CD4+ and CD8+ T cells producing Th1 and Th17 cytokines are involved in the pathogenesis of autoimmune orchitis

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Abstract

Experimental autoimmune orchitis (EAO) is a useful model to study chronic testicular inflammation and infertility. EAO is characterized by severe damage of seminiferous tubules with germ cells that undergo apoptosis and sloughing. We previously reported an increase in CD4+ and CD8+ effector T cells in the testes of rats with EAO. Since cytokine patterns determine T cell effector functions, in the present work we analyzed the cytokines expressed by these cells during disease development. By flow cytometry, we detected an increase in the number of tumor necrosis factor-α (TNF) and interferon -γ (IFNG)-producing CD4+ T cells in the testis at EAO onset. As the severity of the disease progressed, these cells declined while CD8+ T cells producing TNF and IFNG increased, with the predominance of IFNG expression. As a novel finding, we identified by immunofluorescence CD4+ interleukin 17 (IL17)+ and CD8+ IL17+ cells in the testes of EAO rats, with CD4+ and CD8+ T cells predominating at the onset and in the chronic phase of EAO respectively. Moreover, IL17 (western blot) and IL23 content (ELISA) increased in EAO, with maximum levels in the chronic phase. These results suggest the involvement of CD4+ T helper (Th) 1 and Th17 subsets as co-effector cells governing EAO onset, as well as the central contribution of CD8+ T cells producing Th1 and Th17 cytokines in the maintenance of chronic inflammation. The expression of T-bet and RORγt (western blot) in the testis over the course of disease also supports the presence of Th1 and Th17 cells in the testes of EAO rats.
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Introduction

Male factor infertility affects almost half of infertile couples. Infection and inflammation of noninfectious origin of the male genital tract including the testis are accepted as important etiological factors of infertility in men. However, since chronic inflammatory reactions in the testis are frequently asymptomatic, diagnosis of orchitis and consideration of this pathology as a possible cause of infertility is underevaluated (Schuppe *et al.* 2008).

Basic research on autoimmune diseases of the gonads has contributed significantly to the general knowledge of autoimmune pathogenesis (Tung *et al.* 2002). In this regard, autoreactive CD4+ T helper (Th) 1 cells have long been associated with the development of many organ-specific autoimmune diseases (Liblau *et al.* 1995). However, a large body of evidence now points to the additional involvement, along with Th1, of specific Th17 cells as important co-effectors of autoimmune tissue damage (Steinman 2007). Th1 and Th17 cells are characterized by transcriptional factors required for their differentiation and the production of specific cytokines. Th1 cell differentiation is initiated by activation of cells in the presence of interferon-γ (IFNG) that leads to activation of the Th1-specific transcription factor, T-bet, which, in turn, induces IFNG production, allowing responsiveness to interleukin 12 (IL12; Dardalhon *et al.* 2008). Differentiation of Th17 cells is induced by a combination of IL6 and transforming growth factor-β (TGFB; Betelli *et al.* 2006) and governed by the Th17-specific transcription factor, the orphan nuclear receptor RORγt (Ivanov *et al.* 2006, Manel *et al.* 2008). After activation, Th1 cells produce IFNG and tumor necrosis factor-α (TNF) whereas Th17 cells secrete large quantities of IL17, IL21, and IL22 and express IL23 receptor. IL23 acts on previously differentiated Th17 cells to induce expansion and/or stabilization of the Th17 phenotype (Veldhoen *et al.* 2006). Th1- and Th17-related cytokines promote inflammation by directly causing tissue injury and enhancing secretion of pro-inflammatory cytokines and chemokines by organ resident cells (Dardalhon *et al.* 2008).

Autoimmune orchitis is a T-cell-dependent testicular inflammation-based pathology triggered by antigens or pathogens that disrupt testicular immunoprivilege (Lustig & Tung 2006). We developed an experimental model of

autoimmune orchitis in rats by active immunization with testicular antigens and adjuvants (Doncel *et al*. 1989). Testicular damage in this experimental model is characterized by an increased number of T cells, macrophages, and dendritic cells that infiltrate the interstitium and by seminiferous tubules (ST) showing apoptotic germ cells and different degrees of cell sloughing that results in aspermatogenesis and atrophy (Theas *et al*. 2003, Rival *et al*. 2006, 2008, Jacobo *et al*. 2009).

Early studies in mice have demonstrated by transfer experiments that CD4+ but not CD8+ T cells are required for experimental autoimmune orchitis (EAO) development (Mahi-Brown & Tung 1989, Yule *et al*. 1990, Tung & Teuscher 1995). Moreover, Yule & Tung (1993) showed that TNF-producing CD4+ Th1 cells are the key players in disease induction. However, our previous results in rats showed that the number of CD4+ and CD8+ effector T (Teff) cells significantly increases in the testis in the course of EAO, suggesting involvement of both subsets in pathogenesis of this disease (Lustig *et al*. 1993, Jacobo *et al*. 2009). Consequently, the aim of this work is to elucidate how testis-infiltrating CD4+ and CD8+ Teff cell subsets contribute to autoimmune inflammatory response. Based on the hypothesis that the pattern of cytokines determines their pathogenicity, we analyzed the pro-inflammatory mediators produced by both T cell subsets through *ex vivo* analysis of cells recovered from inflamed testis. We also explored, for the first time, the presence and possible role of CD4+ Th17 cells in the testis of rats undergoing EAO.

