

# Tumour necrosis factor- $\alpha$ released by testicular macrophages induces apoptosis of germ cells in autoimmune orchitis

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**BACKGROUND:** Experimental autoimmune orchitis (EAO) is a model of chronic inflammation and infertility useful for studying testicular immune and germ cell (GC) interactions. In this model, EAO was induced in rats by immunization with testicular homogenate and adjuvants; Control (C) rats were injected with adjuvants. EAO was characterized by an interstitial infiltrate of lymphomonocytes and seminiferous tubule damage, moderate 50 days (focal orchitis) and severe 80 days after the first immunization (severe orchitis). Based on the previous results showing that the number of macrophages and apoptotic GC expressing tumour necrosis factor (TNF) receptor 1 increased in EAO, we studied the role of macrophages and TNF- $\alpha$  in GC apoptosis. **METHODS AND RESULTS:** Conditioned media of testicular macrophages (CMTM) obtained from rats killed on Days 50 and 80 decreased the viability (MTS,  $P < 0.01$ ) and induced apoptosis (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling, TUNEL) of GC obtained from EAO but not from non-immunized, N rats ( $P < 0.001$ ). TNF- $\alpha$  content (enzyme-linked immunosorbent assay) was significantly higher in the CMTM from EAO versus C rats on Day 80 ( $P < 0.05$ ). The apoptotic effect of CMTM from Day 80 rats was abrogated by a selective TNF- $\alpha$  blocker (Etanercept). Moreover, TNF- $\alpha$  *in vitro* induced GC apoptosis. TNF- $\alpha$  expression (by immunofluorescence) was observed in testicular (ED2<sup>+</sup>) and non-resident (ED1<sup>+</sup>) macrophages, the percentage of TNF- $\alpha$ <sup>+</sup> macrophages being similar in focal and severe orchitis. **CONCLUSIONS:** Results demonstrated that soluble factors released from testicular EAO macrophages induce apoptosis of GC, biased by the local inflammatory environment, and that TNF- $\alpha$  is a relevant cytokine involved in testicular damage during severe orchitis.

**Keywords:** autoimmune orchitis; testis; macrophages; tumour necrosis factor- $\alpha$ ; apoptosis

## Introduction

Although the testis is considered an immunoprivileged organ, inflammation, infection and trauma can overwhelm immunosuppressor mechanisms and induce testicular autoimmune disease. In fact, the disruption of immunoprivilege is a matter of clinical importance due to the prevalence of inflammatory reactions among infertile men. Autoimmune orchitis is a model of chronic inflammation that is useful for elucidating pathophysiological mechanisms involved in testicular damage and infertility. We developed an experimental autoimmune orchitis (EAO) in rats by active immunization with spermatogenic antigens (Doncel *et al.*, 1989). EAO is characterized by an interstitial cell infiltrate of macrophages, dendritic cells and lymphocytes and by apoptosis and sloughing of germ cells (GCs) from the seminiferous tubules (Lustig *et al.*, 1993; Theas *et al.*, 2003; Rival *et al.*, 2006).

In the normal adult rat testis, the macrophage population is heterogeneous and includes a subset of resident macrophages (ED2<sup>+</sup> cells) and monocytes recently arrived from circulation

(ED1<sup>+</sup> cells). It has been proposed that circulating monocytes could differentiate into resident macrophages under the testicular immunosuppressor micro-environment through an intermediate cell type that expresses both markers (Dijkstra *et al.*, 1985; Wang *et al.*, 1994; Hedger, 1997). Macrophages are known to produce and secrete a plethora of cytokines such as tumour necrosis factor (TNF)- $\alpha$ , Fas L, interleukin (IL)-1, IL-6 as well as other soluble mediators such as nitric oxide (NO) and reactive oxygen species, which are involved in the orchestration of the inflammatory response. It has been demonstrated that these soluble factors are also involved in the induction of tissue damage in other autoimmune diseases and experimental models of inflammation (Liversidge *et al.*, 2002; Lange-Sperandio *et al.*, 2003; Ponomarev *et al.*, 2005; Yoon and Jun, 2005).

