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Differential changes in CD4$^+$ and CD8$^+$ effector and regulatory T lymphocyte subsets in the testis of rats undergoing autoimmune orchitis

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Abstract

Experimental autoimmune orchitis (EAO) is a useful model to study organ-specific autoimmunity and chronic testicular inflammation. EAO is characterized by an interstitial lymphomononuclear cell infiltration and damage of the seminiferous tubules showing germ cell sloughing and apoptosis. Using flow cytometry, we analysed the phenotype and number of T lymphocytes present in the testicular interstitium of rats during EAO development. A large increase in the number of testicular CD3$^+$ T lymphocytes was detected. The number of CD4$^+$ and CD8$^+$ effector T lymphocytes ($T_{effector}$ cells) dramatically increased in the testis at EAO onset, with the CD4$^+$ cell subset predominating. As the severity of the disease progressed, CD4$^+$ $T_{effector}$ cells declined in number while the CD8$^+$ $T_{effector}$ cell subset remained unchanged, suggesting their involvement in maintenance of the chronic phase of EAO. As a novel finding, we detected by immunohistochemistry and flow cytometry Foxp3 expressing CD4$^+$ and CD8$^+$ regulatory T lymphocytes (T_{regs}) in chronically inflamed testis of EAO rats. The numbers of both T_{reg} cell subsets increased in the testis of rats with orchitis, mainly at the onset of EAO; CD4$^+$Foxp3$^+$ T_{reg} cells were more abundant than CD8$^+$Foxp3$^+$ T_{reg} cells. Unexpectedly, CD25$^-$ T lymphocytes were more abundant than CD25$^+$ cells within CD4$^+$Foxp3$^+$ and CD8$^+$Foxp3$^+$ T_{reg} cell populations. Although T_{reg} subsets are actively accumulated into the testis in EAO rats, these cells are outnumbered by an even more vigorously expanding $T_{effector}$ subset. Further, it is possible that factors present in the inflamed testis might limit the ability of T_{regs} to abrogate tissue damage.

Keywords: Autoimmune orchitis; Testis; T lymphocytes; Regulatory T lymphocytes

1. Introduction

Although the testis is an immunoprivileged organ, inflammation associated with subfertility or infertility can occur. Infection and inflammation of the male genital tract are a frequent cause or co-factor of fertility disturbances in men (Schuppe and Meinhardt, 2005). Experimental autoimmune orchitis (EAO) is an organ-specific model of autoimmunity characterized by the presence of interstitial lymphomononuclear cell infiltrates and severe damage of seminiferous tubules resulting in aspermatogenesis and infertility.

The relevant role of regulatory T lymphocytes (T_{reg} cells) suppressing autoimmunity and preventing graft rejection has been reported in many animal models. The T_{reg} cell family is predominantly composed of CD4$^+$ cell subsets, the thymus-derived natural CD4$^+$CD25$^+$ T_{reg} cells and the adaptive T_{reg} cells generated from CD25$^-$ precursors in peripheral lymphoid organs (Fontenot et al., 2003). The development and function of CD4$^+$CD25$^+$ T_{reg} cells is critically dependent

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on the transcriptional repressor Foxp3 which is currently the most specific marker to identify this T lymphocyte subset (Hori et al., 2003; Fontenot et al., 2003). Although T_{reg} cells are enriched in the $CD4^+CD25^+$ cell subset, there is also evidence for the presence of $CD4^+CD25^-$ T_{reg} cells in the periphery of rodents (Stephens et al., 2004). It has been demonstrated that $CD8^+Foxp3^+$ T_{reg} cells can also suppress autoimmunity (Hahn et al., 2005; Singh et al., 2007).

Few data are available in the literature concerning the presence and functional role of T_{reg} cells in the testis, and to our knowledge, no identification of $Foxp3^+$ T_{reg} cells has been reported. Early work of Mukasa et al. (1995) showed that the depletion of testicular γδ T lymphocytes with regulatory function accelerated inflammatory response in a model of autoimmune orchitis induced by bacterial infection of the contralateral testis. Also, Nasr et al. (2005) demonstrated the role of testicular induced Ag-specific $CD4^+CD25^+$ T_{reg} cells in the maintenance of tolerance to pancreatic islet allografts in testis.

In order to better elucidate alterations of testicular immunoregulation in chronic inflammation we phenotypically characterized, quantified and localized T lymphocyte subsets focusing on autoaggressive effector T lymphocytes ($T_{effector}$ cells) and T_{reg} cells present in testis during EAO development.

2. Materials and methods

2.1. 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 with 14 h light, 10 h 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.

2.2. Induction of EAO

Rats in the experimental group were actively immunized with 3 doses, at 14-day intervals, of testis homogenate emulsified with complete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO, USA) using *Bordetella pertussis* as co-adjuvant as previously described (Doncel et al., 1989). Rats in the control group were injected with an emulsion of saline solution and adjuvants in the same conditions as the experimental group. A third group of normal rats were also studied. Rats in the normal, control and experimental groups were killed 30, 50 and 80 days after the first immunization.

2.3. Histopathology

Testes were fixed in Bouin's solution and embedded in paraffin. The histopathology of the testes was analysed in transversal sections obtained from the poles and equatorial areas of testis and stained with hematoxylin–eosin.

