Diazoxide attenuates autoimmune encephalomyelitis and modulates lymphocyte proliferation and dendritic cell functionality

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ABSTRACT

Activation of mitochondrial ATP-sensitive potassium (K_{ATP}) channels is postulated as an effective mechanism to confer cardio and neuroprotection, especially in situations associated to oxidative stress. Pharmacological activation of these channels inhibits glia-mediated neuroinflammation. In this way, diazoxide, an old-known mitochondrial K_{ATP} channel opener, has been proposed as an effective and safe treatment for different neurodegenerative diseases, demonstrating efficacy in different animal models, including the experimental autoimmune encephalomyelitis (EAE), an animal model for Multiple Sclerosis. Although neuroprotection and modulation of glial reactivity could alone explain the positive effects of diazoxide administration in EAE mice, little is known of its effects on the immune system and the autoimmune reaction that triggers the EAE pathology. The aim of the present work was to study the effects of diazoxide in autoimmune key processes related with EAE, such as antigen presentation and lymphocyte activation and proliferation. Results show that, although diazoxide treatment inhibited *in vitro* and *ex-vivo* lymphocyte proliferation from whole splenocytes it had no effect in isolated $CD4^+$ T cells. In any case, treatment had no impact in lymphocyte activation. Diazoxide can also slightly decrease CD83, CD80, CD86 and major histocompatibility complex class II expression in cultured dendritic cells, demonstrating a possible role in modulating antigen presentation. Taken together, our results indicate that diazoxide treatment attenuates autoimmune encephalomyelitis pathology without immunosuppressive effect.

Key Words: Diazoxide; K_{ATP} channels; EAE mice; spleen; lymphocyte; dendritic cell.

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). It is hypothesized that the disease is caused by an autoimmune reaction orchestrated by myelin-specific $CD4^+$ Th1 that results in demyelination and neuronal injury (Trapp and Nave 2008). T cells are key players in shaping the immune responses by directly secreting soluble mediators or through cell contact-dependent mechanism (Goverman 2009). In experimental autoimmune encephalomyelitis (EAE), the classical animal model for MS, Th1 cells have been shown to promote inflammation within the CNS and facilitate Th17 cell infiltration into the CNS (O'Connor and Anderton 2008). In this context, it has been suggested that encephalitogenic T cells that invade the CNS during disease interact locally with antigen presenting cells (APCs), resulting in both, re-activation of the T cells and activation of the APCs (Shrikant and Benveniste 1996). Different cell types including resident astrocytes and microglia as well as infiltrating macrophages and dendritic cells (DC) are potential APCs inside the CNS (Carson et al. 2006). While APCs constitutively express CD45, CD86 and CD40 at low levels (Carson et al. 1998), expression of CD45, major histocompatibility complex class II (MHC class II), CD80, CD86 and CD40 appear to be upregulated in response to inflammatory stimuli, thus enhancing their ability to function as APCs (Shrikant and Benveniste 1996).

Potassium channels play an important role in the cardiovascular system and in the CNS. They are critically involved in regulating signaling, proliferation, secretion and migration not only in neurons but also in glial cells such as astrocytes and microglia (Olsen and Sontheimer 2008; Skaper 2011; Steinhauser et al. 2012; Rodriguez et al. 2013). Potassium channels have been also described to modulate the immune response. For instance, the relevance of the voltage-gated K^+ channel Kv1.3 (Beeton et al. 2006;

Judge and Bever, Jr. 2006) and the Ca^{2+}-dependent K^+ channel KCa3.1 (Ghanshani et al. 2000) in the immune system has been widely documented. Recently, the TWIK-related acid sensitive potassium channel 2 (K2P5.1, KCNK5, TASK2) has been postulated as new K^+ channel that is a critical player in T-cell effector function (Meuth et al. 2008; Bittner et al. 2010).

ATP-sensitive K^+ (K_{ATP}) channels are large hetero-octameric complexes consisting of four pore-forming inward-rectifying K^+ subunits (Kir6.x) and four regulatory sulfonylurea receptor (SURx) subunits (Mannhold 2004). They are considered metabolic sensors that couple cellular energy metabolism to membrane excitability by regulating potassium flux. These channels act as energy sensors of ATP production and are believed to regulate various physiological functions, such as muscle contraction and insulin secretion, by coupling cell metabolism to membrane potential (Nichols 2006). K_{ATP} channels are also present at the mitochondrial inner membrane (mito-K_{ATP}) where they participate in the regulation of mitochondrial volume and membrane potential ($\Delta\Psi m$). Furthermore, their activity is related to electronic transport, metabolic energy, reactive oxygen species (ROS) production and mitochondrial welfare (Busija et al. 2008; Xie et al. 2010). Diazoxide (7-chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide) is a well-known small molecule that opens K_{ATP} channels with high affinity for mito-K_{ATP} channels. Our previous studies demonstrated that oral administration of diazoxide ameliorated disease progression in EAE and caused neuroprotection (Virgili et al. 2011; Virgili et al. 2013). Diazoxide elicited a significant reduction in myelin and axonal loss accompanied by a decrease in glial activation and neuronal damage without affecting the number of infiltrating lymphocytes positive for CD3 and CD20 in the spinal cord (Virgili et al. 2011). These results demonstrated novel actions of diazoxide as glial mediated anti-inflammatory and neuroprotective agent, although the effects of

K_{ATP} channel openers (KCOs) in the immune system are poorly understood. It has been described that diazoxide suppresses leukemic cell proliferation through depolarization of the mitochondrial membrane and disruption of intracellular Ca^{2+} dynamics (Holmuhamedov et al. 2002). Other studies demonstrated a reduction of leukocyte adhesion and migration by KCOs (nicorandil and diazoxide) in tissue subjected to ischemia/reperfusion (Yasu et al. 2002). Recently, it has been showed that activation of mito-K_{ATP} channels reduced apoptosis on spleen mononuclear cells induced by hyperlipidemia (Alberici et al. 2013). Here, we have addressed the possible peripheral actions of diazoxide modulating specific or non-specific lymphocyte activation. Thus, our aims were: A) To analyze the effect of diazoxide on proliferative response in the EAE model, and B) To analyze the K_{ATP} channel expression in $CD4^+$ T cells and to study proliferative lymphocyte response in specific anti-CD3-CD28 activation lymphocyte model.

MATERIAL & METHODS

Mice

Female C57BL/6J mice, 8 to 10 weeks of age, were purchased from Janvier (Le Genest-Saint-Isle, France) and maintained on a 12:12 hours light:dark cycle, with standard chow and water freely available. Females were used due to their better incidence and clinical course of the disease and also for the recommendation to use females in animal models for Multiple Sclerosis, a disease with higher incidence in females than males (Rahn et al. 2014). Animal were handled according to European legislation (86/609/EU) and procedures were approved by the Ethics and Scientific Committees of the University of Barcelona (UB) and registered at the "Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Spain". All animals used for treatment comparisons were subject to the same conditions, individuals distributed randomly to treatment or control groups and experimental procedures were performed at the same time in order to guarantee maximal homogeneity and avoid the influence of individual factors such estrous cycling that could interfere with data analysis (Rahn et al. 2014).

