CD4$^+$ Foxp3$^+$ Regulatory T Cells in Autoimmune Orchitis: Phenotypic and Functional Characterization

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Keywords
Immunoregulation, inflammation, testis

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Submission April 15, 2014;
accepted August 1, 2014.

Citation
Jacobo P, Guazzone VA, Pérez CV, Lustig L. CD4$^+$ Foxp3$^+$ regulatory T cells in autoimmune orchitis: phenotypic and functional characterization. Am J Reprod Immunol 2014

doi:10.1111/aji.12312

Problem
The phenotype and function of regulatory T (Treg) cells in rats with experimental autoimmune orchitis (EAO) was evaluated.

Method of study
Distribution of Treg cells in draining lymph nodes from the testis (TLN) and from the site of immunization (ILN) was analysed by immunohistochemistry. The number, phenotype and proliferative response (5-bromo-2′-deoxyuridine incorporation) of Treg cells were evaluated by flow cytometry and Treg cell suppressive activity by *in vitro* experiments. TGF-β expression was evaluated by immunofluorescence.

Results
Absolute numbers of Treg cells and BrdU+ Treg cells were increased in LN from experimental compared to normal and control rats. These cells displayed a CD45RC$^-$, CD62L$^-$, Helios$^+$ phenotype. CD4$^+$ CD25^{bright} T cells from TLN of experimental rats were able to suppress T cell-proliferation more efficiently than those derived from normal and control rats. Cells isolated from TLN and ILN expressed TGF-β.

Conclusion
Our results suggest that Treg cells with a memory/activated phenotype proliferate extensively in the inflamed testis and LN of rats with EAO exhibiting an enhanced suppressive capacity. TGF-β may be involved in their suppressive mechanism.

Introduction

Regulatory T (Treg) cells are critical for peripheral immune tolerance against auto-reactive T cells and control of autoimmune diseases.[1] Foxp3$^+$ Treg cell family contains multiple heterogeneous subsets that include CD4$^+$ and CD8$^+$ cells (CD25$^+$ and CD25$^-$), CD4$^+$ CD25$^+$ Foxp3$^+$ cells being the subset most extensively characterized. Most Treg cells are previously activated cells that express CD25, CD62L and CD45RC at low levels. CD62L expression has also been related to functional regulatory status of Treg cells.[2–6]

Microarray studies showed relative up-regulation of the Ikaros family transcription factor Helios in CD4$^+$ Foxp3$^+$ Treg cells. CD4 $^+$ Helios$^+$ Foxp3$^+$ Treg cells were also reported to represent an activated functional subset with enhanced suppressive potential compared to CD4$^+$ Helios$^-$ Foxp3$^+$ Treg cells.[7]

Treg cells can suppress the proliferation and cytokine production of other T cells through cell-contact and/or membrane-bound TGF-β dependent mechanisms.[8,9] The functional characterization of Treg cells has been difficult by the lack of a specific surface marker to distinguish them from the recently activated CD25$^+$ non-Treg cells. Dependence on Foxp3

expression for both development and function of Treg cells[10] has led to it being considered the only truly specific marker of these cells; however, current techniques still preclude the isolation of live Foxp3$^+$ cells. The regulatory function is generally associated with CD4$^+$ T cells expressing CD25 at high levels (CD25^{bright}) and these cells are widely used in the study of functional behaviour of Treg cells. However, functional Treg cells can be also observed within CD4$^+$ T cell subset exhibiting a very broad spectrum of CD25 expression.[2,11,12]

The testis is considered an immunologically privileged site where physiological mechanisms must be active in setting up responses against meiotic and haploid germ cells expressing antigens considered non-self because they first appear at the time of puberty.[13] Despite this immune-privileged status, infection and inflammation of the testis are important aetiological factors in male infertility. Experimental autoimmune orchitis (EAO) is the model classically used for investigation of autoimmune-based inflammatory testicular impairment.[14] In rats with EAO, testicular damage is characterized by an increased number of macrophages, dendritic cells and T cell subsets that include auto-pathogenic and regulatory T cells infiltrating the interstitium and by seminiferous tubules showing apoptotic germ cells, different degrees of germ cell degeneration and sloughing, aspermatogenesis and atrophy.[15–19]

Treg cells have been suggested to contribute to the unique immune environment of the testis, although their functions remain unexplored.[20] Few data are available in the literature concerning the number and functional role of testicular Treg cells both in normal and pathological conditions. In this regard, our previous work was the first to reveal the presence of Foxp3-expressing CD4$^+$ and CD8$^+$ Treg cells in normal rat testis, and their increased number in the inflamed testis of rats with EAO. Evaluation of CD25 expression led us to identify CD25$^+$ and CD25$^-$ cells within CD4$^+$ Foxp3$^+$ and CD8$^+$ Foxp3$^+$ Treg cell subsets; CD4$^+$ CD25$^-$ Foxp3$^+$ being the more abundant Treg cell subset.[18] Fijak et al.[21] reported a role for CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells in the immunomodulatory effect of testosterone in the pathogenesis of EAO. Wheeler et al.[22] showed that CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cell depletion in vasectomized mice leads to CD4$^+$ T cell- and antibody-dependent bilateral orchitis development.

Peripheral tolerance for internal organs relies on the control of auto-reactive T cells by Treg cells in regional lymph nodes (LN) where antigen-specific cells continuously encounter tissue antigens.[23–26] As the microenvironment of different LN may translate into a biologically different behaviour and function of Treg cells, we examined the number, phenotype and functional status of the CD4$^+$ Foxp3$^+$ Treg cell population of LN draining the testis as well as LN draining the site of immunization under physiological and inflammatory (EAO) conditions.

Materials and methods

Animals

Adult male *Sprague-Dawley* rats 50–60 days old were purchased from Bioterio Central, Facultad de Farmacia y Bioquímica (Buenos Aires, Argentina). Animals were kept at 22°C on a 12 hr light, 12 hr dark schedule and fed 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 our local committee.

Induction of EAO

Experimental (E) rats were actively immunized with testicular homogenate (TH) prepared as previously described.[27] TH (0.4 mL) emulsified with complete Freund's adjuvant (0.4 mL; Sigma–Aldrich, St. Louis, MO, USA) was injected subcutaneously into footpads and at multiple sites near LN. E rats were injected three times (200 mg of TH/dose/rat) at 14-day intervals. The first two immunizations were followed by an intravenous injection of *Bordetella pertussis* (0.5 mL, strain 10536, Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by an intraperitoneal injection of 5×10^9 microorganisms. Control (C) rats were injected with an emulsion of saline solution and adjuvant in the same conditions as E group. Normal (N, untreated) rats were also studied. E, C and N rats were killed 50 and 80 days after the first immunization. LN draining the testis (iliac and renal LN; TLN) and LN draining the site of immunization (inguinal and popliteal LN; ILN) were frozen or used for preparation of single cell suspensions.

For histopathological study one testis of each rat was fixed in Bouin's solution, embedded in paraffin, sectioned and stained with haematoxylin–eosin. As we previously described,[26] no testicular damage was observed in E rats killed at 30 days (end of the

immunization period). 50 days after the first immunization, 50% of E rats presented a focal orchitis characterized by mild lymphomononuclear cell infiltrate and several foci of seminiferous tubules showing degeneration and sloughing of germ cells (focal EAO). At 80 days after the first immunization, 75% of E rats showed a severe and extensive damage of most of the seminiferous tubules in which only spermatogonia and Sertoli cells were attached to the tubular wall (chronic EAO). N and C rats presented normal testicular histopathology at any time studied.

5-Bromo-2′-Deoxyuridine Administration *In Vivo*

5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was dissolved in warm water (50 mg/kg) and was administered three times at 24-hr intervals by intraperitoneal injection. Rats were killed 3 hr after the last BrdU injection.

Preparation of Spermatic Antigen

TH was prepared as previously described.[27] Supernatant obtained after TH centrifugation (13,000 ***g***, 30 min, 4°C) was collected and used as spermatic antigen for *in vitro* experiments. The final concentration was 29 mg/mL.

Preparation of Lymph Node Cell Suspension

Lymph nodes were cut with scissors in several parts. Pieces were pressed against bottom of a Petri dish containing 3 mL phosphate buffered saline (PBS) plus 0.1% bovine serum albumin (BSA, fraction V; Sigma Chemical Co.) with the plunger of a syringe until mostly fibrous tissue remained. Clumps were removed by filtration through a fine stainless steel screen followed by filtration through 30 μm mesh size filter (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell suspension was centrifuged (300 ***g***, 10 min, 4°C). Red blood cells were depleted by osmotic lysis with ammonium chloride (0.16M NH4Cl, 0.17M Tris–HCl, pH 7.2) for 5 min at room temperature (RT). Cells were washed, centrifuged and counted in a Neubauer chamber by the trypan blue exclusion method.

