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ORIGINAL ARTICLE

Characterization of dendritic cells in testicular draining lymph nodes in a rat model of experimental autoimmune orchitis

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Summary

The maturation state of dendritic cells (DC) is regarded as a control point for the induction of peripheral tolerance or autoimmunity. Experimental autoimmune orchitis (EAO) serves as a model to investigate inflammatory-based testicular impairment, which ranks as a significant cause of male infertility. This work aimed to determine whether DC enrichment occurs organotypically in testicular draining lymph nodes (TLN) compared with LN draining the site of immunization (ILN) and thus contributes to the pathogenesis of autoimmune orchitis. In this regard, we quantified and characterized the DC from TLN and ILN in rats with EAO. Flow cytometric analysis showed a significant increase in the percentage of DC (OX62+) only in TLN from EAO rats compared with normal (N) and adjuvant control (C) groups. The number of DC from ILN and TLN expressing CD80, CD86 and major histocompatibility complex (MHC) II was comparable among N, C and experimental (E) groups at 30 and 50 days after the first immunization. However, TLN DC from EAO rats (50 days) showed an increase in mean fluorescence intensity for MHC II compared with N, C and E groups (30 days). The mRNA expression level of IL-10 and IL-12p35 was significantly upregulated in enriched DC fraction from TLN in EAO rats with no significant changes observed in ILN DC. The expression of IL-23p19 mRNA remained unchanged. Functional data, using proliferation assays showed that EAO-DC from TLN, but not from ILN, significantly enhanced the proliferation of naïve T cells compared with C-DC. In summary, our data suggest that the DC in TLN from orchitis rats are mature, present antigens to T cells and stimulate an autoimmune response against testicular antigens, thus causing immunological infertility.

Introduction

Dendritic cells (DC) are a heterogeneous cell population that serves as sentinels of the immune system. Antigen uptake in non-lymphatic organs associated with ongoing inflammation and release of 'danger' signals such as pathogen-associated molecular patterns or pro-inflammatory cytokines leads to DC maturation with subsequent migration to the draining lymph nodes (LN) via the lymphatic system. These phenotypical and functional changes maximize the ability of DC to elicit proliferation of T cells (Banchereau & Steinman, 1998). The spectrum of cytokines produced by the DC is a reflection of the functional and immune status of these antigen-presenting cells (APC). The maturation process encompasses the production of bioactive interleukin (IL)-12p70 and tumour necrosis factor (TNF)-α, the upregulation of surface T-cell co-stimulatory molecules (CD80, CD86), major histocompatibility

complex (MHC) class II and the downregulation of endocytic capacity (Hackstein & Thomson, 2004). Production of cytokines such as IL-10 or IL-12 during DC maturation can influence induction of a Th1, Th2 or Th17 immune response. The T-effector (Th) cells are considered major players in the immune response and can be subdivided into three different types based on their cytokine signature: interferon (IFN)-γ-secreting Th1; IL-4- and IL-5-secreting Th2 (Mosmann & Coffman, 1989) as well as IL-17-producing Th17 cells (Steinman, 2007). The differentiation of naïve T cells into Th1 or Th2 cells occurs during their activation (priming) by DC. All three Th subtypes are implicated in the pathogenesis of autoimmune disease.

IL-12 production and release occur very early in the immune response. The bioactive IL-12p70 is a 70-kDa heterodimeric cytokine composed of two disulphide-linked subunits designated p35 and p40, critical to the initiation and progression of Th1 response (Gately *et al.*, 1991; Wolf *et al.*, 1991). The 40-kDa fragment, formed exclusively in DC, macrophages and monocytes is produced constitutively and in excess of the 35-kDa subunit (Albrecht *et al.*, 2004). IL-12 is often the first cytokine released by professional phagocytic and sentinel cells like DC and macrophages after antigen is apparent in the microenvironment. It is pivotal not only for initiation and polarization of the immune response but also for the maintenance of immunoreactivity throughout its duration (Trinchieri, 2003).

IL-23 is a heterodimeric cytokine with many similarities to IL-12 and comprises a p19 subunit that is associated with the IL-12p40 subunit. Biologically active IL-23 is formed by both p19 and p40 subunits within the same cell and expressed predominantly by activated DC and phagocytic cells (Oppmann *et al.*, 2000). Analysis of the cytokine profile of IL-23-dependent pathogenic effector CD4+ T cells indicated that this subset, named Th17, preferentially produced IL-17 rather than IFN-γ (Weaver *et al.*, 2007). Furthermore, in the presence of IL-12p70, Th17 cells produce IFN-γ and proliferate (Acosta-Rodriguez *et al.*, 2007). The development of Th1 vs. Th17 subsets is strongly dependent on the balance between IL-12 and IL-23 production by DC (Goriely & Goldman, 2008). The initial secretion of IL-17 in naïve T cells is independent of IL-23; however, production of IL-17 by memory effector cells is enhanced in the presence of IL-23 (Oppmann *et al.*, 2000).

IL-10 is a multifunctional cytokine that plays a crucial immunosuppressive role during excessive Th1 responses through its ability to inhibit activation and effector function of T cells, monocytes and macrophages (Moore *et al.*, 2001). IL-10 influences also differentiation and function of regulatory T cells, which control immune responses and tolerance in vivo (Akbari *et al.*, 2001).

The major route of DC entry into the LN follows recruitment of DC precursors into the periphery. Signals from their surrounding environment and the differentiation status decide about the residence time in the periphery. As an example, Langerhans cells and dermal DC may have a relatively long residence (Kamath *et al.*, 2002), whereas a subset of DC that migrates to mesenteric LN from the intestine remains only shortly in the periphery (Pugh *et al.*, 1983). The mobilization of DC to LN via lymphatics is mediated by chemokine receptor CCR7 (Ohl *et al.*, 2004). The CCR7 ligands CCL19 and CCL21 are expressed by the lymphatic epithelium and/or within LN by stromal cells, endothelial cells and DC themselves (Martín-Fontecha *et al.*, 2003) with each of them involved in the migration of DC from peripheral tissues to LN (Randolph *et al.*, 2005). DC maturation is paralleled by upregulation of MHC class II and co-stimulatory CD80/CD86 molecules as well as by production of IL-12 (Banchereau & Steinman, 1998).

Experimental autoimmune orchitis (EAO) in rat is a model of testicular chronic inflammation and human immunologically impaired spermatogenesis and infertility. Rat EAO is a progressive disease characterized by interstitial inflammatory cell infiltrates, apoptosis of germ cells followed by aspermatogenesis, production of auto-antibodies against testicular antigens and increased secretion of pro-inflammatory cytokines such as TNF-α, IL-6 or monocyte chemoattractant protein-1 in the testis (Doncel *et al.*, 1989; Fijak & Meinhardt, 2006; Guazzone *et al.*, 2009). In spite of its immunoprivileged status, all classical signs of an inflammatory reaction including accumulation of DC, macrophages and T cells are observed in EAO testis (Fijak *et al.*, 2005; Rival *et al.*, 2006, 2008; Jacobo *et al.*, 2009a). As shown in our previous study, isolated testicular DC from EAO testis revealed a mature immunogenic status before migration to LN to stimulate autoresponses to testicular antigens (Rival *et al.*, 2007).