Reagents

Diazoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (50 mM) of diazoxide were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Solutions for cell treatment were prepared by diluting the stock solution in culture media immediately before being added to the cells (final DMSO concentration: 0.5%). Solutions for animal treatment were prepared by diluting the stock solution at each treatment day (final DMSO concentration: 0.3%).

Mouse splenocytes and $CD4^+$ T cells preparation

Splenocytes were isolated from the spleens of mice. Cells were gently flushed out from the spleens using a syringe plunger with PBS and then centrifuged (1000rpm, 4ºC). The pellet was resuspended and left 5 minutes on ice in ACK lysing buffer (Invitrogen, Eugene, OR, USA) to remove erythrocytes. After further centrifugations, cells were resuspended in 10 ml of complete medium RPMI (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), Hepes 10mM (Invitrogen), Sodium Pyruvate 1mM (Invitrogen), non-essential aminoacids 1% (Sigma-Aldrich) and 50μM β-Mercaptoetanol (Sigma-Aldrich).

$CD4^+$ T cells were isolated using Dynabeads FlowComp Mouse $CD4^+$ kit (DYNAL, Invitrogen) following manufacturer's instructions. After isolation, cells were counted and the viability was determined by trypan blue exclusion. The purity for $CD4^+$ cells (>92%) was assessed by FACS (Gallios BD Bioscience).

EAE induction and treatment

EAE was induced by immunization with > 95% pure synthetic myelin oligodendrocyte glycoprotein peptide 35-55 (rat MOG 35-55, MEVGWYRSPFSRVVHLYRNGK; EspiKem Srl, Florence, Italy). Mice were injected subcutaneously at one side of the flank with 100 μL solution containing 150 μg of rat MOG in complete Freund's adjuvant (Sigma-Aldrich) and 5 mg/mL Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA). Mice also received intraperitoneal injections of 150 ng pertussis toxin (Sigma-Aldrich) in 100 μL PBS immediately after MOG injection and 48 hours later. Mice were scored daily for signs of EAE on a scale of 0 to 6 using the following criteria: 0, no clinical signs; 1, distal limp tail; 1.5, complete limp tail; 2, mild paraparesis of the hind limbs, unsteady gait and impairment of righting reflex; 3, moderate paraparesis, partial hind limb paralysis, voluntary movements still possible

and ataxia; 4, paraplegia and forelimb weakness; 5, tetraparesis; 6, moribund state. When clinical signs were intermediate between two grades of the disease, 0.5 was added to the lower score. Treatment began when the EAE clinical score was ≥ 1 (appearance of clinical signs). The MOG-immunized mice were administered either 0.8 mg/kg diazoxide (treated group) or diluent (0.3% DMSO in water, vehicle group) for 15 days by oral gavage.

Proliferative response of lymphocytes and treatment

Splenocytes were isolated from the spleens of EAE mice after 15 days of diazoxide or vehicle treatment (n=3 per each group) and assayed *ex vivo* for their response to specific lymphocyte stimulus anti-CD3 (KT3, coated at 1μg/ml; Serotec, Oxford, England, UK) and anti-CD28 (37.51, coated at 2.5 μg/ml; eBioscience, San Diego, CA, USA). For *in vitro* studies, splenocytes and $CD4^+$ T cells were isolated from wild type animals. Proliferation assay was carried out by plating 2.10^5cells/well in 96 well plates and exposed to anti-CD3/CD28 for 72 hours. Cells were treated with diazoxide at various concentrations (100-0.1 μM) after plating and the treatment remained during 72 hours. Cell proliferation was determined by colorimetric Bromodeoxyuridine (BrdU) Cell Proliferation kit (Calbiochem, Darmstadt, Germany) following manufacturer's instructions. In brief, 20 μl of BrdU labelling solution diluted 1:2000 in a culture medium was added to each well for the last 18h of culture. After removing the medium, cells were fixed, and the anti-BrdU peroxidase working solution was added to each well and incubated for an hour at room temperature. Following several washes, the substrate solution was added, color was developed, and absorbance was measured with a microplate reader at 450 nm. Proliferation rate was calculated as follows: the absorbance of cells in the presence of anti-CD3-CD28 divided by the absorbance of

cells in the absence of antibodies. Moreover, carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) (2.5 μM) staining was used to confirm proliferation of the CD4-positive population in the whole splenocytes culture.

Generation, maturation and treatment of mouse bone-marrow derived dendritic cells (BMDCs)

Bone marrow from C57BL/6 mice was harvested by flushing femurs. After a washing and a centrifugation step cells were diluted in Petri dish to final concentration of 4.10^6 cell/ml. Bone marrow progenitor cells were cultured in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) to stimulate proliferation and maturation of DCs. Cells were maintained at 37ºC and 5%CO_2. Media and GM-CSF was replaced at day 3, 6 and 8 after. At day 9, lipopolysaccharide (LPS, 1 μg/ml, Sigma) was added for 24 hours to induce DC maturation. To explore if the compound has an effect on DC development, cells were treated from the first day of DC isolation with different concentrations of diazoxide (100-0.1 μM). Moreover, to discriminate if the compound acts on DC development or on DC maturation, on day 9, one diazoxide group was treated with LPS while the other remained unstimulated. On day 10, after 24 hours LPS treatment, DCs were harvest and stained for phenotype and maturation markers for flow cytometry.

Flow cytometry

Cells were resuspended in FACS buffer and blocked with anti-mouse CD16/32 (eBioscience) for 15 minutes and, then, stained with different combinations of primary - conjugated Abs for 20 minutes at 4ºC. After a washing step, cells were resuspended in FACS buffer and analyzed by FACS (FACSGallios, BD Biosciences, San Jose, CA,

USA). The following antibodies were used: For spleen lymphocytes determination, CD4-PE (dil. 1:200) (eBiosciense). For lymphocytes activation studies CD69-FITC, CD25-APC, CD4-PE or -APC (dil. 1:200) (eBioscience). For surface DC staining, CD83-APC, MHCII-FITC, CD80-PerCP-Cy5.5 and CD86-APC-Cy7 (dil. 1:200) (BD Bioscience). 7-AAD was used to determinate the living population (eBioscience).

Isolation of total protein

For isolation of total proteins from cell cultures, after a cold PBS wash, total proteins were recovered in 80 μL per well of RIPA buffer supplemented with complete protease inhibitor cocktail tablets. The samples were sonicated and stored at −80°C. Protein amount was determined by the Lowry assay (Total Protein Kit micro-Lowry, Sigma-Aldrich).

