

**Inhibition of NF- $\kappa$ B enhances the capacity of immature dendritic cells to induce  
antigen-specific tolerance in Experimental Autoimmune Encephalomyelitis.\***

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Abbreviations used in this paper: APC, antigen presenting cell; pMHC, peptide/MHC complex; EAE, experimental autoimmune encephalomyelitis; DTH, delayed type hypersensitivity; PAMPs, pathogen-associated molecular patterns ; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; NF- $\kappa$ B, nuclear factor kappa B; H & E, hematoxylin and eosin stain.

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## ABSTRACT

Autoimmune disorders develop as a result of deregulated immune responses that target self-antigens and cause destruction of healthy host tissues. Due that dendritic cells (DCs) play an important role in the maintenance of peripheral immune tolerance we are interested in identifying means of enhancing their therapeutic potential in autoimmune diseases. It is thought that during the steady state, DCs are able to anergize potentially harmful T cells bearing TCRs that recognize self-peptide-MHC complexes. The tolerogenic capacity of DCs requires an immature phenotype, which is characterized by a reduced expression of costimulatory molecules. On the contrary, activation of antigen-specific naïve T cells is enhanced by DC maturation, a process that involves expression of genes controlled by the transcription factor NF- $\kappa$ B. We evaluated the capacity of drugs that inhibit NF- $\kappa$ B to enhance the tolerogenic properties of immature DCs in the Experimental Autoimmune Encephalomyelitis (EAE) model. We show that andrographolide, a bicyclic diterpenoid lactone, and rosiglitazone, a PPAR- $\gamma$  agonist, were able to interfere with NF- $\kappa$ B activation in murine DCs. As a result, treated DCs showed impaired maturation and a reduced capacity to activate antigen-specific T cells. Further, NF- $\kappa$ B-blocked DCs had an enhanced tolerogenic capacity and were able to prevent EAE development in mice. The tolerogenic feature was specific for myelin antigens and involved the expansion of regulatory T cells. These data suggest that NF- $\kappa$ B blockade is a potential pharmacological approach that can be used to enhance the tolerogenic ability of immature DCs to prevent detrimental autoimmune responses.

## INTRODUCTION

When the immune response mistakenly targets healthy host tissues autoimmune disorders develop. Although the mechanisms responsible for the loss of tolerance to self remain obscure, evidence for both genetic and environmental factors have been provided. Because dendritic cells (DCs) have emerged as key regulators of immune responses by their capacity to modulate the balance between immunity and tolerance (Banchereau et al., 2000), it is likely that alterations in their function can be involved in the pathogenesis of autoimmunity (Iruretagoyena et al., 2006). Therefore, DCs could be exploited to reestablish immune homeostasis by promoting on them a tolerogenic phenotype that could enhance their capacity to interfere with the activation of self-reactive T cells.

Due to their unique ability to efficiently capture antigens and activate naïve T cells to initiate the adaptive immune response, DCs are critical for the defense against infectious agents and tumors (Lanzavecchia and Sallusto, 2001). In addition to activating immune responses, DCs also play a central role in peripheral T cell tolerance, by inducing T cell anergy or unresponsiveness to self-antigens (Steinman et al., 2003). Consequently, it is thought that the interaction between DCs and T cells can lead to at least two distinct outcomes, while immature DCs would induce antigen-specific tolerance, mature DCs would promote immunity by priming naïve T cells (Steinman et al., 2003). The ability of DCs to determine the outcome of antigen-specific immune responses emphasizes their potential key role in preventing autoimmunity. Immature DCs have demonstrated to be useful for inducing tolerance in some autoimmune (Jonuleit et al., 2000) and transplantation models (Sato et al., 2003). However, further research is

required to enhance their tolerogenic capacity and to make them eventually suitable for clinical applications. Interestingly, several studies have shown that the activity of some immunosuppressive drugs could be in part mediated by interfering with DC function, and support the notion that pharmacological interference with DCs could be useful to abridge autoimmune responses (Hackstein et al., 2001; Xing et al., 2002; Iruretagoyena et al., 2005). Those studies have established DCs as targets for classical immunosuppressive drugs and set the stage for the design of pharmacological strategies aimed to induce “tolerogenic” DCs for the treatment of autoimmune diseases.

NF- $\kappa$ B is a transcription factor known to play a central role in immune and inflammatory responses and is involved in the transcriptional regulation of many genes important for DCs function (Ghosh et al., 1998; Li and Verma, 2002). NF- $\kappa$ B can be activated in DCs by several stimuli, including proinflammatory cytokines, LPS, etc. In response to these stimuli, I $\kappa$ B is phosphorylated and targeted for proteasome degradation, allowing NF- $\kappa$ B translocation to the cell nucleus and the subsequent transcription of genes controlled by specific promoter elements (Ghosh et al., 1998). Increased activation of NF- $\kappa$ B has been reported for T cells derived from patients affected by autoimmune disorders (Burgos et al., 2000). Due that most classical DC maturation stimuli mediate their activity by inducing transcription of genes controlled by NF- $\kappa$ B, this transcription factor is a key element on determining the phenotype of DCs (O'Sullivan and Thomas, 2002).

Here we have evaluated whether an immature phenotype on DCs can be promoted by two drugs that interfere with NF- $\kappa$ B function, andrographolide and rosiglitazone. Andrographolide is a bicyclic diterpenoid lactone derived from extracts of *Andrographis*

*paniculata*, for which a variety of anti-inflammatory properties have been reported (Iruretagoyena et al., 2005). Rosiglitazone is a thiazolidinedione agonist for peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ), which is used for the treatment of Type 2 Diabetes. PPAR- $\gamma$  is a ligand activated transcription factor that regulates cell growth, lipid metabolism and insulin sensitivity and recently reported to be anti-inflammatory (Nencioni et al., 2003; Mohanty et al., 2004). Here we show that both, andrographolide and rosiglitazone are able to interfere with the activation of NF- $\kappa$ B. As a result, DCs treated with these drugs fail to mature in response to LPS and to activate antigen-specific T cells *in vitro*. The potential capacity of these drug-treated DCs to prevent an autoimmune response *in vivo* was evaluated in experimental autoimmune encephalomyelitis (EAE), a murine model of Multiple Sclerosis (MS) (Kuchroo et al., 2002). We observed that immature DCs treated *ex vivo* with either andrographolide or rosiglitazone and pulsed with myelin antigens show an enhanced capacity to prevent EAE development in mice. Our data suggest that an immature and tolerogenic phenotype can be promoted in DCs by pharmacologically blocking NF- $\kappa$ B function and that this approach could be useful to enhance the capacity of DCs to promote tolerance to self-antigens.

## METHODS

### *Animals*

Six to eight weeks old female C57BL/6 mice were used in these experiments and kept under pathogen-free conditions at the animal core facility of the Pontificia Universidad Católica de Chile. All animal work was performed according to institutional guidelines.

### *Reagents and synthetic peptides*

Andrographolide was purchased from Sigma-Aldrich Chemical Co. A stock solution was prepared by dissolving andrographolide in dimethylsulphoxide (DMSO) at 50 mM. Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in DMSO (10 mM stock solution). Drugs were diluted in PBS immediately prior to experiments and added to the DCs culture at 10  $\mu$ M, a concentration that can influence DC function without affecting their viability (Iruretagoyena et al., 2005 and data not shown). DMSO controls were included when applicable (data not shown). Myelin oligodendrocyte glycoprotein-derived peptide (MOG<sub>35-55</sub>, MEVGWYRSPFSRVVHLYRNGK), Chicken Egg Ovalbumin (OVA)-derived peptide SIINFEKL (OVA<sub>257-264</sub>, for activation of H-2K<sup>b</sup>-restricted CD8<sup>+</sup> T cells) and OVA-derived peptide TEWTSSNVMEERKIKV (OVA<sub>265-280</sub>, for activation of I-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells) were synthesized by solid-phase method using Fmoc chemistry on an automated 433A peptide synthesizer (Applied Biosystems, Foster City, CA) at the Peptide Synthesis Facility of the Albert Einstein College of Medicine. All peptides were

purified to > 98 % homogeneity by reversed-phase HPLC on a Vydac C-18 column (2.1 or 4.6 mm x 25 cm, 300 Å) using HP-1090M HPLC (Hewlett Packard). The identity of the purified peptide was determined by a tandem quadrupole mass spectrometer (TSQ700, Finnigan MAT, San Jose, CA).

