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Expression of co-stimulatory molecules, chemokine receptors and proinflammatory cytokines in dendritic cells from normal and chronically inflamed rat testis

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The presentation of self antigens by dendritic cells (DC) plays an important role in the initiation and maintenance of autoimmunity. In a model of experimental autoimmune orchitis (EAO), we have previously characterized dominant testicular autoantigens and shown an increase in DC numbers during the course of disease. In this study, we have developed a protocol for the isolation of a highly pure population of DC (∼97%) from the testis of EAO and control rats to analyse the expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD80, CD86), chemokine receptors (CCR2, CCR7) and cytokines (IL-10, IL-12p70, TNF-α). By flow cytometry, we observed similar percentage and intensity levels of MHC class II, CD80 and CD86 expression in testicular DC in all groups. Moreover, by real-time RT-PCR we have detected significantly higher CCR7 mRNA level in isolated testicular DC from rats with EAO compared to controls, whereas the expression of CCR2 was decreased in orchitis. Transcripts of IL-12p40 were observed in DC from all groups, whereas the expression of IL-10 and the rate limiting IL-12 subunit p35 were detectable exclusively in testicular DC from the inflamed testes. In co-culture experiments, testicular DC isolated from EAO animals significantly enhanced naïve T-cell proliferation compared with control DC. Taken together these results suggest that testicular DC in control testis is not mature and functionally tolerogenic, whereas in EAO testis, IL-12 expression and stimulation of T-cell proliferation points to a mature immunogenic state prior imminent migration to the lymph nodes to amplify immune responses against testicular antigens

Keywords: experimental autoimmune orchitis (EAO); dendritic cells; testicular inflammation; co-stimulatory molecules; cytokines

Introduction

Dendritic cells (DC) represent the pacemakers of the immune response. They are crucial for the presentation of proteins and peptides to T and B lymphocytes and are widely recognized as the key antigen presenting cells (APCs). In peripheral organs, DC differentiate and become active in the uptake and processing of antigens with their subsequent presentation on the cell surface linked to major histocompatibility complex (MHC) molecules. Upon receiving an activatory signal associated with pathogens or inflammation, DC undergo further maturation and migrate to secondary lymphoid tissues where they present antigens to naïve T-cells and induce an immune response (Banchereau and Steinman, 1998). Mature DC possesses a very efficient T-cell priming ability as a consequence of the up-regulation of MHC and co-stimulatory molecules on their cell surface.

In recent years, DC have received the increasing attention as key players in both the initiation and maintenance of autoimmune disease and the induction of peripheral tolerance (Thomas and Lipsky, 1996; Banchereau *et al*., 2003; Steinman *et al*., 2003). The maturation and/or activation state of DC are regarded as a control point for the induction of either peripheral tolerance or autoimmunity. Especially immature or semi-mature DC have a potent ability to tolerize T-cells and prevent undesired immune reactions. Immature DC have the highest capacity to internalize antigens, but low T-cell stimulatory activity, whereas mature DC down-regulate their endocytic activity and are excellent T lymphocyte stimulators (Banchereau *et al*., 2000). The maturation process is concomitant with the migration of DC from peripheral sites of inflammation through lymphoid vessels to the lymph nodes (LN).

Expression of stimulatory and co-stimulatory molecules has major consequences on the immune response. Thus, a switch in chemokine receptor expression enables activated DC to migrate to the draining LN from the site of inflammation. In particular, up-regulation of the chemokine receptor CCR7 is a key step required for the entry of DC into LN and their homing to T- and B-cell zones therein (Randolph *et al*., 2005). However, CCR7 also can regulate other functions of DC such as endocytosis, cell survival or maturation and therefore provides a further level of control in the direction and magnitude of immune responses (Sanchez-Sanchez *et al*., 2004, 2006). Mature

DC are characterized by the upregulation of surface co-stimulatory (CD40, CD80 and CD86) and MHC class II molecules, and a higher production of cytokines such as IL-12p70, which modulates T-cell responses promoting either Th1-cell or cytotoxic T-cell development. Bioactive IL-12p70 is a heterodimeric protein composed of the p40 and p35 subunits. As the p35 subunit is expressed at substantially lower levels than p40, the availability of bioactive IL-12 (IL-12p70) is ultimately determined by the p35 subunit production (Babik *et al*., 1999; Trinchieri, 2003). The p40 subunit is shared with IL-23, a crucial cytokine for the maintenance of the novel Th17 lineage involved in the pathogenesis of several autoimmune diseases (Oppmann *et al*., 2000; Cua *et al*., 2003; Langrish *et al*., 2005; McKenzie *et al*., 2006).

Infertility affects one in seven couples worldwide. Infection and inflammation of the male genital tract are a frequent cause or co-factor of fertility disturbances in men [13–15% of all cases of infertility (WHO, 1987; Nieschlag and Behre, 2000)]. Experimental autoimmune orchitis (EAO) serves as a model to study chronic testicular inflammation and organ specific autoimmunity. The histopathology of EAO rat testis is characterized by a substantial increase of interstitial lymphocytes and macrophages, different degrees of germ cell loss caused by apoptosis resulting in aspermatogenesis and atrophy of the seminiferous tubules. Numerous granulomae are also observed (Doncel *et al*., 1989; Lustig *et al*., 1993; Suescun *et al*., 2003).