Western blot

40 μg of proteins from denatured (100°C for 5 minutes) total extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis together with a molecular weight marker (Full Range Rainbow Molecular Weight Marker, Amersham, Buckinghamshire, UK), and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After washing in Tris-buffered saline (TBS: 20 mM Tris, 0.15 M NaCl, pH 7.5) for 5 minutes, dipping in methanol for 10 s and drying in air, the membranes were incubated with the following primary antibodies overnight at 4°C: polyclonal rabbit anti-Kir6.1 or polyclonal rabbit anti-Kir6.2 (both 1:200, Alomone, Jerusalem, Israel), polyclonal rabbit anti-SUR1 (1:100, Alomone) and monoclonal mouse anti-β-actin (1:100000, Sigma-Aldrich) diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% non-fat dry milk). The membranes

were then washed twice in 0.05% Tween-20 in TBS for 15 s and incubated with the following horseradish peroxidase (HRP)-labeled secondary antibodies for 1 hour at 23 to 25°C: donkey anti-rabbit (1:5000, Amersham) or goat anti-mouse (1:5000, Santa Cruz Biotechnology, St. Cruz, CA, USA). After extensive washes in 0.05% Tween-20 in TBS, they were incubated in ECL-Plus (Amersham) for 5 minutes. Membranes were then exposed to the camera and the pixel intensities of the immunoreactive bands were quantified using the percentage adjusted volume feature of Quantity One 5.6.4 software (Bio-Rad Laboratories, Hercules, CA, USA).

Histological and immunohistochemical analysis

Hematoxylin and eosin (H&E) staining was used for the histological spleen studies. For immunohistochemical studies and cell staining, EAE spinal cord sections and $CD4^+$ T cells were first blocked in PBS (0.5% Triton) containing 10% goat serum (Vector) for 2h. Sections were then incubated with anti-CD3 (1:300, Serotec) and anti-Kir6.2 (1:300, Alomone, Jerusalem, Israel) at 4ºC overnight, followed by secondary antibodies Alexa 488 and 596 (1:2000 and 1:500, respectively) for 2h. Nuclear stain Hoechst 34580 (2 μg/mL; Invitrogen) was added prior to final washes and then samples were mounted using ProLong Gold antifade medium (Invitrogen).

Statistical Analysis

Data are expressed as the mean ± SEM unless specified. Statistical analysis of cell treatments was carried out using one-way ANOVA followed by Newman-Keuls post test when three or more experimental groups were compared and t-student for two groups comparison. Data on the effect of EAE treatment on clinical signs were analyzed

using the Mann–Whitney test for nonparametric data. Values of $p<0.05$ were considered statistically significant.

RESULTS

Lymphocyte proliferation is decreased in spleens from diazoxide treated EAE mice

EAE mice were orally treated with 0.8 mg/kg diazoxide from the onset of clinical signs (clinical score > 1) of the disease for a period of 15 days. Diazoxide treatment showed a beneficial effect on the disease severity compared to vehicle treated animals (Figure 1A). The amelioration was significant in the initial and in the chronic phase of the EAE (At day 3, 4 and 15, $p < 0.05$). No adverse effects related to the treatment were observed in any of the treated animals. At the end of the treatment, spleens from EAE animals were dissected and splenocytes isolated after erythrocyte lysis. Afterward, flow cytometry for CD4 positive cells were performed in isolated splenocytes. Results showed that diazoxide did not alter the percentage of CD4 positive events (32.1±1.6 from EAE vehicle *vs.* 31.1±1.7 from EAE diazoxide, t = 0.4, $p > 0.05$, t-test) (Figure 1B). In addition, we examined the proliferative capacity of splenocytes under unspecific T lymphocyte stimulation with anti-CD3 and anti-CD28 antibodies. After 72 hours of stimulation, proliferation was reduced in cells obtained from diazoxide treated animals when compared to vehicle treated EAE mice (4.6 ± 0.6 *vs.* 6.7 ± 0.9 respectively; t = 1.89, $p = 0.035$, t-test) (Figure 1C). Moreover, spleens from vehicle and diazoxide treated EAE mice displayed different morphology. Whereas in control EAE mice spleens showed reduced size and external signs of necrosis, in diazoxide treated animals spleens were better preserved and resembled in size and aspect those from non-immunized animals (Figure 2A). Upon cellular count, results showed an increase of mononuclear cells in diazoxide treated animals compared to vehicle group (EAE diazoxide: $45.10^6 \pm 5.10^6$; EAE vehicle $27.10^6 \pm 4.10^6$ cells, t = 2.9, $p = 0.0067$, t-test) (Figure 2B). H&E staining of histological sections from EAE spleens confirmed the preservation of spleen histoarchitecture in diazoxide treated animals (Figure 2C). In

contrast, spleens from vehicle EAE mice showed clear signs of atrophy, condensed nuclei and intercellular disruption (insert in Figure 2C).

SUR and Kir K_{ATP} channels subunits are expressed in splenocytes, $CD4^+$ isolated cells and CD3 infiltrated lymphocytes.

In order to elucidate if K_{ATP} channels subunits are expressed in lymphocytes, immunofluorescence detection for Kir6.2 and CD3 was performed in both, $CD4^+$ spleen isolated cells and CD3 infiltrated lymphocytes in the spinal cord from EAE animals. Upon microscopic observation, results showed positive expression of CD3 and Kir6.2 in most of $CD4^+$ T cells (Figure 3A). Spinal cord infiltrated T lymphocytes identified as CD3 positive cells also showed Kir6.2 expression (Figure 3B). Kir6.1, Kir6.2 and SUR1 expression was also analyzed in total splenocytes and CD4 positive T cells isolated from spleen by western blotting. Results showed positive signaling for all the K_{ATP} channel subunits studied in both, splenocytes and isolated $CD4^+$ cells (Figure 3C).

Diazoxide does not influence lymphocyte activation

In order to explore if diazoxide could modulate the activation of lymphocytes, we analyzed an early activation surface marker, CD69, and a late activation surface marker, CD25 on splenocytes and $CD4^+$ cells by flow cytometry. Generally, mouse T cells *in vitro* show a relatively low activation status. Anti-CD3-CD28 stimulation after 4h showed a high increase of CD69 expression in T cell population by close to tenfold. Diazoxide treatment did not modulate the percentage of positive CD69 cells at any dose tested in splenocytes cells (100 and 0.1 μM, $F_{(2,8)} = 1.2$, $p > 0.05$) (Figure 4A). Similar

results were observed for the CD25 marker. As shown in the representative flow cytometry diagrams, the anti-CD3-CD28 stimulation after 16h increased CD25 expression by close to tenfold. This activation was not modified by diazoxide treatment (100 and 0.1 μM, $F_{(2,8)} = 4.1$, $p > 0.05$) (Figure 4B). The same results were observed when experiments were performed in $CD4^+$ isolated population for CD69 ($F_{(2,11)} = 0.4$, $p > 0.05$) and CD25 ($F_{(2,11)} = 0.2$, $p > 0.05$) activation T-cell markers (Figure 4 A-B). Taken together, results showed that diazoxide does not influence the activation rate of lymphocytes.