### ***Transient transfection assays***

Using FuGENE® 6 (Roche Diagnostics, Indianapolis, IN), mouse epithelial MLE-12 cells (ATCC # CRL-2110) were co-transfected with the NF-κB-luciferase reporter construct 3xMHC-luc and the renilla luciferase reporter pGL3-hRL. This latter construct was used as an internal control to determine transfection efficiency. 24 h post-transfection, cells were left untreated or incubated with andrographolide (10 μM) or rosiglitazone (10 μM) for 12 h, followed by activation with LPS at the indicated concentrations. Cell lysates were prepared for analysis of luciferase and renilla activities using Dual Luciferase Reporter System (Promega, Madison, WI). Results are expressed as relative luciferase/renilla units.

### ***DC maturation assays***

Bone marrow-derived DCs were prepared as previously described (Inaba et al., 1992). Briefly, DCs were grown from bone marrow progenitors in RPMI 1640 containing 5 % FCS (Hyclone, Utah) supplemented at 3 % vol/vol with supernatant from murine myeloma J558L cell line (ATCC # TIB-6) transduced with murine GM-CSF. Day 5 DCs were treated with andrographolide (10 μM) or rosiglitazone (10 μM) for 24 h (Xia et al., 2004), then the drugs were washed out and maturation was induced with LPS (Sigma, St.



Louis, MI) 1  $\mu$ g/ml for 36 h. Untreated control DCs and DMSO controls (data not shown) were included in all experiments. After LPS-induced maturation, cells were analyzed for expression of surface markers I-A<sup>b</sup>, CD86 and CD40 on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To evaluate DCs maturation, cells were double-stained with anti-CD11c-PE (clone HL3, PharMingen, San Diego, CA) plus either anti-I-A<sup>b</sup>-FITC (clone AF6-120.1, PharMingen, San Diego, CA), anti-CD86-FITC (clone GL1, PharMingen, San Diego, CA), or anti-CD40-FITC (clone 3/23, PharMingen, San Diego, CA), fixed on 1 % paraformaldehyde in PBS and analyzed by FACS. Data was analyzed using WinMDI software.

### ***Antigen-presentation assays***

Day 5 DCs were treated with andrographolide (10  $\mu$ M) or rosiglitazone (10  $\mu$ M) for 24 h. After this time, drugs were washed and DCs were pulsed for 16 h either with OVA protein or OVA peptides (OVA<sub>257-264</sub> for presentation on H-2K<sup>b</sup> or OVA<sub>265-280</sub> for presentation on I-A<sup>b</sup>). After antigen pulse, DCs were washed and co-cultured at different ratios with either 1.0 x 10<sup>5</sup> B3Z or 1.0 x 10<sup>5</sup> OT4H T-cell hybridomas. B3Z and OT4H are specific for H-2K<sup>b</sup>/OVA<sub>257-264</sub> and I-A<sup>b</sup>/OVA<sub>265-280</sub>, respectively and secrete IL-2 upon TCR stimulation (Shastri and Gonzalez, 1993). After 20 h of DC-T cell co-culture, IL-2 was measured on supernatants by cytokine ELISA using purified anti-mouse IL-2 (clone JES6-1A12, BD Biosciences Pharmingen) for capture and biotin rat anti-mouse IL-2 (clone JES6-5H4, BD Biosciences Pharmingen) for detection, as previously described (Kalergis et al., 2001; Iruretagoyena et al., 2005). DC viability was determined by Trypan blue exclusion. For phagocytosis assessment, control and treated DCs were

incubated for 4 h with Dextran-FITC (Molecular Probes, Eugene OR) at 37° or 4° (as negative control) and then evaluated by FACS. Immature DCs were compared to LPS-activated DCs.

### ***EAE induction and assessment***

Six to eight weeks old female C57BL/6 mice were injected *s.c* in the flank with 50 µg of MOG<sub>35-55</sub> peptide emulsified in Complete Freund's Adjuvant (Gibco BRL, NY) supplemented with heat-inactivated *Mycobacterium tuberculosis* H37 RA (Difco Laboratories, Detroit, MI). In addition, mice received *i.p.* injections of 500 ng Pertussis toxin (Calbiochem, La Jolla, CA) at the time of sensitization and 48 h later. Clinical signs of disease were seen usually between day 15 and 18 after sensitization and assessed daily according to the following scoring criteria: 0 = no detectable signs of EAE; 1 = flaccid tail; 2 = hind-limb weakness or abnormal gait; 3 = complete hind-limb paralysis; 4 = paralysis of fore and hind limbs; 5 = moribund or death. In order to prevent unnecessary animal suffering, mice severely affected by the disease were euthanized with the supervision of a veterinarian. Mean clinical score was calculated by adding every day clinical score for all mice in a group and then divided by the total number of mice. Daily mean clinical scores for each group were averaged and are shown for each week after disease induction.

### ***Injection of immature MOG-loaded DCs***

Treatment of mice consisted of 2 *i.v.* injections separated by one week of  $1.0 \times 10^6$  immature DCs, which were previously pulsed with MOG (5 µg/ml) for 24 hours.

Immature DCs injections were given two weeks before EAE induction. Mice received either MOG-pulsed immature DCs or MOG-pulsed immature DCs that were previously treated with andrographolide (10  $\mu$ M) or rosiglitazone (10  $\mu$ M) during 24 hours. As a control, a group of mice received immature DCs and treated immature DCs that were not pulsed with MOG peptide.

### ***MOG-specific T cell activation assays***

Draining inguinal and mesenteric lymph nodes were obtained on day 21 after EAE induction from control or immature DCs treated mice. Cellular suspensions obtained from lymph nodes were cultured ( $5.0 \times 10^5$  cells/well) in RPMI 1640 containing 5 % FCS with increasing concentrations of MOG<sub>35-55</sub> peptide. Cultures were incubated in 96-well round bottom plates for 48 h at 37 °C in a cell culture incubator. IL-2 release in response to MOG<sub>35-55</sub> peptide was determined on culture supernatants by cytokine ELISA as previously described (Kalergis et al., 2001; Iruretagoyena et al., 2005). IFN- $\gamma$  release was also determined by cytokine ELISA, but using purified anti-mouse IFN- $\gamma$  (clone R4-6A2, PharMingen, San Diego, CA) as a capture antibody and biotin rat anti-mouse IFN- $\gamma$  (clone XMG1.2 PharMingen, San Diego, CA) as a detection antibody.

### ***Spinal cord histological analysis***

Demyelination and inflammation of the central nervous system of mice with EAE were evaluated on day 21 by histological analysis. Mice were euthanized by Ketamine (1,5 mg/mice *i.p.*) and perfused by intracardiac infusion of 4% paraformaldehyde in PBS. Spinal cord samples were removed and frozen in Tissue-Tek (Sakura Finetek, CA). 10

µm transverse sections from lower thoracic and lumbar regions of the spinal cord were stained with Luxol Fast Blue and hematoxylin-eosin (H & E). The infiltration of mononuclear cells and loss of myelin was evaluated.

### ***Detection of anti-MOG antibody response***

Serum samples from mice were obtained on day 21 after sensitization with MOG<sub>35-55</sub> peptide and analyzed for the presence of MOG-specific IgG by ELISA. Briefly, ELISA plates (Falcon) were coated at 4 °C overnight with 10 µg/ml MOG<sub>35-55</sub> peptide in 0.1 M NaHCO<sub>3</sub> buffer (pH 8.4) and then blocked with PBS-BSA 1 % for 2 h at room temperature. Serum samples were diluted in PBS-BSA 1 % starting at 1:100 and incubated for 3 h at room temperature. IgG was detected with rabbit anti-mouse IgG antibody conjugated to horse-radish peroxidase (Amersham Pharmacia). After washing, TMB substrate (3,3',5,5' tetramethylbenzidine, Sigma, St. Louis, MI) was added and absorbance (OD<sub>450</sub> nm) was measured on a micro plate reader.

### ***OVA-specific delayed-type hypersensitivity reaction***

Control mice and mice that received immature DCs pulsed with MOG, were immunized subcutaneously with 100 µg of OVA emulsified in Complete Freund's Adjuvant (Gibco, BRL). One week after immunization, animals were *s.c.* challenged in the ear with 50 µg OVA dissolved in 20 µl of PBS. Ear thickness was measured at different times after challenge with a micrometer (Mitutoyo, Japan). Increased ear thickness was expressed as the means of at least four measurements per mouse in millimeters x 10<sup>-2</sup> ± SE.