2.4. Isolation of testicular interstitial cells

Testicular interstitial cells from normal, control and experimental groups of rats were obtained as previously described (Rival et al., 2007). Briefly, decapsulated testes were incubated with type I collagenase (0.3 mg/ml; Worthington Biochemical Corporation, Freehold, NJ) at 34 °C for 15 min. The collagenase was inactivated and seminiferous tubules allowed to settle; the supernatant was washed with phosphate buffered saline (PBS) and red blood cells were depleted by osmotic lysis with ammonium chloride (160 mM NH_4Cl, 170 mM Tris–HCl, pH 7.2). Cells were washed, centrifuged and counted in a Neubauer chamber by the trypan blue exclusion method.

2.5. Flow cytometric analysis

Testicular interstitial cells (1×10^6) were stained with the following combinations of antibodies: CD45-PE-Cy5+CD3-APC, CD3-APC+CD4-FITC+CD8-PerCP, CD3-PE-Cy5+CD4-FITC+CD25-AlexaFluor®647 and CD3-PE-Cy5+CD8-FITC+CD25-AlexaFluor®647.
The appropriate control isotypes were used. To analyse the intracellular expression of Foxp3, cells fixed and permeabilized were incubated with anti-CD16/32-Blocks Fc followed by anti-Foxp3-PE or isotype control according to the manufacturer's staining protocol. All antibodies were purchased from BD Bioscience (BD Pharmingen, San Diego, CA, USA), except for CD25-AlexaFluor®647 and IgG1-AlexaFluor®647 purchased from Serotec Laboratories (Serotec, Raleigh, NC, USA) and Foxp3 staining kit obtained from eBioscience (eBioscience, San Diego, CA, USA). BD FACSCalibur cytometer was used and 30,000 events on $CD45^+$ or $CD3^+$ cell gates were acquired. The absolute number of positive cells per testis was calculated from percentages obtained by flow cytometric analysis and total number of interstitial cells.

2.6. Immunofluorescent technique

Frozen testis sections (5–7 μm) were fixed in acetone–ethanol (1:1) at −20 °C for 10 min, washed in

PBS and incubated with monoclonal antibodies anti-CD4 or anti-CD8 (Cappel Laboratories, West Chester, PA). Then sections were blocked with PBS containing 5% skim milk, 0.1% TritonX-100 and anti-CD16/32-Blocks Fc (eBioscience) for 2 h and incubated overnight with anti-Foxp3-PE-Cy5 or isotype control. Sections were mounted in PBS–glycerin and observed in a confocal laser microscope (FV300 Olympus).

2.7. Statistical analysis

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

3. Results

3.1. Histopathology

As we previously described (Doncel et al., 1989), 50 days after the first immunization the testis of experimental rats presented mild lymphomononuclear cell infiltrate and several foci of damaged seminiferous tubules with different degrees of germ cell sloughing (focal orchitis) (Fig. 1B). From day 80 on we observed an increase in the interstitial cell infiltrate and extensive damage of most of the seminiferous tubules in which only spermatogonia and Sertoli cells were attached to the tubular wall (severe EAO) (Fig. 1C). Lymphocytes were not observed inside the ST. No testicular damage was observed in the control group (Fig. 1A) or normal group of rats at any time studied, or in experimental rats killed on day 30 (at the end of the immunization period).

3.2. Increased number of T lymphocytes in the testis of rats with EAO

To determine the number of T lymphocytes in testis throughout EAO development, two-colour flow cytometric analysis was performed. The CD45 marker was first used to identify the whole population of interstitial leukocytes. Then $CD45^+$ cells were gated and analysed for CD3 expression (Fig. 2B and D). A significant increase in the number of $CD45^+$ cells and $CD3^+$ T lymphocytes was observed in the testis of experimental compared to normal and control rats killed on days 50 and 80 (Fig. 2A and C). No differences between groups were detected on day 30.