Isolation of Testicular Interstitial Cells

Testicular interstitial cells were obtained by collagenase digestion of testis as previously described.[28]

Isolation of $CD4^+$ $CD25^{bright}$ Regulatory T Cells

Lymph nodes were collected and pooled, and cell suspensions were prepared as described above. For immunofluorescent techniques, cells were incubated with anti-CD3(APC), anti-CD8(PerCP) and anti-CD25(PE) for 30 min at 4°C. For functional studies, cells were incubated with anti-CD3(APC), anti-CD4 (FITC) and anti-CD25(PE) in the same conditions. Appropriate control isotypes were used. All antibodies were purchased from BD Bioscience (BD Bioscience, San Diego, CA, USA). Cells were isolated by fluorescence-activated cell sorting (FACS). BD FACSAriaII (BD Bioscience) sorter was used to isolate $CD3^+$ $CD8^-$ ($CD4^+$ cells) and $CD3^+$ $CD4^+$ T cells expressing CD25 at high levels ($CD25^{bright}$) for each technique respectively. Intracellular staining for Foxp3 was performed on cells obtained after sorting as per manufacturer's instructions (eBioscience, San Diego, CA, USA).[18] Purity of the cell preparations analysed using FACS was typically >92%.

Immunohistochemistry

Lymph nodes cryostat sections were fixed with ice-cold acetone to detect Foxp3 and Helios expression or with 4% paraformaldehyde to detect TGF-β expression (10 min, 4°C). Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min. Non-specific labelling was prevented by incubating the sections with 10% normal horse serum in PBS for 30 min at RT (Foxp3 and Helios) or with 5% normal goat serum, 3% BSA and 0.1% Triton X-100 in PBS (solution A). Sections were treated with avidin/biotin blocking solution (ABC Vectastain Kit; Vector Laboratories, Burlingame, CA, USA). After incubation with mouse anti-Foxp3 (0.25 mg/mL) (eBioscience), goat anti-Helios (0.02 mg/mL) or rabbit anti-TGF-β (0.2 mg/mL) (Santa Cruz Biotechnology, Inc., CA, USA) sections were incubated with biotinylated anti-mouse Ig or anti-goat Ig made in horse (Vector Laboratories) diluted in 5% normal horse serum in 3% BSA at RT or biotinylated anti-rabbit Ig made in goat (Vector Laboratories) diluted in solution A. The reaction was amplified using the Vectastain Elite ABC kit (Vector Laboratories) and the reaction product was visualized by adding diaminobenzidine substrate (Vector Laboratories). Finally, sections were counter-stained with haematoxylin. Negative controls were obtained by incubating sections with PBS instead of primary antibodies.

Immunofluorescence

Non-specific labelling was prevented by incubating cells with 5% normal goat serum, 3% BSA and 0.1% Triton X-100 in PBS (solution A) for 30 min at RT. Then, cells were incubated overnight with rabbit anti-rat TGF-β (0.2 mg/mL; Santa Cruz Biotechnology) diluted in solution A. After three washes in 0.1% Triton X-100 in PBS, cells were incubated with goat anti-rabbit(FITC) diluted in solution A for 1 hr at RT. For negative control, the first antibody was omitted. Cells were mounted with a medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Flow Cytometric Analysis

To detect and quantify Treg cells, cells (1×10^6) were incubated with anti-CD3(APC), anti-CD4(FITC) and anti-CD25(PE) for 30 min at 4°C. To analyse intracellular expression of Foxp3, cells fixed and permeabilized were incubated with anti-CD16/32-Blocks Fc followed by anti-Foxp3(PE-Cy5) as we previously described.[18]

For phenotypical characterization of Treg cells, cells were incubated with avidin/biotin blocking solution (Vector Laboratories) followed by incubation with the biotinylated primary antibody anti-CD45RC or anti-CD62L for 40 min at RT. Cells were washed twice with permeabilization buffer. Finally, PE-Cy7-conjugated streptavidin (0.02 μg/mL) was used to detect primary antibodies.

For BrdU incorporation assays, cells were stained using the FITC BrdU Flow Kit. After washing and centrifugation, cells were resuspended in 50 μL ice-cold 0.15M NaCl and fixed by adding ice-cold 95% ethanol dropwise (Merck, Darmstadt, Germany) on ice for 30 min. Cells were washed with PBS and incubated with 1 mL 1% paraformaldehyde 0.01% Tween-20 in PBS for 1 hr at RT. After centrifugation, cells were resuspended in 250 μL of DNAse (100 U/mL; Sigma-Aldrich) diluted in 4.2 mM $MgCl_2$ 0.15M NaCl, pH5 for 10 min at RT. Finally, cells were incubated with 20 μL anti-BrdU(FITC) antibody for 30 min on ice.

The appropriate control isotypes were used. All antibodies and streptavidin were purchased from BD Bioscience except for anti-Foxp3(PeCy5) purchased from eBioscience and anti-CD45RC purchased from Abcam (Cambridge, MA, USA). BD FACSAriaII cytometer was used and 30,000 events with light-scatter characteristics of T cells on $CD3^+$ cell gate were acquired. The absolute number of positive cells was calculated from percentages obtained by flow cytometric analysis and total cell number counted in a Neubauer chamber by the trypan blue exclusion method.

Cell Proliferation Assay

Lymph nodes were collected and pooled. Quintuplicate cultures of 5×10^5 cells/well were stimulated with 100 ng/mL phorbol 12-myristate13-acetate (PMA) plus 4 μg/mL ionomycin (Sigma Chemical Co.) or 0.1 mg/mL spermatic antigen in 96-well plates in RPMI1640 medium (Gibco-BRL Life Technologies, Merelbeke, Belgium) supplemented with 10% heat inactivated foetal calf serum (Gibco-BRL), 10^{-5}M 2-β-mercaptoethanol (Bio Rad), 2 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1× antibiotic–antimycotic solution (Gibco-BRL). The cell cultures were incubated for a total of 72 hr and pulsed with [^{3}H]-thymidine (0.5 μCi/well) for the last 18 hr of incubation. Proliferative response is expressed as the mean ± S.D. [^{3}H]-thymidine incorporation (counts per minute) of quintuplicate wells.

Cell Suppression Assay

Pooled cells from TLN of E rats (responder T cells, Tresp) were co-cultured with or without autologous $CD4^+$ $CD25^{high}$ Treg cells isolated from TLN or ILN of N, C and E rats at various ratios (1:1, 5:1, 10:1, 50:1) in presence or absence of 100 ng/mL PMA plus 4 μg/mL ionomycin (Sigma Chemical Co.) or 0.1 mg/mL spermatic antigen in 96-well plates in RPMI1640 medium (Gibco-BRL) supplemented with 10% heat inactivated foetal calf serum (Gibco-BRL), 10^{-5}M 2-β-mercaptoethanol (Bio Rad), 2 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1× antibiotic–antimycotic solution (Gibco–BRL). The cell cultures were incubated for a total of 72 hr and pulsed with [^{3}H]-thymidine (0.5 μCi/well) for the last 18 hr of incubation. Proliferative response is expressed as the mean ± S.D. [^{3}H]-thymidine incorporation (counts per minute, CPM) of quintuplicate wells.

Statistical Analysis

Results were expressed as mean ± S.E.M. Comparisons of groups were assessed by the non-parametric

Kruskal–Wallis one-way ANOVA or by one-way ANOVA followed by the Bonferroni test when applicable. $P \leq 0.05$ was considered significant.

Results

Distribution of Foxp3$^+$ Regulatory T Cells in Rat Lymph Nodes

By immunohistochemistry, Foxp3$^+$ cells were identified in LN draining the testis (TLN) and in LN draining the site of immunization (ILN) from N, C and E rats killed on days 50 and 80. In TLN and ILN from all rats studied, Foxp3$^+$ cells were localized in the LN cortex around primary follicles in parafollicular thymus-dependent areas and in the subcortical sinus and medulla sinusoids (Fig. 1a–f). Foxp3$^+$ cells were frequently observed in primary follicles of TLN from E rats, but not from N and C rats (Fig. 1c). Positive cells were only occasionally observed in primary follicles of ILN from E rats. No specific staining was observed in absence of the primary antibody (negative control) (Fig. 1g).

