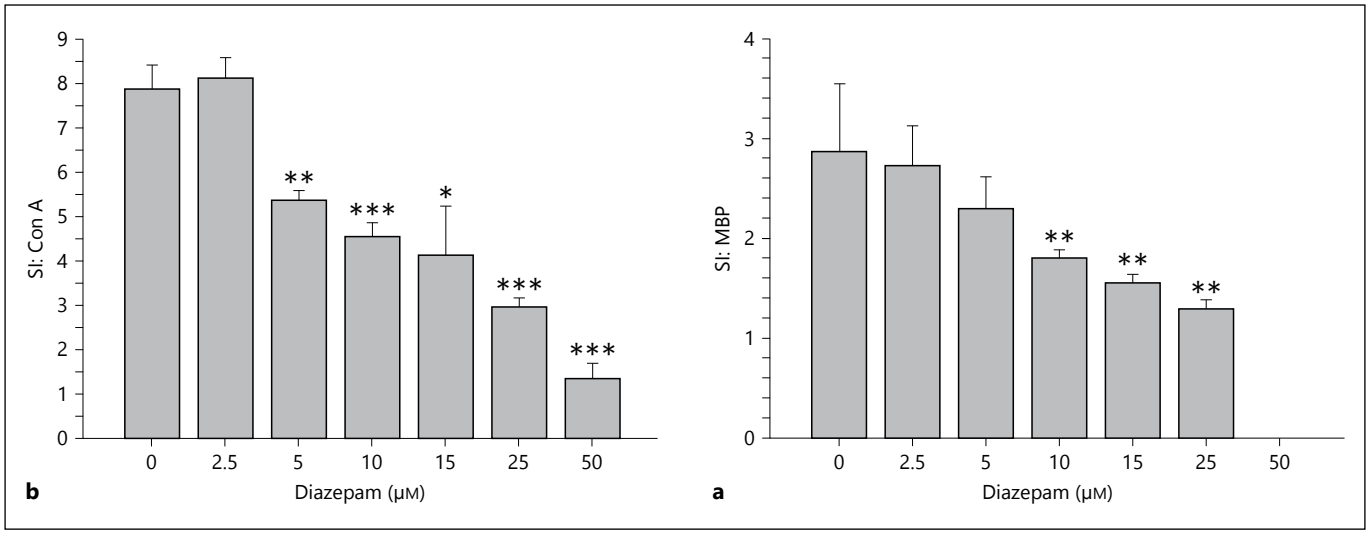

Fig. 4. Effect of diazepam on T-cell proliferation. MNC were cultured with 1 μg/ml Con A for 24 h (**a**) or 100 μg/ml of MBP for 48 h (**b**) in the presence of different concentrations of diazepam. Data indicate the SI of treated cells (means ± SEM of 7 independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA).

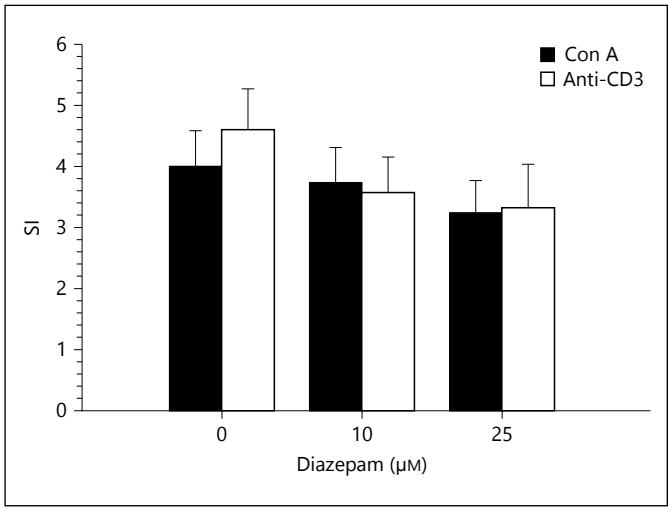

Fig. 5. Effect of diazepam on T-cell anergy. MNCs were incubated for 24 h in the presence of diazepam (0, 10, 25 μM) without any stimuli. Then cells were harvested, washed with PBS to remove diazepam, resuspended in new complete medium and plated for 24 h in the presence of Con A or anti-CD3 antibody. Data indicate the SI of diazepam-preincubated cells (means ± SEM of 5 independent experiments).