### ***foxp3 expression analysis by real-time RT-PCR***

Total RNA was isolated from whole spleens obtained on day 21 after EAE induction from control or immature DC-treated mice, with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RT-PCR was performed using LightCycler RNA Amplification Kit SYBR Green I (Roche Diagnostics, Indianapolis, IN). The sequences for the specific primers used in this study were: *foxp3* 5'-GGCCCTTCTCCAGGACAGA- 3' and 5'-GCTGATCATGGCTGGGTGT- 3'. *β-actin* was used as a house-keeping reference gene and the primers were 5'-AGAGGGAAATCGTGCGTGAC -3' and 5'-GACTCATCGTACTCCTGCTTG -3'. A standard curve was generated with a series of dilutions (1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000) of a reference cDNA sample. We collected data using LightCycler Software version 3.5 (Roche, Mannheim, Germany). Data are expressed as normalized *foxp3* expression, which was obtained by dividing the relative quantity of *foxp3* for each sample by the relative quantity of *β-actin* for the same sample. Expression of *foxp3* transcripts is shown as arbitrary units normalized to *β-actin* signals.

## RESULTS

### *Andrographolide and rosiglitazone inhibit NF- $\kappa$ B activation.*

Treatment with the indicated concentrations of andrographolide or rosiglitazone prevented LPS-induced NF- $\kappa$ B activation in mouse MLE-12 cells co-transfected with an NF- $\kappa$ B-luciferase reporter construct (3xMHC-luc) and a renilla luciferase reporter (pGL3-hRL) (Figure 1). Data shown are ratios between LPS-induced luciferase activity (3xMHC-luc) and constitutive renilla luciferase activity (pGL3-hRL) in transfected MLE-12 cells. Cell viability was not affected either by the drugs or by LPS (not shown). The inhibition of NF- $\kappa$ B-induced gene expression observed here is consistent with recent observations suggesting that while andrographolide interferes with NF- $\kappa$ B activation by covalently modifying some of the members of this family of transcription factors (Xia et al., 2004), rosiglitazone can interfere with NF- $\kappa$ B function by shuttling it back to the cytoplasm (Kelly et al., 2004) or by stimulating the activity of a NF- $\kappa$ B repressor (Pascual et al., 2005).

### *NF- $\kappa$ B blockade interferes with LPS-induced DC maturation.*

To evaluate whether NF- $\kappa$ B blockade by rosiglitazone and andrographolide could interfere with the process of DC maturation induced by LPS, we measured the up-regulation of several maturation surface markers in bone marrow-derived DCs. As shown in Figure 2a, DCs responded to LPS stimulation by up-regulating maturation markers I-A<sup>b</sup>, CD40 and CD86. In contrast both andrographolide- and, rosiglitazone-treated DCs showed no significant increase on the surface expression of maturation markers I-A<sup>b</sup>,

CD40 and CD86 in response to LPS. These data are consistent with our previous observation that andrographolide can prevent DC maturation in response to LPS (Iruretagoyena et al., 2005). No significant alterations to DC viability were observed as a result of the treatment with the drugs (data not shown). The effect of andrographolide or rosiglitazone treatment on the phagocytic capacity of DCs was evaluated by incubation with Dextran-FITC for 4 h and Flow Cytometry. As shown in Figure 2b, although a slight decrease in mean fluorescence intensity for the brighter peak was observed in treated DCs when compared to control DCs, these differences were not statistically significant ( $p = 0.5$ , Student's  $t$  test). As a positive control for inhibition of phagocytosis, DCs were induced to mature with LPS, which significantly reduced their phagocytic capacity (Figure 2b) ( $p < 0.05$  when compared to control, Student's  $t$  test).

***NF- $\kappa$ B blockade can prevent T cell activation by antigen-pulsed DCs.***

To address whether the interference with DC function by andrographolide and rosiglitazone could impair the capacity of these cells to activate MHC class I- and class II-restricted T cells, we performed antigen presentation assays using OVA-specific T cells. For these assays, control DCs, andrographolide- and rosiglitazone-treated DCs were pulsed with OVA protein and co-cultured either with H-2K<sup>b</sup>/OVA<sub>257-264</sub>- or I-A<sup>b</sup>/OVA<sub>265-280</sub>-specific T cell hybridomas (B3Z and OT4H, respectively). As positive controls, DCs were pulsed with OVA peptides SIINFEKL or TEWTSSNVMEERKIKV for presentation on H-2K<sup>b</sup> or I-A<sup>b</sup>, respectively. As shown in Figure 2c, treating DCs with andrographolide (Iruretagoyena et al., 2005) or rosiglitazone before OVA-pulse prevented them from activating both CD4<sup>+</sup> and CD8<sup>+</sup> OVA-specific T cell hybridomas.

This inhibition was only observed when drug-treated DCs were pulsed with whole OVA protein, and not when OVA<sub>257-264</sub> or OVA<sub>265-280</sub> peptides were exogenously added to these cells. These results suggest that andrographolide and rosiglitazone do not affect the activation capacity of T cells, but instead the ability of DCs to generate the MHC molecules loaded with OVA-derived peptides.

***Inhibition of NF- $\kappa$ B on immature DCs enhances their capacity to reduce the severity of EAE in mice.***

Data shown above suggest that andrographolide and rosiglitazone, by reducing NF- $\kappa$ B activation, could interfere with the capacity of DCs to mature and prime antigen specific T cells. To test whether these drug-treated DCs were also able to interfere *in vivo* with a T cell mediated autoimmune response, immature DCs were pulsed with MOG<sub>35-55</sub> peptide, treated either with andrographolide or rosiglitazone and used to induce tolerance in the EAE mouse model. Mice were intravenously injected with a suspension of MOG<sub>35-55</sub> peptide-pulsed immature DCs starting two weeks prior to inducing EAE by immunization with MOG<sub>35-55</sub> peptide emulsified in CFA and clinical scores were determined daily (Figure 3a). A group of DCs were treated either with andrographolide or rosiglitazone during the time that cells were pulsed with MOG<sub>35-55</sub> peptide. As shown in Figure 3a, although MOG<sub>35-55</sub> peptide-pulsed immature DCs were able to reduce the severity of EAE in mice, andrographolide- or rosiglitazone-treated DCs showed an enhanced capacity to interfere with the development of EAE. Furthermore, complete remission of symptoms was observed significantly earlier in animals that received NF- $\kappa$ B-blocked immature DCs (Figure 3a). The specificity of this treatment is supported by



the observation that NF- $\kappa$ B-blocked immature DCs that were not pulsed with MOG failed to prevent the development of EAE in mice (Figure 3b).

Consistent with the clinical score data, lumbar sections of spinal cords obtained from mice suffering from EAE showed increased loss of myelin in animals that did not receive immature DCs (Figure 4b and c). In contrast, the frequency of demyelinating lesions was reduced in mice that had received immature DCs pulsed with MOG<sub>35-55</sub> peptide before induction of EAE (Figure 4d). No signs of demyelination were observed in the spinal cords of mice that had received andrographolide- or rosiglitazone-treated immature DCs pulsed with MOG<sub>35-55</sub> peptide (Figure 4e and 4f, respectively).

***MOG<sub>35-55</sub> peptide-pulsed immature DCs reduced anti-myelin T cell and antibody responses in EAE-induced mice***

On day 21 after EAE induction sera were obtained from control and immature DC-injected mice and anti-MOG IgG titers were measured by ELISA. As shown in figure 5a, a reduced antibody response was observed for mice that had been injected either with immature DCs, andrographolide-treated-immature DCs or rosiglitazone-treated immature DCs, pulsed in each case with MOG<sub>35-55</sub> peptide. In addition, lymph node single cell suspensions were obtained from control or immature DC-injected mice to measure release of IL-2 and IFN- $\gamma$  in response to increasing concentrations of MOG<sub>35-55</sub> peptide. As shown in figure 5b, IL-2 (bottom panel) and IFN- $\gamma$  (top panel) secretion was significantly reduced in lymph node suspensions obtained from treated mice, as compared to control mice suffering from EAE (Figure 5b).