Testicular DC have received little attention in spite of the fact that they might play an important role in testicular immune privilege. Previous reports suggested the presence of DC in the normal testis of mice (Itoh *et al*., 1995; Hoek *et al*., 1997), rats (Head and Billingham, 1985) and humans (Haas *et al*., 1988; Derrick *et al*., 1993). However, the use of markers that cross-reacted with macrophages as the most abundant leukocytes in the testicular interstitium, did not unarguably demonstrate the existence of testicular DC. In our recent study (Rival *et al*., 2006), the presence and numbers of DC in normal and chronically inflamed testes from Wistar and Sprague–Dawley rats were determined for the first time using DC specific markers (OX-62 and CD11c) in combination with immunohistochemistry and stereological analysis. Significantly elevated numbers of DC were observed in the testicular interstitium of animals with EAO (Rival *et al*., 2006). The numbers of DC strongly correlated with the development of inflammatory lesions in the inflamed testes suggesting an important pathogenic role of DC in induction and development of the autoimmune response in EAO.

To understand the role of DC in normal and inflamed testis, we have characterized the maturation state of DC by analysing the expression of co-stimulatory molecules (CD80, CD86), chemokine receptors (CCR2, CCR7), cytokines (IL-10, IL-12p70, TNF-α) and molecules involved in antigen presentation (MHC class II). Moreover, in functional studies the capacity of isolated DC from control and orchitis animals to induce T-cell proliferation was assessed.

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 were approved by local committees (Regierungspraesidium Giessen GI 20/23—Nr. 15/03; Consejo Nacional de Investigaciones Científicas y Tècnológicas, N6022).

Induction of EAO

Wistar rats of experimental (E) group were actively immunized with syngeneic testicular homogenate (TH) as previously described (Doncel *et al*., 1989). Briefly, inbred rats were anesthetized by intraperitoneal (i.p.) administration of 100 mg/kg b.w. Ketamine (Ketavet, Pharmacia, Erlangen, Germany) and 10 mg/kg b.w. Xylazine (Rompun, Bayer Vital, Leverkusen, Germany). Immunization was performed injecting 0.4 ml syngeneic TH mixed with 0.4 ml complete Freund's adjuvant (Sigma–Aldrich, 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 isotonic saline, whereas the third one was followed by an i.p. injection of Bp at a concentration of 5×10^9 in 0.5 ml isotonic saline. Control (C) animals received complete Freund's adjuvant and *B. pertussis*, but no testis homogenate 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. Testes were removed, weighed and testicular single cell suspensions were prepared. Epididymis 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.

Preparation of testicular interstitial cell suspension

Testicular interstitial cells from N, C and E rat testes were obtained by enzymatic digestion. Briefly, decapsulated testes were incubated with type I collagenase (0.3 mg/ml; Worthington Biochemical Corporation, Freehold, NJ) plus 0.1% bovine serum albumin (BSA, fraction V, Sigma Chemical Co.) in a shaking water bath at 34°C for 15 min. The collagenase was inactivated by adding ice cold PBS and the tubule fragments were allowed to settle for 4 min, then the supernatant was centrifuged at 300 g for 10 min at 4°C. The pellet containing interstitial cells was washed with PBS and red blood cells were depleted by osmotic lysis with ammonium chloride (160 mM NH_4Cl, 170 mM Tris–HCl, pH 7.2) for 5 min at room temperature (RT). Final cell suspension was washed with PBS at 300 g for 10 min at 4°C and processed directly for flow cytometric analysis or isolation of DC.

Flow cytometric analysis

The phenotype of freshly isolated testicular interstitial cells was determined using three-color flow cytometry. Monoclonal antibodies (mAb) used for cell surface staining were directly conjugated to either R-phycoerythrin (R-PE) or PE-Cy-Chrome (PE-Cy5) or a secondary antibody conjugated with fluorescein isothiocyanate (FITC) was used to detect unlabeled mAb. A summary of primary antibodies used in this study is presented in Table I. One million interstitial cells from N, C and E testes were incubated with OX-62 mAb for 30 min. After washing in cold PBS with 0.1% azide, 5% fetal bovine sera (FBS) and 2 mM EDTA (PBS/FBS buffer), cells were incubated with anti-mouse FITC conjugated-IgG (1:50; Vector Laboratories, Burlingame, CA, USA) for 30 min. Cells were washed with PBS/FBS buffer and stained for 30 min with the following combination of anti-rat mAb: (i) CD45+CD80, (ii) CD45+CD86 and (iii) CD45+MHC class II. Finally, cells were washed twice with PBS/FBS buffer. Background staining was evaluated using isotype controls: PE or PE-Cy5-conjugated mouse $IgG_{1,k}$ mAb (BD Biosciences PharMingen, San Jose, CA, USA) or mouse $IgG_{1,k}$ mAb (Sigma–Aldrich). The whole procedure was performed at 4°C. Samples were fixed using 2% paraformaldehyde and were run on a FACS Calibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Data were collected for 20–30 000 events on CD45+ gate (Fig. 1).

Isolation and high-purity sorting of DC from rat testis

DC were enriched from the interstitial cell suspension by positive selection using mouse anti-rat OX-62 antibody coupled 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 OX-62 antibody (Table I), followed by incubation with the secondary FITC conjugated anti-mouse IgG antibody (Vector Laboratories) under the same conditions as mentioned above. Stained cells were resuspended in MACS buffer (2 mM EDTA and 0.5% BSA in PBS) and stored on ice until sorting. Fluorescence-activated cell sorting was performed using a

Table 1. Summary of primary antibodies used in the study.

Reagent	Working dilution (ug/ml)	Specificity	Fluorochrome	Origen
CD3	10.0	T-cell receptor αβ or γδ	FITC	BD Pharmingen[a]
CD4	10.0	CD4 antigen	R-PE	BD Pharmingen
CD45	0.7	Leukocyte common antigen	PE-Cy5	BD Pharmingen
OX62	10.0	αE2 integrin (DC)	—	Serotec[b]
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	BD Pharmingen

[a]San Diego, CA, USA. [b]Kidlington, Oxford, UK. DC, dendritic cells; MHC, major histocompatibility complex; FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin; PE-Cy5, phycoerythrin-Cy5.