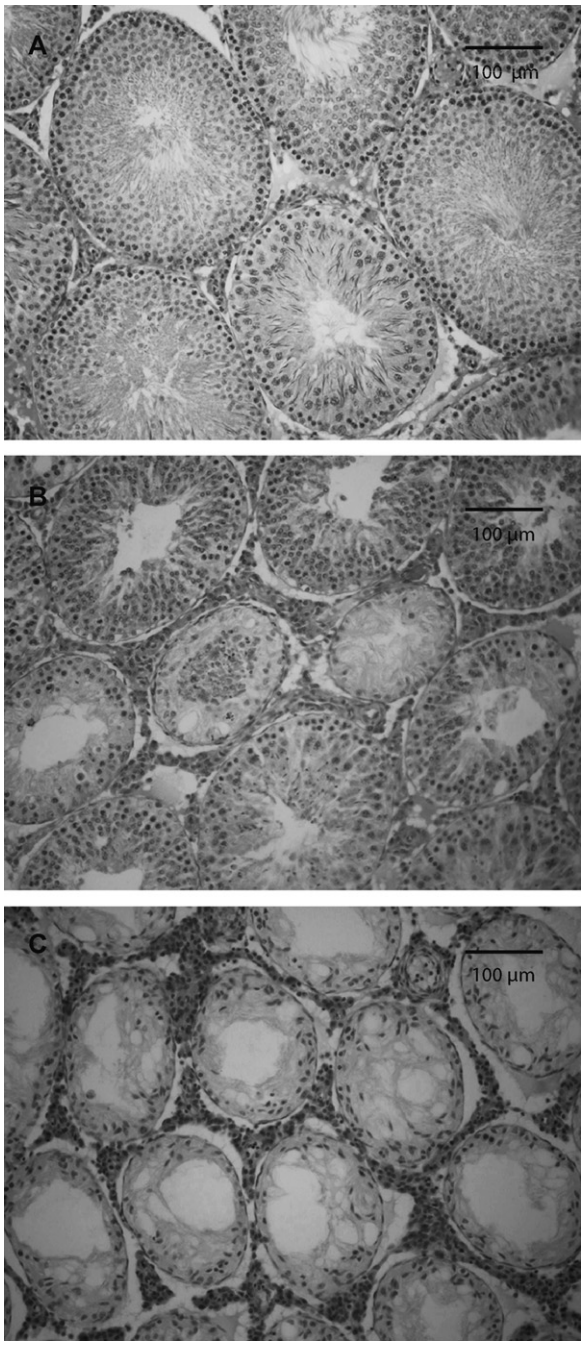

Fig. 1. Testicular histopathology. Foci of seminiferous tubules with germ cell sloughing and moderate interstitial lymphomononuclear cell infiltrate in testis of experimental rat killed on day 50 (focal EAO) (B); more severe and extensive damage of seminiferous tubules with increased interstitial inflammatory cell infiltrate in E rat killed on day 80 (severe EAO) (C). Normal histopathology is observed in testis of control rat killed on day 50 (A). 50×.

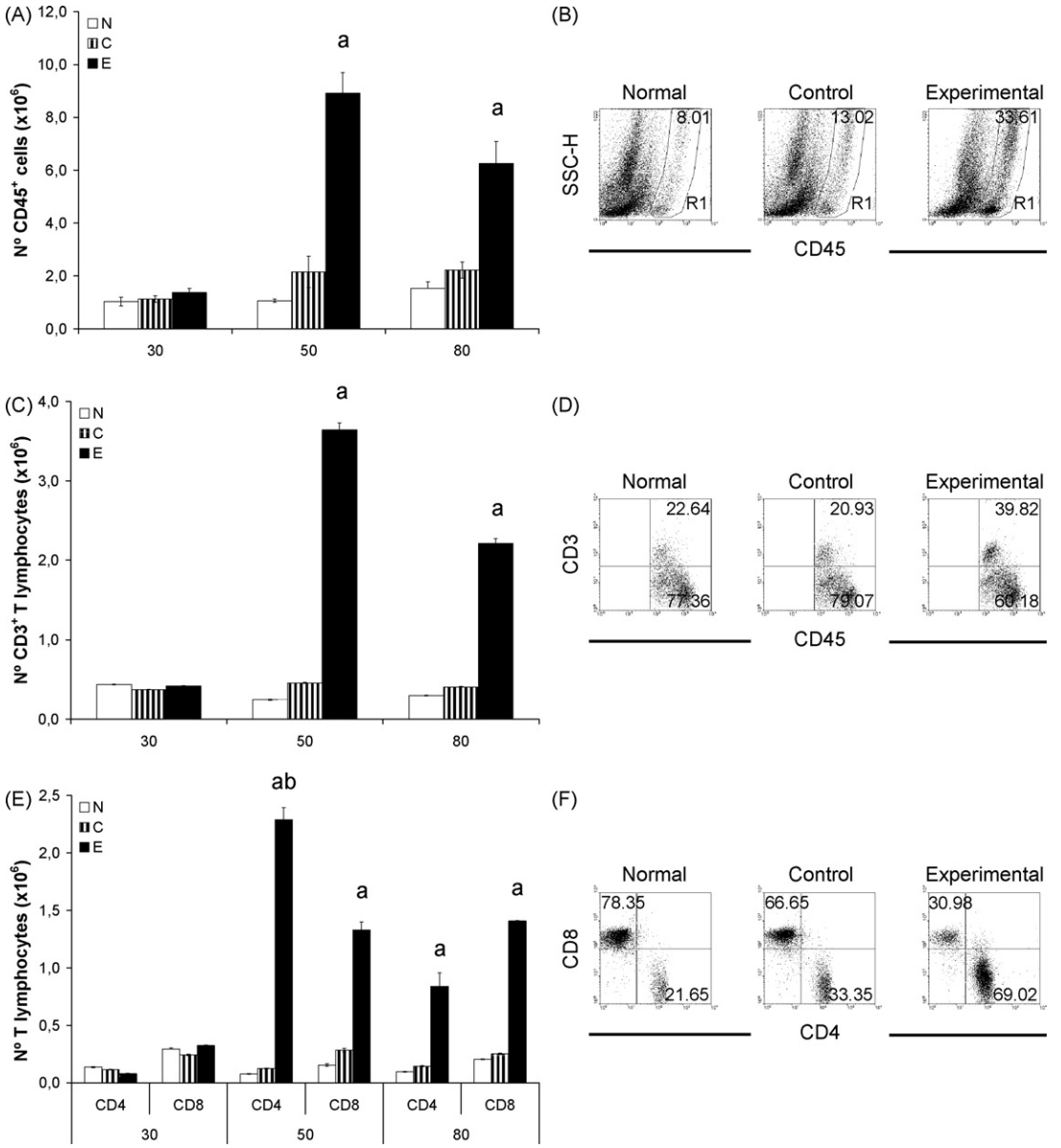

Fig. 2. Increased number of T lymphocytes and chronological changes of $CD4^+$ and $CD8^+$ subsets during EAO development. Absolute number of $CD45^+$ cells (A), $CD3^+$ T lymphocytes (C) and $CD4^+$ and $CD8^+$ T lymphocyte subsets (E) in testis of normal (N), control (C) and experimental (E) rats killed on days 30, 50 and 80. $N = 5–7$ rats/group/time point. Testicular interstitial cells were stained for CD45, CD45+CD3 and CD3+CD4+CD8 and analysed by flow cytometry. A gate was drawn selecting the total leukocyte population ($CD45^+$ cells, R1) based on the isotype control (B). $CD3^+$ T lymphocytes were detected and quantified on R1 (D). Expression of CD4 and CD8 was analysed on the $CD3^+$ gate (F). Representative dot plots show CD45 (B), CD3 (D) and CD4 and CD8 expression (F) in testis of N, C and E rats killed on day 50. Quadrants were set based on the isotype controls. The percentages of cells in the different quadrants are indicated. $^a p < 0.01$ versus respective N and C, $^b p < 0.01$ versus $CD4^+$ E_{80}.