***EAE modulation by andrographolide- or rosiglitazone-treated immature DCs is likely to be mediated by regulatory T cells and is antigen-specific.***

To determine the mechanisms that could be responsible for the induction of tolerance by immature DCs, we measured the abundance of regulatory T cells in the spleens of animals at day 21 after EAE induction. Frequency of regulatory T cells was evaluated by real time PCR measurement of *foxp3* expression, a transcription factor that is specifically expressed by this subset of T cells (Fontenot et al., 2005). As shown in figure 6, we observed significantly increased levels of Foxp3 mRNA in spleens obtained from andrographolide- and rosiglitazone-DC treated mice. These data are consistent with an *in vivo* expansion of regulatory T cells in mice treated with MOG<sub>35-55</sub> peptide-pulsed DCs, in which NF- $\kappa$ B activity has been blocked.

To evaluate whether the effect of andrographolide- or rosiglitazone-treated immature DCs on EAE was antigen-specific, we analyzed another effector T cell response in treated mice. With this aim, delayed type hypersensitivity (DTH) against OVA was induced in control mice and in mice treated with MOG<sub>35-55</sub> peptide-pulsed immature DCs. As shown in Figure 7, treatment with DCs pulsed with MOG<sub>35-55</sub> peptide had no effect on the mouse DTH response against OVA. These data support the notion that the down-modulation of the anti-myelin T cell responses caused by the injection of immature DCs pulsed with MOG<sub>35-55</sub> peptide is restricted to this particular antigen and does not seem to influence the response to other antigens.

## DISCUSSION

During steady state, immature DCs are thought to be responsible for maintaining tolerance to self-antigens (Steinman et al., 2003). This function of DCs is critical for the homeostasis of the immune system and it is likely that alterations in the tolerogenic capacity of these cells could lead to the development of autoimmune disorders. NF- $\kappa$ B activation in DCs in response to maturation stimuli, such as PAMPs, promotes their maturation and immunogenicity (O'Sullivan and Thomas, 2002). Accordingly, NF- $\kappa$ B blockade would reduce DC maturation and their capacity to prime antigen-specific naïve T cells. Here we provide evidence supporting this notion and show that NF- $\kappa$ B inhibition impairs the capacity of DCs to prime effector T cells and enhances their tolerogenic properties, which can be applied to promote antigen-specific tolerance in the EAE model by expanding regulatory T cells.

To inhibit NF- $\kappa$ B activation, DCs were treated with either andrographolide, a bicyclic diterpenoid lactone (Rajagopal et al., 2003), or rosiglitazone, a PPAR  $\gamma$  agonist, commonly used for the treatment of Type 2 Diabetes (Lee et al., 2003). These two drugs were able to independently impair several functions of DCs, such as maturation and antigen presentation to T cells. For andrographolide, it has been reported that this drug can exert an anti-inflammatory effect on immune cells by directly binding to p50 and inhibiting NF- $\kappa$ B dimerization (Xia et al., 2004). We have previously shown that andrographolide can diminish the severity of EAE when injected directly to mice (Iruretagoyena et al., 2005). Here we expand these findings by showing that this drug can improve the tolerogenic capacity of DCs *in vivo*. Rosiglitazone has also been shown to

inhibit NF- $\kappa$ B function (Mohanty et al., 2004). Some studies have suggested that PPAR $\gamma$  would induce NF- $\kappa$ B to shuttle from the cell nucleus back to the cytoplasm, thus preventing this transcription factor from binding to its DNA response elements (Kelly et al., 2004). Other reports have shown that PPAR $\gamma$  stimulation would retain NF- $\kappa$ B in the cytoplasm by increasing expression of I $\kappa$ B inhibitor (Setoguchi et al., 2001; Klotz et al., 2005). Consistent with those previous observations, here we show that andrographolide and rosiglitazone inhibit NF- $\kappa$ B-mediated gene expression induced by LPS in murine MLE-12 cells transfected with an NF- $\kappa$ B reporter system (Figure 1). Inhibition of NF- $\kappa$ B activation by andrographolide and rosiglitazone could account for their capacity to impair DC maturation and antigen presentation to T cells. We observed that blockade of NF- $\kappa$ B in DCs with andrographolide or rosiglitazone leads to a reduced response to LPS, demonstrated by the absence of up-regulation on the surface expression of costimulatory molecules, such as CD40, CD86 and MHC II. The impairment on DC function caused by andrographolide or rosiglitazone led to a reduced DC capacity to prime antigen-specific T cells, without affecting their ability to capture antigens (Figure 2). Our data are in the overall consistent with studies on human DCs (hDCs), in which the effect of rosiglitazone on maturation was evaluated (Gosset et al., 2001). Although an apparent enhancement on CD86 upregulation in response to LPS was shown for rosiglitazone-treated hDCs, a reduced response for all the other maturation parameters analyzed (CD80, IL-12p70, etc) was observed as a result of rosiglitazone treatment. However, in the study by Gosset et al the impact of rosiglitazone on the capacity of DCs to prime T cells was not evaluated.

The phenotypic changes induced by NF- $\kappa$ B blockade on DCs were consistent with the notion that reduced maturation and T cell priming could promote the tolerogenic

capacity of DCs. It has been reported that immature DCs can induce anergy or tolerance to the antigens they are presenting (Jonuleit et al., 2000; Dhodapkar et al., 2001; Yamazaki et al., 2003). Interestingly, several studies have recently demonstrated that “semimature” DCs can also induce tolerance in autoimmune models (Menges et al., 2002; Kleindienst et al., 2005; Verginis et al., 2005). These semimature DCs, generated by TNF $\alpha$  stimulation, were pulsed with antigen, and used to prevent EAE development in mice (Menges et al., 2002). However, the question as to what DC maturation state is more efficient at inducing antigen-specific tolerance still remains unanswered. In our system, we evaluated whether the tolerogenic capacity of DCs could be enhanced by NF- $\kappa$ B blockade. Using the EAE as a model for autoimmunity, we observed that MOG-pulsed immature DCs were able to significantly reduce severity of disease in mice (Figure 3a). NF- $\kappa$ B blockade by andrographolide and rosiglitazone significantly enhanced the tolerogenic capacity of immature DCs pulsed with MOG. Indeed, mice that received NF- $\kappa$ B-impaired DCs showed almost no clinical signs of disease (Figure 3a). In addition, EAE demyelinating lesions and anti-MOG IgG titers were significantly reduced in mice treated with NF- $\kappa$ B-blocked DCs (Figure 4 and 5). Similarly, the frequency of T cells secreting IL-2 and IFN- $\gamma$  in response to MOG was also significantly reduced in mice receiving MOG-pulsed immature DCs (Figure 5). Our data are consistent with previous studies showing that NF- $\kappa$ B-blockade can benefit the therapeutic capability of DC administration to interfere with unwanted T cell responses (Martin et al., 2003), such as Type 1 Diabetes (Ma et al., 2003) and transplant rejection (Saemann et al., 2004; Tomasoni et al., 2005). Here we provide new evidence supporting the notion that

pharmacological inhibition of NF- $\kappa$ B function on DCs can promote or reestablish antigen-specific tolerance.

To evaluate if the tolerogenic properties of the immature DCs could be mediated by the expansion of regulatory T cells, we decided to determine the expression of the transcription factor Foxp3. This transcription factor has been implicated as a key controller of development and function of regulatory T cells (Fontenot et al., 2005). To evaluate whether regulatory T cells were expanded with immature DCs we measured the relative levels of Foxp3 RNA expression in spleens of control and DC-treated mice. We observed that, although MOG-pulsed immature DCs led to a slight increase of Foxp3 RNA levels in the spleen compared to control mice, NF- $\kappa$ B-blockade significantly enhanced the capacity of DCs to increase splenic Foxp3 RNA levels (Figure 6). These data suggest that immature DCs in which NF- $\kappa$ B-driven transcription has been inhibited, can promote tolerance in the autoimmune EAE model by stimulating the expansion of regulatory T cells expressing Foxp3. The slight increase on the splenic *foxp3* levels shown by mice treated with MOG-pulsed immature DCs was apparently sufficient only to abolish the MOG-specific antibody, but not to completely prevent EAE. The relative contribution of regulatory T cells to different parameters of the immune response, such as antibody and T cell responses or the clinical EAE response, have not been dissected. Thus, it might be possible that an increase in Foxp3 could influence at distinct extents the MOG-specific antibody response and the EAE clinical response.