Results showed the presence of CD4+ Th1 and Th17 subsets in the induction of EAO and the presence of CD8+ T cells producing Th1 and Th17 cytokines in the chronic phase of the disease.

Results

Histopathology

As we previously described (Doncel *et al*. 1989), 50 days after the first immunization the testes of experimental (E) rats presented a focal orchitis characterized by mild lymphomononuclear cell infiltrate and several foci of ST showing germ cell sloughing and degenerated spermatocytes and spermatids intermingled with normal ST (EAO onset; Fig. 1A). From day 80 on we observed severe and extensive damage of most of the ST in which only spermatogonia and Sertoli cells were attached to the tubular wall. Although the areas of dense lympho-mononuclear infiltrates may be present in the inter-stitium, these cells were not observed inside the ST (EAO chronic phase; Fig. 1B). Granulomae were frequently detected. No testicular damage was observed in the testes of control (C) (Fig. 1C) or normal (N) rats at any time studied.
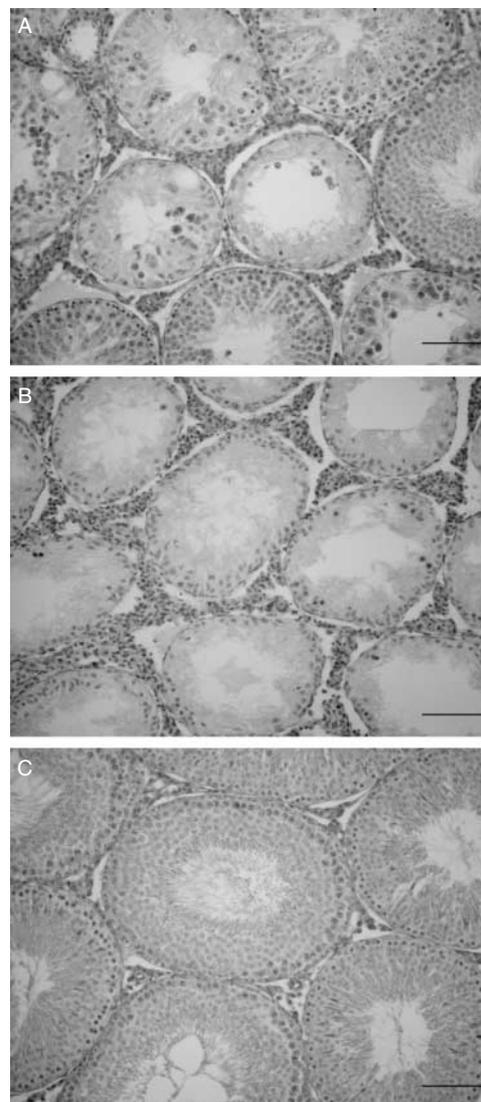

Figure 1 Testicular histopathology. (A) Foci of seminiferous tubules (ST) with germ cell sloughing intermingled with normal ST and interstitial lymphomononuclear cell infiltrates in the testis of an experimental (E) rat killed on day 50 (focal EAO, EAO onset). (B) Severe and extensive damage of ST in the E rat killed on day 80 (severe EAO, chronic EAO). Note that only spermatogonia and Sertoli cells are attached to the tubular wall. (C) Normal histopathology is observed in the testis of a control rat killed on day 50. Scale bars indicate 100 μm.

Th1 and Th17 cytokines were increased in the testes of rats with EAO

Cytokine production by T cells dictates their effector function. In order to determine the pathogenic role of CD4+ and CD8+ Teff cells that infiltrate the testis during EAO development, we investigated the pattern of cytokine expression of these subsets.

By flow cytometry we first analyzed the intracellular expression of cytokines TNF, IFNG, IL4, and IL10 by CD3+ FOXP3− CD4+ (CD4+ Teff) and CD3+ FOXP3− CD8+ (CD8+ Teff) T cell subsets, isolated

from the testes of rats killed on days 50 and 80 after *in vitro* activation with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A. We compared the cytokine profiles of CD4+ and CD8+ Teff cells from the testes of E rats to those of C and N rats. Intracellular detection of cytokines was possible in testicular-stimulated Teff cells, whereas no cytokines were detectable in unstimulated cells. As a control we verified that the percentage of FOXP3− T cells did not differ after mitogen treatment.

Results showed that CD4+ and CD8+ Teff cell subsets from the testes of N, C, and E rats have the potential to produce pro-inflammatory cytokines such as TNF and IFNG. A significant increase in the number of TNF-producing CD4+ and CD8+ Teff cells was detected in the testis of E compared to N and C rats on days 50 and 80. Although both subsets were found to express this cytokine, we detected a higher number of CD4+ cells producing TNF than CD8+ cells in the EAO onset and vice versa for the EAO chronic phase. IFNG expression by CD4+ Teff cells during EAO onset followed a course similar to TNF. However, the number of cytokine-producing CD8+ Teff cells did not differ compared to N and C rats. In contrast, an increase in the number of IFNG-producing cells was detected within CD4+ and CD8+ Teff cell subsets in the EAO chronic phase, CD8+ cells predominating (Fig. 2). No IL4 production was detected in the testes of E rats.