We have previously shown that, in rats undergoing EAO, the number of apoptotic GCs expressing TNF receptor (TNFR1) increases in association with an increased number of interstitial lymphomononuclear cells. Although we are aware

that different immune testicular cells (dendritic cells, mast cells and lymphocytes) are able to secrete TNF- $\alpha$  (Lustig, 1995, personal communication, unpublished results; Rival *et al.*, 2007), the aim of this work was to evaluate the role of macrophages and the involvement of TNF- $\alpha$  secreted by these cells on GC apoptosis in rats with autoimmune orchitis.

## Materials and Methods

### Animals

Male adult Sprague–Dawley rats were kept at 22°C with a 12 h light, 12 h dark schedule and fed standard food pellets and water ad libitum. Animal handling and experimentation were done in accordance with the NIH Guide for the Care and Use of Experimental Animals.

### Immunization schedule

Rats of the EAO group were immunized with testicular homogenate (TH) prepared as previously described (Doncel *et al.*, 1989). Briefly, rat testes were decapsulated, an equal volume of saline solution was added and they were disrupted in an Omni mixer for 30 s. The TH final concentration was 500 mg/ml. A total of 0.4 ml of TH emulsified with 0.4 ml of complete Freund's adjuvant (CFA) was injected intradermally in footpads and at multiple sites near ganglionic regions, three times at 14 day intervals. The first two immunizations were followed by an i.v. injection of 0.5 ml of *Bordetella pertussis* (Bp) (strain 10 536; Instituto Malbrán, Buenos Aires, Argentina) containing  $10^{10}$  micro-organisms and the third by an i.p. injection of  $5 \times 10^9$  micro-organisms. Rats from the Control (C) group were injected with an emulsion of saline solution, CFA and Bp in the same conditions as the EAO group. EAO and C rats were killed at different time periods (15–30, 50–60 or 70–80 days) after the first immunization. We also studied a group of non-immunized Normal (N) rats killed at the same time as rats from the other groups. Rats were killed and weighed; testes were removed and weighed. One testis was fixed in Bouin's solution and embedded in paraffin for histopathology and the other was processed for macrophage, GC or seminiferous tubule isolation and culture.

### Isolation and culture of testicular macrophages

The isolation procedure was performed under sterile and low endotoxin conditions, as previously described (Yee and Hutson, 1983). Briefly, rats from N, C and EAO groups were perfused with cold sterile saline solution until tissues became pale. For each rat, one testis was incubated with Type I collagenase (0.3 mg/ml; Worthington Biochemical Corporation, Freehold, NJ, USA) in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (A-4553, fraction V, Sigma–Aldrich, St Louis, MO, USA) for 15 min at 34°C in a Dubnoff shaking water bath. After adding PBS to inactivate collagenase, the seminiferous tubules were allowed to settle at 4°C and the supernatant centrifuged at  $300 \times g$  for 5 min at 4°C. The pellet was resuspended in PBS (2 ml), plated both on 35 mm diameter polystyrene Petri dishes (Nunc Inc., Naperville, IL, USA) to obtain conditioned media of testicular macrophages (CMTM) and on round coverslips placed on 24-well plates for immunofluorescence (IF) and then incubated for 2 h at 34°C in a humidified atmosphere with 5% CO<sub>2</sub>. Dishes and coverslips were rinsed several times in PBS to remove unattached cells. Cells attached to coverslips were fixed with 2% paraformaldehyde for immunoassays. In order to obtain CMTM, cells attached to the dishes were cultured in Dulbecco's modified Eagle's medium-nutrient mixture F12 (1:1; DMEM/F12, 12500-039, Gibco–BRL, Rockville, MD, USA) supplemented with L-glutamine (2 mM; G-8540, Sigma–Aldrich), insulin–transferin–selenium-A supplement (1 $\times$ ; ITS-A, 51300-044, Gibco–BRL), sodium DL-lactic acid (1 mM; L-4263, Sigma–Aldrich) and

antibiotic–antimycotic solution (1 $\times$ ; 5240-096, Gibco–BRL) (DMEM/F12-S) for 20 h at 34°C in a humidified atmosphere with 5% CO<sub>2</sub>. CMTM was collected, centrifuged and stored at –70°C until use. Adherent cells were harvested and counted to determine the number of macrophages/dish; cell viability was also determined by the trypan blue dye exclusion test. Purity of macrophage preparations was 90–95% as evaluated by IF with macrophage markers (ED1 + ED2) performed on cells attached to coverslips.