Disturbances during the presentation process of sequestrated self-antigens to T cells are known as a possible mechanism responsible for the development of autoimmune disease. In this context, the maturation status and ability to stimulate naïve T cells are critical to both initiation and progression of autoimmune disease or maintenance of immunological tolerance. Previous reports (Scheinecker *et al.*, 2002; Bode *et al.*, 2008; Wheeler *et al.*, 2009) showed that immune cells from draining and non-draining LN are characterized by an organotypical marker expression imprinted by the drained areas. Thus, we examined the phenotype and functional status of the DC population of the testicular draining LN (renal and iliac; TLN) as well as LN draining the site of immunization

(inguinal and popliteal; ILN) in control and EAO rats to elucidate the contribution of DC to the development and progression of rat EAO.

Materials and methods

Animals

Adult male inbred Wistar rats aged 50–70 days were purchased from Bioterio Central Facultad de Farmacia y Bioquímica (Buenos Aires, Argentina) and Charles River Laboratories (Sulzfeld, Germany). Animals were kept at 22 °C with 14 h light, 10 h dark schedule and fed with standard food pellets and water ad libitum. The use of rats followed NIH guidelines for care and use of experimental animals and were approved by local committees (Regierungspraesidium Giessen GI 20/23 – No. 32/2008; CICUAL-Facultad de Medicina).

Induction of EAO

Wistar rats of the experimental (E) group were actively immunized with syngeneic testicular homogenate (TH) as described previously (Doncel *et al.*, 1989). Immunization was performed by injecting 0.4 mL syngeneic of TH mixed with 0.4 mL of complete Freund's adjuvant (CFA; Sigma-Aldrich, St Louis, MO, USA) into the hind footpads (subcutaneously) and in different sites near popliteal LN and the neck area (intradermally). These injections were repeated twice at 14-day intervals. The first two immunizations were followed by an intravenous injection of 10^{10} inactivated *Bordetella pertussis* (Bp) bacteria (strain DSM 4952; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany or strain 10536, kindly provided by Instituto Malbrán, Buenos Aires, Argentina) dispersed in 0.5 mL of isotonic saline, whereas the third one was followed by an intraperitoneal injection of Bp at a concentration of 5×10^9 in 0.5 mL of isotonic saline. Control (C) animals received CFA and Bp, but no TH otherwise following the same scheme. E and C group rats were killed 30 and 50 days after the first immunization. Normal (N) untreated rats were also studied. Popliteal, inguinal, renal and iliac LN were removed and single cell suspensions were prepared. Testes of all rats were fixed in Bouin's solution to evaluate the degree of germ cell sloughing as an indirect parameter of seminiferous tubule damage. As reported previously, a normal histopathology was observed in E rats killed at 30 days (end of the immunization period), whereas E rats killed at 50 days showed multifocal orchitis characterized by interstitial lymphomononuclear cell infiltrates, germ cells sloughing and seminiferous tubule atrophy. N and C rats revealed normal testicular histopathology (Doncel *et al.*, 1989). Only rats from the E group developing orchitis after 50 days were studied.

Immunohistochemistry

Mouse monoclonal antibody anti-OX-62 (dilution 1 : 75, MCA1029G; Serotec, Oxford, UK; Brenan & Puklavec, 1992), which is specifically expressed by rat DC and gamma delta T cells, was used to identify the DC population of inguinal, popliteal, iliac and renal LN. The staining procedure was performed as described previously (Rival *et al.*, 2007).

Preparation of LN cell suspension

LN cells from N, C and E group rats were obtained by enzymatic digestion. Briefly, a pool of popliteal and inguinal LN (ILN) and a pool of iliac and renal LN (TLN) were incubated with type I collagenase (1 mg/mL; Worthington Biochemical Corporation, Freehold, NJ, USA) plus 0.1% bovine serum albumin (BSA, fraction V; Sigma Chemical Co.) in a Dubnoff shaking water bath at 37 °C for 20 min. Collagenase was inactivated by adding ice-cold phosphate-buffered saline (PBS) and the cell suspension was centrifuged at 300 ***g*** for 10 min at 4 °C. Red blood cells were depleted by osmotic lysis with ammonium chloride (0.16 M NH_4Cl, 0.17 M Tris-HCl, pH 7.2) for 5 min at room temperature. Final cell suspension was washed with PBS, centrifuged at 300 ***g*** for 10 min at 4 °C and processed directly for flow cytometric assay or enrichment of DC. The DC were separated from the LN cell suspension by positive selection using mouse anti-rat OX-62 antibody conjugated with magnetic beads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, enriched DC populations were stained with the primary mouse anti-rat-CD11c-Alexa Fluor 647 antibody (AbD Serotec, Oxford, UK) followed by incubation with mouse anti-rat CD3, MHC II and CD45 RA (B-cell marker) antibody (BD Biosciences, Heidelberg, Germany) and analysed by flow cytometry (Table 1). The DC-enriched cell preparation of LN had a purity of ~70% as assessed by flow cytometric analysis. The contaminating population was essentially composed of T cells (~30%) and very few B cells (~3%; Fig. 1). To check whether the contaminating T cells in the enriched DC fraction express noteworthy amounts of the investigated cytokines IL-10, IL-12p35, IL-12p40 and IL-23p19, a highly purified fraction of T cells (~95%) was examined by reverse transcription-polymerase chain reaction (RT-PCR). No significant quantities of investigated transcripts were detected (data not shown).

Table 1 Summary of primary antibodies used in the study

Reagent	Working dilution (μg/mL)	Specificity	Fluorochrome	Origin
CD3	1.6	T-cell receptor αβ or γδ	FITC or PE	BD Pharmingen
CD4	10.0	CD4 antigen	R-PE	BD Pharmingen
CD11c	2.5	Integrin alpha x chain	Alexa Fluor 647	Serotec
OX-62	10.0	Alpha E2 integrin (DC)	–	Serotec
CD45 RA (Ox-33)	6.0	CD45 antigen on B lymphocytes	FITC	BD Pharmingen
CD80 (B7-1)	10.0	Co-stimulatory molecule	R-PE	BD Pharmingen
CD86 (B7-2)	10.0	Co-stimulatory molecule	R-PE	BD Pharmingen
MHC class II	3.3	MHC class II	R-PE or PerCP	BD Pharmingen

PerCP, peridinin chlorophyll protein.