Quantification and Phenotypical Characterization of Regulatory T Cells

To quantify and phenotypically characterize Treg cells in TLN and ILN from N rats and E rats throughout EAO development, we performed a flow cytometric assay. We determined the percentage of phenotypically distinct CD25$^+$ Foxp3$^+$ and CD25$^-$ Foxp3$^+$ Treg cell subsets within CD3$^+$ CD4$^+$ cell population with light scatter characteristics of T cells from N, C and E rats killed on days 50 and 80 (Fig. 2).

At 50 and 80 days, a significant increase in the percentage of CD3$^+$ CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cell subset was detected in TLN from E compared to N and C rats, but not in ILN. No differences between groups of rats were detected for percentage of CD3$^+$ CD4$^+$ CD25$^-$ Foxp3$^+$ Treg cell subset in both LN types. Absolute numbers of CD3$^+$ CD4$^+$ CD25$^+$ Foxp3$^+$ and CD3$^+$ CD4$^+$ CD25$^-$ Foxp3$^+$ Treg cell subsets were increased in TLN and ILN from E compared to N and C rats (Fig. 3). These numeric variations correlate with the dramatic expansion of T cells in ILN after immunization and in TLN during EAO development. No differences between N and C rats were detected in percentages or absolute numbers for each Treg cell subset at any time studied.

In order to define naïve/memory phenotype and regulatory status of each Treg cell subset in both LN types and in testis of N rats and E rats during EAO development, we performed a flow cytometric assay to investigate expression of CD45RC and CD62L. Results showed that most of the CD4$^+$ CD25$^+$ Foxp3$^+$ and CD4$^+$ CD25$^-$ Foxp3$^+$ Treg cells from TLN and ILN of all rats studied expressed little or no CD45RC or CD62L. The same result was obtained for both Treg cell subsets in the testis of N, C and E

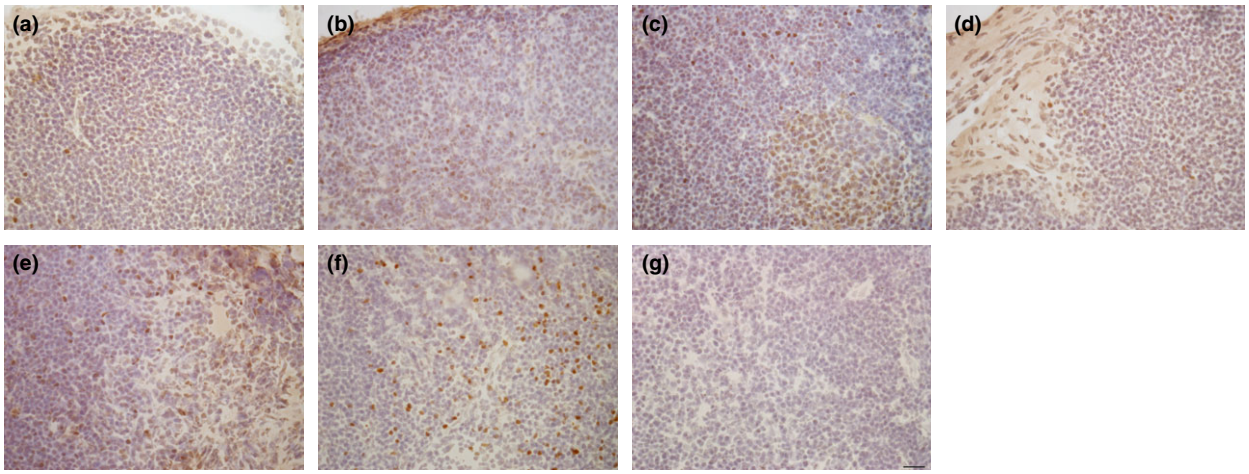

Fig. 1 Representative immunostaining for Foxp3 in cryostat sections of lymph node draining the testis (TLN) (a, b, c) and lymph node draining the site of immunization (ILN) (d, e, f) from normal (a, d), control (b, e) and experimental (c, f) rats. Omission of primary antibody show negative staining (g). Scale bar indicates 20 μm.

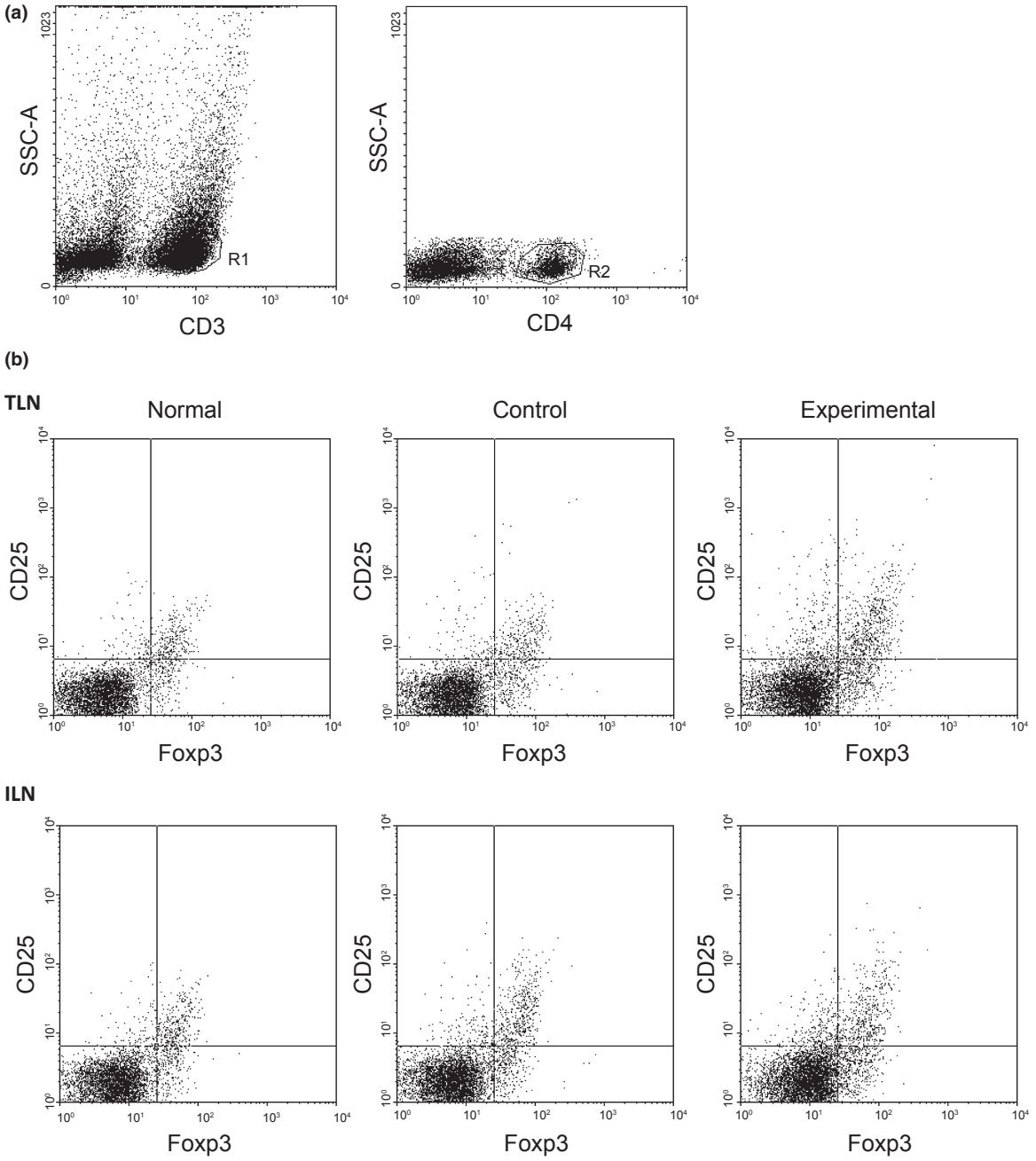

Fig. 2 Representative flow cytometry dot plots of lymph node cells obtained from an experimental rat. Analysis gate (R1) was set on T cell population selected by scatter properties and CD3 expression. A gate (R2) was drawn selecting $CD4^+$ cells within R1 based on the isotype control (a). Representative flow cytometry dot plots illustrating Foxp3 and CD25 expression on gated $CD3^+$ $CD4^+$ T cells (R1 and R2) in cell suspensions of lymph nodes draining the testis (TLN) and lymph nodes draining the site of immunization (ILN) from normal, control and experimental rats (b). Quadrants were set based on isotype controls.

rats (data not shown). As a control we verified the expression of CD45RC and CD62L within $CD4^+$ $CD25^+$ $Foxp3^-$ non-Treg cell population from TLN of N, C and E rats (Figure S1). Our results are consistent with the notion that all Treg cells evaluated have an activated/memory phenotype.