FACSVantage SE DiVA cell sorter (BD Biosciences). Sorting gates were set on the low side scatter (SSC)/OX62 positive cell population. Cell debris was excluded by appropriate gate setting. Cells were sorted in RPMI 1640 (PAA, Germany) plus 10% FCS (fetal calf serum, PAA, Germany). Data acquisition and analysis were performed using the BD FACSDiva Software package (BD Biosciences).

RNA isolation and real-time RT-PCR

The 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) for 15 min at RT. The total mRNA obtained from up to 5×10^5 DC together with 2 µl oligo$(dT)_{15}$ primer was pre-incubated at 70°C for 10 min. The reaction mixture for RT-PCR (RT buffer, 10 mM each dNTP, 40 U recombinant RNasin ribonuclease inhibitor) was pre-heated at 42°C for 2 min before pre-incubated mRNA and oligo$(dT)_{15}$ primer and 200 U of M-MLV reverse transcriptase were added and incubation continued for 75 min at 42°C. The reaction was inactivated by incubation at 70°C for 15 min. All RT-PCR reagents were provided by Promega (Mannheim, Germany). The cDNA quality was checked by amplifying the GAPDH message.

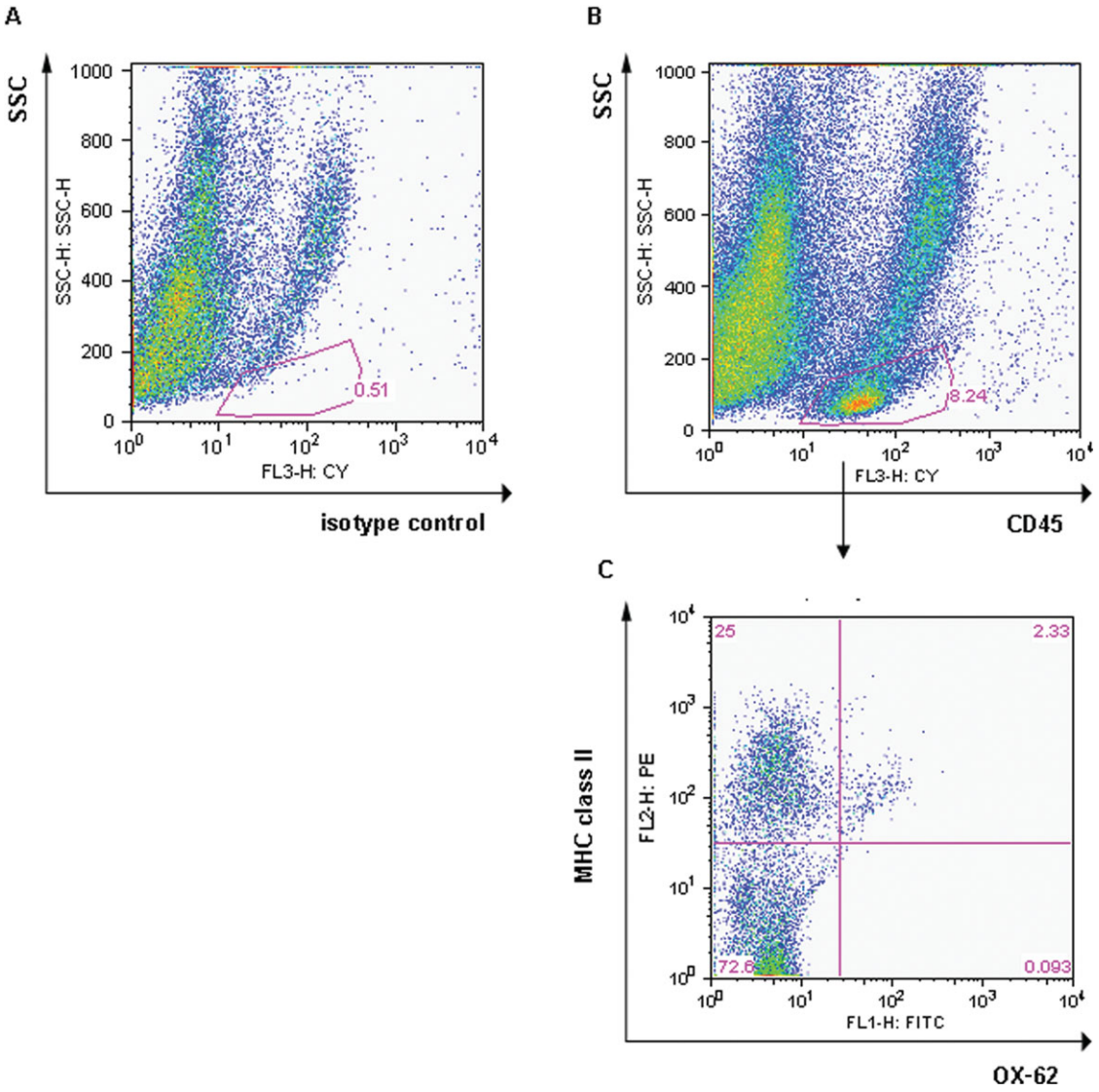

Figure 1: FACS analysis of MHC class II expression in testicular DC
(**A**) Isotype IgG conjugated with PE-Cy5. (**B**) Representative dot plot showing gating on the CD45+ cells, (**C**) Representative dot plot of the CD45+ population revealing MHC class II expression of OX62+ cells. Red numbers represent the percentages of CD45 positive cells (A, B) or Ox62 and MHC class II positive cells (C) within the CD45 positive fraction

Table II. RT-PCR primers used in this study.