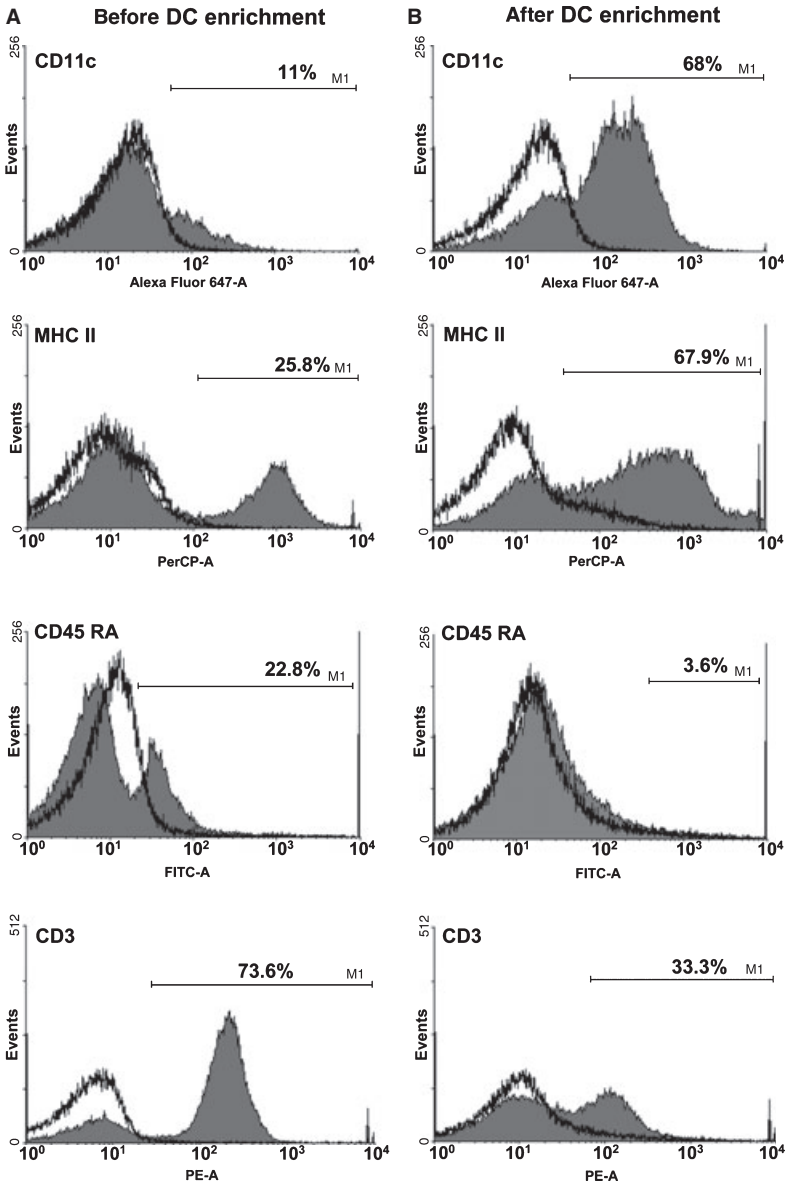

Figure 1 Flow cytometric characterization of the dendritic cell (DC)-enriched population of testicular draining lymph node (LN) cells before (A) and after (B) Ox-62-coupled magnetic beads separation. Filled histograms represent all viable cells in the LN single cell suspension labelled with fluorescent conjugated antibodies directed against CD11c (DC), major histocompatibility complex II, CD45 RA (B cells) or CD3 (T cells). The thick black lines indicate the respective isotype controls. All data are representative of at least three independent experiments with similar results.

Flow cytometric analysis

The phenotype of freshly prepared LN cells was determined using two-colour flow cytometry. Monoclonal antibodies (mAb) used for cell surface staining were directly conjugated to R-phycoerythrin (R-PE) or a secondary antibody conjugated with fluorescein isothiocyanate (FITC) was used to detect unlabelled mAb. A summary of primary antibodies used in this study is provided in Table 1. About 2×10^6 LN cells from N, C and E group rats were incubated with OX-62 mAb for 30 min. After washing in cold PBS with 0.03% azide and 1% BSA (PBS/BSA), cells were incubated with anti-mouse FITC-conjugated immunoglobulin G (IgG, 1 : 20; Vector Laboratories, Burlingame, CA, USA) for 30 min. Cells were washed with PBS/BSA buffer and stained for 30 min with anti-rat mAb MHC II, CD80 or CD86. Finally, cells were washed with PBS/BSA buffer and fixed with 0.5% paraformaldehyde. Background staining was evaluated using isotype controls: PE-conjugated mouse $IgG_{1,k}$ mAb or mouse $IgG_{1,k}$ mAb (BD PharMingen, San Diego, CA, USA). The whole procedure was performed at 4 °C. Samples were run on a fluorescence activated cell sorting (FACS) Calibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Data were collected for 10 000 events on a 'DC gate' based on the light scanner properties.

RNA isolation and real-time RT-PCR

Total RNA was obtained from isolated DC using RNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To remove genomic DNA contamination, isolated RNA samples were treated with RNase-free DNase I (Qiagen). The total mRNA obtained from up to 5×10^5 DC was purified and analysed by real-time quantitative PCR as described previously (Rival *et al.*, 2007). The primers for rat IL-10, IL-12p35, IL-12p40 and IL-23p19 were purchased as QuantiTect Primer Assays from Qiagen. Primer sequences and amplicon sizes are shown in Table 2. All analyses were performed in triplicate and the mean was used for further calculations. The mRNA expression of all investigated genes was normalized with β-actin and HPRT1 (hypoxanthin-phosphoribosyltransferase 1) as a housekeeping gene (HKG). The relative expression levels were calculated by the equation

$$2^{-(S\Delta C_t - C\Delta C_t)},$$

where ΔC_t is the difference in the C_t value between the gene of interest (GOI) and the HKG as calculated by (GOI C_t)−(HKG C_t). $S\Delta C_t - C\Delta C_t$ is the difference between the $S\Delta C_t$ ($S\Delta C_t$ is the experimental or adjuvant control) and the control ΔC_t ($C\Delta C_t$ is the untreated control). Comparison of both HKG genes showed no significant differences between experimental groups. Data for each animal from the E and adjuvant C groups are expressed in relation to the expression in the corresponding untreated (N) control animal. All data are presented as the mean ± SEM of 5–7 different testicular DC samples for each treatment group.

Preparation of T cells

Allogeneic T cells from Sprague-Dawley rats were isolated after centrifugation of splenocyte suspension on Ficoll–Paque PLUS gradient (GE Healthcare, Uppsala, Sweden). Splenocytes were prepared by injecting the spleen with RPMI-1640 medium and grinding through 100-μm nylon mesh. Spleen erythrocytes were osmotically lysed. Leucocyte-enriched fraction was washed and further purified using magnetic beads coated with monoclonal mouse anti-rat pan T-cell antibody and an MACS column (Miltenyi Biotec). The positively selected cells were collected as T cells. Cells were washed and suspended in complete RPMI-1640 medium supplemented with 10% foetal calf serum, 1% Non-Essential-Amino Acid Solution (MEM) (Sigma), 1 mM of sodium pyruvate (Gibco, Paisley, UK), 10 mM of HEPES (Gibco), 100 U/mL of penicillin, 100 μg/mL of streptomycin (PAA Laboratories, Cölbe, Germany) and 50 μM of 2-mercaptoethanol (Gibco). Purity (>95%) was examined by flow cytometry using FITC-conjugated anti-CD3 and R-PE-conjugated anti-CD4 antibodies (Table 1).