### Immunoassay for rat TNF- $\alpha$

A commercial rat-specific TNF- $\alpha$  enzyme-linked immunosorbent assay (ELISA) kit (KRC6011, Biosource International, Inc., CA, USA) was used to quantify TNF- $\alpha$  content in the CMTM. Samples were measured by triplicate. The minimum detectable concentration of rat TNF- $\alpha$  was <4 pg/ml. All procedures followed the manufacturer's instructions.

### Isolation and culture of GCs

Testes from EAO rats were killed on 50 days after the first immunization and from N rats were decapsulated and digested with type II collagenase (0.3 mg/ml, Worthington Biochemicals Corporation) in PBS with 0.1% BSA for 15 min at 34°C in a Dubnoff shaking water bath. After adding PBS, seminiferous tubules were allowed to settle and then washed three times with DMEM/F12 medium. After mechanical dispersion of the seminiferous tubules with a Pasteur pipette, cell debris was eliminated by pressing the cell suspension against a fine stainless steel screen. Isolated GCs ( $10^5$  cells/well) were plated into a 96-well culture plate in DMEM/F12-S. To each well, different volumes of CMTM (diluted in DMEM/F12-S) were added. Final incubation volume was 100  $\mu$ l/well. The plates were incubated for 18 h at 34°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Assessment of GC viability

After the culture period, GC viability was evaluated using the MTS Cell Titer Cell Proliferation assay (G5421, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. This assay is a colorimetric method to determine the number of viable cells. MTS is bioreduced by dehydrogenase enzymes found in metabolically active cells into a formazan product soluble in the cell culture medium. The quantity of formazan product is directly proportional to the number of living cells in the culture. Optical density (OD) was read at a wavelength of 490 nm in a microplate reader.

### Isolation and culture of seminiferous tubule segments

Testes removed from EAO rats were killed on 50 days after the first immunization and from N rats were decapsulated and the seminiferous tubule segments (STS) microdissected under a transillumination stereomicroscope as previously described (Parvinen and Hecht, 1981). The isolated STS ( $\approx$  2 mm in length) were transferred to a 96-well culture plate in DMEM/F12-S medium. To each well, 50  $\mu$ l of DMEM/F12-S alone or containing recombinant human TNF- $\alpha$  (final concentration: 50 ng/ml, G5241, Promega Corporation) or CMTM was added. To neutralize TNF- $\alpha$  in the CMTM, a dimeric fusion protein consisting of the p75 TNFR linked to an immunoglobulin (Ig)G Fc (Etanercept, Embrel<sup>®</sup>) was used (Scallon *et al.*, 2002). The final incubation volume was 200  $\mu$ l/well. The plates were incubated for 18 h at 34°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Assessment of apoptosis

After the culture period, STS were squashed in order to obtain the GCs and fixed (Henriksen *et al.*, 1995). Apoptosis was determined by the TUNEL assay. The squash preparations were irradiated in a

microwave oven (370 W for 5 min) in 10 mM sodium citrate buffer, pH 6.0 and permeabilized with 0.1% Triton-X 100 (T-9284, Sigma–Aldrich) in 0.1% sodium citrate for 5 min at 4°C. Non-specific labelling was prevented by incubating the preparations with blocking solution (5% blocking reagent; 11 096 176 001, Roche Molecular Biochemicals GmbH, Mannheim, Germany, in 150 mM NaCl and 100 mM maleic acid, pH 7.5) for 30 min at room temperature. After 10 min incubation with TdT buffer (11 966 006 001, Roche) containing 1.25 mg/ml BSA, the apoptotic DNA was three-end labelled with digoxigenin-11-dideoxy-uridine triphosphate (4  $\mu$ M Dig-dd-UTP; 11 363 905 910, Roche) by the incubation with TdT (0.18 U/ml TdT; 33 335 740 01, Roche) in TdT buffer for 1 h at 37°C. As control of the assay, the TdT enzyme was replaced with the same volume TdT buffer. The preparations were then incubated with the blocking solution (2% blocking reagent in 150 mM NaCl and 100 mM maleic acid, pH 7.5) for 30 min at room temperature, followed by the detection of the Dig-dd-UTP with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; 11 093 274 910, Roche) incubated for 2 h at room temperature. Squash preparations were rinsed and equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM MgSO<sub>4</sub>, pH 9.5) containing 1 mM levamisole (L-9756, Sigma–Aldrich). Then, alkaline phosphatase substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, 11 697 471 001, Roche) were added for 60 min. The reaction was stopped by washing the preparations with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Squashes were light counterstained with eosin, dehydrated and mounted. In each experiment, STS were individually squashed and analyzed.