Finally, to evaluate the antigenic specificity of the tolerance induced by MOG-pulsed immature DCs, mice that received these cells were then sensitized with OVA and the DTH response against this antigen was evaluated. No reduction on the OVA-specific

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DTH response was observed in mice that received MOG-pulsed immature DCs, supporting the notion that induction of tolerance is antigen specific in our system (Figure 7). In contrast, we had previously reported that treatment of mice directly with andrographolide led to a significant inhibition of an anti-OVA DTH response (Iruretagoyena et al., 2005), due probably to an antigen-independent tolerogenic effect of andrographolide on mouse DCs.

The findings reported here suggest that blockade of NF- $\kappa$ B activation can be used to downmodulate DC immunogenicity. The features of immature DCs with blocked NF- $\kappa$ B function were compatible with an efficient induction of antigen-specific T cell tolerance in the EAE model, which significantly reduced the severity of disease in mice. Our data support the notion that NF- $\kappa$ B blockade could be considered as an important pharmacological approach to promote DC-mediated tolerance to auto-antigens and prevent detrimental autoimmune responses.

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## FOOTNOTES

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## LEGENDS FOR FIGURES

**Figure 1.** Andrographolide and rosiglitazone inhibit NF- $\kappa$ B-driven gene expression. Mouse MLE-12 cells were cotransfected with an NF- $\kappa$ B-luciferase reporter construct (3xMHC-luc, (McKay and Cidlowski, 2000)) and Renilla luciferase reporter (pGL3-hRL, (Stoneley et al., 2000)) as an internal control. 24 h post-transfection, cells were left untreated or incubated with andrographolide (10 $\mu$ M) or rosiglitazone (10  $\mu$ M) for 12 h followed by LPS stimulation at the indicated concentrations. Cell lysates were analyzed using a luciferase assay kit (Hermoso et al., 2004). Data are expressed as luciferase/renilla units and are means of 3 independent experiments (\*  $p < 0.05$  when compared to control, Student's  $t$  test). Error bars represent standard deviations.

**Figure 2.** Blockade of NF- $\kappa$ B activation downmodulates DCs maturation and antigen presentation to T cells. **A)** Up-regulation of DC maturation markers in response to LPS is inhibited by either rosiglitazone or andrographolide treatment. Bar graphs show the fold increase of mean fluorescence intensity for MHC II, CD86 and CD40 in CD11c<sup>+</sup> cells after 36 h of stimulation with LPS (1  $\mu$ g/ml). Data shown are means of 5 independent experiments (\*  $p < 0.05$ , when compared to control DCs, Student's  $t$  test). Error bars represent standard deviations. **B)** Andrographolide and rosiglitazone treatments do not alter the phagocytic capacity of DCs. For phagocytosis assessment, control and treated DCs were incubated with Dextran-FITC for 4 h and then evaluated by FACS. Histograms show Dextran-FITC uptake by DCs when incubated at 4° C (shaded histograms) and at 37° C (clear histograms). Immature DCs were compared to LPS-activated DCs. Data

shown are representative FACS histograms from 3 independent experiments. **C)** T cell activation by antigen-pulsed DCs is suppressed by andrographolide or rosiglitazone treatment. Control DCs (top panel) and andrographolide- (middle panel) or rosiglitazone-treated (bottom panel) DCs were pulsed with OVA protein (10 µg/ml) and cocultured either with H-2K<sup>b</sup>/OVA<sub>257-264</sub>- (left panel) or I-A<sup>b</sup>/OVA<sub>265-280</sub>-specific (right panel) T cell hybridomas. As controls, DCs were pulsed with OVA-derived antigenic peptides for presentation on H-2K<sup>b</sup> or I-A<sup>b</sup>, respectively. Data shown are means of 4 independent experiments. Error bars represent standard deviations.

**Figure 3.** NF-κB blockade enhances the tolerogenic capacity of MOG-pulsed immature DCs and their ability to prevent EAE development in the mouse. **A)** C57BL/6 mice received two *i.v.* injections of MOG-pulsed immature DCs (10<sup>6</sup>) previously treated with the indicated NF-κB inhibitors, 7 and 14 days prior to EAE induction. MOG-pulsed untreated immature DCs were included as controls. EAE was induced with MOG<sub>35-55</sub> peptide in CFA and clinical score was determined daily. (\*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ , Mann-Whitney rank sums two-tailed test). Error bars represent standard error. **B)** Mice received immature and andrographolide- or rosiglitazone-treated DCs that were not pulsed with MOG, 7 and 14 days prior to EAE induction. Results are shown as the week average of the mean daily clinical scores for each group. Data shown are means of 5 independent experiments consisting of groups of 4 mice each.

**Figure 4.** Histological analysis of spinal cord sections of mice suffering from EAE. H & E and Luxol Fast blue staining (Goto, 1987) of lumbar spinal cord sections of mice in

which EAE was not induced (**A**), control mice suffering from EAE (**B and C**), mice that received immature MOG-pulsed DCs (**D**), andrographolide-treated DCs-injected mice (**E**) and rosiglitazone-treated DCs-injected mice (**F**). Areas of inflammation and loss of myelin are indicated by arrows. Data shown are representative staining of sections obtained from 4 independent experiments.

**Figure 5.** Reduced anti-myelin T cell and antibody responses in immature DC-injected mice. **A)** Three weeks after EAE induction, sera were obtained from mice and anti-MOG IgG titers were measured by ELISA, as previously described (Iruretagoyena et al., 2005). Data shown are means of 3 independent experiments (\*  $p < 0.05$  when compared to control, Student's  $t$  test). There is no statistical difference between control DCs and drug-treated DCs. Error bars represent standard deviations. **B)** Single cell suspensions obtained from lymph nodes derived from control or immature DC-injected mice were tested for IL-2 and IFN- $\gamma$  secretion in response to MOG peptide. Cytokine release was determined by ELISA, as previously described (Kalergis et al., 2001). Data shown are means of 3 independent experiments. Error bars represent standard deviations.

**Figure 6.** NF- $\kappa$ B blockade enhances the capacity of MOG-pulsed immature DCs to increase the level of Foxp3 mRNA in mice suffering from EAE. Total RNA was isolated from spleens obtained on day 21 after EAE induction from control or immature DCs-injected mice. Real Time PCR was performed using LightCycler, as described in the Material and Methods section. Expression of *foxp3* is shown as arbitrary units normalized

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to  $\beta$ -actin expression. (\*  $p < 0.05$  when compared to control, Student's  $t$  test). Data shown are means of 4 independent experiments. Error bars represent standard deviations.

**Figure 7.** Modulation of EAE by MOG-pulsed immature DCs is antigen specific. DTH against OVA was induced in control mice and mice that received immature DCs loaded with MOG peptide. One week after sensitization with OVA-CFA, animals were challenged in the ear with OVA in PBS. Data shown are means of 2 independent experiments. Error bars represent standard deviations.

Figure 1.