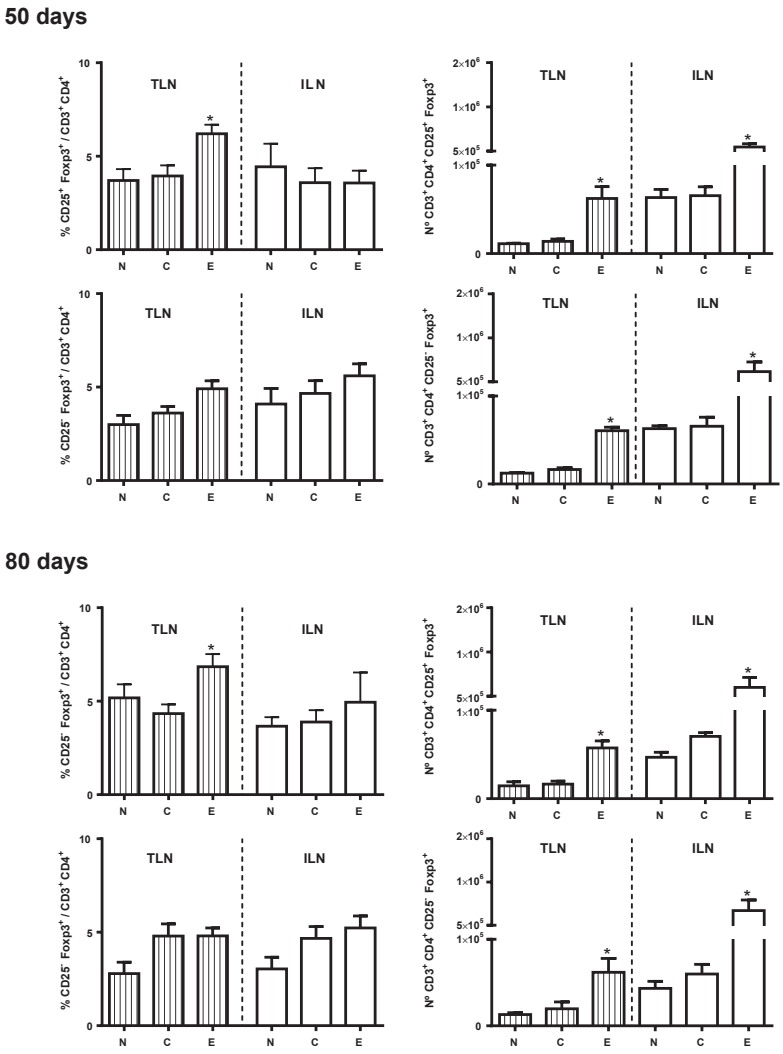

Fig. 3 Quantitative assessment of percentage and absolute number of $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ regulatory T cell subsets in cell suspension of lymph nodes draining the testis (TLN) and lymph nodes draining the site of immunization (ILN) from normal (N), control (C) and experimental (E) rats. Data are representative of $n = 7$–10 rats per group. $*P < 0.01$ versus respective N and C.

Given that $Helios^+$ Treg cells represent a functional subset with enhanced suppressive potential compared to $Helios^-$ Treg cells,[7] we examined Helios expression. Since commercial anti-rat Helios antibody suitable for flow cytometric analysis is unavailable, Helios expression was investigated by immunohistochemistry on sections of TLN and ILN from N, C and E rats killed on days 50 and 80. Helios expression was detected in the three groups of rats at any time studied. Similarly to distribution of $Foxp3^+$ cells, Helios+ cells were localized in the TLN and ILN cortex, in the subcortical sinus and medulla sinusoids and around primary follicles (Fig. 4a–f). Primary follicles with positive cells were only observed in TLN from E rats (Fig. 4c). No specific staining was observed in absence of the primary antibody (negative control) (Fig. 4g).

In situ Proliferation of Regulatory T Cells

In order to investigate the ability of Treg cells to locally proliferate in LN (TLN and ILN) and in testis under normal and inflammatory conditions, we performed a BrdU incorporation assay. N, C and E rats were administered BrdU daily for 3 days before sacrifice on days 50 or 80, and $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cells which went through divisions during this period incorporated this nucleoside analogue into their DNA and were quantified by flow cytometry using an anti-BrdU antibody (Fig. 5).

In LN from rats killed on days 50 and 80, a similar percentage of BrdU+ cells within $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cell subset was visible in TLN

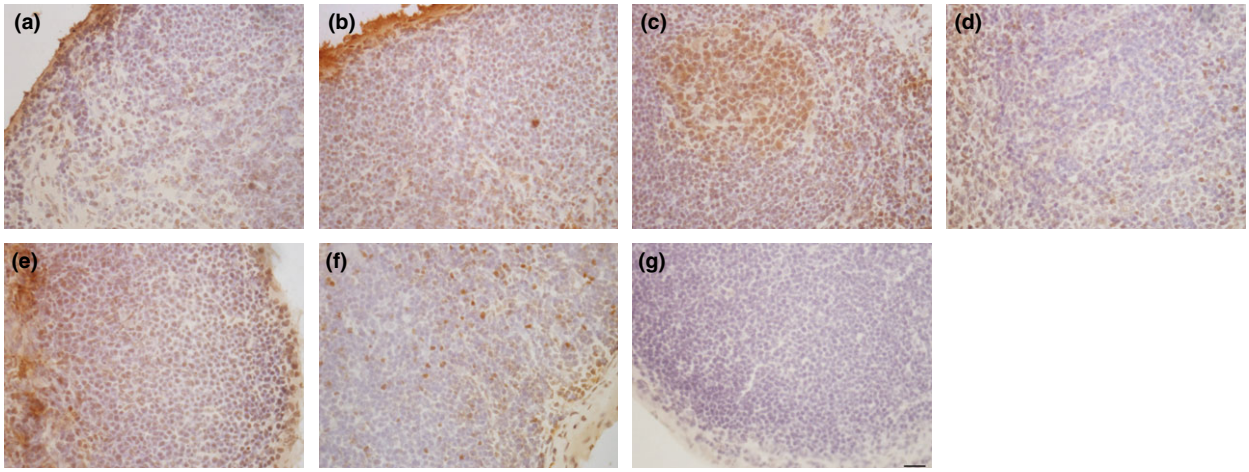

Fig. 4 Representative immunostaining for Helios in cryostat sections of lymph node draining the testis (TLN) (a, b, c) and lymph node draining the site of immunization (ILN) (d, e, f) from normal (a, d), control (b, e) and experimental (c, f) rats. Omission of primary antibody show negative staining (g). Scale bar indicates 20 μm.

and ILN from N, C and E rats. In contrast, a significant increase in the percentage of BrdU+ cells within $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cell subset was detected both in TLN and ILN from E compared to N and C rats. Absolute numbers of each proliferating Treg cell subset were increased in TLN and ILN from E compared to N and C rats. The fraction of BrdU+ cells was higher within ILN Treg cell subsets (Fig. 6).

In testis, the percentage of BrdU+ cells was significantly higher within $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cell subset from E compared to N and C rats killed on days 50 and 80. Absolute numbers of proliferating cells increased in each of the Treg cell subsets analysed in testis of E compared to N and C rats (Fig. 7).

Our results indicate that spermatic antigen-specific Treg cells proliferate extensively in ILN in response to antigenic challenge supplied by immunization and in TLN and testis by antigens released from damaged seminiferous tubules.

Suppressive Activity of Regulatory T Cells

For functional analysis, $CD3^+$ $CD4^+$ T cells expressing CD25 at high levels ($CD25^{bright}$) were purified from TLN and ILN from N, C and E rats killed on days 50 and 80 by FACS. Subsequent analysis of Foxp3 expression showed that these cell populations contained over 92% of $Foxp3^+$ cells (Fig. 8). Quantitative assessment of percentage of $CD3^+$ $CD4^+$ $CD25^{bright}$ T cells in TLN from N, C and E rats showed similar values; however, an increase in the percentage of these cells was detected in ILN from E compared to N rats (*TLN*: N: 0.55 ± 0.04, C: 0.67 ± 0.04, E 50 days: 0.66 ± 0.13, E 80 days: 0.72 ± 0.31; *ILN*: N: 0.19 ± 0.02, C: 0.32 ± 0.08, E 50 days: 0.51 ± 0.11**, E 80 days: 0.65 ± 0.21**, $^{**}P < 0.01$ versus N, mean ± S.E.M.).