#### ED1/ED2 and TNF- $\alpha$ double IF

To detect TNF- $\alpha$  expression in testicular macrophages, a double IF assay was performed. A mouse monoclonal antibody, ED1, which recognizes a cytoplasmic antigen in rat monocytes, macrophages and dendritic cells and a mouse monoclonal antibody, ED2, which recognizes a membrane antigen of tissue macrophages, were used to identify testicular non-resident and resident macrophages, respectively. Isolated testicular macrophages obtained as described above were permeabilized with 0.1% Triton-X 100 in PBS containing 3% BSA for 30 min at room temperature. Cells were then reacted with a rabbit polyclonal antibody anti-rat TNF- $\alpha$  (200  $\mu$ g/ml, P350, Endogen, Rockford, IL, USA) for 1 h at 4°C. Non-specific binding sites were blocked with 3% normal horse serum in PBS for 30 min at room temperature. Then, the cells were incubated with ED1 (25  $\mu$ g/ml, 554954, BD Pharmingen, San Diego, CA, USA) and/or ED2 (12.5  $\mu$ g/ml, 550573, BD Pharmingen) antibodies. Immunoreactivity for TNF- $\alpha$  was developed using an anti-rabbit biotinylated-IgG (4  $\mu$ g/ml, BA-1000, Vector Laboratory) in 5% BSA for 1 h at room temperature, followed by incubation with Rhodamine Avidin DCS (2  $\mu$ g/ml, A 2012, Vector Laboratory) in 10 mM HEPES buffer containing 5% BSA for 30 min at room temperature. Macrophage antigens were revealed with an anti-mouse fluorescein isothiocyanate conjugated-IgG (5  $\mu$ g/ml, FI-2001, Vector Laboratory). The nucleus were counterstained using 4',6-diamidino-2-phenylindole (0.5 mg/ml DAPI, Roche 10 236 276 001). For negative controls, primary antibodies were replaced by PBS. Cells were observed and counted using epifluorescence optics with an Axiophot microscope.

#### Statistical analysis

Data from cell viability (MTS assay) and TNF- $\alpha$  content (ELISA) were expressed as mean  $\pm$  SEM and were evaluated by Student's *t*-test. Differences were considered significant at the  $P < 0.05$  level. Data of apoptotic cell number (TUNEL assay) were expressed as the percentage  $\pm$  95% confidence limits of TUNEL<sup>+</sup> cells of the

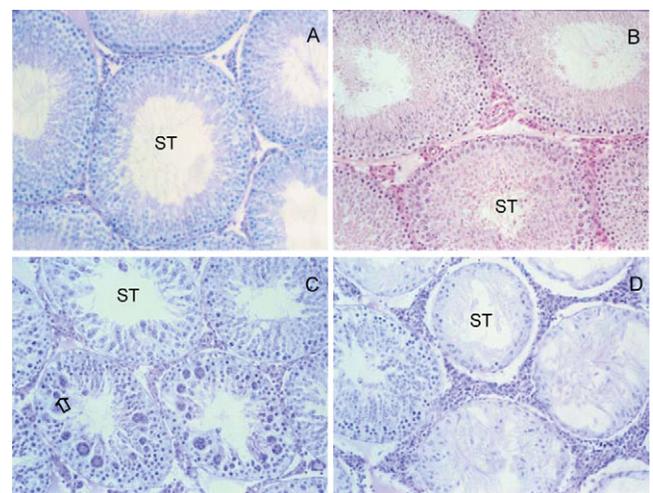
total number of cells counted from STS incubated under each experimental condition. Confidence intervals for proportions were analysed by the  $\chi^2$ -test, differences between proportions were considered significant if  $P < 0.05$  (Jaita *et al.*, 2005).