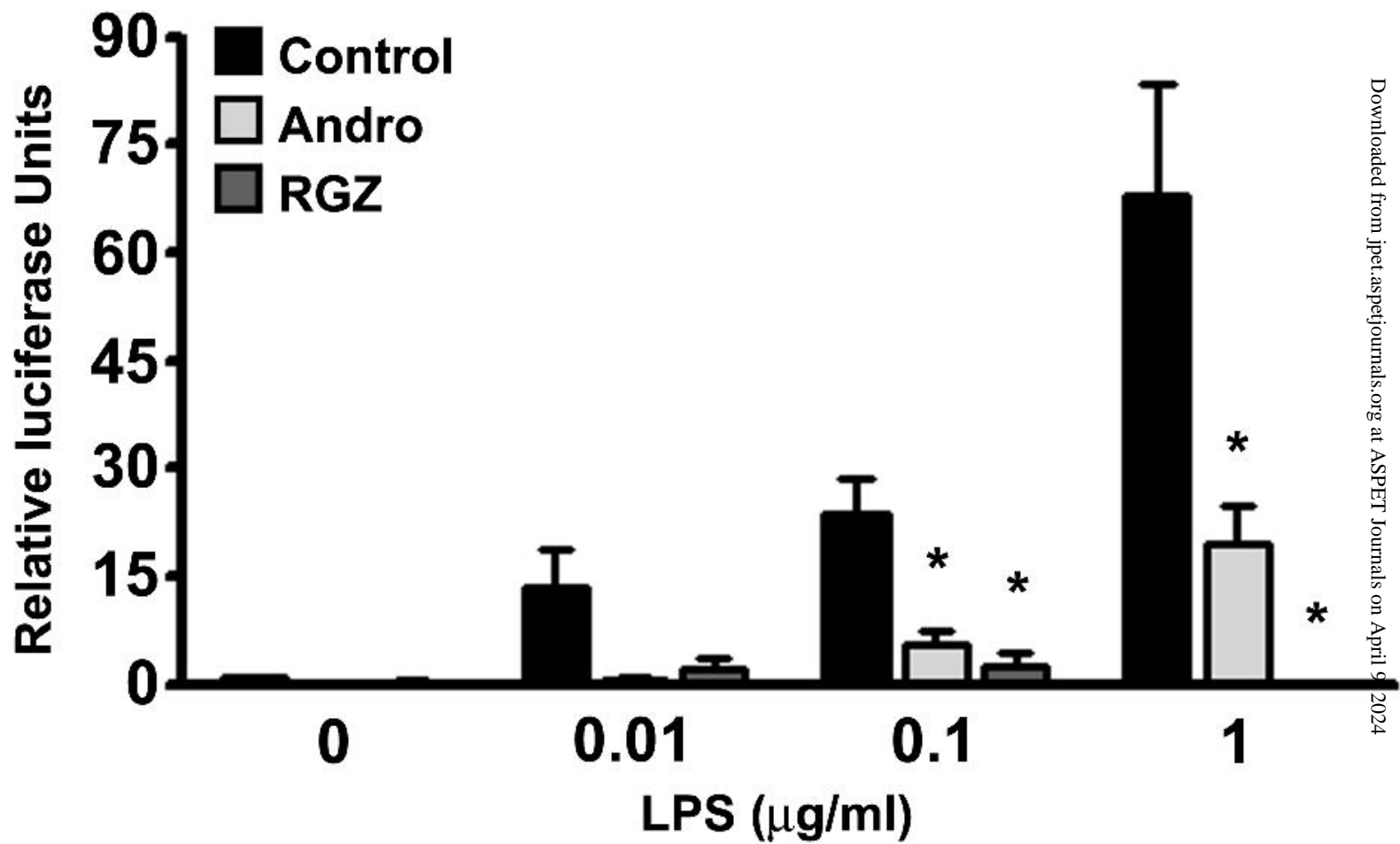
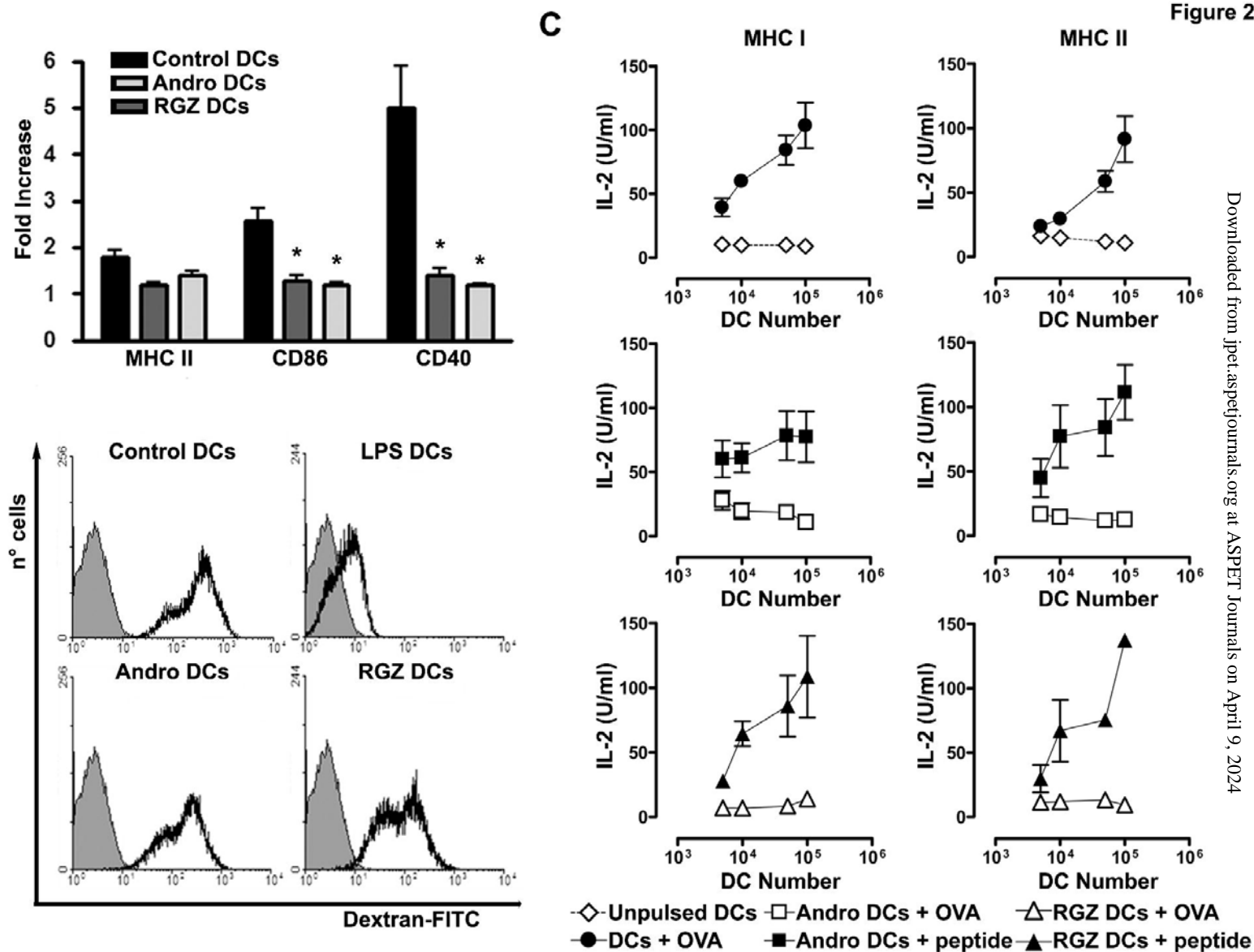
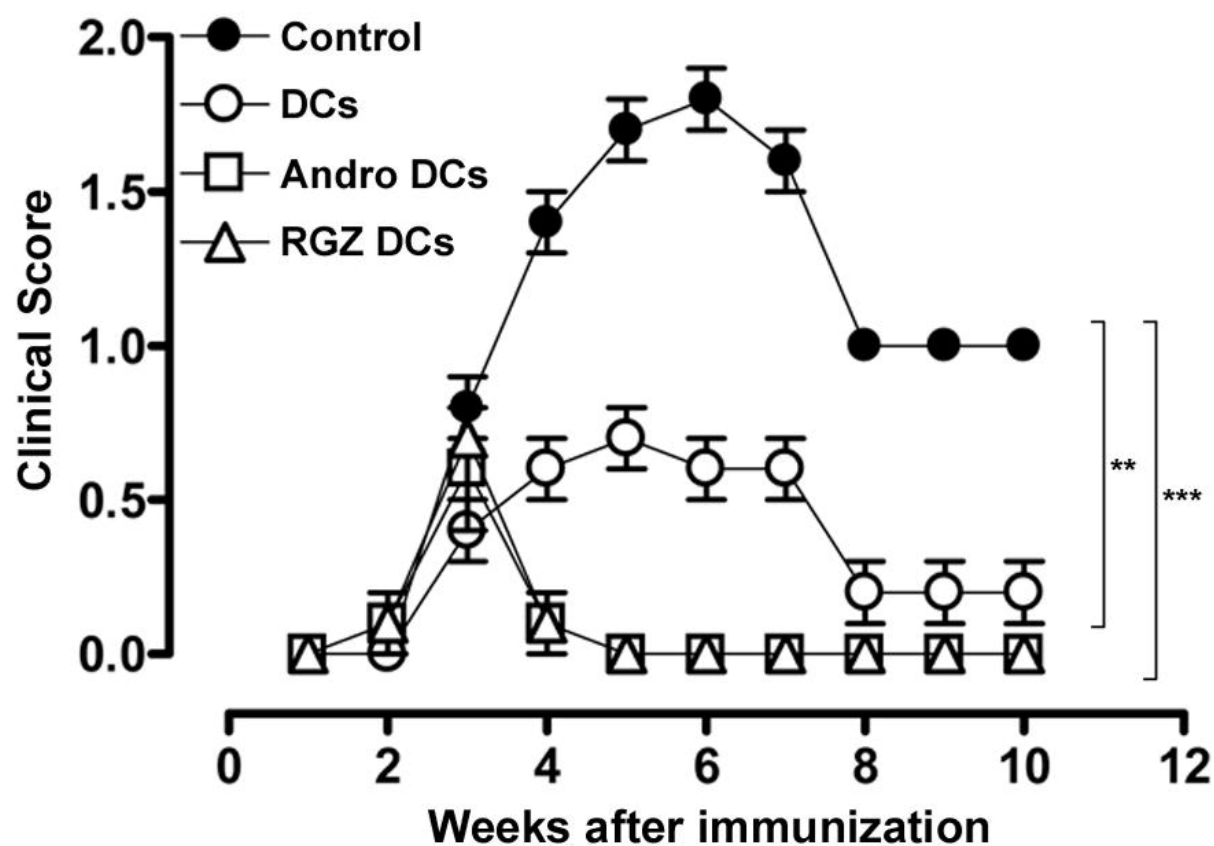