Gene	Primer	Katalog-No (Qiagen)	Entrez gene ID	Product length (bp)
GAPDH	sense CATTGTTGCCATCAACGACC	—	24383	340
	antisense TCACACCCATCACAAACATG	—		
CCR2	QuantiTect Primer Assay	QT00459473	60643	79
CCR7	QuantiTect Primer Assay	QT00391664	287673	94
TNFα	QuantiTect Primer Assay	QT00178717	24835	75
IL10	QuantiTect Primer Assay	QT00177618	25325	69
IL12p35	QuantiTect Primer Assay	QT00191023	84405	122
IL12p40	sense TCACCTGGACCTCAGACCAGA	—	64546	239
	antisense GAACCGTCCGGAGTAGTTTGG	—		

Real-time quantitative PCR was performed using the I-cycler IQ detection system (Bio-Rad, Munich, Germany) in combination with the IQ SYBR-Green Real-time PCR Supermix (Bio-Rad) or QuantiTect SYBR Green PCR Master Mix (Qiagen). The thermal cycling program consisted of initial denaturation in one cycle of 15 min at 95°C, followed by 45 cycles of 15 s at 94°C, annealing 10 s at 63°C for IL-12p40 or 30 s at 55°C for others and 30 s at 72°C. The primers for rat CCR2, CCR7, IL-10, IL-12p35 and TNF-α were purchased as QuantiTect® Primer Assays from Qiagen. Primer sequences and amplicon sizes are shown in Table II. All analyses were done in triplicate and the mean was used for further calculations. The mRNA expression of all investigated genes was normalized with ß-actin and GAPDH as a housekeeping gene (HKG). The relative expression levels were calculated by the equation $2^{-(S\Delta Ct - C\Delta Ct)}$, where ΔCt is the difference in the threshold (Ct value) between the gene of interest (GOI) and the HKG as calculated by (GOI Ct)–(HKG Ct). SΔCt–CΔCt is the difference between SΔCt (SΔCt, experimental or adjuvant control) and the control ΔCt (CΔCt, untreated control). Comparison of both HKGs showed no significant differences between experimental groups. Data for each animal from experimental (E) and adjuvant control (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 splenocytes

Splenocytes from untreated Wistar rats were used as positive control for APC in the *in vitro* proliferation assay. Splenocytes were prepared by injecting the spleen with RPMI-1640 medium and grinding through 100 μm nylon mesh. Spleen erythrocytes were osmotically lysed. The remaining cells were washed and treated with 100 μg/ml mitomycin C (Sigma) at 37°C for 50 min. Cells were washed and suspended in complete RPMI-1640 medium supplemented with 10% FCS, 1% MEM (Sigma), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (PAA) and 50 μm 2-mercaptoethanol (Gibco) at a cell concentration of $1 \times 10^6 ml^{-1}$ for all experiments.

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). Leukocyte enriched fraction was washed and further purified using magnetic beads coated with monoclonal mouse anti-rat pan T-cell antibody and a MACS column (Miltenyi Biotec). The positively selected cells were collected as T-cells. Purity (>95%) was examined by flow cytometry using FITC-conjugated anti-CD3 and R-PE-conjugated anti-CD4 antibodies (Table I).

T-cell proliferation assay

In order to test the capacities of DC to stimulate naïve T-cells proliferation a radiometric assay based on ^{3}H-thymidine incorporation was applied. For *in vitro* experiments, triplicate aliquots (200 μl) of purified testicular DC (5×10^4) collected from testis of EAO animals 50 days after injection and adjuvant control group or splenocytes from untreated animals (5×10^4) were co-cultured with allogeneic T-cells (1×10^5) in 96-well round-bottomed microtiter plates (Nunc, Wiesbaden, Germany) at 37°C. Moreover, phytohemagglutinin (PHA, final concentration 10%) was added into appropriate wells containing T-cells only as positive control for 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. At the end of the incubation period, the medium was removed, cells were washed twice with cold PBS and then solubilized in 0.5 M NaOH. The amount of radioactivity incorporated into DNA was determined by liquid scintillation spectrometry (Tri-Carb 1500, Packard) and expressed as counts per minute (cpm).

Statistical analysis

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

Results

Histopathology

In rats with EAO, numerous immature and degenerated germ cells sloughed from the seminiferous epithelium were observed in the lumen of epididymal tubules in contrast with N (untreated rats) and C (rats injected with saline and adjuvants) groups in which degenerated or immature germ cells were present only occasionally (Fig. 2). Most of the sloughed cells showed alterations in nuclear condensation. The previous observation (Theas *et al*., 2003) of a strong correlation between reduced testis weight and a significant increase in the number of germ cell sloughed in the epididymal duct of rats with EAO compared to N and C groups was also evident in this study.

MHC class II, CD80 and CD86 expression on testicular DC

For the analysis of flow cytometry data, the gate was set on the CD45+ fraction selected by 90° SSC and PE-Cy5-fluorescence (FL) (Fig. 1B). In the CD45+ population, we analysed the expression of MHC class II, CD80 and CD86 molecules on DC (OX62+). Fig. 1C is a representative dot plot of MHC class II expression on testicular DC. Flow cytometric analysis showed a significant increase in the number of testicular DC in rats with orchitis killed at 50 days after the first immunization compared to C rats, confirming the stereological data using OX62 and CD11c obtained previously (Rival *et al*., 2006). Using flow cytometry a 3.5-fold increase in the number of

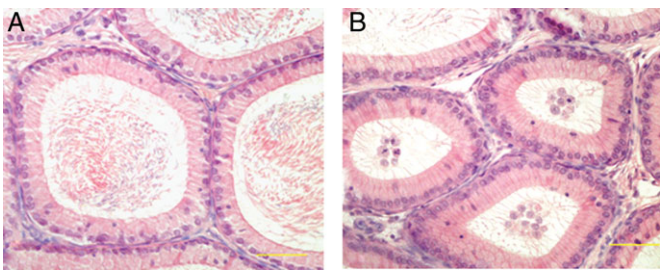

Figure 2: Microphotographs of hematoxylin and eosin stained caput or cauda epididymis sections in control rat
(**A**) numerous spermatozoa are visible in the epididymal lumen, while prematurely released degenerated germ cells are seen in rats with orchitis (**B**). Scale bar represents 50 μm

DC was observed in rats with EAO compared to C rats ($P = 0.001$). Moreover, an additional 2-fold increase in the number of testicular DC from rats with EAO killed at 50 days compared to those killed at 30 days ($P = 0.005$) was noted (data not shown).