3.3. Temporal changes in the number of $CD4^+$ and $CD8^+$ T lymphocytes in EAO

Three-colour flow cytometry was performed to assess the $CD4^+$ and $CD8^+$ T lymphocyte subsets in the testis during EAO development. A significant increase in the number of $CD4^+$ and $CD8^+$ T lymphocytes was detected in testis of experimental compared to normal and control rats on days 50 and 80. The number of $CD4^+$ T lymphocytes increased to its maximum value on day 50 then decreased significantly on day 80. The number of $CD8^+$ T lymphocytes increased on day 50, to a lesser extent compared to $CD4^+$ cells and remained unchanged on day 80 (Fig. 2E and F).

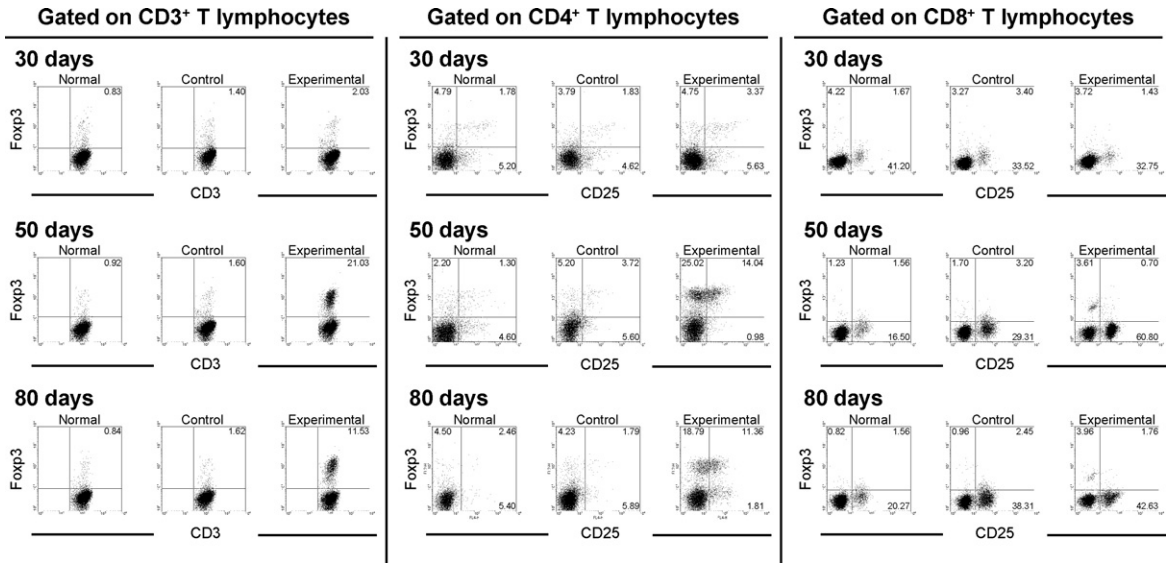

Fig. 3. Representative flow cytometry dot plots of the testicular effector and regulatory T lymphocytes. Testicular interstitial cells were stained for CD3+Foxp3, CD3+CD4+CD25+Foxp3 and CD3+CD8+CD25+Foxp3 and analysed by flow cytometry. Expression of Foxp3 was analysed on the $CD3^+$ cell gate (left panel). Expression of CD25 and Foxp3 was analysed on the $CD3^+CD4^+$ cell gate (middle panel) and on $CD3^+CD8^+$ cell gate (right panel). $Foxp3^+$ regulatory T lymphocytes (T_{reg} cells), $CD25^+Foxp3^+$ T_{reg} cells, $CD25^-Foxp3^+$ T_{reg} cells, $Foxp3^-$ effector T lymphocytes ($T_{effector}$ cells) and $CD25^+Foxp3^-$ $T_{effector}$ cells are shown. Quadrants were set based on the isotype controls. Percentages of cells in the different quadrants are indicated.

We next quantified $T_{effectors}$, defined by the absence of Foxp3 expression, within $CD4^+$ and $CD8^+$ cell populations. Both effector subsets increased in number in testis of experimental rats compared to normal and control rats on days 50 and 80 (Fig. 3 and Fig. 4A and B). Chronological changes observed in the number of $CD4^+$ and $CD8^+$ $T_{effector}$ cells in the testis of experimental rats resembled those observed in the whole $CD4^+$ and $CD8^+$ cell populations. Analysing CD25 expression we detected an increase in the number of activated $CD25^+$ $T_{effector}$ cells (both $CD4^+$ and $CD8^+$ T cell subsets) in the testis of experimental compared to normal and control rats on days 50 and 80. Interestingly, activated $T_{effector}$ cells were predominantly $CD8^+$ (Fig. 3 and Table 1). No differences between groups were detected on day 30.

3.4. Increased number of testicular regulatory T lymphocytes in EAO

At the onset of EAO we observed numerous $CD4^+$ and scarce $CD8^+$ T lymphocytes widespread in the testicular interstitium, mainly in the subalbuginea and peritubular

Table 1
Number of testicular activated $CD4^+$ and $CD8^+$ effector T lymphocytes ($\times 10^5$).