Figure 2.

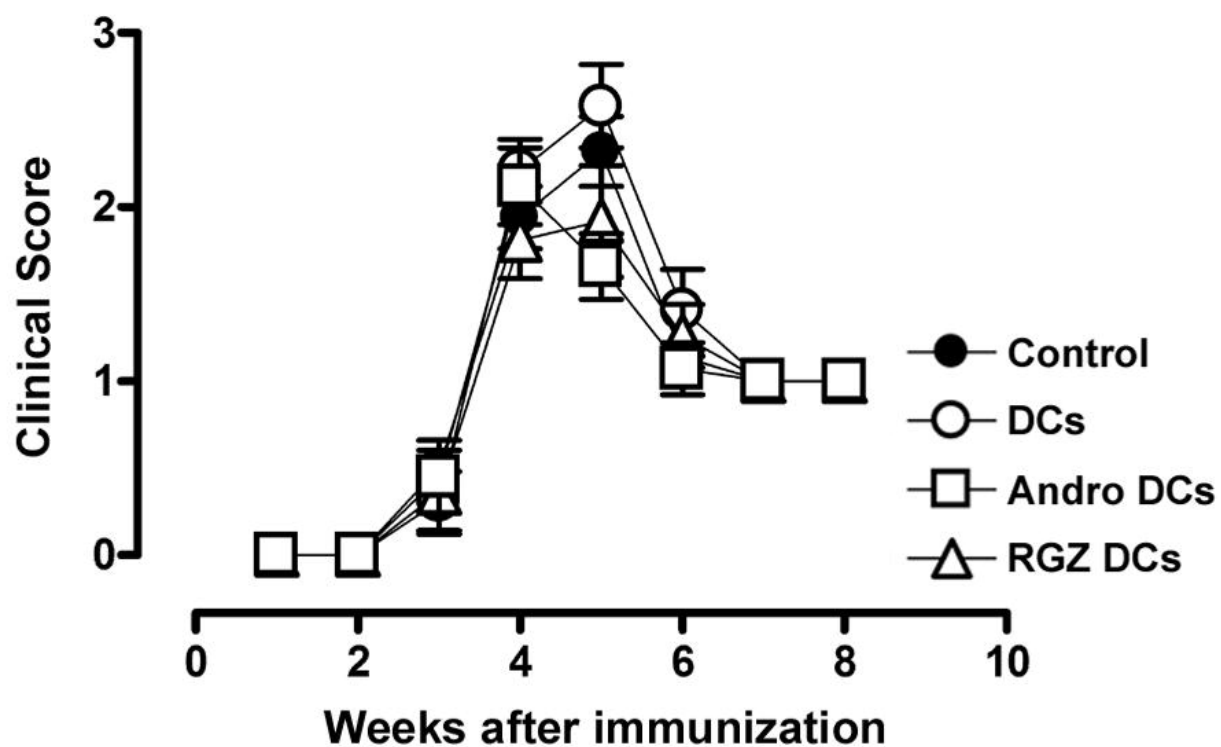


**Figure 3.**

**A**

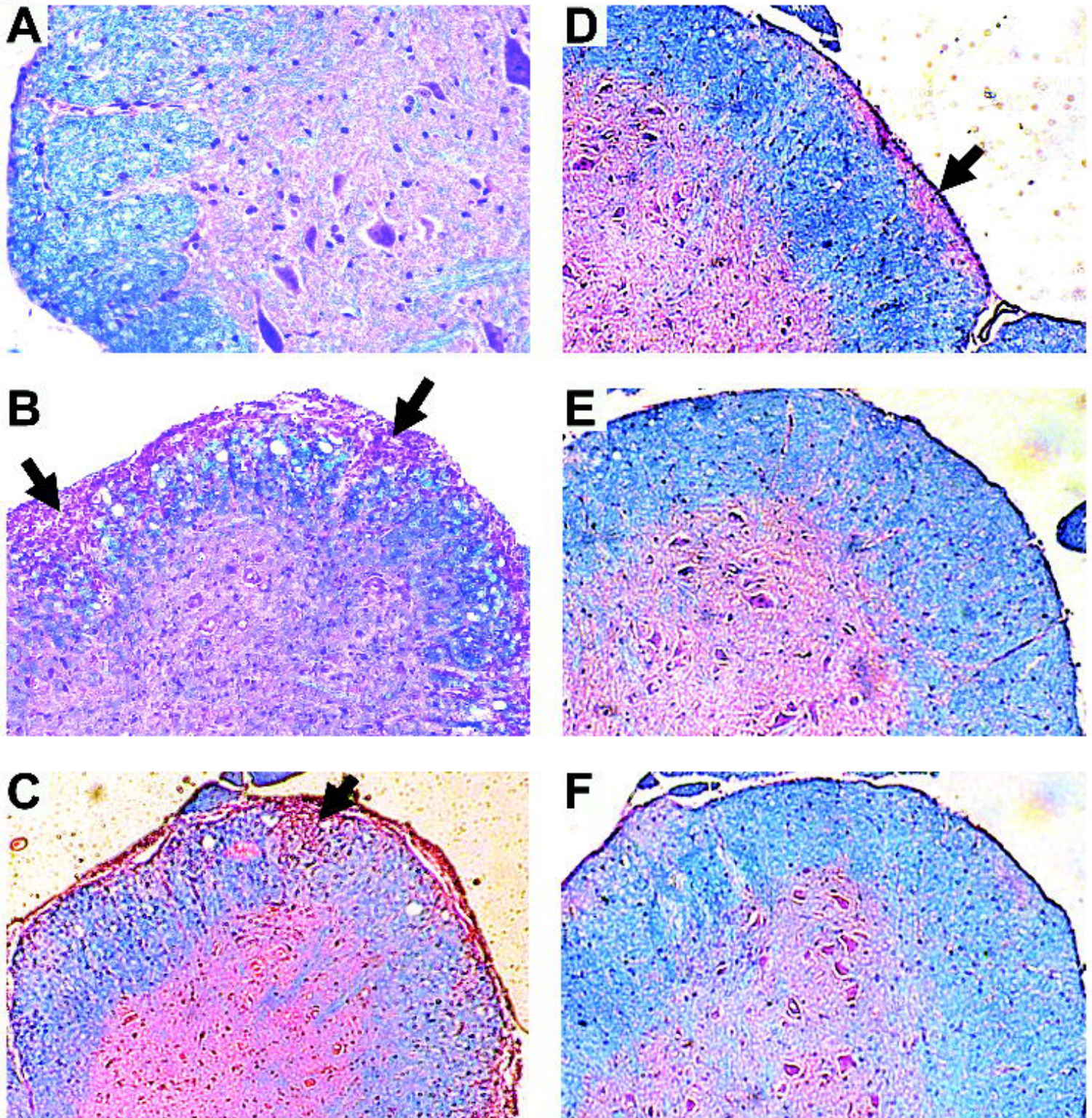


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