Cells obtained from TLN and ILN of N, C and E rats as well as each fraction of purified $CD4^+$ $CD25^{bright}$ T cells were assessed for their proliferative response to mitogen (PMA and ionomycin) and spermatic antigen. As indicated in Fig. 9a, cells derived from TLN and ILN of rats killed on day 50 proliferate in response to polyclonal stimuli such as mitogens. Interestingly, cells derived from TLN were the only ones that showed a potent antigen-specific proliferative response to spermatic antigens in all groups of rats studied. In contrast, ILN cells proliferated in response to spermatic antigens when they were derived from E rats indicating that cells undergo a strong proliferative response to immunization. As expected, all $CD4^+$ $CD25^{bright}$ T cells fractions were anergic to polyclonal and *in vitro* antigen-specific stimulation. These results indicate that a response to spermatic antigens occurs specifically in the TLN environment under normal and inflammatory conditions. Similar results were obtained in rats killed on day 80 (Figure S2a).

In order to comparatively analyse the regulatory status of Treg cells from TLN and ILN of each group of rats, co-culture experiments were performed in which TLN cells obtained from E rats (responder T cells, Tresp) were stimulated with mitogens (PMA and ionomycin) or spermatic antigen in absence or

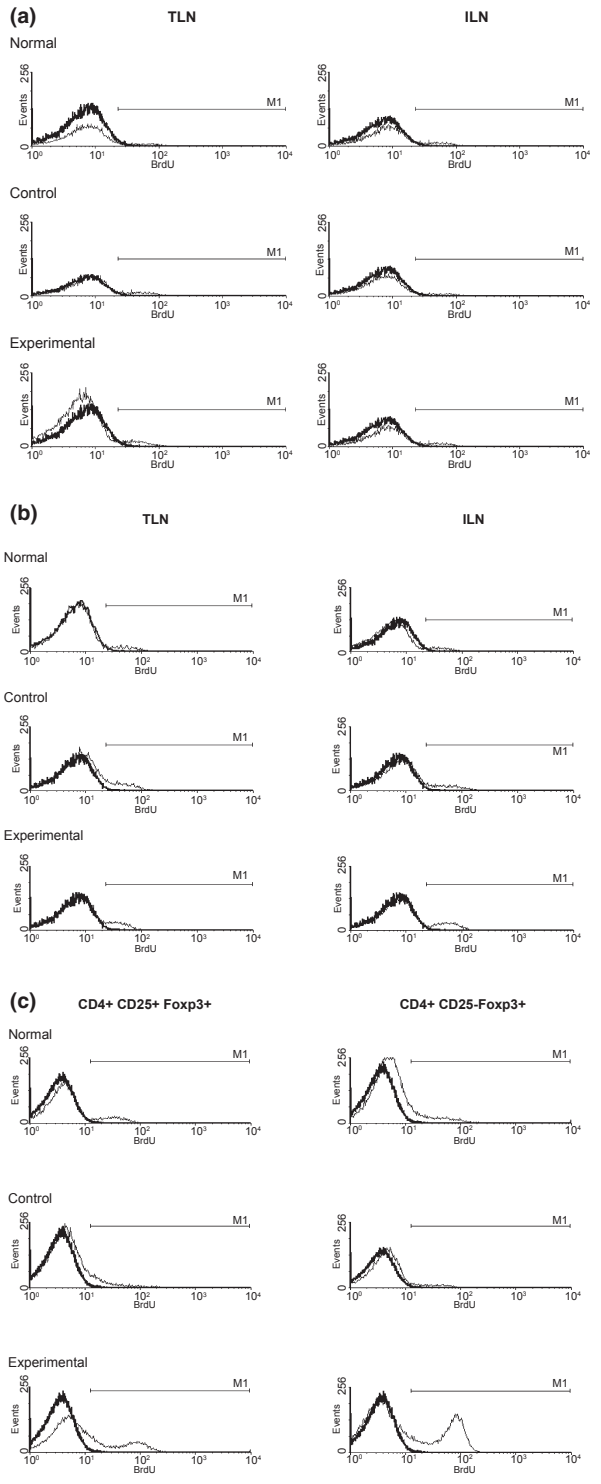

Fig. 5 Representative flow cytometry histogram plots illustrating bromodeoxyuridine (BrdU) incorporated into DNA of dividing cells in gated $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ (a) and $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ (b) Treg cells in cell suspension of lymph nodes draining the testis (TLN) and lymph nodes draining the site of immunization (ILN) from normal, control and experimental rats. Representative histogram plots showing BrdU staining in $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cell subsets in cell suspensions of testicular interstitium of normal, control and experimental rats (c). Thicker lines represent background BrdU staining levels obtained from rats non-treated with BrdU.

presence of purified $CD4^+$ $CD25^{bright}$ Treg cells from TLN and ILN of N, C and E rats at a 1:1, 5:1, 10:1 and 50:1 ratio. Relative difference in proliferative response of Tresp cells observed in the absence and presence of Treg cells was calculated for each condition and indicated as percentage of inhibition. As indicated in Fig. 9b, $CD4^+$ $CD25^{bright}$ T cells purified from TLN from N, C and E rats killed on day 50 are able to suppress Tresp proliferation. However, $CD4^+$ $CD25^{bright}$ T cells isolated from E rats were much more efficient than those derived from C and N rats, both at a low (1:1) and a high dose (10:1) of $CD4^+$ $CD25^{bright}$ T cells. These results suggest that spermatic antigen-specific Treg cells are enriched in TLN where testicular antigens are continuously presented and Treg cells exhibit their higher suppressive potential. Similar results were obtained in rats killed on day 80 (Figure S2b).

Expression of TGF-β1 in Regulatory T Cells in Rat Lymph Nodes

By immunohistochemistry, we analysed the expression of TGF-β within $CD4^+$ $CD25^{bright}$ T cell fraction isolated by FACS from TLN and ILN of N, C and E rats killed on days 50 and 80. Cells isolated from TLN and ILN expressed TGF-β in all groups of rats at any time studied (Fig. 10). TGF-β+ cells were also observed in tissue sections of TLN and ILN from N, C and E rats (data not shown).

Discussion

Although the testis is an immunoprivileged organ, infection and inflammation may overwhelm immunosuppressor mechanisms inducing autoimmune reactions against spermatic antigens which result in aspermatogenesis and infertility. Regional LN is a location where Treg cells control effector T cell responses.[29] Since quantitative and/or qualitative deficiencies of these cells contribute to development of autoimmune diseases, we undertook this study to analyse the number, phenotype and functional