MBP, without affecting cell viability or inducing apoptosis, we sought to evaluate if it could also affect the production of pro- and anti-inflammatory cytokines. MNC were cultured for 24–48 h in the presence of a stimulus and different concentrations of diazepam (0, 5, 10 and 25 μM), and ELISA was performed to determine the production of IFN-γ and IL-17 in culture supernatants. One-way ANOVA of the IFN-γ concentration showed a significant dose-dependent reduction in the presence of diazepam in cells stimulated with Con A or MBP (fig. 6a, b). Levels of IL-17, an important cytokine involved in autoimmune-allergic processes, were diminished by diazepam when cells were stimulated with a nonspecific (Con A) or specific antigen related to the disease like MBP (fig. 6c, d). With intracellular cytokine marking, IFNγ and IL-17 also decreased in cells stimulated with Con A and incubated in the presence of 25 μm diazepam compared to cells incubated with Con A only (table 1).

Diazepam Reduces the Percentage of Activated CD8+ T Cells

Diazepam reduces the CD8+CD25+ T-cell population when unspecifically stimulated. CD25 is the α-chain of the IL-2 receptor. It is a type-I transmembrane protein present on activated T and B cells, some thymocytes, myeloid precursors and oligodendrocytes. This chain associates with CD122 to form a heterodimer that can act as a high-affinity receptor for IL-2 [24]. IL-2 is a small molecule that can acts a a growth factor, and it is involved in the survival and proliferation of T lymphocytes. In this assay, we evaluated the population of CD4+CD25+ and CD8+CD25+ T cells in culture at different doses of diazepam with or without stimulation with Con A for 24 h (fig. 7). MNC isolated from popliteal lymph nodes cul-