Table 2 Reverse transcription-polymerase chain reaction primers used in this study

Gene	Primer	Catalogue no. (Qiagen)	Entrez gene ID	Product length (bp)
β-actin	Sense: ATGGTGGGTATGGGTCAGAA	–	81 822	232
	Antisense: GGGTCATCTTTTCACGGTTG	–		
HPRT-1	Sense: TCT GTC ATG TCG ACC CTC AG	–	24 465	109
	Antisense: CCT TTT CCA AAT CTT CAG CA	–		
IL-10	QuantiTect Primer Assay	QT00177618	25 325	69
IL-12p35	QuantiTect Primer Assay	QT00191023	84 405	122
IL-12p40	QuantiTect Primer Assay	QT00188839	64 546	88
IL-23p19	QuantiTect Primer Assay	QT0042560	155 140	85

T cell proliferation assay

To test the capacities of DC to stimulate naïve T-cell proliferation, a radiometric assay based on [^{3}H]-thymidine incorporation was applied. For in vitro experiments, triplicate aliquots (200 μL) of enriched DC (5×10^4) were collected from TLN or ILN of EAO animals 50 days after first immunization. C and N groups were co-cultured with allogeneic T cells (1×10^5) in 96-well round-bottomed microtitre plates (Nunc, Wiesbaden, Germany) at 37 °C. Moreover, ready-to-use phytohemagglutinin solution (PHA, final concentration 10%; PAA Laboratories) was added into appropriate wells containing T cells only as positive control for stimulating T-cell proliferation. After 48 h of incubation, the cells were pulsed with 0.25 μCi of [^{3}H]-thymidine (GE Healthcare) for 15 h. Subsequently, the medium was removed; cells were washed twice with cold PBS and then solubilized in 0.5 M of NaOH. The amount of radioactivity incorporated into DNA was determined by liquid scintillation spectrometry (Tri-Carb 1500; Packard, Meriden, CT, USA) and expressed as counts per minute (cpm).

Statistical analysis

Results are expressed as mean ± SEM. Comparisons between groups were made using the non-parametric Kruskal–Wallis one-way ANOVA or the one-way ANOVA accompanied by the Bonferroni test when applicable. $p \leq 0.05$ was considered significant.

Results

Identification and localization of DC in LN

By immunohistochemistry, OX-62+DC were identified in popliteal, inguinal, iliac and renal LN from rats of C and EAO groups. We observed that OX-62+DC were predominantly localized in the cortex around the lymphatic nodules and also in the lymphatic sinuses (Fig. 2). A stronger labelling intensity was visible in LN from EAO animals in comparison with control LN.

Quantification of DC in LN

To quantify the percentage of OX-62+DC in ILN and TLN from N rats and C and E groups sacrificed at 30 and 50 days after the first immunization, a flow cytometry assay was performed. In ILN, a similar percentage of OX-62+DC was visible in the different groups studied (Fig. 3A). However, the percentage of OX-62+DC in TLN was significantly higher in rats of E group compared with N and C groups on days 30 and 50 (Fig. 3B). No differences between rats of E group sacrificed at 30 or 50 days were detected.

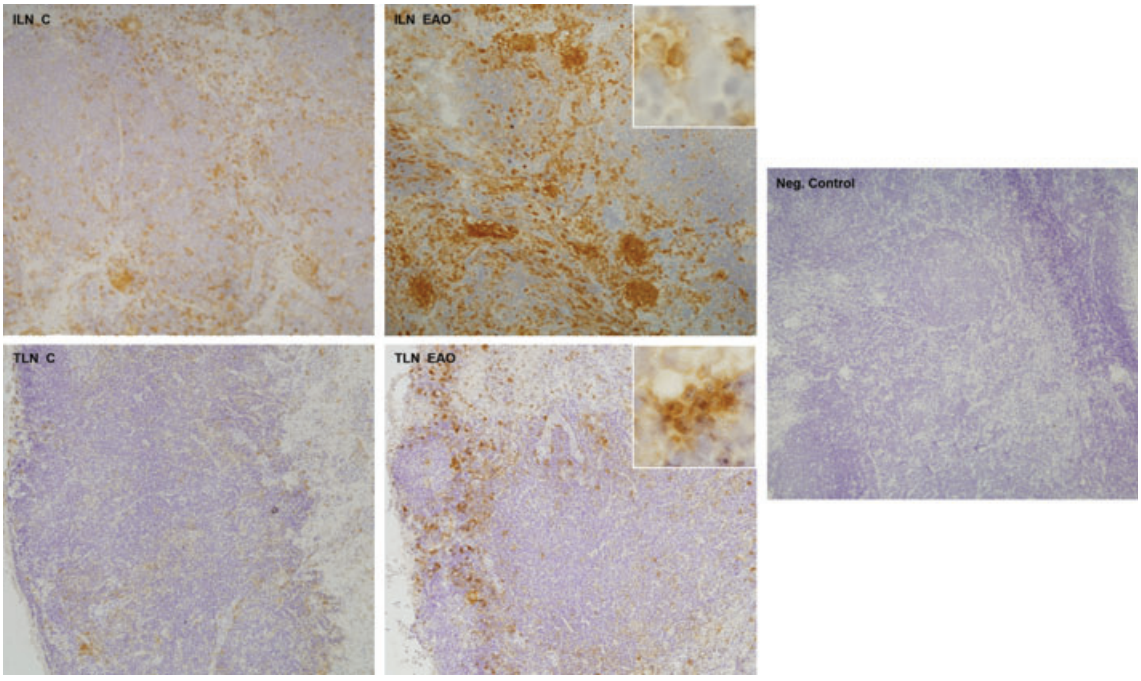

Figure 2 Representative immunostaining of dendritic cells with the anti-OX-62 antibody in cryostat lymph node (LN) sections from control (C) and orchitis (EAO) rats 50 days after first immunization. Positive cells are mainly located in the cortex, around the lymphatic nodules and also in the lymphatic sinuses. Omission of primary antibody showed negative staining (negative control). TLN, testicular draining LN; ILN, LN draining the site of immunization. Magnification: ×100 and ×1000 (insert).

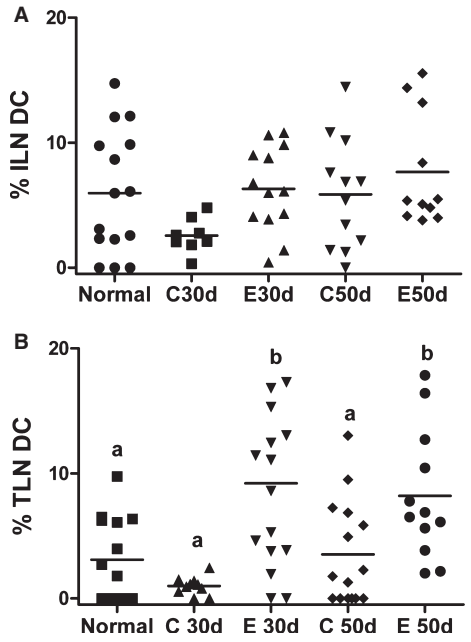

Figure 3 Quantitative assessment of percentage of OX-62+DC in cell suspension of draining lymph node (LN) from the immunization site (ILN; A) and from the testis (TLN; B) evaluated in normal, control (C) and experimental (E) groups using FACS analysis. Analysis was performed on the total dendritic cell fraction. Each dot represents one rat. Values with different superscript letters differ significantly compared with the normal and control groups ($p < 0.05$); d indicates days after the first immunization.