## Results

### Histopathology

Rats from N and C groups showed similar testicular weight, whereas rats from the EAO group were killed on 50–60 and 70–80 days after the first immunization showed a significant decrease in testicular weight associated with testicular damage (15–30 days—C: 1.60  $\pm$  0.09 g, EAO: 1.49  $\pm$  0.07, N: 1.58  $\pm$  0.02,  $n = 4$ ; 50–60 days—C: 1.76  $\pm$  0.05, EAO: 1.21  $\pm$  0.10\*\*\*,  $n = 8-9$ , N: 1.72  $\pm$  0.03,  $n = 6$ ; 70–80 days—C: 1.68  $\pm$  0.03, EAO: 0.87  $\pm$  0.03\*\*\*,  $n = 11-13$ , N: 1.76  $\pm$  0.06,  $n = 4$ , \*\*\* $P < 0.001$ ; mean  $\pm$  SEM, unpaired *t*-test with Welch correction). Body weight of N, C and EAO rats were killed at the time periods studied did not differ significantly (15–30 days—C: 351.50  $\pm$  8.50 g, EAO: 370.50  $\pm$  29.50, N: 368.93  $\pm$  11.95; 50–60 days—C: 489.63  $\pm$  43.88, EAO: 480.52  $\pm$  19.91, N: 492.98  $\pm$  14.81; 70–80 days—C: 512.67  $\pm$  18.57, EAO: 552.50  $\pm$  9.39, N: 525.35  $\pm$  37.77; mean  $\pm$  SEM).

Histopathology of testes from N and C rats showed no lesion (Fig. 1A and B). Rats from the EAO group showed testicular damage as we previously described (Doncel *et al.*, 1989; Theas *et al.*, 2006). Fifty days after the first immunization testis sections of EAO rats presented mild lymphomononuclear cell infiltrate and foci of seminiferous tubules with degenerating GCs intermingled with normal seminiferous tubules (focal orchitis) (Fig. 1C); 80 days after the first immunization, most of the seminiferous tubules were aspermatogenic due to the GC



**Figure 1:** Histopathology of testis sections from N (A), C (B) and EAO (C and D) rats.

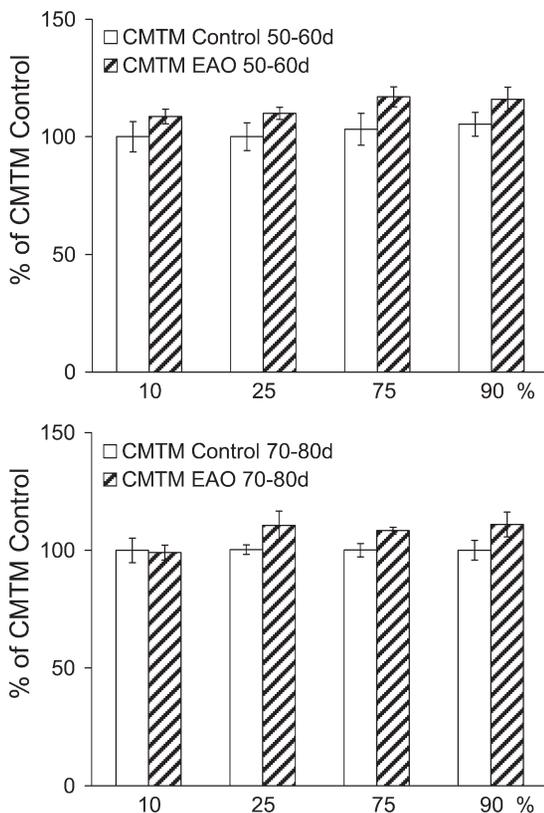
Seminiferous tubules (ST) with normal spermatogenesis and lack of interstitial immune cell infiltration are observed in the testis of N (A) and C (B) rats. Focal damage of ST together with moderate interstitial cell infiltrate is present in the testis of EAO rats killed on Day 50 (C); a more severe and extended damage with increased interstitial cell density is observed on Day 80 (D). Note GC degeneration and sloughing (arrow) in the ST of EAO rats ( $\times 100$  magnification).

loss associated with an increased interstitial lymphomononuclear cell infiltrate (severe orchitis) (Fig. 1D).