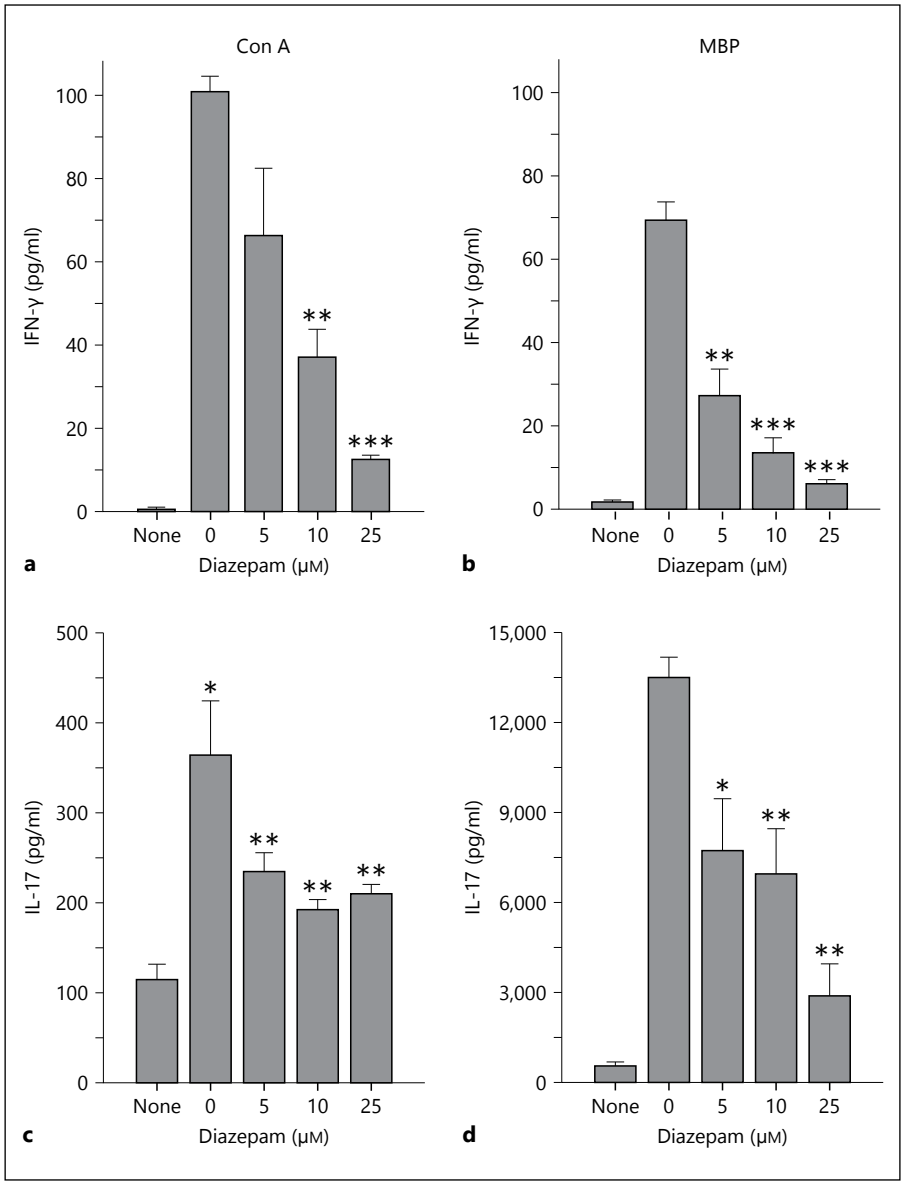

Fig. 6. Determination of cytokine levels in vitro. MNC were cultured with 1 μg/ml Con A for 24 h (**a**, **c**) or 100 μg/ml of MBP for 48 h (**b**, **d**) in the presence of different concentrations of diazepam. The concentrations of IFN-γ (**a**, **b**) and IL-17 (**c**, **d**) were determined using matching antibodies in a sandwich ELISA. Data indicate the levels of produced cytokines (means ± SEM of 4 independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA).

Table 1. Determination of intracellular cytokines

Cell type	Cells, %	Con A, %	Con A + diazepam	
			%	% diff. Con A
CD4+IFNγ+	2.33±0.33	8.00±1.15	4.33±1.20*	–45.88
CD8+IFNγ+	2.33±0.33	9.33±1.33	5.33±1.20*	–42.87
CD4+IL-17+	3.00±0.58	13.67±1.45	9.33±1.33*	–31.75
CD8+IL-17+	6.33±0.68	18.33±3.18	14.33±3.84	–21.82

Data of quantitative variables are expressed as group mean of total cells ± SEM. * $p < 0.05$, vs. Con A only.