IL4+ cells were occasionally detected in the testes of N and C rats, which appeared to be produced by a very scarce number of CD4+ Teff cells (data not shown). Moreover, we were unable to detect IL10 in any of the groups of rats studied.

We next focused on the analysis of Th17 cytokine expression. Since commercial anti-rat IL17 antibody suitable for flow cytometric analysis is unavailable, IL17 expression was investigated by different technical approaches. By immunofluorescence we detected IL17+ cells within a pool of CD4+ and CD8+ T cell subsets isolated by fluorescence-activated cell sorting (FACS) from the testicular interstitium of three E rats killed on days 50 and 80 (Fig. 3A). At EAO onset, the percentages of CD4+ IL17+ T cells were higher than CD8+ IL17+ T cells whereas the opposite occurred during the chronic phase of the disease (50 days – CD4+ IL17+: 39.75±2.96%, CD8+ IL17+: 10.28±0.70%; 80 days – CD4+ IL17+: 19.45±2.16%, CD8+ IL17+: 44.6±3.19%). In N and C rats, none of the T cell subsets showed IL17 expression at any time studied.

Accordingly, IL17+ as well as RORγt+ cells with lymphocyte morphology were localized by immunohistochemistry in the testicular interstitium of E rats, mainly within and around blood vessels and in the subalbuginea area. No positive cells were detected in N or C rat testes. No staining was observed when primary antibody was omitted (Fig. 3B and C).
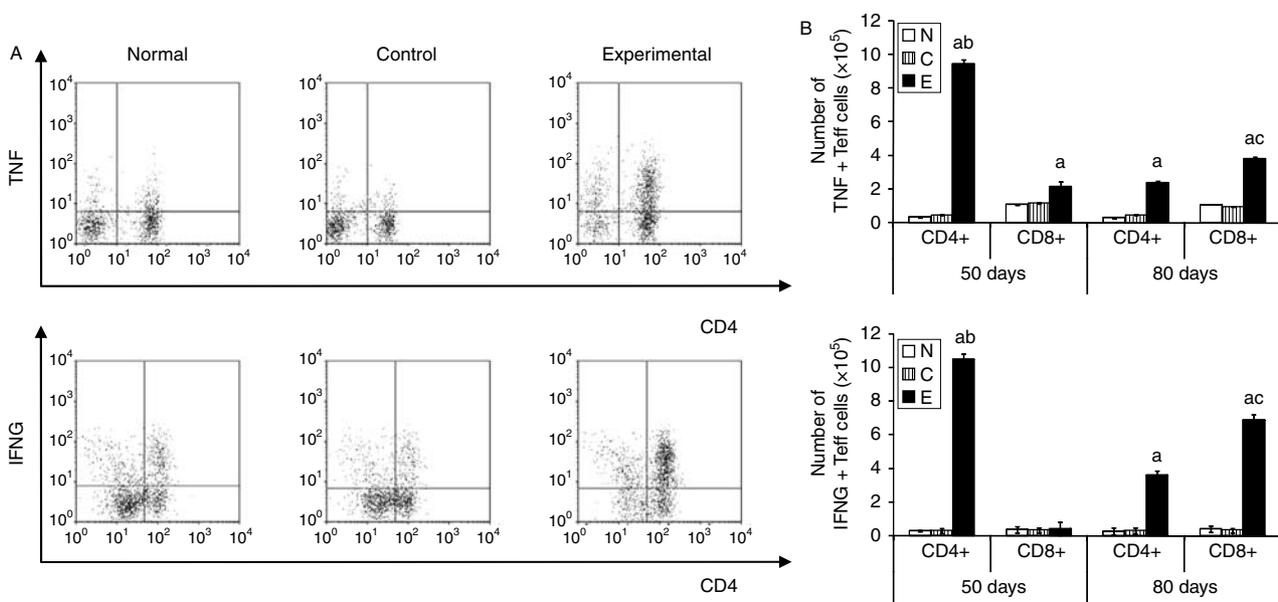

Figure 2 Th1 cytokines in the testes of rats with EAO. (A) Flow cytometric representative dot plots of intracellular cytokine production in CD4+ (upper right quadrant) and CD8+ (upper left quadrant) effector T (Teff) cells from the testis of normal (N), control (C), and experimental (E) rats killed on day 50 after the first immunization. Isolated testicular interstitial cells stained with anti-CD3 (APC) and anti-CD4 (FITC) were cultured for 6 h with or without phorbol myristate acetate and ionomycin in the presence of brefeldin A. After fixation and permeabilization, cells were stained with anti-FOXP3 (Pe-Cy5) in combination with (PE)-labeled antibodies against TNF or IFNG. An analysis gate was set on lymphocytes by scatter properties and CD3 expression. Data are representative of staining of 5–7 rats/group per time. (B) Absolute number of CD4+ and CD8+ effector T (Teff) cells producing TNF or IFNG in the testes of N, C, and E rats killed on days 50 and 80 after the first immunization. [a]$P<0.01$ versus respective N and C, [b]$P<0.01$ CD4+ E 50 days versus CD4+ E 80 days, and [c]$P<0.01$ CD8+ E 80 days versus CD8+ E 50 days.