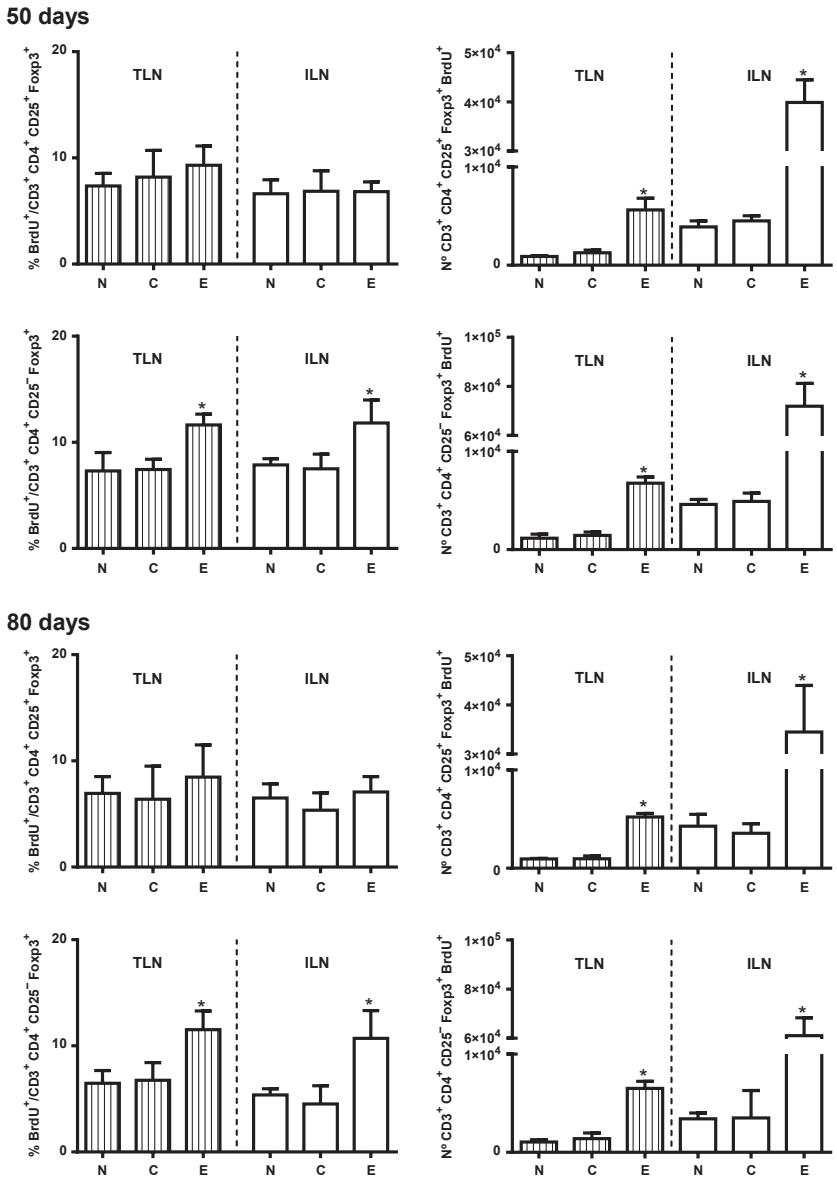

Fig. 6 Quantitative assessment of percentage and absolute number of $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ $BrdU^+$ and $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ $BrdU^+$ regulatory T cell subsets in cell suspension of lymph nodes draining the testis (TLN), lymph nodes draining the site of immunization (ILN) from normal (N), control (C) and experimental (E) rats. Data are representative of n = 7–10 rats per group. *$P < 0.01$ versus respective N and C.

response of Treg cells from TLN and ILN throughout EAO development and compared them with N and C rats.

Concording with our previous data in the inflamed testis,[18] flow cytometric results showed that the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells from TLN, but not from ILN, increased in rats with focal and severe EAO compared to N and C rats. In line with reports of Wheeler et al.[30] supporting LN-specific distribution of antigen-specific Treg cells in normal mice, our results suggest that TLN is enriched for spermatic antigen-specific Treg cells with the potential to inhibit autoimmune attack to the specific organ that drains to it.

Since LN hypertrophy is one of the most basic clinical signs related to inflammation, we consider important to evaluate variations in cell absolute numbers between different LN types along EAO course to better understand the *in vivo* status of rats within each group and time period. Similarly to previous results,[31,32] rats with focal and severe EAO showed an increase in cellularity of both ILN and TLN compared to N and C rats. Although cell numbers increased in both LN types, total cellularity of

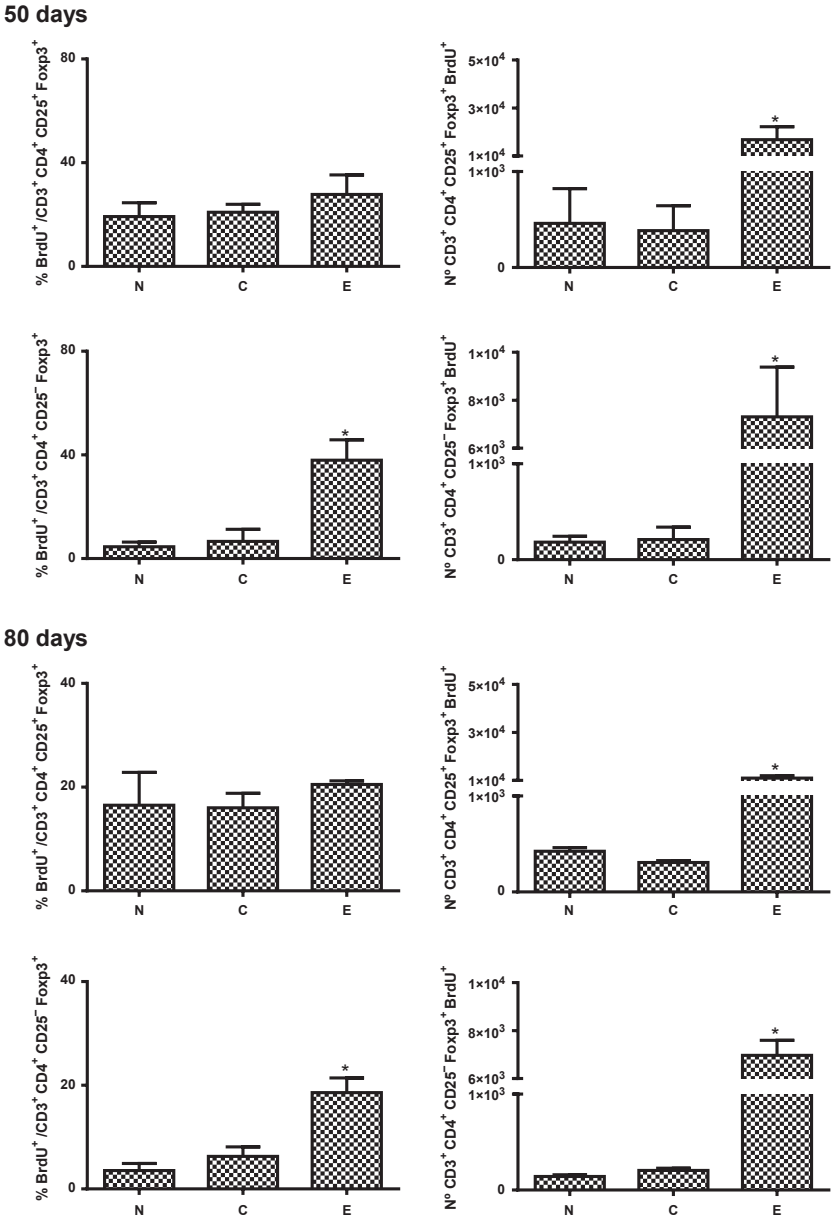

Fig. 7 Quantitative assessment of percentage and absolute number of $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ $BrdU^+$ and $CD3^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ $BrdU^+$ regulatory T cell subsets in cell suspension of testicular interstitium from normal (N), control (C) and experimental (E) rats. Data are representative of n = 7–10 rats per group. *$P < 0.01$ versus respective N and C.

ILN was higher than TLN. These effects had two direct consequences: (i) an increase in absolute numbers of all Treg cell subsets in TLN and ILN of E rats, in comparison with N and C rats, even though the percentage of some of these populations was not significantly modified, (ii) absolute numbers of each Treg cells subset was higher in ILN than in TLN. Results showing increased absolute numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cells subsets in ILN and TLN of E rats agree with our previous finding in the testis of rats undergoing EAO.[18]

Present results in rats with EAO are consistent with the fact that challenge of regional LN by spermatic antigens provided by immunization and/or progressively released from damaged seminiferous tubules as disease progresses triggers the increase in the number of Treg cells in ILN and TLN respectively. However, only specific LN that drains testis is strategically enriched for antigen-specific Treg cells.

Increased numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cells at sites of inflammation have been reported in other organ-specific autoimmune disease models.[33–35] Comparative analysis

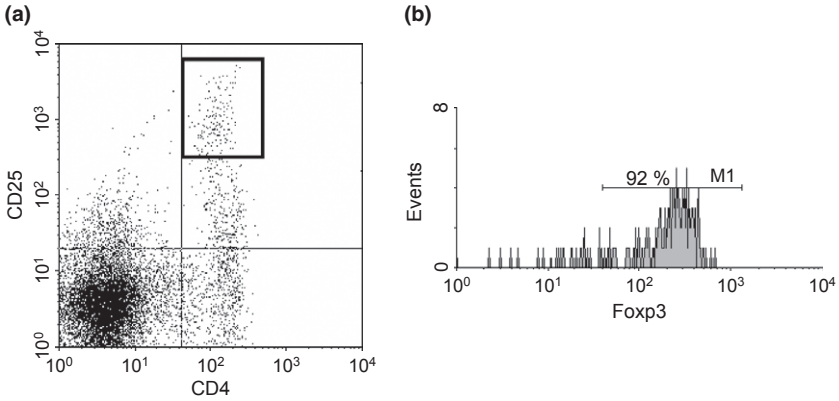

Fig. 8 Representative flow cytometry dot plot illustrating CD4 and CD25 expression in gated $CD3^+$ cells obtained from lymph nodes draining the testis of an experimental rat. A gate was drawn selecting cells with the highest expression of CD25 (a). $CD4^+$ $CD25^{bright}$ cells were isolated by fluorescence-activated cell sorting and analysed for Foxp3 expression. Representative flow cytometry histogram plot illustrating $Foxp3^+$ expression on sorted $CD3^+$ $CD4^+$ $CD25^{bright}$ T cells (b). Data are representative of at least three independent experiments with similar results.

of $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cell subsets in patients with systemic lupus erythematosus and healthy controls revealed that $CD4^+$ $CD25^-$ $Foxp3^+$ cells phenotypically resemble conventional Treg cells but possess functional defects. These cells suppressed T cell proliferation but were unable to reduce interferon-gamma production *in vitro*.[12]

Based on the fact that most of the Treg cells from TLN and ILN of all rats studied lacked CD45RC and CD62L expression, our results suggest that these cells exhibit an antigen-experienced status. Previous studies indicated that $CD62L^-$ Treg cells could migrate efficiently to non-lymphoid tissues whereas $CD62L^+$ Treg cells migrate mainly to secondary lymphoid tissues.[3,36] In this line, our results suggest that Treg cells from TLN are able to migrate to the testis where they may encounter their cognate antigen.