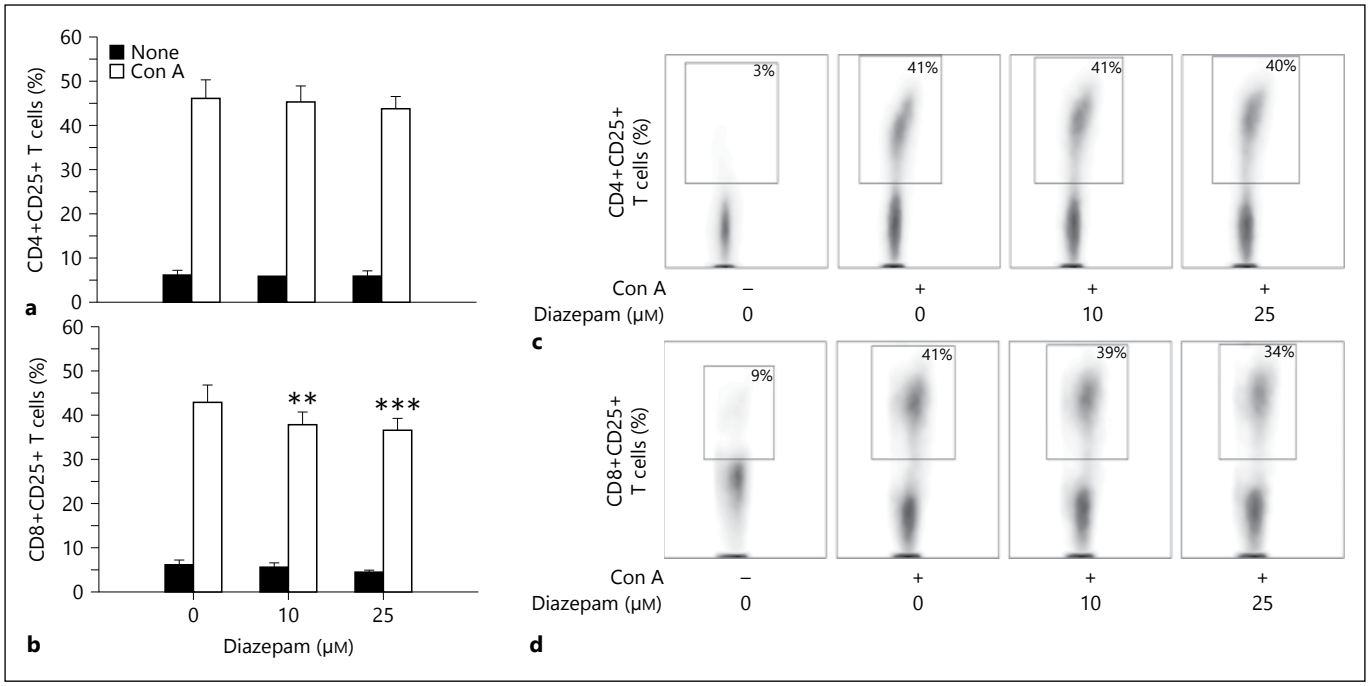

Fig. 7. Determination of CD25 expression. The expression of the surface activation marker CD25 was measured in cultured cells in the presence or absence of Con A and the indicated doses of diazepam. Bar graphs show the mean percentages of CD25+ on CD4+ (**a**) and CD8+ (**b**) T-cell populations from data accumulated of 5 independent experiments; the two-dimensional dot plots (**c**, **d**) are the correspondent representative individual determinations. ** $p < 0.01$, *** $p < 0.001$ (Student's t test for paired samples).

tured with different doses of this benzodiazepine reduced the CD8+CD25+ T-cell population in a dose-dependent manner after stimulation with Con A, whereas no changes were observed in the CD4+CD25+ T-cell population at any dose of diazepam. The percentages of CD4+ and CD8+ populations were not altered at the different doses of diazepam (data not shown).

Discussion

Lymphocytic activation is a complex process that involves the induction of gene transcription, surface antigen expression, cytokine secretion and mitotic activity resulting in proliferation that plays a key role in the onset and development of many autoimmune diseases [21]. Previous results demonstrated that the treatment of rats with 0.1 mg/kg/day diazepam during the inductive phase of EAE (6–11 dpi) reduces the incidence and clinical signs in animals challenged with the disease [13]. In treated animals, we also observed a reduction in some immunological parameters, such as DTH reaction, antibody production and proliferation induced by MBP in MNC isolated from popliteal lymph nodes. The effect of diazepam during the in vivo treatment could be mediated, at least in part, by a direct effect on lymphocytes. In this study, we demonstrate that diazepam has a direct inhibitory effect on cells involved in adaptive immune responses without affecting cell viability or exerting a proapoptotic or toxic effect, at least up to 25 μM, on MNC isolated from popliteal lymph nodes of EAE animals during the acute state of the disease.

Proliferation is a key event during the development of EAE; it mainly happens in lymph nodes where antigen-presenting cells, mostly dendritic cells that migrated from the site of induction, mature and present antigens to naïve T lymphocytes. After the antigen-presenting process, T cells proliferate, start to produce proinflammatory molecules and augment the expression of integrins, which allows these cells to interact with endothelial cells, cross the blood-brain barrier, enter the CNS and exert damage [25]. In this context, it is important to note that diazepam reduced the proliferation of T lymphocytes in a dose-dependent manner when administered in vitro in the presence of a specific (MBP) or unspecific (Con A) stimulus. In agreement with these results, previous studies showed that diazepam inhibited IFN-γ production by human peripheral blood MNC induced by anti-CD3 in a dose-de-

pendent manner. The inhibitory effect of diazepam on IFN-γ production is similar to that of R05-4864, a selective PBR ligand. Collectively, these results suggested that diazepam suppressed human T-cell function through PBR [10].

Cytokines are messenger molecules that play a critical role in the immune system. They are potential targets for immunomodulation and are divided into two subsets: Th1 (proinflammatory) and Th2 (anti-inflammatory) cells. Evaluation of the production of proinflammatory cytokines in culture supernatants from isolated cells stimulated with Con A or MBP revealed that diazepam reduce the production of IFN-γ in a dose-dependent manner. IFN-γ is a proinflammatory cytokine that is produced by T lymphocytes and natural killer cells. Its main function is to activate macrophages in innate and adaptive immune responses to produce TNF-α and IL-1 [26]. This effect was also observed using another technique in a study by Wei et al. [10]. When cells were specifically and unspecifically activated with MBP and Con A, respectively, diazepam reduced the levels of IL-17, as expected. This proinflammatory cytokine activates T cells and other immune cells to produce a variety of cytokines, chemokines and cell adhesion molecules. This cytokine is augmented in the sera and/or tissues of patients with contact dermatitis, asthma, rheumatoid arthritis and also in multiple sclerosis [27].