Furthermore, the expression of MHC class II, CD80 and CD86 molecules on the surface of OX-62+ DC within interstitial single cell suspension were assessed by flow cytometry. Fig. 3 shows that 95–100% of testicular DC from N, C and E rats killed at 30 or 50 days after the first immunization expressed these molecules. Analysis of mean fluorescence intensity (MFI) showed similar expression levels of MHC II, CD80 and CD86 molecules in testicular DC in every group studied (Fig. 3). We also evaluated the MHC class II and CD80 expression in DC from spleen, lung and LN. Highest levels were found in DC from LN (MFI $>$ 250), with substantially lower, but similar levels observed in testis, lung and spleen (MFI $<$ 35).

Isolation of testicular DC

A newly developed two-step isolation procedure originating from an interstitial single cell suspension obtained from N, C and E group rat testes resulted in a very highly purified fraction of testicular DC (Fig. 4). The whole interstitial single cell suspension contained between 1% and 3% of DC in C and E rat testes, respectively (Fig. 4A). After separation with OX-62 coated magnetic beads the enriched fraction revealed ~15% of DC (Fig. 4C). Final purification by cell sorting resulted in a purity of the DC population between 90% and 97% (Fig. 4D). However, the total number of isolated DC per animal was relatively low ranging $1-2 \times 10^5$ in N and C rats and $3-9 \times 10^5$ in E group. The highly pure fraction of isolated DC was used for mRNA isolation and subsequent real-time RT-PCR analysis of chemokine receptors (CCR2, CCR7) and cytokines (IL-10, IL-12p35, IL-12p40 and TNF-α) as well as for *in vitro* T-cell proliferation assay.

Expression of chemokine receptors CCR2 and CCR7 on testicular DC

The relative mRNA expression of chemokine receptors CCR2 and CCR7 in testicular DC from N, C and E group was quantified by real-time RT-PCR. CCR2 expression was comparable in all investigated groups with the exception of rats with orchitis (E group-50 days) showing a significant decrease compared to C rats (Fig. 5A). The mRNA expression for CCR7 was significantly up-regulated in rats with orchitis (E group, 50 days) compared to E 30 days and control groups (Fig. 5B).

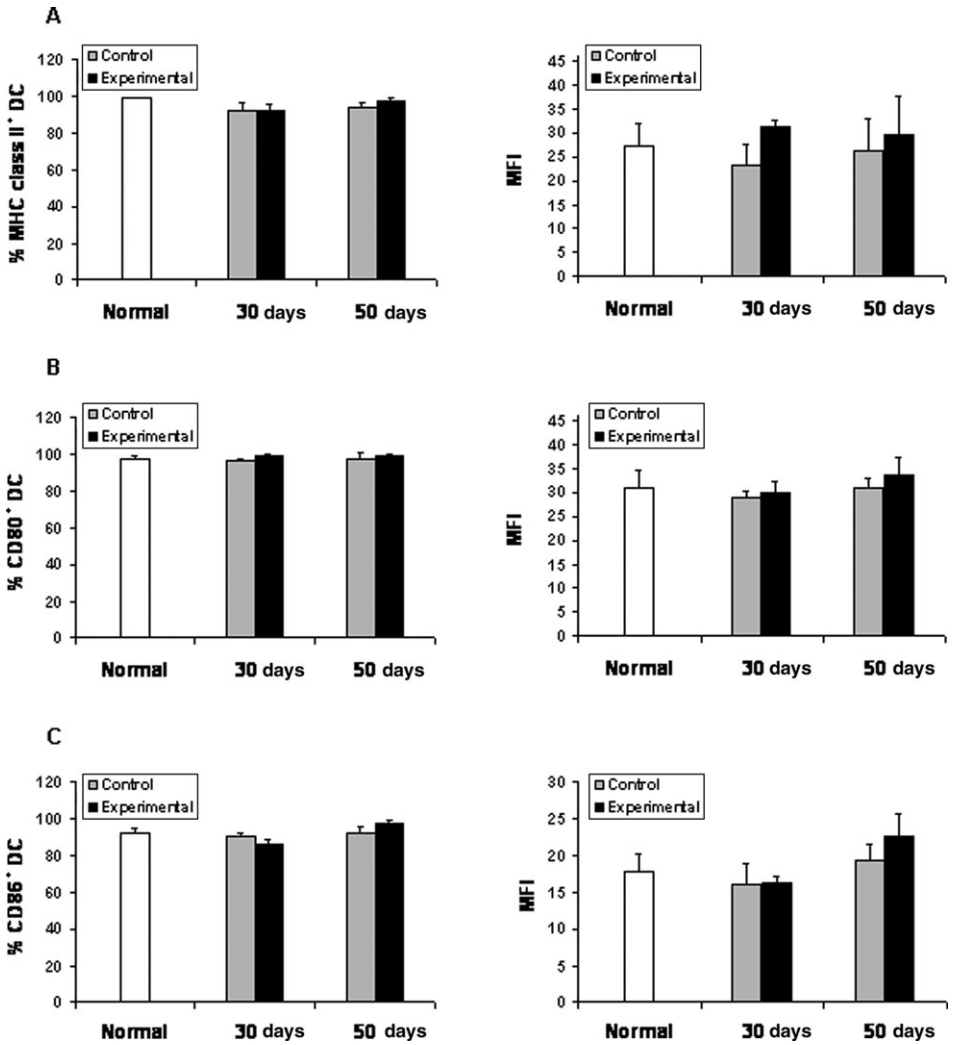

Figure 3: Quantitative assessment of testicular OX62+ DC expressing MHC II (**A**) CD80 (**B**) and CD86 (**C**) in interstitial single cell suspension from normal, control and EAO rats using FACS analysis. Left panel: percentage of positive cells in total DC population; right panel: MFI of the sample/MFI of isotype control IgG