A CD4+ IL17+ T cells   CD8+ IL17+ T cells

B IL17+ cells   Negative control

C RORγt + cells   DAPI

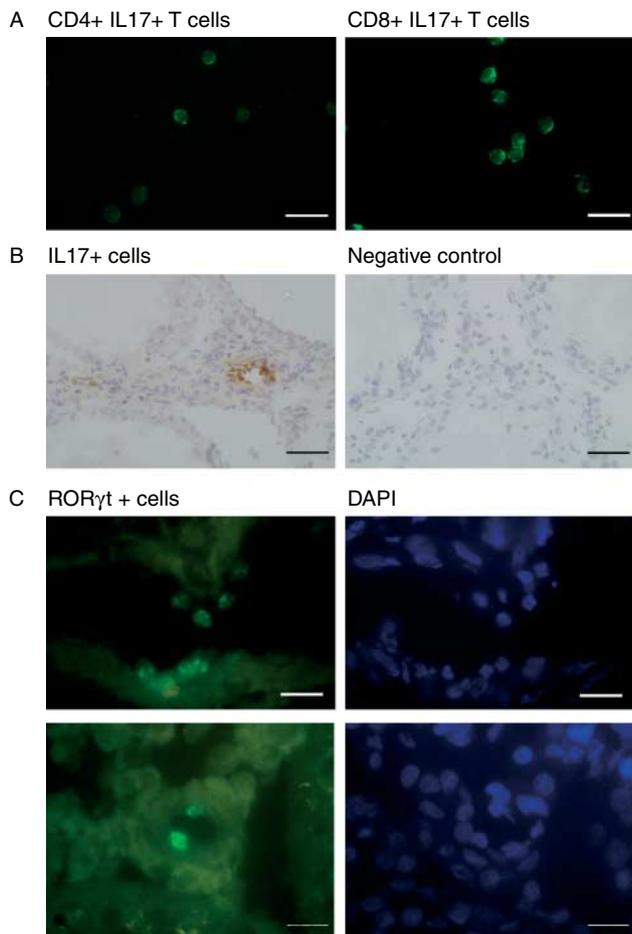**Figure 3** Immunostaining of IL17 and RORγt in the testes of EAO rats. (A) IL17 expression in testicular-pooled CD4+ and CD8+ T cells isolated by fluorescence-activated cell sorting from the testes of three rats with severe EAO. Secondary antibody conjugated with FITC was used to detect anti-IL17. Scale bar indicates 20 μm. (B and C) Immunohistochemical detection of IL17 and RORγt in the cryostat testis sections from a rat with focal EAO. Groups of IL17+ and RORγt+ cells were located in the interstitium, mainly within blood vessels or perivascular in subalbuginea areas. In negative controls, the first antibody was omitted (B). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; C). Scale bar indicates 50 μm (B) and 20 μm (C).

Western blot analysis performed on interstitial cells isolated from the testis showed that E rats expressed IL17. Interestingly, IL17 content significantly increased in the testis during disease progression to reach a maximum in the EAO chronic phase. A slight expression of IL17 was occasionally detected in N and C rat testes ($n=2/6$; Fig. 4A and B).

By ELISA we measured the concentration of secreted IL23 in the testicular fluids of N, C, and E rats killed on days 50 and 80. A significant increase in IL23 content was observed in the testicular fluid of E rats compared to C rats. Tallying with the higher expression of IL17 in E rats, IL23 concentration significantly increased in E rats killed on day 80 compared to day 50 (Fig. 4C). IL23

expression was not detected in the testicular fluids of N rats at any time studied.

Our results show that Teff cells expressing Th1 and Th17 cytokines infiltrate the testis during the development of EAO, CD4+ and CD8+ being the predominant subsets expressing these cytokines at the onset and chronic phase of the disease respectively. Th17 response may be sustained and strengthened by the increased levels of IL23 locally secreted in the EAO testis.

Transcriptional regulators T-bet and RORγt were expressed in the testes of rats with EAO

To confirm the presence of CD4+ Th1 and Th17 cell lineages at the onset and during the chronic phase of EAO we performed western blot analysis of T-bet and RORγt on lysates from the interstitial cells isolated from the testes of N, C, and E rats killed on days 50 and 80.

We showed that E rats expressed T-bet and RORγt throughout the two phases of EAO development, while no expression was detected in N and C rats at any time studied. Although RORγt content in the testes of E rats did not differ during the course of disease, we detected variable levels of T-bet expression as the disease progressed: maximum at EAO onset and decreasing during the chronic phase. Interestingly, our results showed that expression of both T-bet and RORγt dominates the onset of EAO while RORγt expression is predominant during the chronic phase of the disease (Fig. 5A and B).