We also evaluated the levels of CD25, the α-subunit of the IL-2 receptor. This cytokine is a feature of the Th1 response and is involved in survival and cell proliferation. CD25 is also marker of activated T lymphocytes (CD4+ and CD8+), the same as CD69. CD25 levels markedly increase in cells cultured in the presence of Con A for 24 h. Incubation with Con A and diazepam reduced CD25 levels, but only in CD8+ T cells, with no effect on the CD4+ T cell population. This may explain our observation in vivo when analyzing DTH responses in diazepam-treated animals compared to vehicle-injected animals [13]. On days 9 and 12, diazepam-treated animals showed less thickness associated to swelling 24 h after the injection of MBP in the corresponding ear. Taking into account that CD8+ T cells are the main cells involved in hypersensitivity responses in many pathologies such as contact dermatitis, asthma and autoimmune diseases [28], and our results indicating that incubation with diazepam reduced the percentage of activated CD8+ T cells following Con A stimulation for 24 h, we hypothesized that the in vivo effect on DTH responses could result from a direct action of diazepam on CD8+ T cells.

As mentioned before, the effect observed as a result of diazepam treatment during the inductive phase of the disease could be at least in part mediated by a direct effect of diazepam on lymphocytes based on our in vitro observations, but other possible events could be operating in vivo. It is possible that diazepam affects other cells involved in the inductive phase of immune responses, such as macrophages or dendritic cells, for example. In agreement with many published records [29] and results from our laboratory [30], diazepam and other benzodiazepines, such as midazolam, also affect antigen-presenting cells, thus reducing the production of proinflammatory cytokines, oxygen and nitrogen reactive species, and the expression of costimulatory molecules. Also, we do not discard the fact that this benzodiazepine modulates EAE in vivo by its action on glucocorticoid-producing cells via increases in the levels of immunomodulatory molecules and modifications in many immunological parameters [12].

In conclusion, we provide evidence that diazepam has a direct inhibitory effect on cells involved in adaptive immune responses and thus has beneficial effects during the development of EAE. Future experiments aiming to analyze the effects of this drug on antigen-presenting cells and possible underlying intracellular pathways and receptors are required.

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References

1 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven MA: Biomarkers for the effects of benzodiazepines in healthy volunteers. Br J Clin Pharmacol 2003; 55:39–50.

2 Olsen RW, Sieghart W: $GABA_A$ receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology 2009;56: 141–148.

3 Riss J, Cloyd J, Gates J, Collins S: Benzodiazepines in epilepsy: pharmacology and pharmacokinetics. Acta Neurol Scand 2008;118: 69–86.