MHC class II, CD80 and CD86 expression on LN DC

Using flow cytometry, the expression of MHC class II, CD80 and CD86 molecules on DC (OX-62+) from ILN and TLN was analysed. As indicated in Figs 4 and 5, the majority of ILN and TLN DC express these molecules and the percentage was comparable between N, C and E groups sacrificed at 30 or 50 days after the first immunization. Analysis of mean fluorescence intensity (MFI) also showed similar expression levels of MHC class II, CD80 and CD86 in ILN DC in every group studied (Fig. 4). Similar results were obtained for TLN DC expressing CD80 and CD86 molecules (Fig. 5). Only TLN DC from EAO rats (50 days) showed an increase in MFI for MHC class II compared with N, C and E groups studied at day 30 (Fig. 6).

Quantitative mRNA expression of IL-10, IL-12p35, IL-12p40 and IL-23p19 in DC from rat LN draining the site of immunization

As no significant changes in the expression of co-stimulatory (CD80, CD86) and MHC class II molecules on DC from TLN as well as ILN 30 days after the first immunization were detected, further quantitative mRNA analysis of cytokine profiles expressed by DC was performed exclusively at the time point of 50 days after the first immunization in C and EAO groups as well as in N animals. The mRNA cytokine expression was assessed by quantitative real-time RT-PCR analysis. The expression profile of IL-10, IL-23p19 and IL-12p35 subunits in the enriched fraction of DC from ILN revealed no significant changes between the analysed groups (Fig. 7A–C). Only the mRNA expression of IL-12 subunit p40 in DC from the C and EAO groups was significantly downregulated compared with the N untreated group (Fig. 7D), an effect that can be attributed as a side effect of Bp treatment (McGuirk & Mills, 2000).

Expression profile of cytokines IL-10, IL-12p35, IL-12p40 and IL-23p19 in DC from rat TLN

Enriched fraction of DC isolated from TLN showed significantly upregulated mRNA expression of IL-10 as well as subunit p35 of bioactive IL-12p70 in EAO (50 days) group compared with DC from C group (Fig. 8A,C). However, the IL-12p40 mRNA in DC from TLN in EAO and C rats was significantly decreased in comparison with the N group (Fig. 8D). Relative mRNA expression of IL-23 subunit p19 in DC from TLN in all investigated groups remained unchanged (Fig. 8B).

Proliferation assay

To analyse the stimulatory capacity of DC from LN, enriched DC fractions were co-cultured in mixed lymphocyte reaction with naïve allogeneic T cells. Functional data from this in vitro assay showed that EAO-DC from TLN significantly enhanced the proliferation of T cells compared with DC from C or N TLN (Fig. 9B). The level of proliferation was about fourfold higher compared with control DC. In contrast, EAO-DC from ILN induced similar proliferation level like DC from C or N ILN (Fig. 9A).

Discussion

DCs are key players in antigen presentation and T-lymphocyte activation exerting a pathogenic role in autoimmune reactions. In our previous study, we reported an increase in the number of testicular DCs in rats undergoing autoimmune orchitis. In this model, DC revealed a mature immunogenic state prior to imminent migration to the LN (Rival *et al.*, 2006, 2007). In this work, we phenotypically and functionally characterized the DC in rat TLNs and ILNs during the course of EAO. The aim was

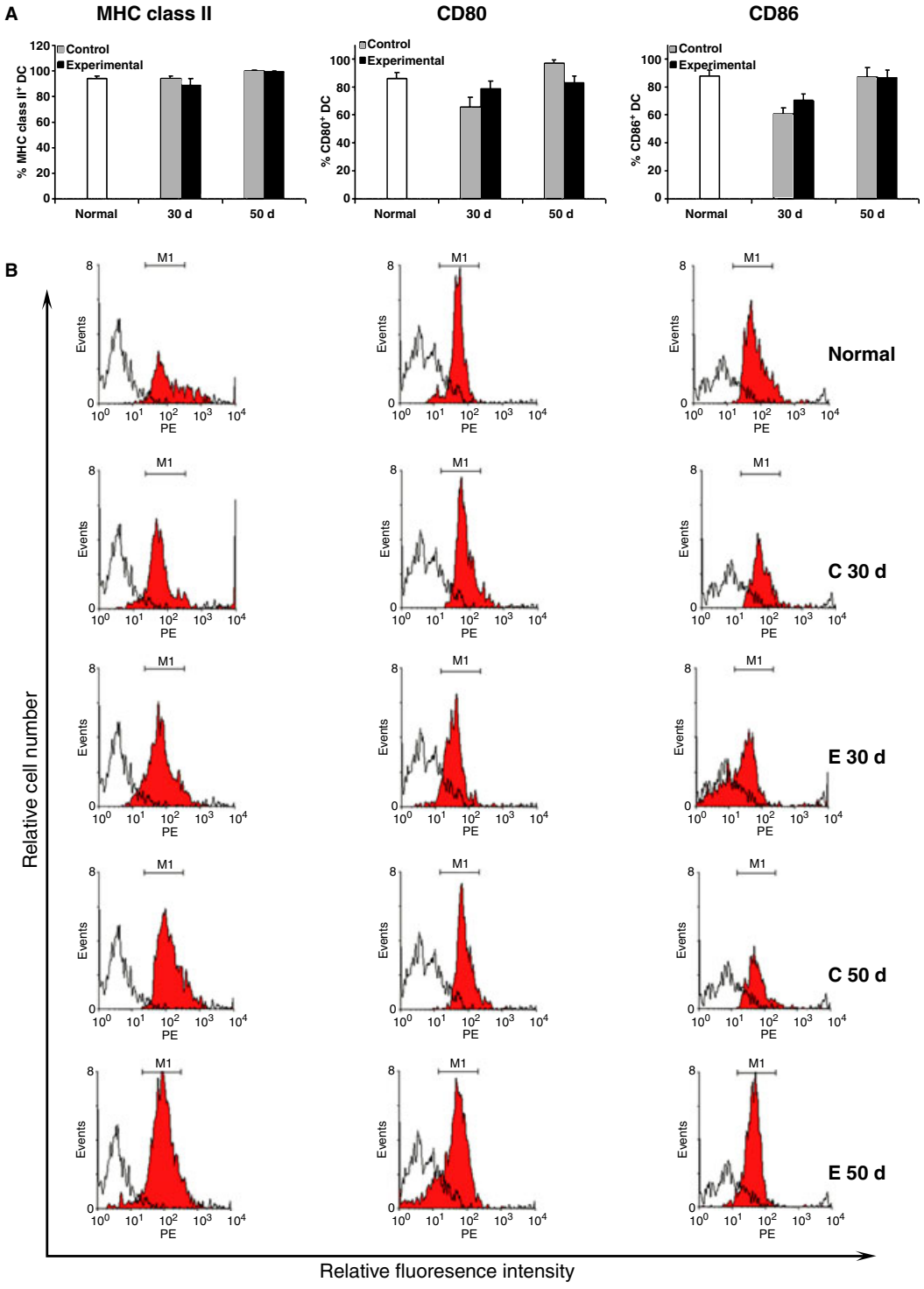

Figure 4 Major histocompatibility complex (MHC) class II, CD80 and CD86 expression on dendritic cells (DC) from lymph nodes (LN) draining the site of immunization (ILN). (A) Quantitative assessment of the percentages of OX-62+DC expressing MHC class II, CD80 and CD86 molecules. Data are presented as the mean ± SEM. (B) Representative histogram plots illustrating MHC class II, CD80 and CD86 expression in OX-62+DC from ILN of normal, control (C) and experimental (E) groups. *n* = 7–10 rats per group; d indicates days after the first immunization.