### **Testicular macrophages and GC apoptosis**

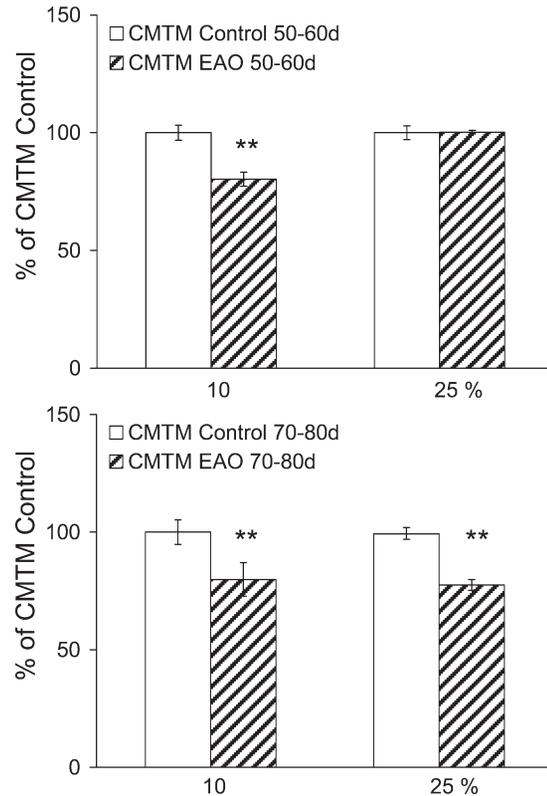
In order to determine the involvement of macrophages in GC apoptosis, we first studied the effect of different concentrations of CMTM on the viability of GCs from N rats. CMTM obtained from EAO rats killed on either 50–60 or 70–80 days after the first immunization did not affect the viability of normal GCs at any concentration analysed (Fig. 2). However, when GCs from EAO rats were used CMTM from rats with orchitis significantly decreased cell viability (Fig. 3); these results were also confirmed by the trypan blue exclusion test (data not shown). Since 10% of CMTM obtained from testis of 50–60 and 70–80 EAO rats decreased GC viability, we chose this CMTM dilution to study GC apoptosis. We have no explanation for the different effects of 10 and 25% CMTM dilution on GC viability. However, it has been reported that increasing concentrations of CMTM do not necessarily correlate with an increased biological response and they can even induce an opposite effect (Afane *et al.*, 1998; Suescun *et al.*, 2003).

We demonstrated that CMTM obtained from EAO rats killed on 50–60 and 70–80 days after the first immunization significantly increased the percentage of TUNEL<sup>+</sup> GCs obtained from



**Figure 2:** Effect of CMTM on the viability of Normal GCs (MTS assay).

GCs obtained from N rats were incubated for 18 h with different percentages (%) of CMTM obtained from C and EAO rats killed on 50–60 or 70–80 days after the first immunization. The value of OD measured after incubating GCs with CMTM of C rats was considered 100%. Each column represents the mean  $\pm$  SEM of 7–15 wells from two separate experiments.



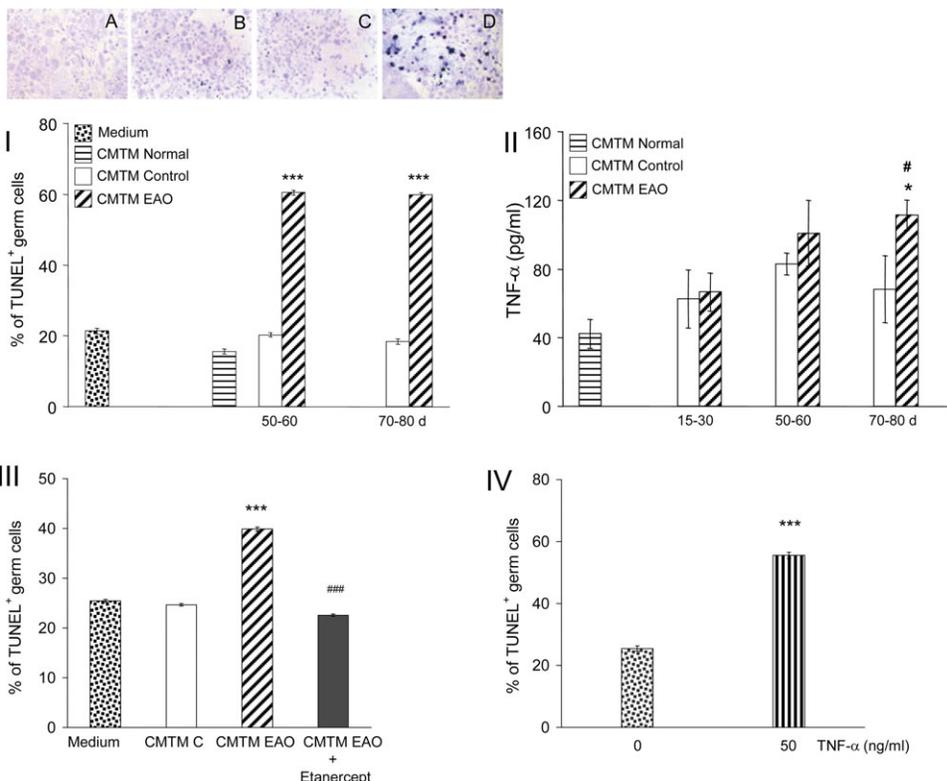
**Figure 3:** Effect of CMTM on the viability of EAO GCs (MTS assay).