4 Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapère J-J, Lindemann P, Norenberg MD, Nutt D, Weizman A, Zhang M-R, Gavish M: Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006;27:402–409.
5 Venneti S, Lopresti BJ, Wiley CA: The peripheral benzodiazepine receptor (Translocator protein 18kDa) in microglia: from pathology to imaging. Prog Neurobiol 2006;80:308–322.
6 Casellas P, Galiegue S, Basile AS: Peripheral benzodiazepine receptors and mitochondrial function. Neurochem Int 2002;40:475–486.
7 Descotes G, Mazue G, Richez P: Drug immunotoxicological approaches with some selected medical products: cyclophosphamide, methylprednisolone, betamethasone, cefoxitine, minor tranquillizers. Toxicol Lett 1982;13:129–137.
8 Zavala F: Benzodiazepines, anxiety and immunity. Pharmacol Ther 1997;75:199–216.
9 Kalashnikov SV, Kalashnikova EA, Kokarovtseva SN: Immunomodulating effects of tofizopam (Grandaxin) and diazepam in vitro. Mediators Inflamm 2002;11:53–59.
10 Wei M, Li L, Meng R, Fan Y, Liu Y, Tao L, Liu X, Wu C: Suppressive effect of diazepam on IFN-γ production by human T cells. Int Immunopharmacol 2010;10:267–271.
11 Schlumpf M, Lichtensteiger W, Ramseier H: Diazepam treatment of pregnant rats differentially affects interleukin-1 and interleukin-2 secretion in their offspring during different phases of postnatal development. Pharmacol Toxicol 1993;73:335–340.
12 Bento de Lima C, Sakai M, Oliveira Latorre A, de Moraes Moreau RL, Palermo-Neto J: Effects of different doses and schedules of diazepam treatment on lymphocyte parameters in rats. Int Immunopharmacol 2010;10:1335–1343.
13 Bibolini MJ, Chanaday NL, Báez NS, Degano AL, Monferran CG, Roth GA: Inhibitory role of diazepam in autoimmune inflammation in rats with experimental autoimmune encephalomyelitis. Neuroscience 2011;199:421–428.
14 Núñez-Iglesias MJ, Novío S, Almeida-Dias A, Freire-Garabal M: Inhibitory effects of alprazolam on the development of acute experimental autoimmune encephalomyelitis in stressed rats. Pharmacol Biochem Behav 2010;97:350–356.
15 Gilani AA, Dash RP, Jivrajani MN, Thakur SK, Nivsarkar M: Evaluation of GABAergic transmission modulation as a novel functional target for management of multiple sclerosis: exploring inhibitory effect of GABA on glutamate-mediated excitotoxicity. Adv Pharmacol Sci 2014;2014:632376.
16 Ludwin SK: The pathogenesis of multiple sclerosis: relating human pathology to experimental studies. J Neuropathol Exp Neurol 2006;65:305–318.
17 Slavin DA, Bucher AE, Degano AL, Soria NW, Roth GA: Time course of biochemical and immunohistological alterations during experimental allergic encephalomyelitis. Neurochem Int 1996;29:597–605.
18 Vilcaes AA, Furlan G, Roth GA: Inhibition of Ca^{2+}-dependent glutamate release in cerebral cortex synaptosomes of rats with experimental autoimmune encephalomyelitis. J Neurochem 2009;108:881–890.
19 Cid MP, Vilcaes AA, Rupil LL, Salvatierra NA, Roth GA: Participation of the GABAergic system on the glutamate release of frontal cortex synaptosomes from rats with experimental autoimmune encephalomyelitis. Neuroscience 2011;199:421–428.
20 Degano AL, Roth GA: Passive transfer of experimental autoimmune encephalomyelitis in Wistar rats: dissociation of clinical symptoms and biochemical alterations. J Neurosci Res 2000;59:283–290.
21 Xiong Y, Zhang S, Lu J, Sun S, Song B, Xu L, Yang Z, Guan S: Investigation of effects of farrerol on suppression of murine T lymphocyte activation in vitro and in vivo. Int Immunopharmacol 2013;16:313–321.
22 Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 1995;184:39–51.
23 Guasconi L, Serradell MC, Masih DT: *Fasciola hepatica* products induce apoptosis of peritoneal macrophages. Vet Immunol Immunopathol 2012;148:359–363.
24 Brisslert M, Bokarewa M, Larsson P, Wing K, Collins LV, Tarkowski A: Phenotypic and functional characterization of human CD25+ B cells. Immunology 2006;117:548–557.
25 Sospedra M, Martin R: Antigen-specific therapies in multiple sclerosis. Int Rev Immunol 2005;24:393–413.
26 Hill N, Sarvetnick N: Cytokines: promoters and dampeners of autoimmunity. Curr Opin Immunol 2002;14:791–797.
27 Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y: IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol 2006;177:566–573.
28 Kalish RS, Askenase PW: Molecular mechanisms of CD8+ T cell-mediated delayed hypersensitivity: implications for allergies, asthma, and autoimmunity. J Allergy Clin Immunol 1999;103:192–199.
29 Ohta N, Ohashi Y, Takayama C, Mashimo T, Fujino Y: Midazolam suppresses maturation of murine dendritic cells and priming of lipopolysaccharide-induced T helper 1-type immune response. Anesthesiology 2011:114:355–362.
30 Falcón CR, Fernández Hurst N, Cervi LC, Monferran CG, Roth GA: The inhibitory effect of diazepam on induced LPS-dendritic cell activation. LXI Reunión Científica Anual de la Sociedad Argentina de Inmunología (SAI) 2013;86:102.