Diazoxide modulates proliferation of splenocytes but not of isolated $CD4^+$ T cells

In order to clarify if diazoxide has a direct effect on lymphocytes proliferation, an *ex vivo* BrdU incorporation proliferation assays were performed on splenocytes from healthy spleen mice. Lymphocyte proliferation was induced by direct T cell receptor (TCR) engagement using anti-CD3-CD28 stimulation. Stimulated splenocytes showed a 5 fold increased proliferation compared to unstimulated cells (Figure 5A). Our results showed a diazoxide-related decrease in proliferation rate compared to CD3-CD28 stimulated splenocytes (up to 45 ± 7% for 100 μM and 37 ± 9% for 0.1 μM, $F_{(4,19)} = 8.0$, $p = 0.0021$) (Figure 5A). CFSE labeling was used to confirm these results and to prove that proliferating cells were indeed CD4-positive T cells (data not shown). To explore if this decrease in proliferation was a direct effect on the $CD4^+$ T cell population, the same proliferation experiments were performed using sorted $CD4^+$ T cells. Stimulated $CD4^+$ T cells showed a 5.8 fold increased proliferation compared to unstimulated cells (Figure 5B). However, when $CD4^+$ T cells were treated with different concentrations of diazoxide, the effect was abolished and no significant changes in proliferation rate were observed ($F_{(4,19)} = 1.7$, $p > 0.05$) (Figure 5B). No change of viability was observed by

flow cytometry using PI (data not shown). These results indicate that diazoxide does not have a direct effect on isolated lymphocytes but could modulate immune intercellular communication affecting splenocyte proliferation.

Diazoxide modulates dendritic cell development and maturation

The results presented above indicate that diazoxide may modulate T cell response by influencing accessory cells such as antigen-presenting cells (APCs). Dendritic cells (DCs) are professional APCs that play a key role in initiating and regulating the immune response. To explore the effect of diazoxide on DCs differentiation and antigen presenting capacity, DCs were generated *in vitro* from mouse bone marrow progenitor cells in the presence of GM-CSF. To explore if diazoxide has an effect on DCs development, cells were treated from the first day of isolation with different concentrations of compound (100 - 0.1 μM). Moreover, to discriminate if the compound acts on DC development or on DC maturation, one of the DC cultures was treated on day 9 with 1μg/ml LPS for 24 hours, while the other remained unstimulated. Afterwards, the percentage of DCs positive for CD83, used as marker for DC development, was analyzed. Results showed a percentage decrease of CD83 in DCs treated with diazoxide compared to DCs control. This decrease was mainly observed at low diazoxide doses in both LPS stimulated DCs (0.1 μM: 13 ± 1%, $F_{(3,11)} = 12.4$, $p = 0.0055$) and unstimulated DCs (0.1 μM: 33 ± 7%, $F_{(3,11)} = 5.4$, $p = 0.0382$) (Figure 6 A-B).

Next, the expression on DCs of the co-stimulatory markers CD80, CD86 as well as the expression of MHCII were analyzed by flow cytometry. Results showed that, the percentage of positive events for the markers was slight decreased in diazoxide treated

DCs when compared with the control DCs. This decrease was significant for CD80 at 0.1 and 10 μM diazoxide ($F_{(4,34)} = 4.1$, $p = 0.0107$) but not for CD86 ($F_{(4,34)} = 1.4$, $p > 0.05$) and MHCII ($F_{(4,29)} = 0.7$, $p > 0.05$) (Figure 6 C). Moreover, the amount of molecules expressed on a single cell as reflected by mean fluorescence intensity (gMFI) was slight decreased by the treatment (data not shown). These results indicate that diazoxide, particularly at low doses, can attenuate DC development and antigen presenting capacity.

DISCUSSION

K_{ATP} channel activators such as diazoxide are well known compounds with approved use for the treatment of hypoglycemia and hypertension and with well documented cardio and neuroprotective properties (Miura and Miki 2003; Yamada and Inagaki 2005; Judge and Smith 2009). However, little is known about their effects on the immune system. Therefore, the present work focused on the immune effects of diazoxide during MOG_{35-55}-induced EAE. Our results demonstrate that diazoxide, at tested doses, promoted a significant improvement of the clinical course of the EAE, without suppressing lymphocyte activation and CNS infiltration. Nevertheless, spleens from treated animals showed a better preservation and lower proliferative capacity under lymphocytic stimulation *ex vivo*, indicating a general reduced T cell responsiveness in the treated animals. In addition, our results demonstrated for the first time the expression of Kir6.1, Kir6.2 and SUR1 of K_{ATP} channel subunits in $CD4^+$ lymphocytes. However, *in vitro* addition of diazoxide inhibited T cell proliferation but only when total splenocytes were used and not when isolated $CD4^+$ cells were treated. Yet, in the *in vitro* cultures using whole splenocytes, the proliferating lymphocytes were identified as $CD4^+$ by flow cytometry. Diazoxide shows no effects in lymphocyte activation but can succinctly affect DC differentiation and their expression of CD80 molecules. Even though the observed changes were modest, the effect was constant and significant. As the anti-CD3-CD28 stimulation is independent of antigen presentation, the decreased T cell proliferation observed in splenocytes could be caused by diazoxide-induced factors in cells other than $CD4^+$ T cells. This effect could be for instance attributed to IL-10, which may enhance regulatory T cell response and, thereby, downregulate $CD4^+$ proliferation after antigenic stimulus (Marshall et al. 2003). However, further analysis of soluble factors is required to explain the acute anti-

proliferative effect of diazoxide as long as, although the activation may have not been affected, the differentiation may have been altered. Additional analysis on the MOG-specific $CD4^+$ T cells ex vivo would be also interesting.