Helios expression is associated with T cell activation and proliferation.[37–39] Moreover, Helios+ Treg cells have recently been shown to be capable of trafficking into inflamed tissues and exerting their suppressive function at these sites in patients with active systemic lupus erythematosus.[40] In our model, the fact that a large number of $Helios^+$ cells was observed in TLN, mainly in E rats, concords with their activated phenotype and migratory ability.

Under normal conditions, blood-testis barrier limits the access of antibodies to the adluminal compartment and the passage of most, but not all, germ cell autoantigens to the interstitium, reducing its interaction with immune cells. At tubuli recti and rete testis, modified Sertoli cells form a weak barrier relevant for immune tolerance mechanisms since continuous spermatic antigen leakage occurs. These sites are also vulnerable to autoimmune attack.[41,42] Phenotypic characterization of Treg cells from N rats tallies with the concept by which antigen-specific Treg cells are continuously activated by self-antigens in regional LN. As was postulated by Fisson et al.,[43] the existence of activated Treg cells in lymphoid tissues suggested that these cells may exert a basal and permanent suppression on T cell activation. Concomitant with seminiferous tubule damage and blood-testis barrier impairment in testis during EAO development,[44] expanded Treg cell populations with an activated/memory phenotype have the potential to migrate from TLN to the inflamed testis in order to down-regulate local inflammatory response.

BrdU incorporation experiments showed that a fraction of each Treg cell subset is proliferating in LN from N, C and E rats killed on days 50 and 80. Interestingly, a higher percentage of BrdU+ cells was observed within $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cell subset from TLN and ILN during EAO development showing the ability of these cells to proliferate in the presence of spermatic antigens. Accordingly, percentage of BrdU+ proliferative cells within $CD4^+$ $CD25^-$ $Foxp3^+$ Treg cell subset increased in the testis of E compared to N and C rats. As expected, the number of BrdU+ cells within $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD4^+$ $CD25^-$ $Foxp3^+$ Treg subset increased in LN and in testis under inflammatory conditions

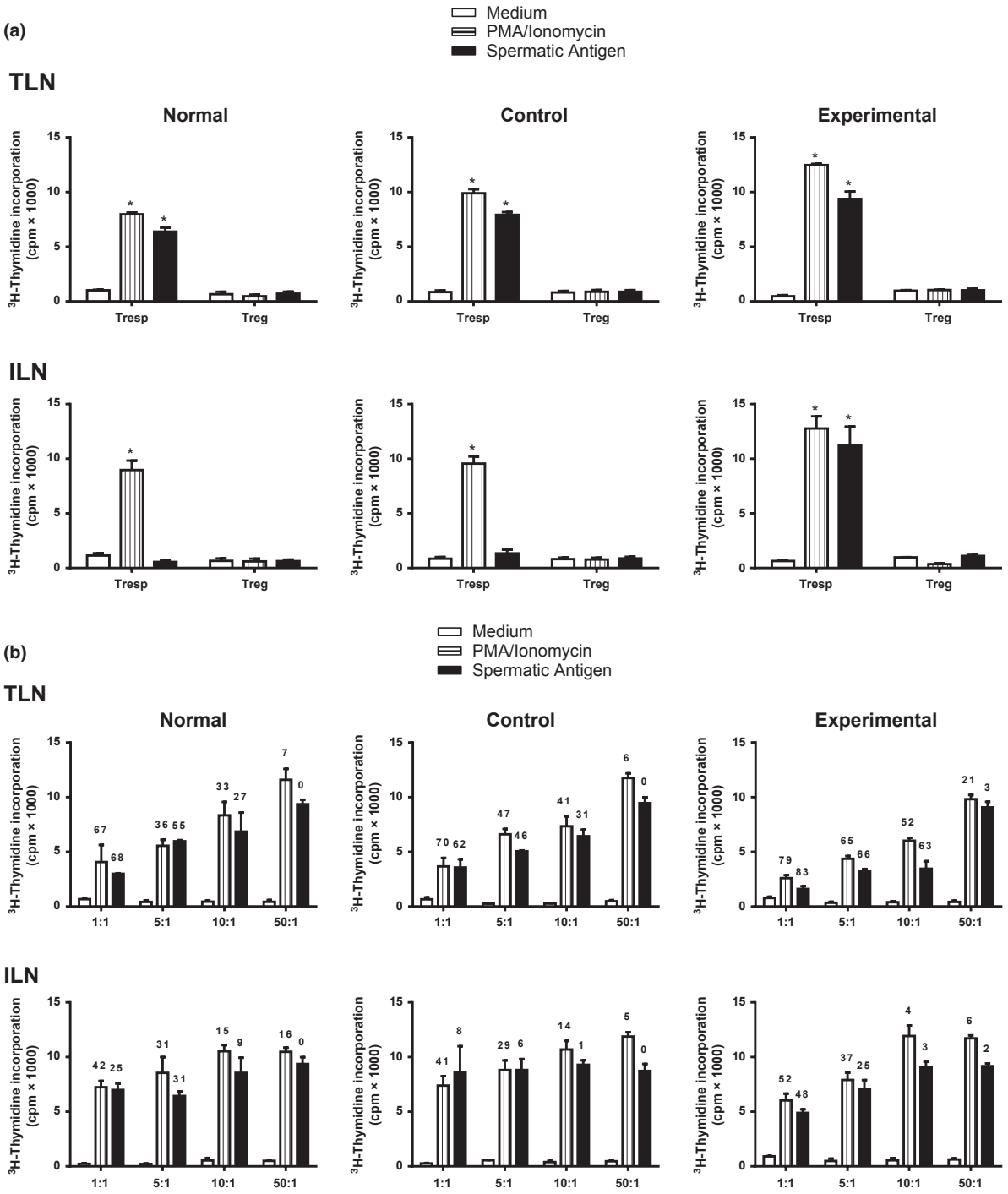

Fig. 9 Proliferative response of cells from lymph nodes draining the testis (TLN) and draining the site of immunization (ILN) of normal, control and experimental rats killed on day 50 in absence/presence of mitogens or spermatic antigens (a). Proliferative response of cells from TLN of E rats (Tresp) co-cultured with the indicated ratio of $CD4^{+}$ $CD25^{bright}$ Treg cells from TLN and ILN of N, C and E rats killed on day 50 in absence/presence of mitogens or spermatic antigens (b). Proliferation of T cells assessed by standard [^{3}H]-thymidine incorporation is shown as counts/minute (CPM). Representative result (mean ± S.E.M) of quintuplicate wells from three independent experiments. Percent inhibition is indicated. *P < 0.01 versus respective medium.