Group of animals	Days	$CD4^+CD25^+$ $T_{effectors}$	$CD8^+CD25^+$ $T_{effectors}$
Normal	30	0.07 ± 0.04	1.20 ± 0.02
Control		0.05 ± 0.02	0.80 ± 0.06
Experimental		0.02 ± 0.01	1.05 ± 0.06
Normal	50	0.04 ± 0.01	0.24 ± 0.03
Control		0.07 ± 0.02[a]	0.82 ± 0.10[a]
Experimental		0.30 ± 0.03[b,c]	8.02 ± 0.75[b,c]
Normal	80	0.04 ± 0.01	0.41 ± 0.05
Control		0.08 ± 0.01[a]	0.96 ± 0.19[a]
Experimental		0.14 ± 0.01[b,c]	5.92 ± 0.07[b,c]

$n = 5$–7 rats/group/time point.
[a] $p < 0.001$ versus respective N.
[b] $p < 0.001$ versus respective control.
[c] $p < 0.001$ versus respective normal.

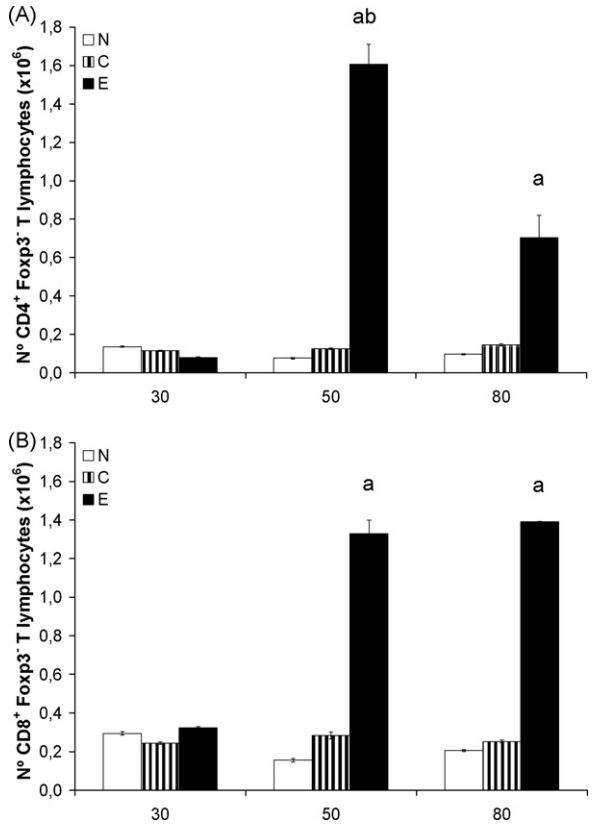

Fig. 4. Increased number of $CD4^+$ and $CD8^+$ effector T lymphocytes in testis of rats with EAO. Absolute number of $CD4^+Foxp3^-$ (A) and $CD8^+Foxp3^-$ (B) effector T lymphocytes in testis of normal (N), control (C) and experimental (E) rats killed on days 30, 50 and 80. $N = 5–7$ rats/group/time point. [a] $p < 0.01$ versus respective N and C, [b] $p < 0.01$ versus $CD4^+$ E_{80}.

area. Nuclear localization of Foxp3 was detected within $CD4^+$ and $CD8^+$ T lymphocytes principally localized in the subalbuginea area. A very scarce number of $CD8^+Foxp3^+$ cells was observed (Fig. 5A and B).

By flow cytometry we quantified $Foxp3^+$ T_{reg} cells in the $CD3^+$ T lymphocyte population and within $CD4^+$ and $CD8^+$ cell subsets. An increase in the number of $Foxp3^+$ T_{reg} cells was detected in the testis of experimental compared to normal and control rats on days 50 and 80 (Fig. 5C). Interestingly, Foxp3 expressing $CD4^+$ and $CD8^+$ T_{reg} cells were detected in all groups studied. The number of both T_{reg} cell subsets significantly increased in testis of experimental compared to normal and control rats killed on days 50 and 80. Of note, $CD4^+Foxp3^+$ T_{reg} cells were more abundant than their $CD8^+Foxp3^+$ counterpart (Fig. 5D and E). Several reports (Bonelli et al., 2009; Graca et al., 2002) showed that $CD4^+CD25^-Foxp3^+$ T_{reg} cells can be peripherically generated *in vivo* and are able to induce immunologic tolerance. Thus, we analysed CD25 expression to differentiate between $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^-Foxp3^+$ T_{reg} cells. Both T_{reg} cell subsets increased in number in testis of experimental compared to normal and control rats killed on days 50 and 80 (Table 2). Of the $CD4^+$ subsets, $CD4^+CD25^-Foxp3^+$ T_{reg} cells were predominant indicating that the main increase of $CD4^+Foxp3^+$ T_{reg} cells occurred within the $CD25^-$ cell subset.

We also analysed CD25 expression on the $CD8^+$ T_{reg} subset. A significant increase in the number of $CD8^+Foxp3^+$ (both $CD25^+$ and $CD25^-$) cells was observed in testis of experimental compared to normal and control rats killed on days 50 and 80. However the degree of increase was much lower than the $CD4^+$ cell subset (Table 2).

Since the balance between autoaggressive $T_{effectors}$ and T_{regs} might determine the course of autoimmune response we analysed the ratio $T_{effectors}/T_{regs}$ within $CD4^+$ and $CD8^+$ subsets. During the development of EAO, the ratios shifted in favor of $CD4^+Foxp3^+$ T_{regs} and $CD8^+Foxp3^-$ $T_{effectors}$ within $CD4^+$ and $CD8^+$ populations, respectively (Table 3). Although T_{regs} increased in number these cells were outnumbered by an even higher increase of $T_{effectors}$.