GCs obtained from EAO rats killed on 50 days after the first immunization were incubated for 18 h with different percentages (%) of CMTM obtained from C and EAO rats killed on 50–60 or 70–80 days after the first immunization. The value of OD measured after incubating GCs with CMTM of C rats was considered 100%. Each column represents the mean  $\pm$  SEM of 7–10 wells from two separate experiments. \*\* $P < 0.01$  versus respective C.

seminiferous tubules of EAO rats compared with CMTM from C rats. CMTM obtained from C rats did not modify the basal percentage of TUNEL<sup>+</sup> GCs (cells incubated with medium only: 21.30%  $\pm$  (0.82–0.79)) (Fig. 4I). CMTM from EAO rats had no apoptotic effect on GCs from seminiferous tubules segments obtained from N rats (percentage of TUNEL<sup>+</sup> GCs 23.89  $\pm$  (0.23–0.24)  $n = 10\ 000$  total cells counted).

### **TNF- $\alpha$ production by testicular macrophages**

We have previously shown that testicular macrophages from rats with severe orchitis and atrophic testes killed on 100 days after the first immunization release higher levels of TNF- $\alpha$  compared with C rats (Suescun *et al.*, 2003). In order to evaluate TNF- $\alpha$  production by testicular macrophages along the induction and development of EAO, we measured the TNF- $\alpha$  released (ELISA) by these cells during the immunization period (15–30 days) and when focal (50–60 days) and severe orchitis (70–80 days) developed. The conditioned medium was obtained by culturing the macrophages from a whole testis of each rat. We showed that the TNF- $\alpha$  content in the CMTM obtained from EAO rats (70–80 days) was significantly higher than the TNF- $\alpha$  content in the CMTM from C rats (Fig. 4II). Moreover, the TNF- $\alpha$  content increased



**Figure 4:** Effect of CMTM and tumour necrosis factor (TNF)- $\alpha$  on GC apoptosis.

(I) STS obtained from EAO rats killed on 50 days (d) after the first immunization were incubated for 18 h with Dulbecco's modified Eagle's medium (DMEM)/F12-S medium (see Materials and Methods) or 10% CMTM obtained from N, C or EAO rats killed on 50–60 or 70–80 days after the first immunization. Each column represents the percentage  $\pm$  confidence limits of TUNEL<sup>+</sup> GCs of the total number of cells counted ( $n = 10\,000$ – $20\,000$  cells/group from three independent experiments). \*\*\* $P < 0.001$  versus respective C. Microphotographs of the TUNEL assay performed on STS incubated with Medium (A), CMTM from N (B), C (C) or EAO rats (D) and stained with eosin. TUNEL<sup>+</sup> cells are blue ( $\times 140$  magnification). (II) TNF- $\alpha$  production by testicular macrophages. TNF- $\alpha$  was measured by enzyme-linked immunosorbent assay in the CMTM obtained from rats sacrificed on different days after the first immunization. Each column represents the mean  $\pm$  SEM of 4–7 rats/group. \* $P < 0.05$  versus respective C, # $P < 0.05$  versus EAO 15–30 days. (III) Effect of neutralizing the TNF- $\alpha$  present in the CMTM on GC apoptosis. STS obtained from EAO rats killed on Day 50 were incubated for 18 h with DMEM/F12-S medium or 10% CMTM obtained from EAO rats killed on Days 70–80 in the presence or absence of the selective TNF- $\alpha$  blocker, Etanercept (1  $\mu\text{g/ml}$ ).  $n = 40\,000$ – $60\,000$  cells/group from three independent experiments. \*\*\* $P < 0.001$  versus CMTM C. ### $P < 0.001$  versus CMTM EAO. (IV) Effect of TNF- $\alpha$  on GC apoptosis. STS obtained from EAO rats killed on Day 50 were incubated for 18 h with DMEM/F12-S medium with or without TNF- $\alpha$ .  $n = 5000$ – $10\,000$  cells/group from two independent experiments. \*\*\* $P < 0.001$ .