Discussion

We previously described the chronological changes in the number of CD4+ and CD8+ Teff cells infiltrating the testis during EAO progression (Jacobo *et al.* 2009). However, the specific functions of these subsets in the pathogenic process of tissue inflammation remained unexplored. Given that cytokine production by T cells dictates their effector function, in this work we determined the expression of cytokines TNF, IFNG, IL4, IL10, and IL17 by testicular CD4+ and CD8+ Teff cell subsets during EAO development. At disease onset, the number of TNF and IFNG-producing CD4+ Teff cells dramatically increased whereas the number of TNF-producing CD8+ cells also increased but to a lesser extent compared to CD4+ cells. During the chronic phase of EAO, the increase detected in the number of both CD4+ and CD8+ Teff cells producing TNF and IFNG corresponded mainly to the CD8+ subset. The fact that none of the Teff cell subsets detected in EAO testis showed evidence of significant production of IL4 may be due to the inhibitory effect of Th1 cytokines on proliferation and cytokine production by Th2 cells (Gajewski & Fitch 1988). Although Teff cells did not express IL10, our previous results showing *IL10* mRNA expression in testicular and lymph node dendritic cells
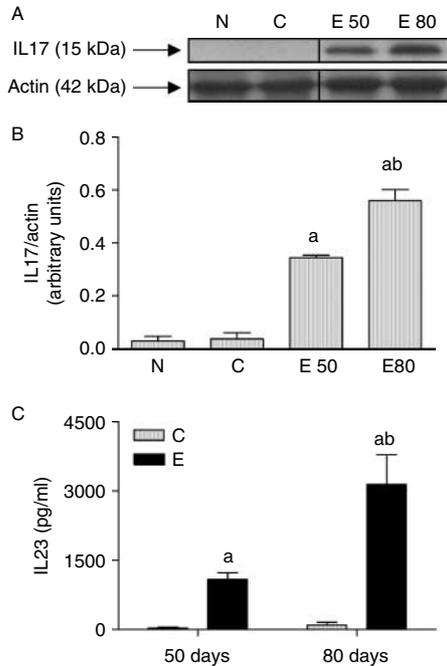
Figure 4 Th17 cytokines in the testes of rats with EAO. (A) Representative western blot of IL17 performed on lysates of interstitial cells isolated from the testes of normal (N), control (C), and experimental (E) rats killed on days 50 (E 50) and 80 (E 80) after the first immunization. (B) Semiquantitative results of IL17 content obtained by densitometric analysis of western blots, $n=5$ rats per group at each time period. [a]$P<0.01$ versus respective N and C and [b]$P<0.01$ versus E 50 days. (C) IL23 was measured by ELISA in the testicular fluids from N, C, and E rats killed 50 and 80 days after immunization. No IL23 was detected in the testicular fluids of N rats. Values are mean±S.E.M. of eight rats per group. [a]$P<0.01$ versus respective C and [b]$P<0.01$ versus E 50 days.

of EAO but not N and C rats demonstrate that this cytokine is overexpressed under inflammatory conditions (Rival *et al.* 2007, Guazzone *et al.* 2011).

As a novel finding, we identified IL17+ cells in the testes of rats undergoing EAO. These cells were detected within CD4+ and CD8+ T cell populations isolated from the testicular interstitium. Interestingly, CD4+ T cells represented the major subset producing IL17 in the testis during EAO onset whereas CD8+ IL17+ T cells predominated in the chronic phase of the disease. These results suggest the involvement of CD4+ and CD8+ Teff cells producing Th1 and Th17 cytokines as the major effector subsets that govern the onset and chronic phase of EAO respectively.

In order to evaluate the relative importance of CD4+ Th1 and Th17 subsets over the course of EAO we analyzed the expression of T-bet and RORγt, which respectively regulate Th1 and Th17 phenotypes. Although RORγt content was found to be stable throughout EAO, a higher level of T-bet expression was detected at the onset compared to the chronic phase of the disease confirming results obtained by cell cytokine analysis. Although it is not clear whether and in what way Th1 and Th17 cells interact to promote autoimmune

tissue injury, it is possible that these cells may orchestrate recruitment of other effector cells, particularly activated macrophages and CD8+ Teff cells, highly increased in the testes of EAO rats (Rival *et al.* 2008, Jacobo *et al.* 2009). Concordantly, Park *et al.* (2005) demonstrated that IL17 induces monocyte chemoattractant protein-1 (CCL2) expression, which plays a prominent role in recruiting immune cells to the testes in rats undergoing EAO (Guazzone *et al.* 2003). Moreover, the fact that RORγt+ and IL17+ cells were detected in the testicular interstitium of the subalbuginea area, where the first signs of histopathological damage appear, suggest their pathogenic role in EAO induction.

The highest number of testicular CD4+ Teff cells producing type 1-cytokines at the onset of EAO tallies with the major role attributed to these cells in the induction of several organ-specific autoimmune diseases, including murine EAO (Mahi-Brown & Tung 1989, Yule & Tung 1993, Itoh *et al.* 1998). In this line, our previous results showing a significant increase in *IL12p35* mRNA expression by the dendritic cells from rats with focal EAO (Guazzone *et al.* 2011) provide evidence in support of a Th1 response.