4. Discussion

These experiments show that in association with damage of the ST, a large increase in the number of T lymphocytes was observed in the testis throughout EAO development. At EAO onset the number of $CD4^+$ $T_{effector}$ cells dramatically increased, then decreased as the disease progressed. In contrast, the number of $CD8^+$ $T_{effector}$ cells also increased at EAO onset but remained stable through the chronic phase of the disease. The increase of both subsets associated with the initiation of testicular damage suggests their involvement in the induction of EAO. The highest number of $CD4^+$ $T_{effector}$ cells at the onset of EAO in rats tallies with the major role attributed to this subset in the development of murine EAO (Mahi-Brown et al., 1989). Previous data of our laboratory (Lustig et al., 1993) and present results showing a sustained increase of $CD8^+$ $T_{effector}$ cells throughout EAO development suggest that this subset plays a major role in the maintenance of the chronic phase of the disease. Different experimental models have also shown the pathogenic role of $CD8^+$ T lymphocytes in the induction of autoimmunity (Kelly et al., 1986; Huseby et al., 2001). Frucht et al. (2001) reported that IFN-γ, a powerful activator of $CD8^+$ T lymphocytes and macrophages, could help to perpetuate

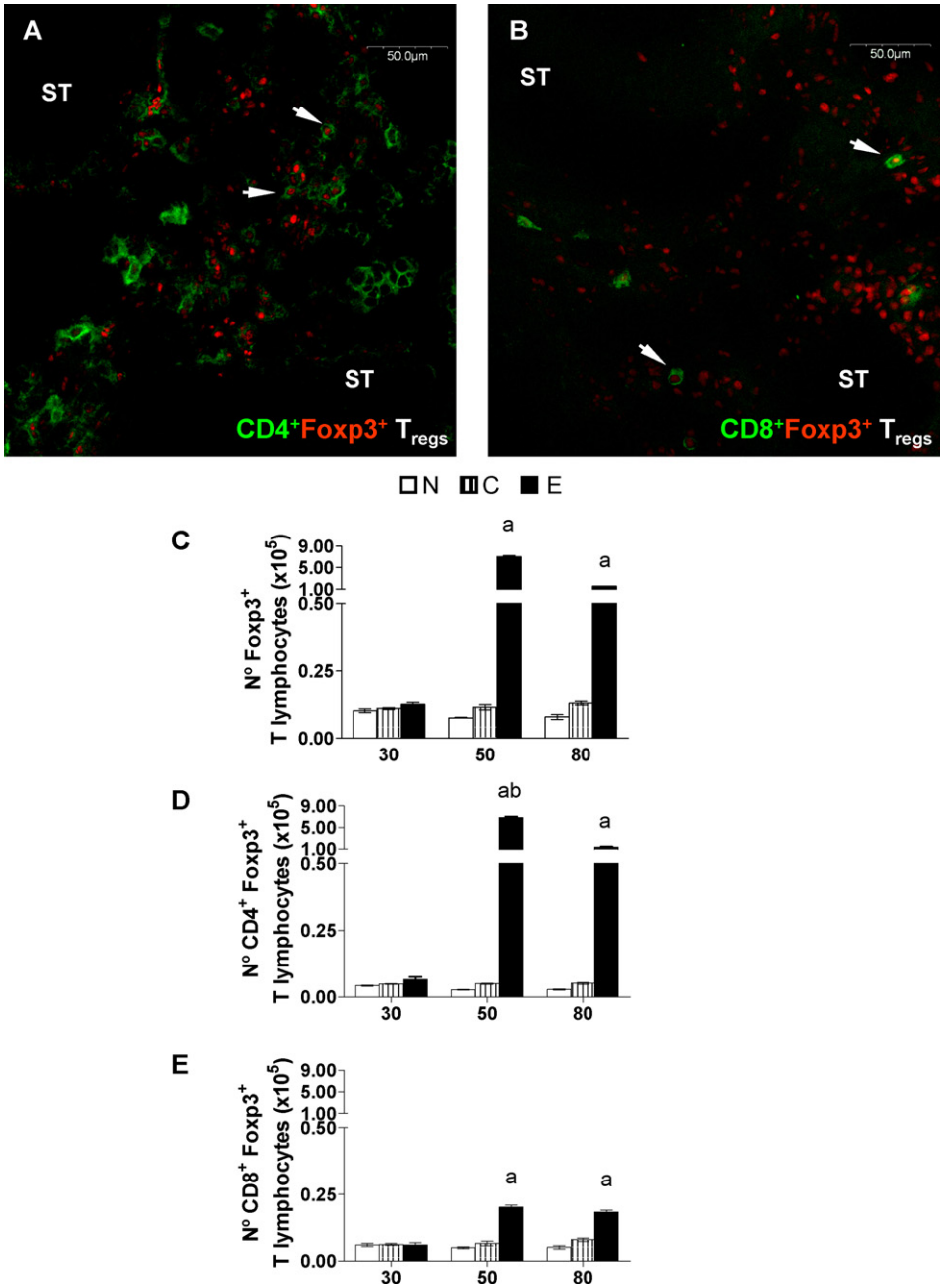

Fig. 5. Increased number of $CD4^+$ and $CD8^+$ regulatory T lymphocytes in testis of rats with EAO. Immunostaining for CD4 or CD8 (green) and Foxp3 (red) in frozen testicular sections from rats with focal EAO (50 days). Groups of $CD4^+Foxp3^+$ regulatory T lymphocytes (T_{reg} cells) (arrow) (A) and scarce number of $CD8^+Foxp3^+$ T_{reg} cells (arrow) (B) are distributed in the testicular interstitium mainly in the subalbuginea and peritubular areas. Absolute number of $Foxp3^+$ (C), $CD4^+Foxp3^+$ (D) and $CD8^+Foxp3^+$ (E) T_{reg} cells in the testis of normal (N), control (C) and experimental (E) rats killed on days 30, 50 and 80. $^a p < 0.01$ versus respective N and C, $^b p < 0.01$ versus $CD4^+$ E_{80}. ST: seminiferous tubule.