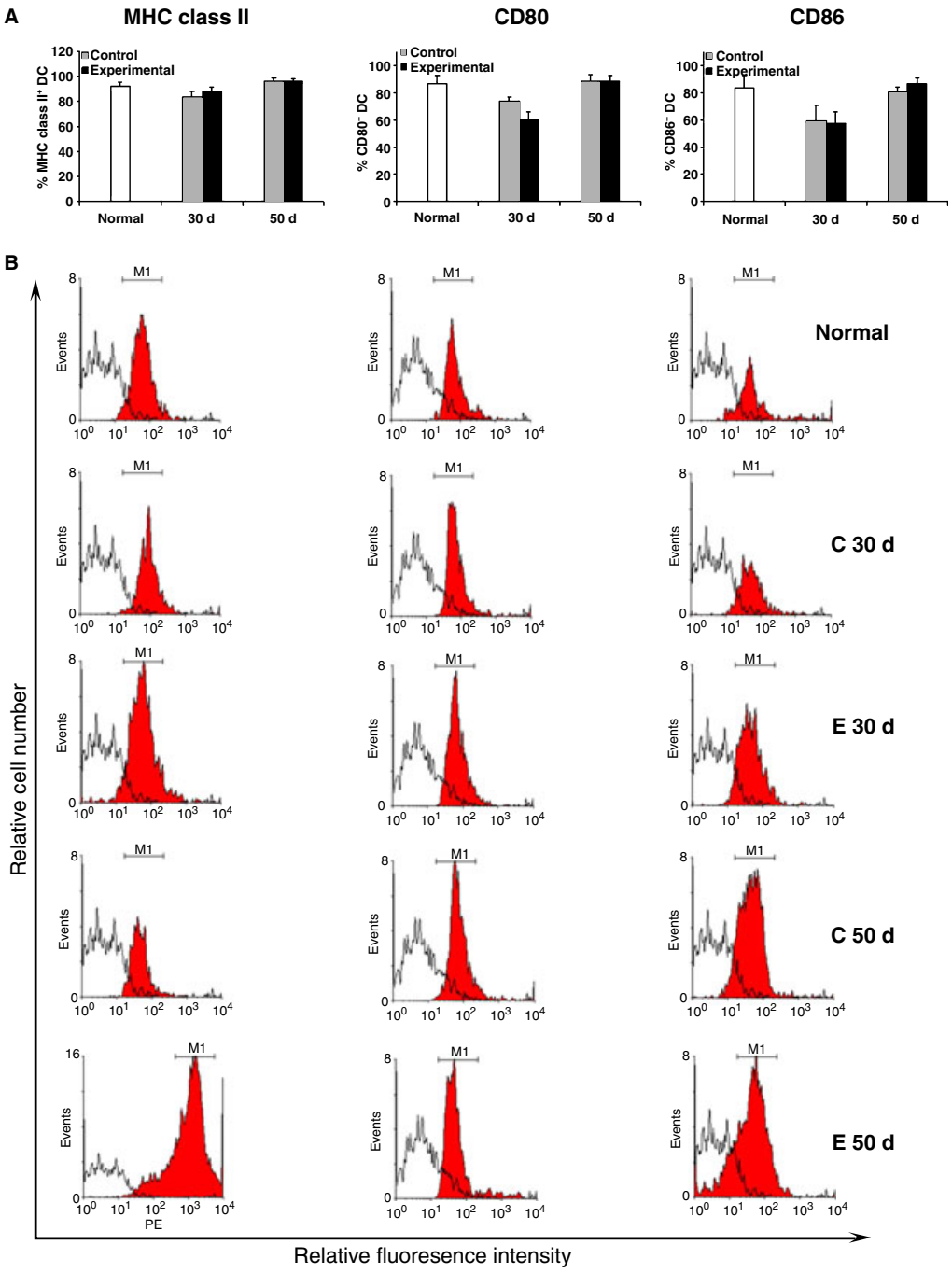

Figure 5 Major histocompatibility complex (MHC) class II, CD80 and CD86 expression on dendritic cells (DC) from testicular draining lymph nodes (TLN). (A) Quantitative assessment of the percentages of OX-62+DC expressing MHC class II, CD80 and CD86 molecules. Data are presented as the mean ± SEM. (B) Representative histogram plots illustrating MHC class II, CD80 and CD86 expression in OX-62+DC from TLN of normal, control (C) and experimental (E) groups. Note the higher relative fluorescence intensity of MHC class II in DC from rats with orchitis (E: 50d). *n* = 7–10 rats per group; d indicates days after the first immunization.

to collect evidence about putative site-specific differences that reflect a role of DC in the initiation and progression of testicular autoimmune responses.

In all studied rats (C and E), DCs were localized mainly in the LN cortex around primary follicles in parafollicular thymus-dependent areas and occasionally at the

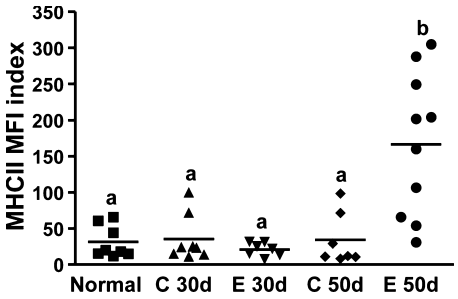

Figure 6 Mean fluorescence intensity (MFI) of major histocompatibility complex (MHC) class II molecules expressed on OX-62+DC from testicular draining lymph nodes analysed in normal, control (C) and experimental (E) rats at 30 and 50 days (d) after first immunization. MFI index was calculated as MFI positive/MFI of negative population. Each dot represents one rat. Values with different superscript letters differ significantly compared with the normal and C groups ($p < 0.05$).

subcortical sinus and medulla sinusoids. A similar localization of DC was observed by different authors in other regional LN (Trinite *et al.*, 2000; Yrlid & Macpherson, 2003). Flow cytometry results showed that the number of DCs from TLN increased in rats with orchitis 50 days after the first immunization and also at the end of the immunization period (30 days). Similarly, in vivo administration of lipopolysaccharide (LPS) has previously been shown to correlate with increased numbers of DCs in T-cell areas of draining mesenteric LN (Turnbull & MacPherson, 2001). In our model, the increase in TLN DC from rats sacrificed 30 days after immunization, when histopathological testicular damage is hardly visible, can be related to an increase in APC around the epithelial cells of the straight tubules, where signs of disease usually occur and the blood testis barrier is less developed (Tung *et al.*, 1987). With the progression of the disease, a significant passage of spermatic antigens to the interstitium occurs and DC with engulfed antigens could migrate to the regional LN.