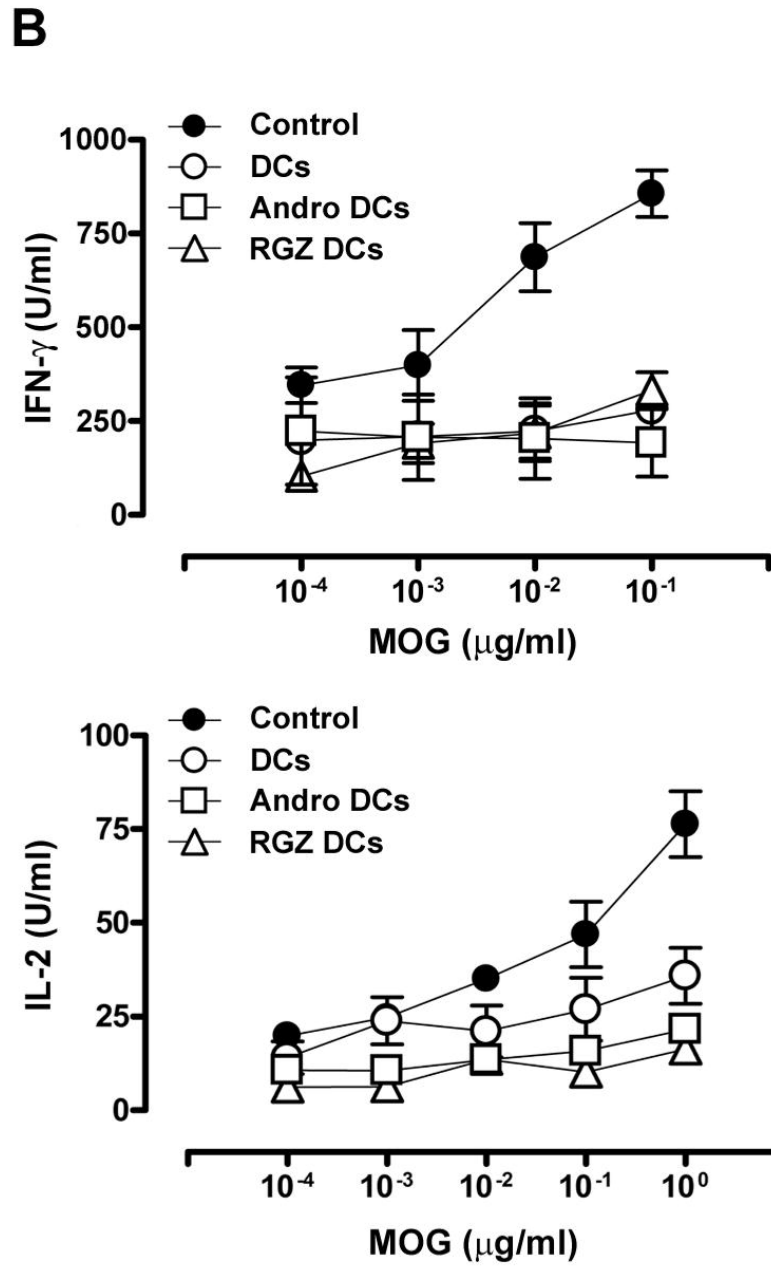
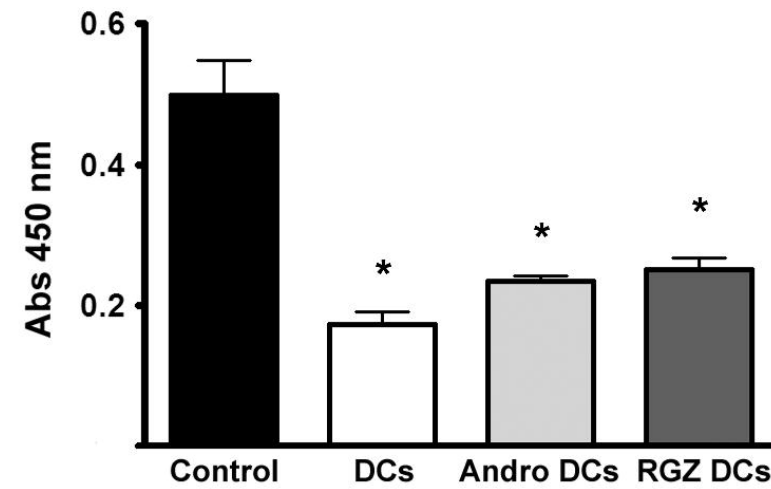




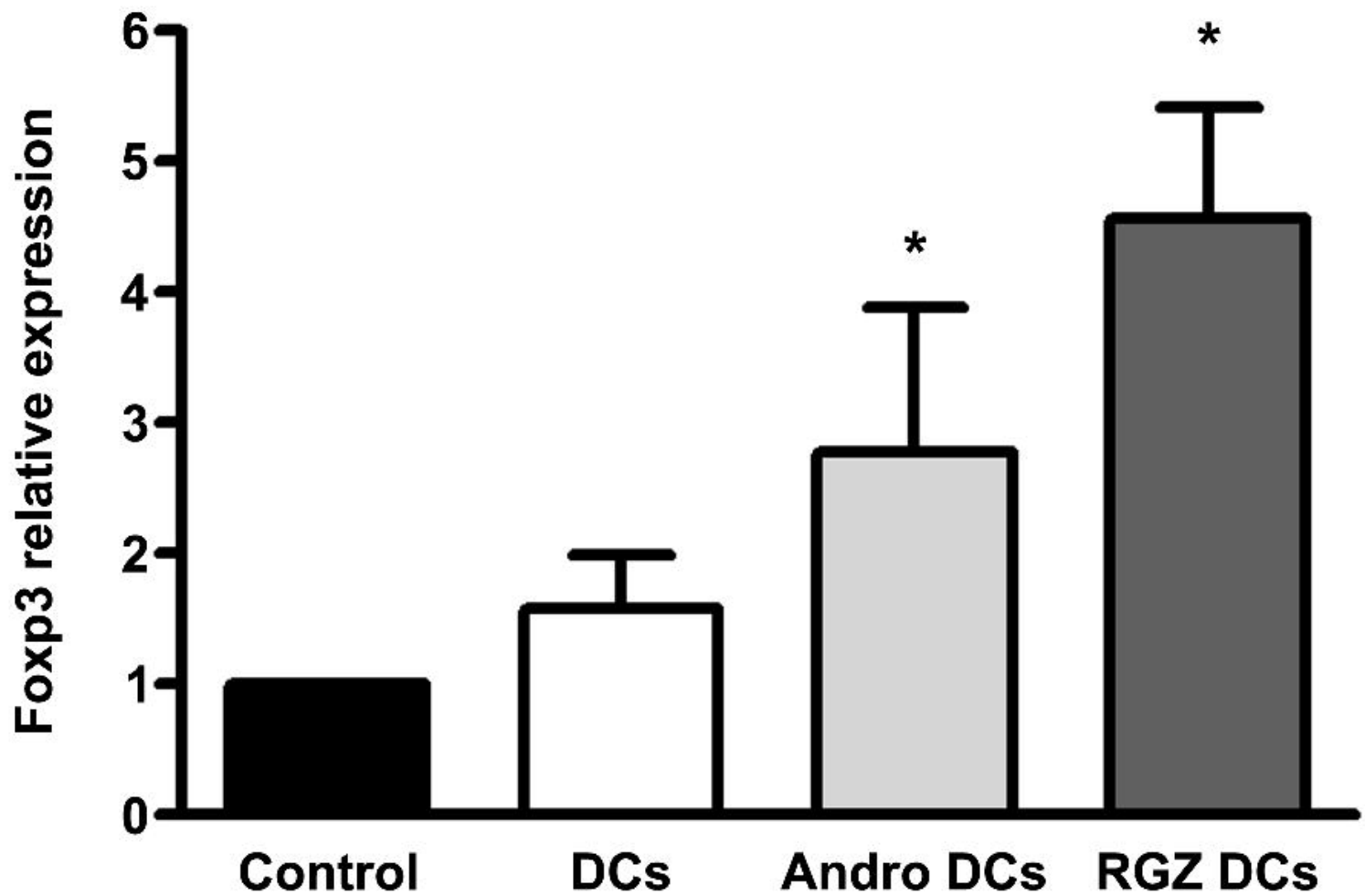
**Figure 4.**







**Figure 6.**



**Figure 7.**