T cell imprinting and lymphocyte activation by antigen presentation is the key step for inducing an effective adaptive (auto) immune response (Mora et al. 2005; Abadie et al. 2009). Besides other factors, calcium signaling, modulated by mitochondrial activity and bioenergetic metabolism can modify both, the antigen presenting cell behavior and lymphocyte activation (Carrasco-Marin et al. 1998; Quintana et al. 2009). In this context, potassium channels such as Kv1.3 or $K_{Ca}3.1$ has been postulated as possible targets for the treatment of autoimmune disorders (Toldi et al. 2011; Gocke et al. 2012; Kuras et al. 2012; Wang et al. 2013). K_{ATP} channels could regulate extracellular calcium signaling linking cell metabolism with membrane electric potential and, thus, emerging as an interesting target for immune modulation. Diazoxide has been described to suppress Jurkat proliferative rate through mitochondrial targeting by inhibiting influx Ca^{2+} release-activated Ca^{2+} channels (I_{CRAC}) dependent calcium mobilization (Holmuhamedov et al. 2002). These results, although they are difficult to extrapolate to physiological lymphocyte activity, demonstrate the capacity of KCOs to modulate lymphocytic behavior. K_{ATP} channel activators have been proposed to modulate inflammatory activity in different situations: diazoxide has been described to favorably shift the systemic cytokine balance after coronary artery bypass towards an anti-inflammatory response increasing the ratio of IL-10 in relation to pro-inflammatory cytokines (Wang et al. 2004). Moreover, nicorandil and diazoxide were shown to regulate leukocyte activation by inhibiting polymorphonuclear leukocyte migration upon ischemia reperfusion (Yasu et al. 2002). Interestingly, regarding the local CNS inflammatory activity, various authors showed that diazoxide exerts an anti-

inflammatory effect in microglia (Zhou et al. 2008; Virgili et al. 2011), the resident macrophage of the CNS and one of the main immune effectors during EAE (Almolda et al. 2011). Our results now suggest that, although diazoxide could not suppress lymphocyte activation it could attenuate autoimmune damage by modulating innate immune response, splenocytes proliferation rate and antigen presentation. These results agree with the previous reported effects of oral diazoxide administration in EAE animals, where preventive administration of the compound did not suppress the generation of autoimmunity or lymphocyte infiltration but reduce the area affected by the inflammatory autoimmune attack (Virgili et al. 2011). The effects of diazoxide as immunomodulator could be related to its protective capacities against cellular stress (Ichinose et al. 2003; Huang et al. 2006; Liu et al. 2006). For instance, spleens from EAE treated mice showed high level of preservation probably as a consequence of a reduced oxidative stress. In this context, a recent study demonstrated that activation of the mito-K_{ATP} channel reduced apoptosis of spleen mononuclear cells induced by hyperlipidemia (Alberici et al. 2013). During immune activation, it is well known that cellular stress and apoptosis could generate a positive feedback loop that would increase inflammatory damage (Freund et al. 2010; Gallo and Gallucci 2013). Moreover, atrophy of the thymus, spleen and lymph nodes has been demonstrated to be associated to an increased Th1 versus Th2 response and an enhanced MOG antibody production in EAE mice (Tsunoda et al. 2005). In this context, apoptosis inhibition, mitochondrial stabilization and anti-oxidative mechanisms, which are well-described actions of diazoxide (Ichinose et al. 2003; Liu et al. 2006; Moghtadaei et al. 2012), may contribute to control the autoimmune reaction also in the absence of immunosuppression.

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COMPETING INTERESTS

NV, PM, MP, JFEP have applied for a PCT application ‘‘Diazoxide for use in the treatment of a central nervous system (CNS) autoimmune demyelinating disease’’ (application number PCT/EP2011/050049). MP is CEO in Neurotec Pharma and also holds shares in the company. NV, PM, BW, JFEP are employed in Neurotec Pharma. CC, AW, MJR and CID declares no competing interests.

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FIGURE LEGENDS

Figure 1. Diazoxide ameliorates EAE course and decreases splenocyte proliferative capacity in the MOG_{35-55} EAE mice

Oral 0.8 mg/Kg/day diazoxide treatment from the disease onset ameliorated EAE clinical course at some days of the experiment (n=14 mice/group) (A). Flow cytometry analysis for $CD4^+$ T cells in total splenocytes from diazoxide and vehicle treated EAE animals showed no differences in the percentage of positive events (n=12 mice/group) (B). T cell proliferation after splenocytic stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours showed a significant reduction in the proliferation rate of cells obtained from diazoxide treated animals when compared to vehicle treated EAE mice (C). Results showed as mean ± SEM. *: $p<0.05$; **: $p<0.01$ compared to Vehicle group

Figure 2. Diazoxide treatment of MOG_{35-55} EAE mice preserves spleen integrity

Macroscopic images of non-immunized healthy mice (left), vehicle control EAE mice (center) and 0.8 mg/Kg/day diazoxide treated EAE mice (right) showed hypoplasia and signs of degeneration in control EAE but not in diazoxide treated EAE mice when compared to healthy animals (A). Upon splenocyte isolation, cell count demonstrated a significant increase of splenocytes in diazoxide treated EAE mice (n=12 mice/group) (B). Microscopic reconstruction of a spleen section stained with the H&E from control EAE mouse showed clear signs of necrosis (black arrows) and intercellular disruption (inferior square, higher magnification) whereas in the spleen from diazoxide treated EAE mouse, a normal tissue composition was observed (C). Scale bar for A: 5 mm. Scale bar for B: 1,5mm (for reconstruction) and 500 μm (for magnification). **: $p<0.01$ compared to Vehicle group.

Figure 3. K_{ATP} channel subunits are expressed in $CD4^+$ $CD3^+$ lymphocytes

Double immunofluorescent staining demonstrated positive signal for Kir6.2 (green) and CD3 (red) staining in spleen $CD4^+$ cells isolated with magnetic beads (A). Infiltrated T lymphocytes in the spinal cord white matter from EAE mice identified by CD3 positive signal (red) showed co-localization with Kir6.2 immunosignal (green, white arrows) (B). Western blot using protein from total splenocytes but also from spleen isolated $CD4^+$ lymphocytes demonstrated positive expression for Kir6.1, Kir6.2 and SUR1 K_{ATP} channel subunits (C). Scale bar: 25 μm for A and 100 μm for B.

Figure 4. Diazoxide does not modify lymphocytes activation in anti-CD3-CD28 T cell activation model

Representative flow cytometry results on CD69 and CD25 expression. After 4 hours stimulation, splenocytes and $CD4^+$ T cells increased CD69 expression. Treatment with diazoxide (100 and 0.1 μM) did not change CD69 profile (A). The late activation marker, CD25, increased in splenocytes and $CD4^+$ T cells after 16 hours of stimulation. Treatment with diazoxide (100 and 0.1 μM) did not modify the expression of this marker (B). A minimum of three experiments were performed for each condition.

Figure 5. Diazoxide modulates proliferation from splenocytes but not from $CD4^+$ T cells

Cell proliferation was determined by colorimetric BrdU incorporation assay. *Ex vivo* proliferation response of lymphocytes stimulated with anti-CD3-CD28 for 72 hours increased at least five fold times in splenocytes and $CD4^+$T cells compared to unstimulated lymphocytes (Cell proliferation rate for unstimulated cells =1, Not stimulated bar). Pre-treatment with diazoxide showed a significant decrease at all dose

tested compared to anti-CD3-CD28 stimulated splenocytes (A). In contrast when the same experiments were performed with $CD4^+$ T cell isolated cells diazoxide effect was abolished and there were no differences in proliferation rate compared to control (B). Results showed as mean ± SEM. *: $p<0.05$; **: $p<0.01$ treated cells compared to untreated CD3-CD28 stimulated cells. n= 4-5 independent experiments.