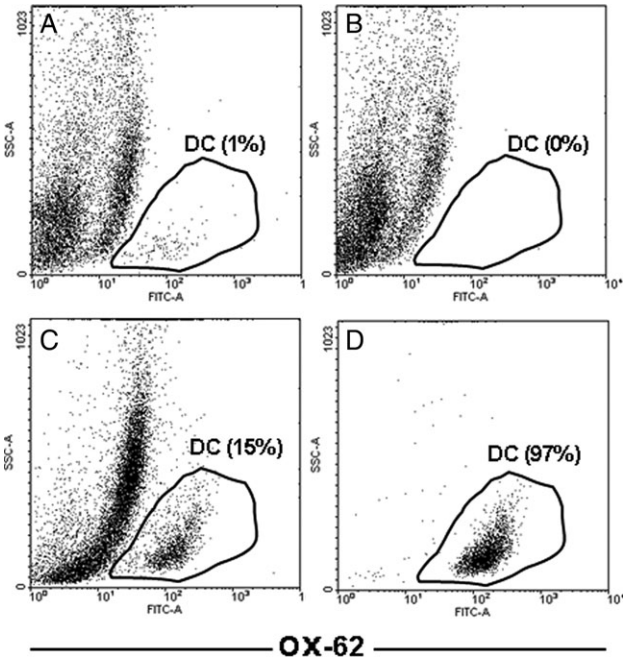

Figure 4: Purity of DC from E rats before and after isolation steps
(**A**) DC in whole interstitial cell suspension (1%). (**B**) Isotype control for OX-62 antibody; (**C**) Enriched fraction of DC after isolation with OX-62-conjugated magnetic beads (15%); (**D**) Highly purified fraction of DC after FACS sorting (97%)

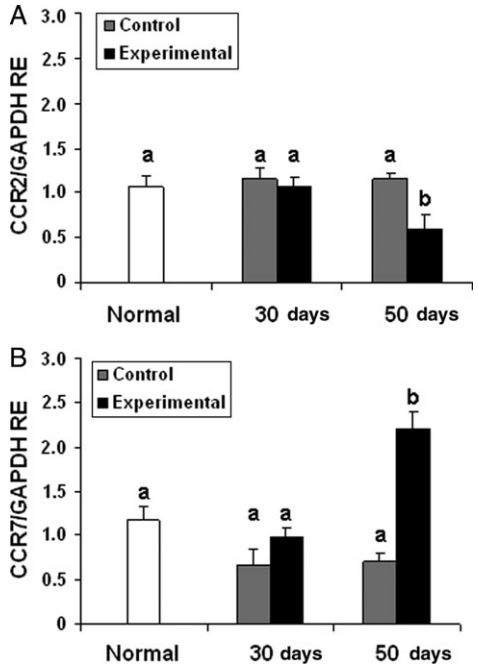

Figure 5: Comparison of mRNA expression of chemokine receptors determined by real-time RT-PCR
(**A**) CCR2/GAPDH and (**B**) CCR7/GAPDH relative expression in DC from normal, adjuvant control and experimental rat testes collected at 30 and 50 days after first immunization. GAPDH is used for normalization of expression data. Values are mean ± SEM of relative expression units (RE) of results for 5–7 animals in each group. Values with different superscript letters differ significantly ($P < 0.05$) compared to respective controls

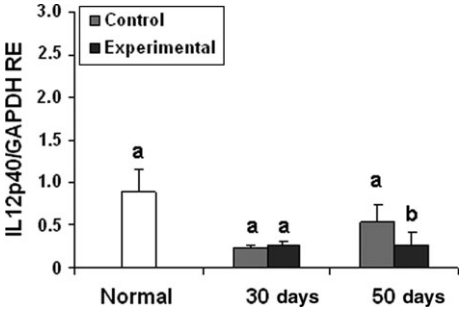

Figure 6: Relative mRNA expression of IL-12p40 subunit in DC from normal, adjuvant control and experimental rat testes analysed by real-time RT-PCR
Values are mean ± SEM of relative expression units (RE) of results for 5–7 animals in each group. Values with different superscript letters differ significantly ($P < 0.05$) compared to normal group

Expression profile of cytokines IL-10, IL-12p35, IL-12p40 and TNF-α in rat testicular DC

The cytokine mRNA expression profiles of testicular DC were assessed utilizing quantitative real-time RT-PCR analysis. Relative mRNA expression of IL-12p40 form showed a decrease in C and E groups from 30 and 50 day rats compared to N group. A significant decrease of IL-12p40 was detected in 50 day E group compared to N group (Fig. 6). TNF-α mRNA was expressed at very low level in all investigated groups. A slight, but not significant decrease in E and C rats at 50 days was noticed compared to N and 30 day C and E group (data not shown).

Clearly visible PCR products for IL-10 and IL-12p35 were observed only in testicular DC from E rats with orchitis (50 days), whereas mRNA levels of these cytokines were undetectable in testicular DC from C rats sacrificed at 50 days as well as N, 30 day E and C group (Fig. 7). Owing to the *de novo* expression of IL-10 and IL-12p35 only in DC isolated from 50 days orchitis testes samples were not further quantified by real-time RT-PCR.