Our results in EAO showed that CD8+ Teff cells predominate in the chronically inflamed testis, these cells being the main producers of pro-inflammatory cytokines at that phase of the disease. These results are also consistent with our previous demonstration of a highly increased number of testicular CD8+ Teff cells expressing the activation marker CD25 compared to the CD4+ subset (Jacobo *et al.* 2009).

Although CD8+ Teff cells have been largely ignored in autoimmunity research, Huseby *et al.* (2001) attributed a pathogenic role to CD8+ Teff cells producing IFNG in the development of an experimental model of multiple sclerosis. In addition, Ford & Evavold (2005) demonstrated that CD8+ T cells might play a role in the chronic phase of experimental autoimmune encephalomyelitis.

Accumulating evidence has demonstrated the importance of CD4+ Th17 cells in the pathogenesis of
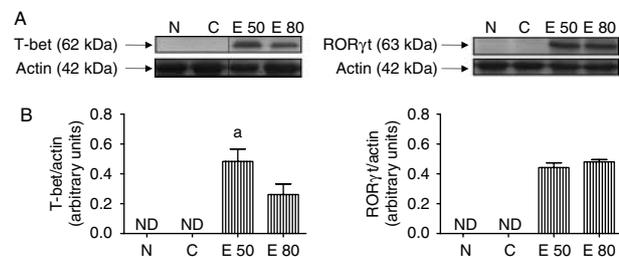

Figure 5 Expression of T-bet and RORγt in the testes of EAO rats. (A) Representative western blot of T-bet and RORγt performed on lysates of interstitial cells isolated from the testes of normal (N), control (C), and experimental (E) rats killed on days 50 (E 50) and 80 (E 80) after the first immunization. (B) Semiquantitative results of T-bet and RORγt content obtained by densitometric analysis of western blots, $n=5$ rats per group at each time period. [a]$P<0.01$ versus E 80 days. ND, not detected.

organ-specific autoimmune inflammation (Komiyama *et al.* 2006, Garret-Sinha *et al.* 2008, Jandus *et al.* 2008). However, few data are available concerning the presence and possible role of CD8+ IL17+ Teff cells in the induction of autoimmune diseases. Recently, CD8+ T cells were found to express IL17 contributing to the pathogenesis of human chronic inflammatory diseases of the nervous system (Friese & Fugger 2009, Madia *et al.* 2009) and the skin (Ortega *et al.* 2009). Moreover, it has been shown that CD8+ T cells are a significant source of IL17 through an IL23-dependent mechanism (Happel *et al.* 2003). Thus, the increased number of CD8+ IL17+ Teff cells detected in rats with EAO could be explained by the high level of IL23 produced in the inflamed testis, mainly at the chronic phase of the disease. IL17 production by CD8+ Teff cells reinforces the involvement of this subset in the pathogenesis of the disease.

Present results showed a low number of Teff cells expressing TNF and IFNG in the testes of N and C rats. Pro-inflammatory cytokines are known to play a relevant role in the development and normal function of the testis (Calkins *et al.* 1990, De *et al.* 1993, Xiong & Hales 1993, Mauduit *et al.* 1998, Hales *et al.* 1999, Pentikainen *et al.* 2001). In testis inflammation, TNF induces apoptosis of germ cells (Suescun *et al.* 2003, Theas *et al.* 2008) and migration of inflammatory cells to the organ contributing to immune response amplification (Riccioli *et al.* 1995). Importantly, Li *et al.* (2006) demonstrated that acute *in vivo* administration of recombinant TNF to adult rat testes disrupted the blood–testis barrier (BTB). Thus, locally increased TNF production in the testes of rats undergoing EAO might enhance the transport of spermatic antigens out of the BTB to reach interstitial antigen presenting cells amplifying the autoimmune response. In turn, the enhanced passage of TNF and other factors through the disrupted BTB may account for the increased number of apoptotic germ cells observed during EAO progression.

On the other hand, IFNG may participate as inducer of apoptosis in EAO testis. This cytokine could activate macrophages and CD8+ Teff cells, which in turn produce more IFNG (Rival *et al.* 2008) that favors T cell infiltration within the testis by increasing vascular permeability. In addition, IFNG may contribute to granuloma formation through its effect on macrophages (Wahlström *et al.* 2001).

Although specific actions of IL17 on testicular cells are still unexplored, this cytokine is known to act on a broad range of cell types to induce the expression of pro-inflammatory cytokines, chemokines, and metalloproteinases, contributing to recruitment, activation, and migration of inflammatory cells to the target organ (Kolls & Linden 2004, Onishi & Gaffen 2010). IL17 alone has the capacity to induce pro-inflammatory factors; however, its activity is vastly increased when combined with other cytokines, particularly TNF, IFNG, and IL1B (Onishi & Gaffen 2010). We could speculate that

in EAO testis, synergistic action of TNF, IFNG, and IL17 directs inflammation to chronicity.