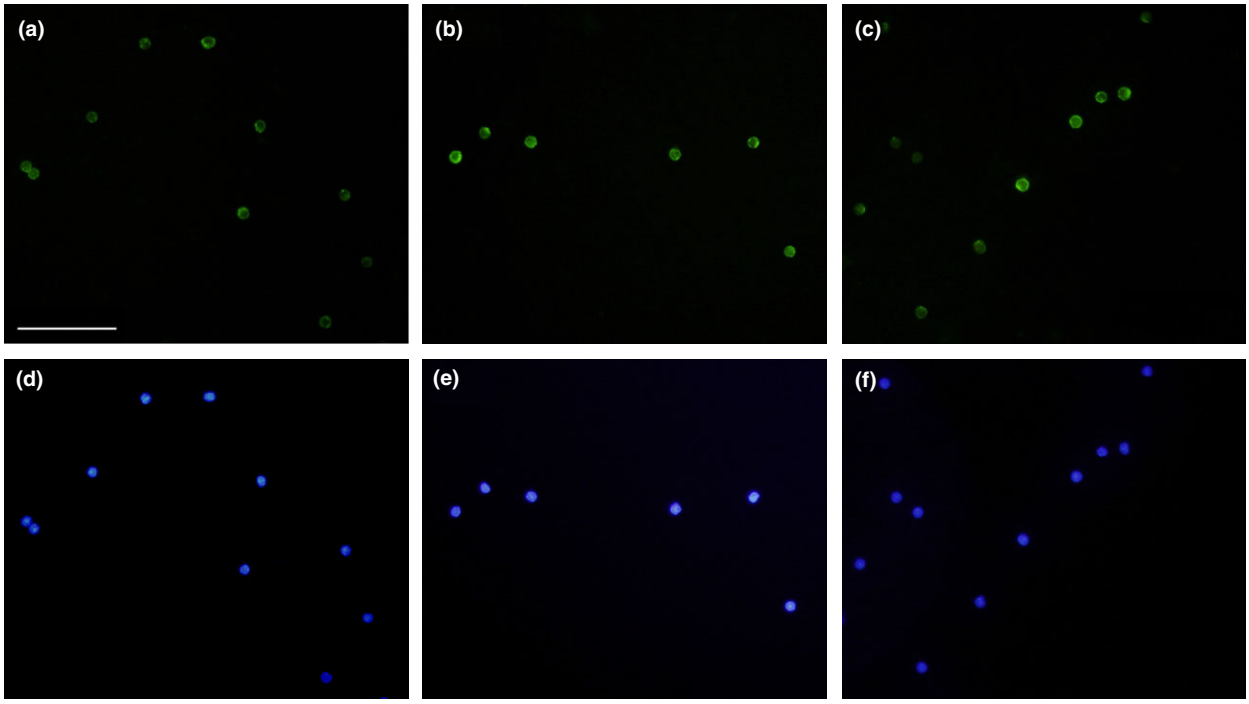

Fig. 10 Representative immunostaining for TGF-β in CD3$^+$ CD4$^+$ CD25^{bright} T cells isolated from lymph nodes draining the testis of a normal (a, d), control (b, e) and an experimental rat (c, f). Secondary antibody conjugated with fluorescein isothiocyanate was used to detect TGF-β (a, b, c). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (d, e, f). Scale bar indicates 50 μm.

reflecting enhanced spermatic antigen stimulation. These results also support the existence of Treg cells displaying a phenotype of activated cells.

CD4$^+$ CD25$^-$ Foxp3$^+$ cells seemed to be the most frequent Treg cell subset in the inflamed testis[18] as well as the most proliferative subset in the LN of rats with EAO. However, the source of CD4$^+$ CD25$^-$ Foxp3$^+$ Treg cells remains unknown. The relationship between CD25$^+$ and CD25$^-$ Treg cells in the periphery has not yet been established, and could result either from the instability of this marker on a single Treg cell subset, from different lineages of Treg cells that might have different mechanisms of action[11] or from a pre-existing pool that arises under certain autoimmune or inflammatory conditions following auto-antigen stimulation.[12] We were able to detect differences in the percentages of BrdU+ cells within CD4$^+$ CD25$^-$ Foxp3$^+$ subset but not within CD4$^+$ CD25$^+$ Foxp3$^+$ subset in LN and testis of rats undergoing EAO. However, without knowing the level of possible conversions from CD25$^+$ to CD25$^-$ phenotype and vice-versa, a definitive conclusion cannot be made. Some of CD4$^+$ CD25$^-$ Foxp3$^+$ BrdU$^+$ could have incorporated BrdU while they were CD4$^+$ CD25$^+$ Foxp3$^+$ cells.

Increase in Treg cell number at the site of inflammation is presumably a general host responses to chronic inflammation.[45,46] Our results in rats with EAO point to the fact that increased percentage of CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells in TLN and in their drained organ could be due to *in situ* proliferation of *de novo* Treg cells where cells are continuously challenged by inflammation.

In humans and rodents, regulatory function associates with CD4$^+$ T cells expressing CD25 at high levels. Other reports showed that cells with intermediate CD25 expression may represent recently activated T cells.[30,34,47] In the present work, we investigated the functional status of Foxp3$^+$ Treg cells contained within CD4$^+$ CD25^{high} T cell population of different LN. Although cells derived from TLN and ILN proliferate in response to polyclonal stimuli, cells derived from TLN were the only ones to show a potent antigen-specific proliferative response in all groups of rats studied. Cells from ILN proliferated in response to spermatic antigen only when derived from rats with EAO. These results suggest that specific response against spermatic antigens occurs in the TLN environment where Treg cells are continuously challenged by their cognate antigen.

Isolated $CD4^+$ $CD25^{bright}$ T cells did not respond to *in vitro* stimulation, in line with previously described functional properties of Treg cells.[48]

Functional analysis showed that Treg cells derived from TLN of E rats exhibited a stronger antigen-specific proliferative response and suppressive capacity compared to their N and C counterparts. In contrast, these cellular responses were observed only for Treg cells derived from ILN of E, but not from N and C rats. These results suggest two important issues: (i) under normal and inflammatory conditions, immunological response to testicular antigens occurred specifically at regional TLN, (ii) Treg cells were overactivated *in vivo* by inflammation, and this led to enhanced suppressive activity. Our results agree with the concept that microenvironment of different LN may translate into biologically different behaviours and functions of Treg cells.

Evidence has been provided that surface-bound TGF-β might be a key mediator of suppression acting by means of direct T cell interaction.[10] The fact that Treg cells from TLN and ILN display membrane-bound TGF-β[9] suggests that this cytokine might be a specific mediator of suppression. It is known that other cytokines, like IL-2[49], IL-10[50] and IL-35[51] are also involved in Treg cell immunoregulation. Importantly, Terayama et al.[51] demonstrated the contribution of IL-12/IL-35 common subunit p35 in maintaining testicular immunoprivilege.

During EAO development, inflammation and tissue destruction progress despite the presence of increased number of functional Treg cells at the site of inflammation. Multiple mechanisms by which immune regulation may be lost have been postulated: (i) inadequate numbers of Treg cells, (ii) Treg cells′ intrinsic functional defects, (iii) resistance to Treg cells by Teff cells, and (iv) impairment of Treg cell function by proinflammatory cytokines. Th17 cells, a T cell subset involved in the pathogenesis of EAO, may be refractory to Treg cell suppression.[48,52] TNF-α and IL-6, cytokines commonly elevated in the testis of rats with EAO, can reduce the suppressive function of Treg cells suggesting that the role of Treg cells might be compromised locally.[13,53–56]

Since Foxp3 expression in T cells derived from inflamed tissues is not confined to $CD25^{bright}$ compartment[18,53,56], we cannot rule out the possibility that Treg cells contained within $CD4^+$ T cell population expressing CD25 at intermediate levels and even $CD25^-$ cells[18,56], might have functional defects.

Conclusions

Our functional data revealed that Treg cells from rats with focal and severe EAO are able to proliferate and suppress proliferation of conventional T cells. These cells are significantly increased in LN draining the testis were they may encounter with their cognate antigen in order to down-regulate local inflammatory response. Nevertheless, Treg cells fail to effectively suppress inflammation. Our previous and present data argue against a general functional defect in Treg cells and instead suggest the importance of the inflammatory milieu in setting the threshold for immune regulation. The wide-range involvement of Treg cells in immunoprivilege and in inflammatory/autoimmune pathology offer an attractive target for better understand testicular immune homeostasis and pathogenesis/treatment of EAO.

Acknowledgements

This work was supported by the Universidad de Buenos Aires and Consejo Nacional de Investigación Científica y Tecnológica. We thank the Instituto Nacional de Microbiología "A. Malbrán", División Vacunas Bacterianas, for their generous donation of *Bordetella pertussis*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative flow cytometry dot plot illustrating Foxp3 and CD25 expression on gated. $CD3^+$ $CD4^+$ T cells from lymph nodes draining the testis of an experimental rat. R1 and R2 gates were set on CD3+CD4+CD25+Foxp3- and CD3+CD4+CD25+Foxp3+ T cells, respectively (a). Representative histogram plots showing CD45RC and CD62L staining in CD3+CD4+CD25+Foxp3- non-Treg cells (b) and CD3+CD4+CD25+Foxp3+ Treg cells (c). Thicker lines represent isotype controls.

Figure S2. Proliferative response of cells from lymph nodes draining the testis (TLN) and draining the site of immunization (ILN) of normal, control and experimental rats killed on day 80 in absence/presence of mitogens or spermatic antigens (a). Proliferative response of cells from TLN of E rats(Tresp) co-cultured with the indicated ratio of $CD4^+$ $CD25^{bright}$ Treg cells from TLN and ILN of N, C and E rats killed on day 80 in absence/presence of mitogens or spermatic antigens (b). Proliferation of T cells assessed by standard [3H]-thymidine incorporation is shown as counts/minute (CPM). Representative result (mean±SEM) of quintuplicate wells. Percent inhibition is indicated.