chronic disease, thus establishing a link between innate and adaptive immunity. Our preliminary results showed that both $CD4^+$ and $CD8^+$ T lymphocytes produce IFN-γ, mainly in the chronic phase of EAO, the $CD8^+$ subset being the most involved. Also, we recently reported an increase of macrophage-secreted IFN-γ in rats with orchitis (Rival et al., 2008). Further functional assays might elucidate the precise role of $CD8^+$ T lymphocytes during onset and the chronic phase of EAO in rats.

It is widely accepted that T_{reg} cells play a pivotal role in peripheral tolerance and prevention of autoimmunity. As a novel finding, we detected $Foxp3^+$ T_{reg}

Table 2
Number of testicular $CD4^+$ and $CD8^+$ ($CD25^+$ and $CD25^-$) regulatory T lymphocytes ($\times 10^4$).

Group of animals	Days	$CD4^+$ T_{regs}		$CD8^+$ T_{regs}	
		$CD25^+$	$CD25^-$	$CD25^+$	$CD25^-$
Normal		0.23 ± 0.03	0.30 ± 0.04	0.36 ± 0.11	0.25 ± 0.06
Control	30	0.24 ± 0.02	0.34 ± 0.01	0.33 ± 0.01	0.29 ± 0.05
Experimental		0.27 ± 0.07	0.38 ± 0.03	0.32 ± 0.08	0.27 ± 0.04
Normal		0.26 ± 0.01	0.21 ± 0.01	0.28 ± 0.01	0.21 ± 0.04
Control	50	0.29 ± 0.01	0.31 ± 0.06	0.21 ± 0.13	0.25 ± 0.04
Experimental		21.82 ± 1.74[a,b]	46.57 ± 0.90[a,b]	0.92 ± 0.03[a,b]	1.10 ± 0.11[a,b]
Normal		0.21 ± 0.02	0.28 ± 0.04	0.23 ± 0.01	0.27 ± 0.03
Control	80	0.24 ± 0.04	0.38 ± 0.05	0.32 ± 0.01	0.36 ± 0.02
Experimental		4.51 ± 0.61[a,b]	9.07 ± 0.32[a,b]	0.65 ± 0.02[a,b]	1.00 ± 0.11[a,b]

$n = 5$–7 rats/group/time point.
[a] $p < 0.0001$ versus respective control.
[b] $p < 0.0001$ versus respective normal.

cells in normal and inflamed rat testis. Interestingly, the number of $CD4^+Foxp3^+$ T_{reg} cells and to a lesser extent of $CD8^+Foxp3^+$ T_{reg} cells increased in testis of rats with EAO. Overall results show that cells expressing Foxp3 were predominantly $CD4^+$ cells. In contrast to $CD4^+Foxp3^-$ $T_{effector}$ cells which were widespread in the testicular interstitium, groups of $CD4^+Foxp3^+$ T_{reg} cells were mainly localized in the subalbuginea and peritubular area. Scarce $CD8^+Foxp3^+$ T_{reg} cells were observed in the same localization.

Increased numbers of $CD4^+CD25^+Foxp3^+$ T_{reg} cells at sites of inflammation have been reported in other organ-specific autoimmune disease models (Cao et al., 2003; Möttönen et al., 2005; Dejaco et al., 2005). Although natural $CD4^+CD25^+Foxp3^+$ T_{reg} cells were mostly studied, a regulatory role for *in vivo* peripherically induced T_{reg} cells with a $CD4^+CD25^-Foxp3^+$ phenotype was also described (Graca et al., 2002; Yoon et al., 2008). Degauque et al. (2007) showed that these induced T_{reg} cells contribute to maintain immune homeostasis and tolerance, and are able to attenuate autoimmune responses. These findings, and others reported by Zheng (2008) showing that natural but not inducible T_{reg} cells can be converted *in vivo* into pathogenic Th17 cells prompted us to examined CD25 expression within T_{reg} subset. Interestingly, $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ T_{reg} cells increased in number in the testis of EAO rats, the $CD4^+CD25^-Foxp3^+$ T_{reg} cells being the major subset. Similarly, Fowell and Mason (1993) reported in rats that the $CD4^+$ T lymphocyte subset contains $CD25^-$ T_{reg} cells. These cells are capable of preventing autoimmune diabetes development (Stephens and Mason, 2000). Ono et al. (2006) showed that both $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ T_{reg} cells contribute to prevent a variety of autoimmune diseases in mice. Discrepant results have been reported concerning the relative abundance of $CD4^+CD25^+$ and $CD4^+CD25^-$ T_{reg} cells. Our findings are similar to those of Graca et al. (2002) who showed in mice made tolerant to skin grafts that the number of $CD4^+CD25^-$ T_{reg} cells was higher than that of $CD4^+CD25^+$ T_{reg} cells suggesting that both populations have a significant role in maintaining transplantation tolerance.