Several authors reported that inflammatory cytokines direct T_{regs} to differentiate into Th17 cells. In fact, murine T_{regs} are converted to Th17 under the influence of IL6 (Afzali *et al.* 2009). Increased intratesticular IL6 content in EAO testis (Rival *et al.* 2006) makes conversion of testicular T_{regs} to the Th17 phenotype possible.

IL23 has a supporting role in the stimulation and survival of Th17 cell in the periphery (Cua *et al.* 2003, Harrington *et al.* 2006). The increase in IL23 content in the testes of EAO rats shown by our results may result in full activation of inflammatory function of Th17 cells.

Overall results suggest that a complex network of cytokines produced by CD4+ Th1 and Th17 together with CD8+ Teff cells modifies the normal immunosuppressor microenvironment of the testis inducing inflammation, germ cell damage, and infertility.

New therapeutic approaches specifically targeting pathogenic Th17 cells may provide effectives strategies for controlling chronic testicular inflammation.

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 with 14 h light:10 h darkness schedule, and fed standard food pellets and water *ad libitum*. The handling of rats followed the NIH guidelines for care and use of experimental animals.

Induction of EAO

Rats in the E group were actively immunized with testicular homogenate (TH) prepared as previously described (Doncel *et al.* 1989). Briefly, rat testes were decapsulated, mixed with an equal volume of saline, and disrupted in an Omni mixer for 30 s. The final concentration was 500 mg/ml wet weight. TH (0.4 ml) emulsified with 0.4 ml complete Freund's adjuvant (Sigma–Aldrich, St Louis, MO, USA) was injected s.c. in footpads and at multiple sites near the popliteal lymph nodes and the neck area. E rats were injected three times with 200 mg of TH/dose per rat at 14-day intervals. The first two immunizations were followed by an i.v. injection of 0.5 ml *Bordetella pertussis* (strain 10536, Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by an i.p. injection of 5×10^9 microorganisms. Rats in the C group were injected with an emulsion of saline solution and adjuvant in the same conditions as the E group. A third group of N rats were also studied. E, C, and N rats were killed at different time periods (50 and 80 days) after the first immunization. Rats were killed and weighed; one testis was removed, weighed, and quickly frozen for immunohistochemistry or processed for interstitial cell isolation. The other testis was processed for histopathology.

Histopathology

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

Isolation of testicular interstitial cells

Testicular interstitial cells from N, C, and E 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, USA) at 34 °C for 15 min. Collagenase was inactivated by adding ice-cold phosphate-buffered saline (PBS) and ST were allowed to settle; the supernatant 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). Cells were washed, centrifuged, and counted in a Neubauer chamber by the trypan blue exclusion method.

Isolation of CD4+ and CD8+ T cells from the testis

Testicular interstitial cells were incubated with saturating concentrations of anti-CD3 (APC) and anti-CD8 (Per-CP; BD Bioscience, San Diego, CA, USA) for 30 min at 4 °C. Appropriate control isotypes were used. Cells were washed twice with PBS and isolated by FACS. BD FACSAria II cytometer was used to isolate CD3+ CD8− (CD4+ T cells) and CD3+ CD8+ T cells.

Collection of testicular fluid

Testicular fluid was collected as previously described (Sharpe *et al.* 1984). Drained fluid was collected after 16 h at 4 °C in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml aprotinin, Sigma–Aldrich). Testicular fluids were stored at −70 °C prior to use.

Western blot analysis of T-bet, RORγt, and IL17

Testicular interstitial cells were homogenized in three volumes (100 µl) of ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 0.5% sodium desoxycholate, and 1% NP-40, pH 7.4) containing protease inhibitors (1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin, Sigma–Aldrich). Homogenates were centrifuged at 13 000 *g* for 30 min at 4 °C. To assess equal loading, protein concentration in lysates was determined by the Lowry method (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA, USA). Extracts were dissolved with sample buffer (500 µM Tris–HCl, pH 6.8, 10% SDS, 30% glycerol, and 0.5% bromophenol blue) containing 0.5% β-mercaptoethanol for T-bet and RORγt or without β-mercaptoethanol for IL17, boiled for 5 min (except samples for IL17), and immediately placed on ice. Equal amounts of proteins (100 µg/lane) were resolved in 10% SDS–PAGE for T-bet and RORγt and 12% SDS–PAGE for IL17 at 120 V for 90 min. Proteins were electroblotted at 66 mA overnight for T-bet, 150 V for 60 min for RORγt, or 100 V for 90 min for

IL17. Membranes were blocked with blocking solution (3% (w/v) of nonfat dry milk in Tris-buffered saline Tween-20 (TBST) buffer: 10 mM Tris, 154 mM NaCl, 0.1% Tween-20 (v/v), pH 7.5) for 90 min. Blots were incubated overnight with mouse MAB against T-bet (1:100; eBioscience, San Diego, CA, USA), rabbit polyclonal antibody against RORγt (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit polyclonal antibody against IL17 (1:100; Santa Cruz Biotechnology). As internal loading control, a rabbit polyclonal antibody anti-β-actin (1:6000; Sigma–Aldrich) was used. After six washes (5 min each) in TBST buffer, blots were incubated with an anti-mouse (1:2000; Vector Laboratories, Burlingame, CA, USA) or anti-rabbit biotinylated antibody (1:3000; Vector Laboratories) depending on the primary antibody used. Then the reaction was enhanced with HRP–streptavidin-peroxidase conjugates (Chemicon International, Temecula, CA, USA) and chemiluminescence was used to detect HRP-labeled protein onto Kodak BioMax MS film (NEN Life Science Products, Boston, MA, USA).