Figure 6. Diazoxide modulates dendritic cell development and maturation

Flow cytometry analysis after diazoxide treatment during DCs development and maturation. Representative panels show CD83 analysis in DCs stimulated and unstimulated with LPS (A). Results showed that the decrease in CD83 surface expression in diazoxide treated cells was independent from LPS stimulation. This decrease was more pronounced for the lower dose tested (0.1 μM) (B). Surface markers for maturation were also analyzed in DCs treated with LPS. Results showed that diazoxide treatment decrease the percentage of positive cells, compared to control. This decrease was significant for CD80 expression (C). Results showed as mean±SEM. *: $p<0.05$; **: $p<0.01$ compared to control DCs. n= 3-6 independent experiments.

Figure 1
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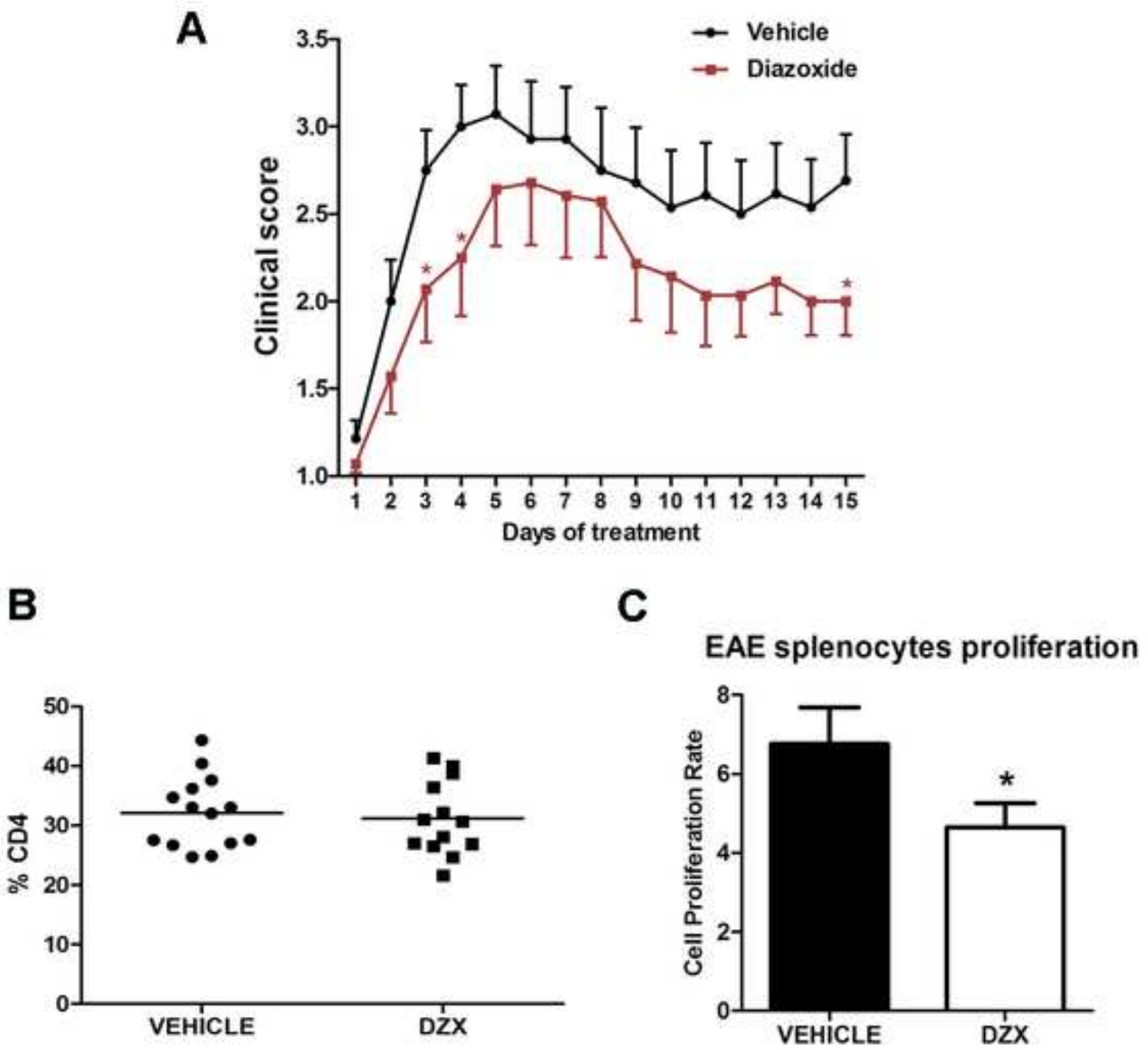

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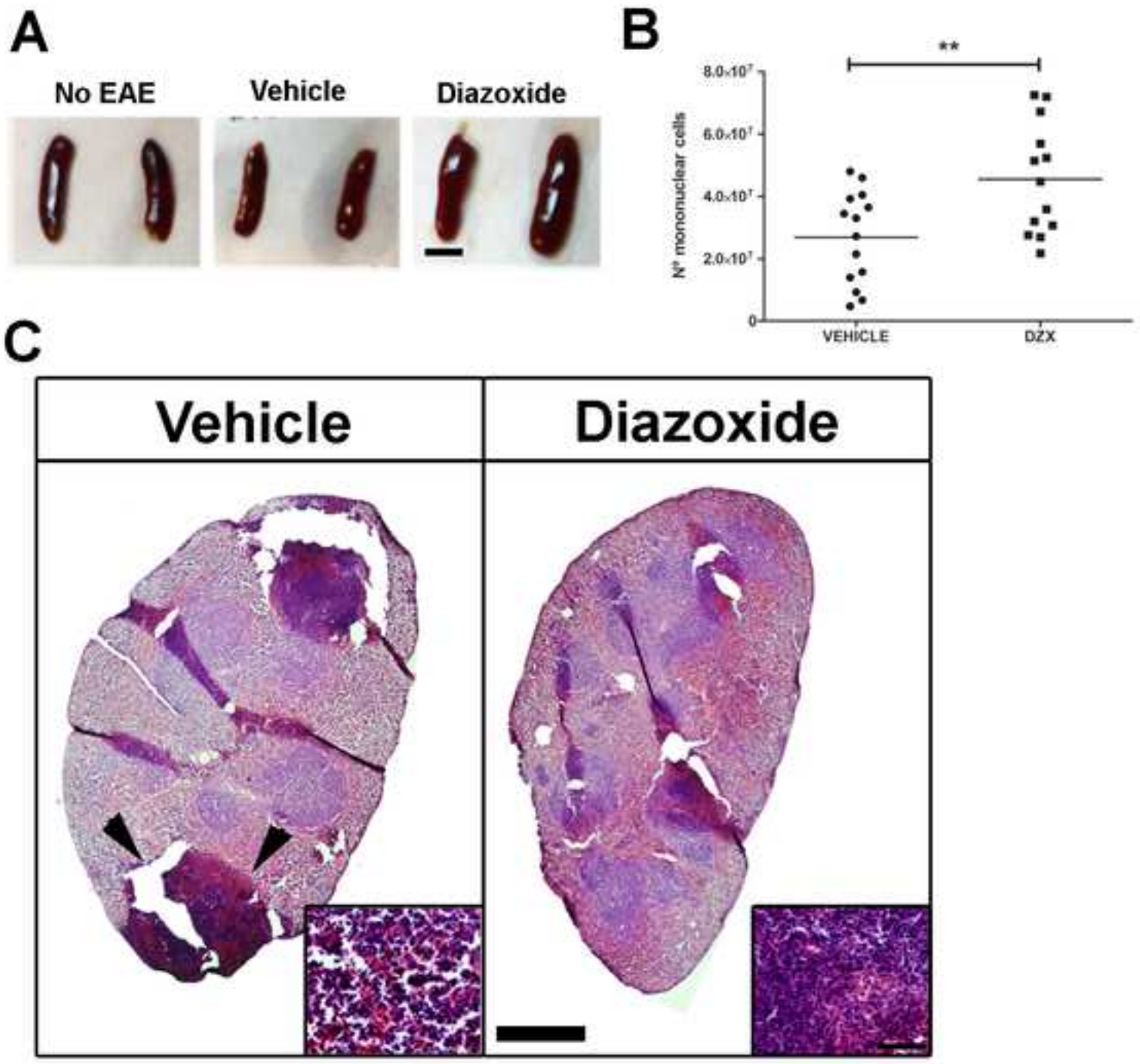