Functional differences have also been reported between $CD4^+CD25^+$ and $CD4^+CD25^-$ T_{reg} cells; $CD4^+CD25^-Foxp3^+$ T_{reg} cells might exhibit less efficacy in suppressing autoimmunity (Ono et al., 2006; Stephens and Mason, 2000; Lehmann et al., 2002; Annacker et al., 2001). Also, Lehmann et al. (2002) showed that $\alpha_E\beta_7$-expressing

Table 3
Ratios of $Foxp3^-$ effector and $Foxp3^+$ regulatory T lymphocytes within $CD4^+$ and $CD8^+$ subsets.

Group of animals	Days	$CD4^+$ $T_{effectors}/T_{regs}$	$CD8^+$ $T_{effectors}/T_{regs}$
Normal		32	48
Control	30	24	39
Experimental		12	54
Normal		29	31
Control	50	25	43
Experimental		3	66
Normal		34	40
Control	80	28	32
Experimental		3	76

Ratios were calculated based on cell numbers presented in Fig. 4A and B and Fig. 5D and E.

$CD4^+CD25^+$ and $CD4^+CD25^-$ T_{reg} cells represent functionally distinct T_{reg} cells with different abilities to produce cytokines. Whereas $\alpha_E\beta_7{}^+CD25^+$ T_{reg} cells produce almost no cytokines except for IL-10, $\alpha_E\beta7^+CD25^-$ represent a unique subset in which high IL-2, IFN-γ and Th2-cytokine production is linked to suppressive function. Of interest, both T_{reg} subsets protect mice from colitis in the severe combined immunodeficient model *in vivo*. Although T_{regs} are enriched in the $CD4^+CD25^+$ T subset both in thymus and periphery, there is evidence of presence of $CD4^+CD25^-$ T_{regs} in the periphery of rats. The relationship between $CD25^+$ and $CD25^-$ T_{reg} cells in the periphery has not yet been established, and could result either from the instability of this marker on a single T_{reg} cell subset or from different lineages of T_{reg} cells that might have different mechanisms of action (Stephens et al., 2004).

There is evidence that different subsets of $CD8^+$ T lymphocytes possess regulatory functions in humans and mice (Shevach, 2006; Singh et al., 2007). In our model we detected an increased number of $CD8^+Foxp3^+$ T_{reg} cells, both $CD25^+$ and $CD25^-$, mainly during the chronic phase of EAO. Most of the $CD8^+$ T_{reg} cells present within testis were $CD25^-$. Chaput et al. (2008) reported that the number of $CD8^+CD25^+Foxp3^+$ T_{reg} cells is increased in tumor tissue of patients with colorectal cancer. Also, Liu et al. (2004) detected an increase of $CD8^+Foxp3^+$ T_{regs} mediating tolerance to allogeneic heart transplants in rats. Poitrasson-Rivière et al. (2008) characterized natural $CD8^+Foxp3^+$ T_{regs} which may account for the regulation of $CD8^+$ T lymphocyte autoreactivity against tissue-specific auto-Ags. This finding could be relevant in our model in which a pathogenic role could be attributed to testicular $CD8^+$ $T_{effector}$ cells.

The increased number of T_{reg} cells seen in testis of rats throughout EAO development may reflect their continued recruitment from the periphery, local generation or expansion in testis. Different authors reported increased recruitment of $CD4^+CD25^+Foxp3^+$ T_{reg} cells at sites of inflammation in autoimmune diseases (Cao et al., 2003; Möttönen et al., 2005; Dejaco et al., 2005). In this line, Lehmann et al. (2002) reported expression of receptors for inflammatory chemokines and increased levels of adhesion molecules on T_{reg} cells, suggesting that these cells migrate to act directly at sites of inflammation. Dal Secco et al. (2008) demonstrated that *in vitro* interaction of $CD4^+CD25^-Foxp3^-$ T lymphocytes with IFN-γ-stimulated Sertoli cells resulted in their conversion into $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Previous studies from our laboratory suggested that testicular APCs might present antigens activating T lymphocytes in testis (Rival et al., 2007, 2008) contributing to local induction of T_{reg} cells. It is known that T_{reg} cell populations can be expanded *in vivo* by antigen, particularly in the presence of inflammatory signals as up-regulated costimulatory molecules on activated APCs. In fact, it has been demonstrated that acquisition of function by polyclonal T_{reg} cells to suppress an autoimmune disease is a process that critically depends on the presence of endogenous autoantigens (Setiady et al., 2006).

Our findings provide evidence that different T_{reg} cell subsets accumulate within the testis of EAO rats, suggesting a role in down-regulation of the local immune response. Nevertheless, these cells fail to effectively suppress inflammation. Multiple mechanisms by which immune regulation may be lost have been suggested, e.g., inadequate numbers of T_{reg} cells, a T_{reg} cell intrinsic functional defect or the presence of pathogenic $T_{effector}$ cells resistant to T_{reg} cell control (Schneider et al., 2008). The first possibility could offer a partial explanation for the development of orchitis. Although T_{reg} cells increase in number, these cells are outnumbered by an even more vigorously expanding $T_{effector}$ cell subset. Also, based on reports by Pasare and Medzhitov (2003), Schneider et al. (2008) and Ruprecht et al. (2005), we speculate that high levels of TNF-α, IL-6 and IL-12 present in testis of EAO rats (Rival et al., 2007; Theas et al., 2008) may substantially impair the suppressive function of T_{reg} cells. However, it remains to be established whether other local mechanisms present in the inflamed testis may reduce the efficacy of T_{reg} cells to control an excessive inflammatory response. In the future, functional studies of T_{reg} cells will help to unravel the role of these cells in the pathogenesis of EAO and the maintenance of testicular immunoprivilege.

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