Flow cytometric analysis

Testicular interstitial cells (1×10^6) were incubated with saturating concentrations of anti-CD3 (APC) and anti-CD4 (FITC) or anti-CD3 (APC) and anti-CD8 (FITC) for 30 min at 4 °C. Cells were washed twice with PBS.

To detect intracellular cytokines in single interstitial testicular cells, cells were stimulated with a combination of 50 ng/ml PMA plus 750 ng/ml ionomycin (Sigma–Aldrich) in the presence of 10 µg/ml brefeldin A (BD Bioscience) for 6 h at 34 °C in a humidified atmosphere with 5% CO_2. Concentrations of mitogens were determined as previously described by Openshaw *et al.* (1995). Brefeldin A was used to enhance flow cytometric analysis of intracellular cytokine staining through its inhibitory effect of protein secretion by interference with the function of the Golgi apparatus (Openshaw *et al.* 1995, Picker *et al.* 1995, Morita *et al.* 1998). Cells were washed with PBS and fixed with 2% paraformaldehyde (PFA) in PBS for 10 min at 4 °C. After washing with PBS, cells were preincubated with 0.1% saponin in PBS for 10 min at 4 °C, then incubated with saturating concentrations of anti-CD16/32-Blocks Fc followed by anti-FOXP3 (PE-Cy5; eBioscience) in combination with anti-TNF (PE), anti-IFNG (PE), anti-IL10 (PE), or anti-IL4 (PE; BD Bioscience) for 30 min at 4 °C. Finally, cells were washed twice with 0.1% saponin in PBS and then with PBS without saponin, resuspended in PBS, and analyzed by flow cytometry. Appropriate control isotypes were used. BD FACSAria II cytometer was used and 30 000 events on 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.

Immunohistochemical detection of RORγt+ and IL17+ cells

To identify RORγt+ cells, testis cryostat sections (5–7 µm) were fixed with 4% PFA for 10 min. After blocking with 3% normal goat serum in 3% BSA in PBS (blocking solution A) for 30 min at room temperature (RT), sections were incubated overnight with rabbit anti-rat RORγt (0.2 mg/ml; Santa Cruz

Biotechnology) and diluted in blocking solution A at 4 °C in a humidified chamber. After three washes in PBS, sections were incubated with goat anti-rabbit (FITC; Vector Laboratories) and diluted in blocking solution A for 1 h at RT. For negative control, the first antibody was omitted. Finally, sections were mounted with a medium containing 4′-6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

To identify IL17+ cells, testis cryostat sections (5–7 μm) were fixed with 4% PFA and incubated with 5% nonfat dry milk, 0.1% Triton X-100 in PBS (blocking solution B) for 30 min at RT followed by avidin/biotin blocking solution (Vector Laboratories). After three washes with PBS, sections were incubated overnight with rabbit anti-rat IL17 (8 μg/ml; Santa Cruz Biotechnology) and diluted in blocking solution B at 4 °C in a humidified chamber. A biotinylated goat anti-rabbit IgG (4 μg/ml) diluted in blocking solution B was used as secondary antibody. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min. The reaction was amplified using the Vectastain Elite ABC kit (Vector Laboratories) and the reaction product was visualized by adding diaminobenzidine substrate (Vector Laboratories). For negative control, the first antibody was omitted. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted.

To detect IL17+ cells within testicular CD4+ and CD8+ T cell subsets, cells isolated from the testis were fixed with 2% PFA in PBS for 10 min and incubated with 3% normal goat serum, 3% BSA, and 0.1% Triton X-100 (blocking/permeabilization solution) in PBS for 30 min at RT. Then, cells were incubated overnight with rabbit anti-rat IL17 (8 μg/ml; Santa Cruz Biotechnology) diluted in blocking/permeabilization solution at 4 °C in a humidified chamber. After three washes in 0.1% Triton X-100 in PBS, sections were incubated with goat anti-rabbit (FITC) and diluted in blocking/permeabilization solution for 1 h at RT. For negative control, the first antibody was omitted. Cells were mounted with a medium containing DAPI (Vector Laboratories).

Determination of IL23 by ELISA

Commercial ELISA kit specific for rat IL23 (Shanghai Transhold Co. Ltd, Shanghai, People's Republic of China) was used to quantify the content of this cytokine in testicular fluids. The minimum detectable concentration is 100 pg/ml. All procedures followed the manufacturer's instructions.

Statistical analysis

Results are expressed as mean±S.E.M. Comparisons of the groups were assessed by the nonparametric Kruskal–Wallis one-way ANOVA or by one-way ANOVA followed by the Bonferroni test when applicable. $P \leq 0.05$ was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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