Ability of testicular DC to stimulate T-cell proliferation

Functional data from *in vitro* assays showed that purified testicular DC from 50 day E animals exhibited a significantly elevated capacity to stimulate the proliferation of naïve T-cells ($P < 0.05$) compared to DC isolated from C group animals (Fig. 8). This clearly suggests a mature phenotype of DC from animals with orchitis. Of note, the proliferative responses of naïve T-cells after stimulation with DC of E animals even recorded 1.5-fold higher than T-cells stimulated with phytohemagglutinin PHA or splenocytes, respectively (Fig. 8).

Discussion

In spite of their potential importance in maintaining the balance of the testicular immune status between tolerance (immune privilege) and (auto-) immunogenic reply, DC in the male gonad have been largely neglected in the past. Recently, we found a significant increase in the number of DC in the testis of rats with autoimmune orchitis using immunohistochemical techniques (Rival *et al*., 2006). These morphometric results were confirmed by the flow cytometric analysis in this study showing that DC together with macrophages, that also increase in EAO (Suescun *et al*., 2003), constitute the main population of APC within the inflamed rat testis. To elucidate the functional status of DC in the pathogenesis of inflammatory testicular diseases, we have successfully developed an isolation protocol in this study combining the use of magnetic beads and cell sorting. This method allowed the collection of a highly purified fraction of testicular DC (90–97%

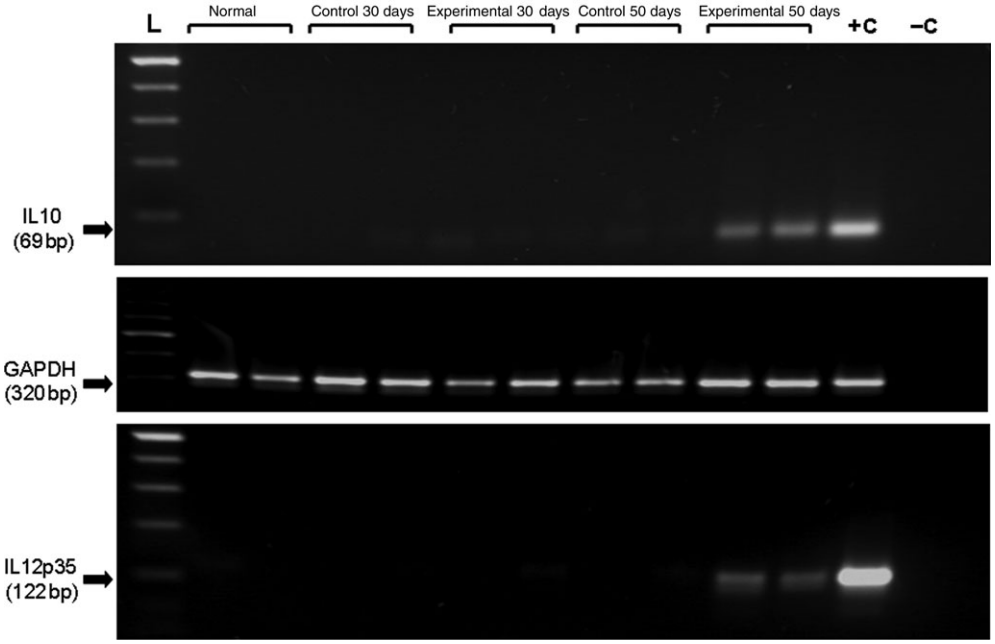

Figure 7: IL-10 and IL-12p35 expression in testicular DC is restricted to Day 50 experimental testes, with no transcript detected in the other groups. GAPDH was used as loading control. +C, positive control; −C, negative control; L, 100 bp DNA ladder

purity). Nonetheless the low yield set limitations in the analysis of cytokine secretion by Western blot or ELISA and requested the use of highly sensitive techniques such as real-time RT-PCR for the subsequent analysis. However, cell numbers were sufficient for functional mixed lymphocyte assays to examine the T-cell stimulatory capacity of isolated DC.

The phenotype of DC plays an important role in the initiation of the immune response, i.e. immature DC are believed to induce tolerance to self antigens whereas mature DC promote immunity to foreign and self antigens (Steinman, 1991). The absence of co-stimulatory molecules on APCs during the antigen presentation has been shown to induce clonal anergy, and consequently peripheral tolerance in other systems (Wolf *et al*., 1994). Known to protect germ cells and foreign tissue grafts from autoimmune attack, the 'Immunoprivilege' of the normal testis was, beside other factors, also attributed to a clonal anergy of T lymphocytes (Fijak and Meinhardt, 2006). However, our observation that most testicular DC from normal and inflamed rat testis express MHC class II, CD80 and CD86 at similar levels suggests that the DC-dependent triggering of activation of naïve T lymphocytes by binding to their specific antigens is at the very least possible in the rodent testis. Presence of co-stimulatory molecules in the testis was previously reported (Sainio-Pollanen *et al*., 1996). These authors could not detect the expression of CD80 and CD86 molecules by immunohistochemistry in the normal testis of BALB/c mice, but observed some CD80+ and CD86+ interstitial cells in 14–22-week-old NOD mice. Although this discrepancy to our results could be explained by differences between mice and rats, it is more likely that the development of better tools and antibodies allowed us to detect the expression of these co-stimulatory molecules even in the normal testis. Unexpectedly, we found that the expression of co-stimulatory molecules was not modified during testicular inflammation. However, we cannot exclude the possibility that other co-stimulatory molecules than those studied in the present report were modulated in testicular DC in EAO. As reported by Sanchez *et al*. (Sanchez *et al*., 2007) *in vivo* there is up-regulation of the 'novel' co-stimulatory molecule CD70 in DC after stimulation with Toll like receptor ligands and CD40 without a significant variation in the expression of the classical co-stimulatory molecules CD80 and CD86.