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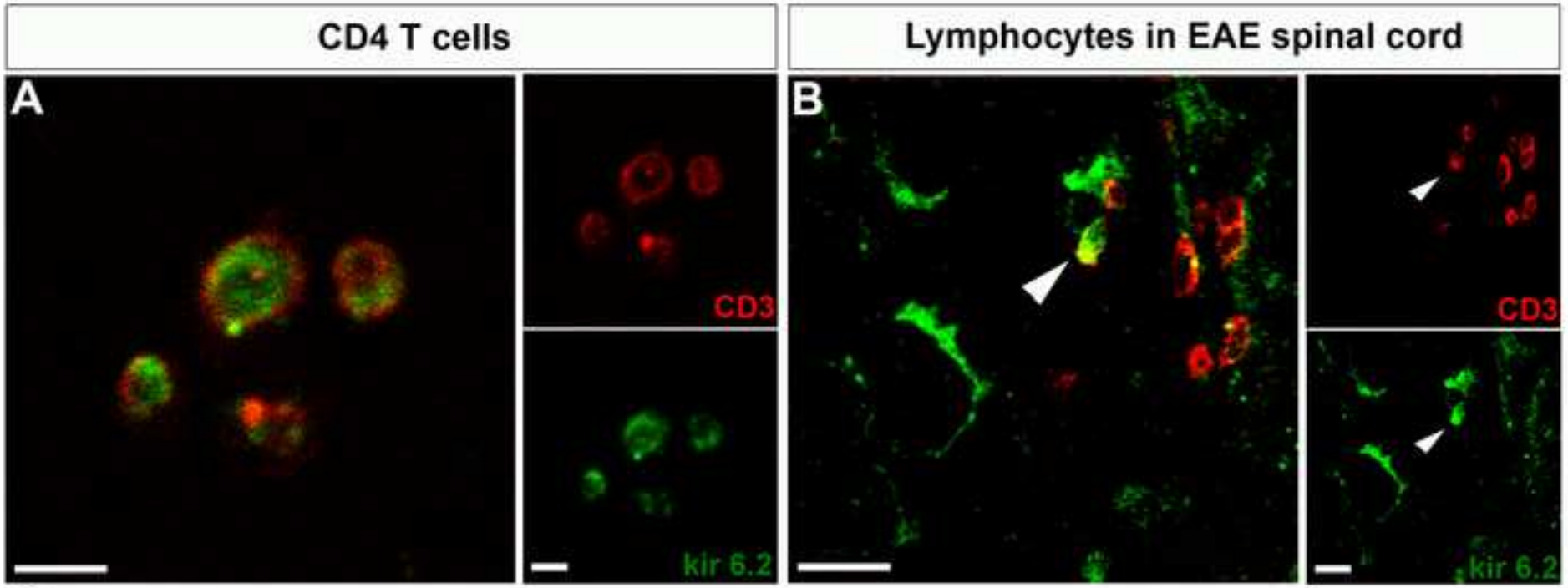