No changes in the number of ILN DCs were observed among the N, C and EAO rats. This could be based on the short mean life of LN DC in rats (3–4 days) (Yrlid & Macpherson, 2003) and their high turnover which prevent detection 30 and 50 days after the first immunization. The majority of DC from LN (ILN and TLN) of all groups of rats studied expressed CD80 and CD86 molecules with a similar MFI. This does not necessarily reflect unchanged T-cell stimulatory capacity as an increase in other co-stimulatory molecules such as CD70 and CD40 DC without changes in CD80 and CD86 expression as reported in mice after stimulation of Toll-like receptor ligands (Sanchez *et al.*, 2007). In contrast to the data of co-stimulatory molecules, a significant increase in MHC class II expression/cell was observed in TLN from rats with EAO pointing to their ability in antigen presentation. In this case, the MFI was higher than that of testicular DC (Rival *et al.*, 2007). Investigating key cytokines, no significant differences in the expression of IL-12p35, IL-23p19 and IL-10 mRNA expression by ILN DC from N, C and E rats were found. In contrast, we observed a significant increase in the expression of IL-12p35 mRNA in TLN DC from EAO rats compared with N and C rats suggesting the presence of immunogenic DC. Moreover, we demonstrated the ability of TLN DC to induce T-cell proliferation. IL-12 is

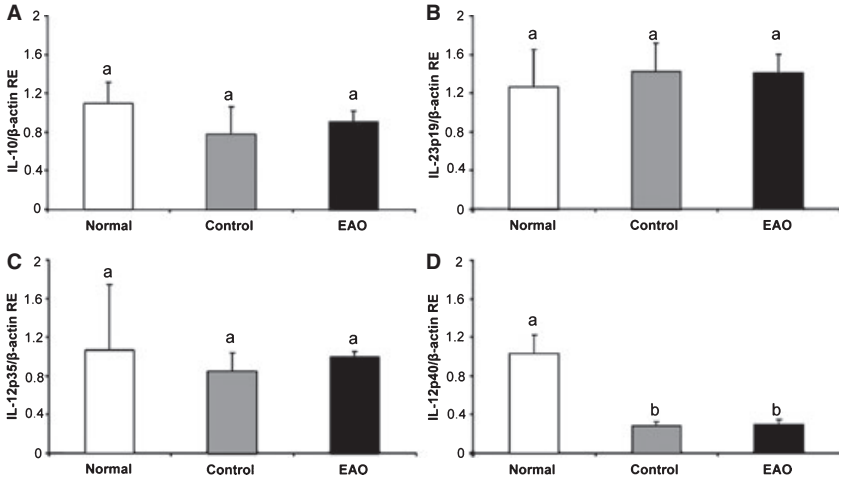

Figure 7 Relative mRNA expression of cytokines in dendritic cells (DC) from lymph nodes (LN) draining the site of immunization (ILN) analysed by real-time reverse transcription-polymerase chain reaction. Interleukin (IL)-10 (A), IL-23p19 (B), IL-12p35 (C) and IL-12p40 subunit (D) mRNA expression in DC collected from ILN of normal, control and orchitis (EAO) rats (50 days). β-actin is used for normalization of expression data. Values are mean ± SEM of relative expression units (RE) of results for 7–10 animals in each group. Values with different superscript letters differ significantly ($p < 0.05$) compared with the respective controls.

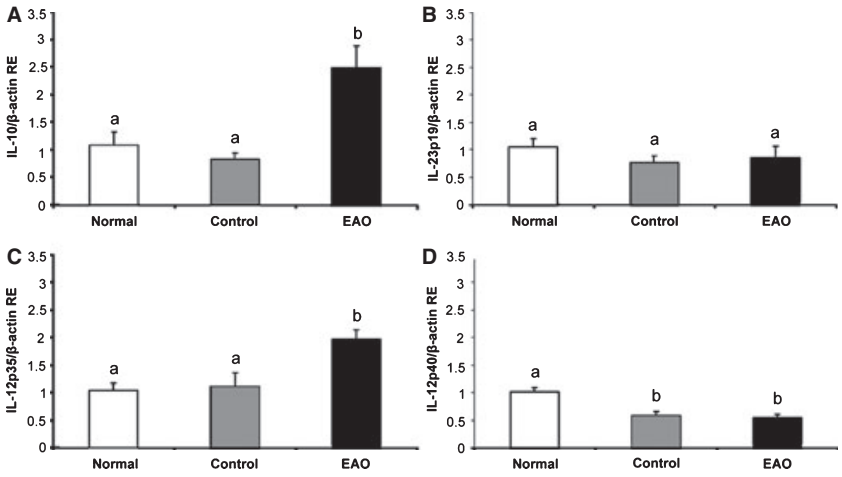

Figure 8 Comparison of the relative mRNA expression levels of interleukin (IL)-10 (A), IL-23p19 (B), IL-12p35 (C) and IL-12p40 subunits (D) determined by real-time reverse transcription-polymerase chain reaction in dendritic cells (DC) collected from rat testicular draining lymph nodes (LN) obtained from normal, adjuvant control and orchitis (EAO) rats at 50 days after first immunization. β-actin is used for normalization of expression data. Values are mean ± SEM of relative expression units (RE) of results for 7–10 animals in each group. Values with different superscript letters differ significantly ($p < 0.05$) compared with the respective controls.