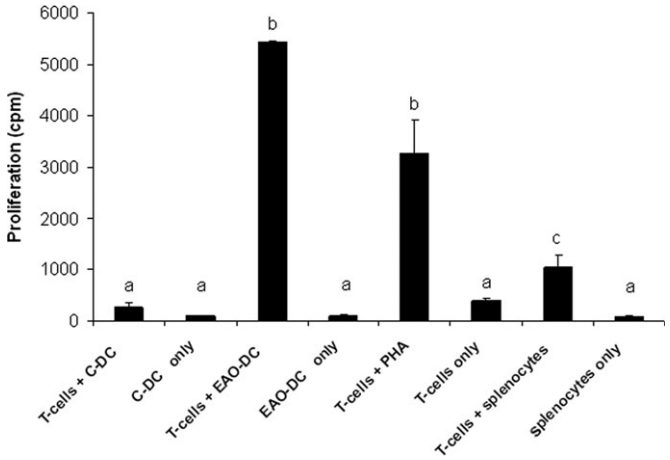

Figure 8: T-cell proliferation induced by testicular DC, splenocytes or PHA stimulation as detected by [^{3}H]-thymidine incorporation.
T-cells (1×10^5) from Sprague–Dawley rats were co-cultured with testicular DCs (5×10^4) from experimental (EAO-DCs) or control group (C-DCs), splenocytes (5×10^4) or PHA (10%) for 63 h at 37°C. Proliferation of T-cells was assessed by standard thymidine incorporation and is displayed as cpm. Data are presented as the mean ± SEM of triplicate samples in one representative of three independent experiments with similar results. Values with different letters superscript differ significantly ($P < 0.05$)

A switch in chemokine receptor expression enables activated DC to leave the site of inflammation and to migrate to the draining LN. CCR7 is a key regulator in this process and governs trafficking of DC under both inflammatory and steady-state conditions (Ohl *et al*., 2004). CCR2 is a marker of immature resident DC. To better characterize the migratory status of testicular DC, we studied the expression of CCR2 and CCR7 by quantitative RT-PCR in isolated testicular DC.

In DC isolated from orchitis testis, we observed an up-regulation of CCR7 and a down-regulation of CCR2 mRNA level compared with controls suggesting that in the inflamed testis these cells acquire a migratory phenotype. The increase of CCR7 expression in DC of rats from the experimental group is concomitant with the onset of testicular damage at 50 days. In combination, analysis of co-stimulatory molecules and chemokine receptors suggests that the DC in the chronically inflamed testis are in a ready migratory state and underwent partial maturation. It was shown that TNF-α is able to induce a semi-mature DC phenotype, which then requires further stimuli to differentiate to mature DC (Menges *et al*., 2002). Interestingly, increased levels of TNF-α were found in testis of rats with orchitis (Suescun *et al*., 2003). This intermediate phenotype was defined for CCR7+ DC with a poor ability to secrete proinflammatory cytokines and high expression of MHC class II, CD80 and CD86 molecules (Lutz and Schuler, 2002). *In vivo*, these semi-mature DC are actively tolerogenic by inducing IL10+CD4+ regulatory T-cells in an antigen specific manner. Thus, MHC II and co-stimulatory molecules expression on its own as found in normal and adjuvant control rats in this study are not sufficient to induce T-cell immunity *in vivo*.

However, beside the expression of co-stimulatory molecules, maturation of DC and the actual outcome of immune responses is further controlled by the production of cytokines which either stimulate a cell-mediated immunity (type 1 cytokines, e.g. TNF-α and IL-12) or cytokines which suppress cell-mediated responses (type 2 or type 3 cytokines, e.g. IL-10 and transforming growth factor β). Fully mature immunogenic DC are best characterized by the production of IL-12, which like IL-10 is absent in semi-mature or immature cells (Lutz and Schuler, 2002). Strikingly, only DC from 50 day EAO testis showed both considerable expression of IL-10 and IL-12p35 as detected by RT-PCR. In contrast control DC and cells isolated from Day 30 EAO were either completely negative for IL-12p35 or showed only very discreet expression of IL-10. This is a clear indication for a mature state of DC from EAO testis, in spite of unchanged levels of co-stimulatory molecule expression. Because of the lower abundancy of p35 transcripts even in activated inflammatory cells, the expression of the p35 subunit is determining the production of bioactive heterodimeric IL-12p70 (Babik *et al*., 1999; Trinchieri, 2003). Our results point out that between 30 and 50 days after induction of EAO, DC undergo a maturation step which parallels the development of severe inflammatory damage in the testis (Rival *et al*., 2006) and auto-antibody production (Fijak *et al*., 2005). Although some caution is necessary as mRNA data may not represent changes on the protein level, our functional analysis of naïve T-cell stimulatory capacity of isolated testicular DC clearly supports the notion that control DC are tolerogenic whilst those from orchitis testis are immunogenic.

In conclusion, our flow cytometric results plus the absence of CCR7 expression and proinflammatory cytokine production show that DC of normal testis are not mature (semi-mature or immature). Semi-maturation of DC represents a unique developmental tolerogenic stage for DC and without further stimulation is not necessarily 'dangerous' for the immune system of the testis. Cells appear comparable to steady-state migratory veiled DCs within the lymphatics, which seem to continuously tolerize T-cells in LN against tissue-derived self-antigens or apoptotic cells (Lutz and Schuler, 2002). During the development of chronic testicular inflammation, the DC population acquires the capacity to move to LN as indicated by high expression of CCR7, thereby stimulating antigen specific T-cell responses. It is challenging to hypothesize that the phenotypical proximity of DC in the normal and EAO testes could represent one possible explanation why immunological infertility in the male is a considerable entity in spite of the immune privileged status of the testis.

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