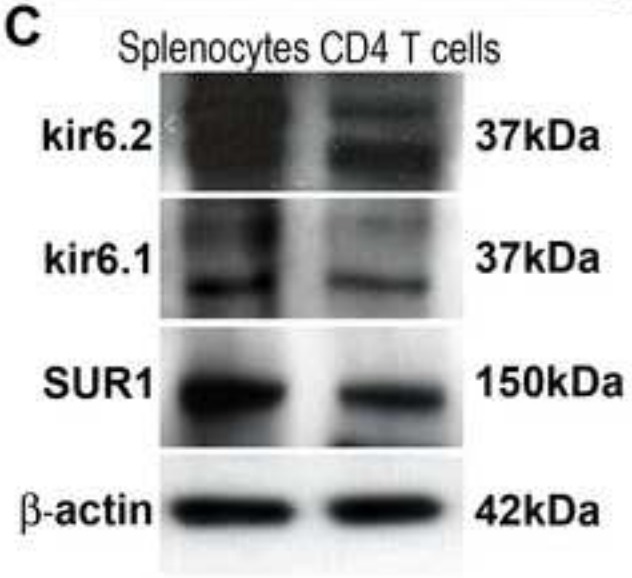

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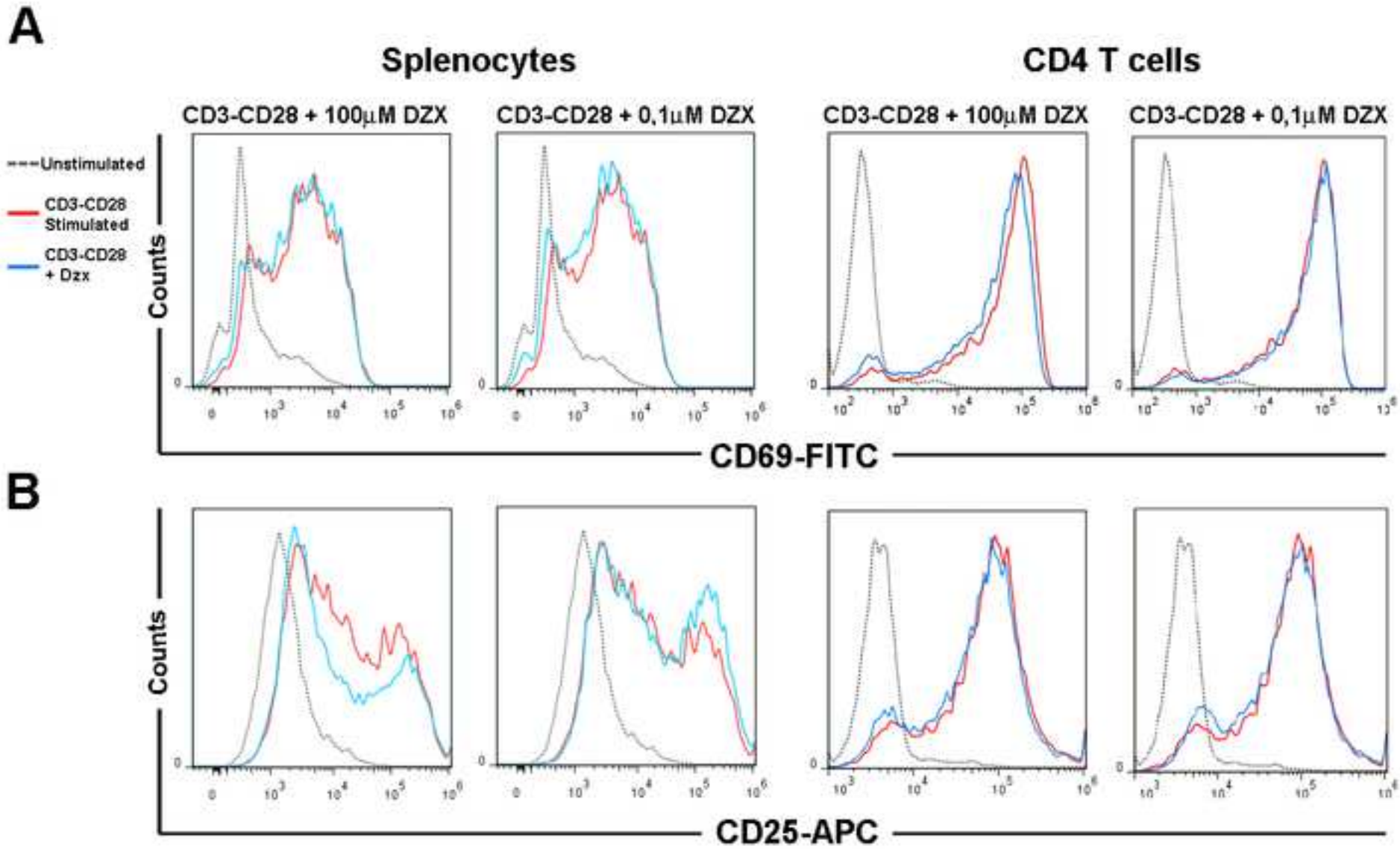

Figure 5 Revised
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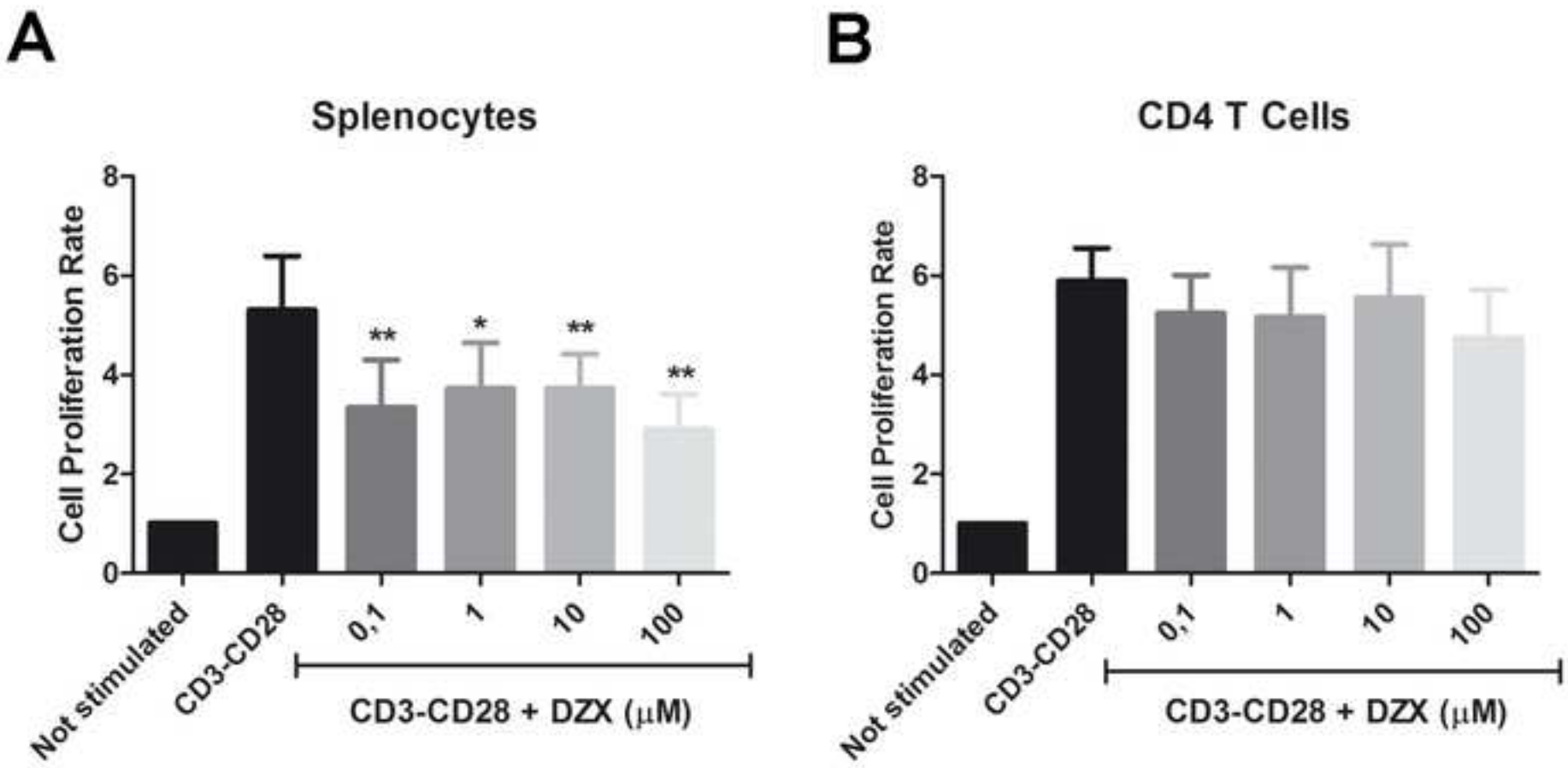

Figure 6 Revised

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