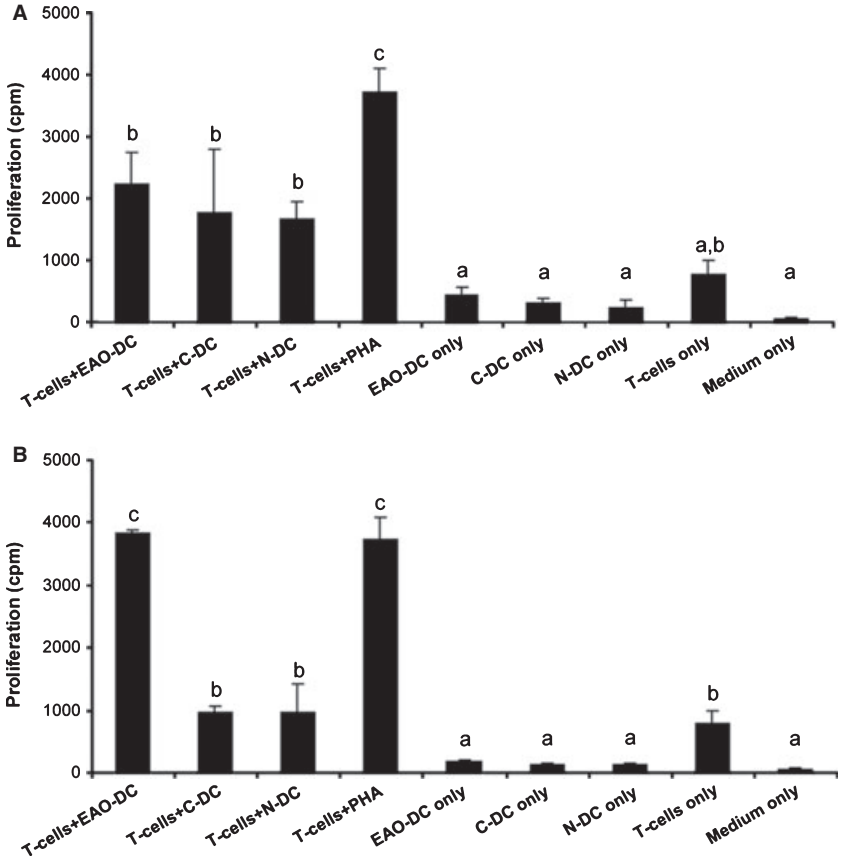

Figure 9 Proliferative responses of naïve T cells (1×10^5) from Sprague-Dawley rats co-cultured with 5×10^4 dendritic cells (DC) from lymph nodes (LN) draining the site of immunization (A) or from testicular draining LN (B) isolated from normal (N-DC), control (C-DC) or experimental (EAO-DC) for 63 h at 37 °C. T cells treated with phytohemagglutinin (10%) were used as a positive control. Proliferation of naïve T cells was assessed by standard [^{3}H]-thymidine incorporation and is displayed as counts per minute. Data are presented as the mean ± SEM of six animals in each group. Values with different superscript letters differ significantly ($p < 0.05$).

a typical mediator released by activated DC that can govern the development of type I immunity characterized by Th1 cell or cytotoxic T-lymphocyte development. Furthermore, because of the lower abundance of p35 transcripts even in activated inflammatory cells, the expression of the p35 subunit determines the production

of bioactive heterodimeric IL-12p70 (Babik *et al.*, 1999; Trinchieri, 2003).

The significant increase in IL-12p35 mRNA expression in DC from E rats at the onset of EAO (50 days) indicates the production of bioactive IL-12p70, a cytokine that promotes a Th1 immune response and induces high IFN-γ production in naïve T cells. Indeed, at this time of EAO development, testicular DC from E rats acquire the ability to activate naïve T lymphocytes (Rival *et al.*, 2007) secreting IFN-γ (Jacobo, personal communication). Furthermore, we observed an increase in IL-10 mRNA expression in DC of E rats compared with N rats, a step which can be ascribed to an increase in IL-12 (Huang *et al.*, 2001).

Interestingly, these results showed no changes in mRNA expression of p19, a subunit of IL-23 in DC from C or E rats at the onset of EAO (50 days) indicating that IL-23 is probably not relevant in the modulation of immune response, at least not at this time point. IL-23 secreted by APC is a central player in the expansion and survival of Th17 cells associated with the development of autoimmunity and inflammatory response (Langrish *et al.*, 2005) and the production of IL-17 is ameliorated by IL-10 (Schaefer *et al.*, 2010). Th1 and Th17 cells represent distinct effector cell subsets that develop in differential conditions of IL-12 and IL-23 (Weaver *et al.*, 2007), and both cell lineages are necessary to induce tissue inflammation. Our preliminary results (Jacobo *et al.*, 2009b) suggest that immune response with a Th17 profile occurs in E rats during late severe stage of the EAO, 80 days after the first immunization.

Studies in other models of autoimmunity such as experimental autoimmune encephalomyelitis in rodents have shown that microinjection of antigen-pulsed DC leads to migration of DC from the brain to the draining cervical LN, recruitment of antigen-specific T cells to the brain and enhancement of the systemic humoral response against myelin oligodendrocyte glycoprotein, a major immunogenic myelin antigen (Carson *et al.*, 1999; Karman *et al.*, 2004; Hatterer *et al.*, 2008). Further emphasizing the role of tissue-derived DC in stimulating the activation of naïve T cells after migrating to draining LN, in non-obese diabetic (NOD) mice, DC purified from pancreas draining LN were highly MHC II class positive and revealed a very strong capacity to induce clustering of T cells as compared with control LN (Clare-Salzler & Mullen, 1992). An important factor in the maturation of DC is TNF-α, which is also found increased in EAO (Suescun *et al.*, 2003). In vivo, neonatal treatment of NOD mice with TNF-α caused an increase in the expression of maturation markers on DC from pancreatic LN, whereas treatment with anti-TNF-α caused a decrease in expression of these markers in the CD11c+CD11b+subpopulation (Lee *et al.*, 2005). Moreover, neonatal application of anti-TNF-α antibodies induced tolerance to islet cell proteins released by apoptosis. The authors concluded that TNF-α plays a crucial role in the initiation of type 1 diabetes in NOD mice by regulating the maturation of DC and thus the activation of islet-specific pancreatic lymph node T cells. Mechanistically, it was proposed that β-cell proteins released by apoptosis are picked by macrophages and DC and transported to the draining pancreatic LN. A similar mechanism seems to be possible in EAO testis, where high levels of TNF-α induce apoptosis of germ cells with severe impairment of spermatogenesis (Theas *et al.*, 2008). Obviously, as shown for autoimmune gastritis tissue destruction by immune cells markedly augments the number of antigen-containing DC in the draining LN as well as their antigen presentation function, inducing epitope spreading and disease amplification. In contrast, constitutive presentation of a natural tissue autoantigen under non-inflammatory conditions does not induce autoimmunity in the gastritis model (Scheinecker *et al.*, 2002).

On the basis of our previous and present data, we speculate that during the immunization period, the autoimmune response initiates in the ILN, where DCs capture the antigen. Subsequently, DC-sensitized lymphocytes migrate to the testis where they contribute to testicular inflammation thus causing tissue damage. Spermatic antigens released to the interstitium during the course of the disease are processed by inflammatory testicular DC that migrate to TLN and activate T lymphocytes. How testicular DC initiate autoimmune response and testicular damage can be explained by the danger model theory (Matzinger, 1994), where stressed and necrotic cells release 'danger' signals in the form of, for example, heat shock proteins (Hsp), which enhance the maturation of DC. In agreement, Hsp 60 and Hsp 70 as testicular autoantigens in EAO were identified in a previous study (Fijak *et al.*, 2005). We hypothesize that under inflammatory conditions with increased levels of TNF-α and release of Hsp, immature testicular DC overcome immune tolerance (privilege), migrate to the LN and activate T cells. This view is supported by the finding that testicular DC in EAO showed increased CCR7 expression (Rival *et al.*, 2007), whereas TLN express the CCR7 ligand CCL19, a chemokine involved in DC traffic (Guazzone & Mardirosian unpublished data). Progressive amplification of the autoimmune response finally leading to chronification of EAO may result from continuous antigen presentation of DC to lymphocytes in TLN and the testis. This model is substantiated by findings in mice autoimmune diabetes islet inflammation that results in an increase in mature islet antigen-presenting DC in the pancreatic LN that may lead to further priming of β-cell antigen-reactive T cells (Melli *et al.*, 2009).

In summation, our results extend the current understanding of the role of DC in EAO pathogenesis and suggest that DC provide a positive feedback in the initiation of inflammatory and autoimmune response in the testis. Identification of key factors and cytokines involved in DC–lymphocyte interactions will provide valuable information on ways to address autoimmune inflammation therapeutically.

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