significantly in the CMTM obtained from EAO rats killed on Days 70–80 compared with those killed during the immunization period (Fig. 4II). Since we counted the number of macrophages per dish, we were able to express TNF- $\alpha$  production per  $10^5$  macrophages. Although the total number of macrophages was higher in both focal and severe orchitis compared with C rats (data not shown), this analysis showed that the macrophages from EAO rats killed on Days 70–80, released significantly less TNF- $\alpha$  per macrophage than C rats. There were no differences between C and EAO rats at the other periods of time studied (TNF- $\alpha$  pg/ml/ $10^5$  macrophages, 15–30 days—C:  $11.67 \pm 4.64$ , EAO:  $18.42 \pm 6.33$ , N:  $9.14 \pm 5.49$ ; 50–60 days—C:  $20.10 \pm 7.31$ , EAO:  $13.11 \pm 2.91$ , N:  $20.61 \pm 2.14$ ; 70–80 days—C:  $26.12 \pm 10.17$ , EAO:  $8.50 \pm 1.99^*$ , N:  $13.86 \pm 4.56$ ; mean  $\pm$  SEM;  $P < 0.05$ , Student's *t*-test).

**Effect of TNF- $\alpha$  neutralization on GC apoptosis**

In order to determine if the TNF- $\alpha$  present in the CMTM obtained from EAO rats killed on 70–80 days after the first

immunization was involved in GC apoptosis, we incubated STS with Etanercept (1  $\mu\text{g/ml}$ ). Neutralization of TNF- $\alpha$  significantly blocked the increase in the percentage of TUNEL<sup>+</sup> GCs induced by the CMTM obtained from EAO rats (Fig. 4III). The TNF- $\alpha$  content in the CMTM from EAO rats was significantly higher than that in the CMTM from C rats (C:  $25.22 \pm 4.04$  pg/ml, EAO:  $85.52 \pm 6.44$ , mean  $\pm$  SEM,  $P < 0.01$  Student's *t*-test). Etanercept (1  $\mu\text{g/ml}$ ) *per se* did not modify the percentage of basal TUNEL<sup>+</sup> GCs (DMEM/F12-S medium plus Etanercept:  $19.56\% \pm (0.21-0.20)$ , DMEM/F12-S medium  $17.90\% \pm (0.18-0.18)$   $n = 8000$ – $30\,000$  total cells counted).

**Effect of TNF- $\alpha$  in vitro**

The cell death inductor effect of TNF- $\alpha$  on EAO GCs was then studied by TUNEL. TNF- $\alpha$  significantly increased ( $\sim 2.2$  times compared with the basal values) the percentage of TUNEL<sup>+</sup> GCs (Fig. 4IV).

### Expression of TNF- $\alpha$ in testicular macrophages

By double IF performed on isolated testicular macrophages, we showed that both macrophage subsets, non-resident ED1<sup>+</sup> and resident ED2<sup>+</sup>, expressed TNF- $\alpha$ . However, ED1<sup>+</sup> macrophages showed a more intense immunoreactivity for TNF- $\alpha$  (Fig. 5). The percentage of total macrophages (ED1<sup>+</sup> + ED2<sup>+</sup>) expressing TNF- $\alpha$  was similar in focal and severe orchitis (50–60 days—65.12%  $\pm$  (0.36–0.41); 70–80 days—58.86%  $\pm$  (0.30–0.40),  $n = 1000$ –1300 total cells counted from two independent experiments).

### Discussion

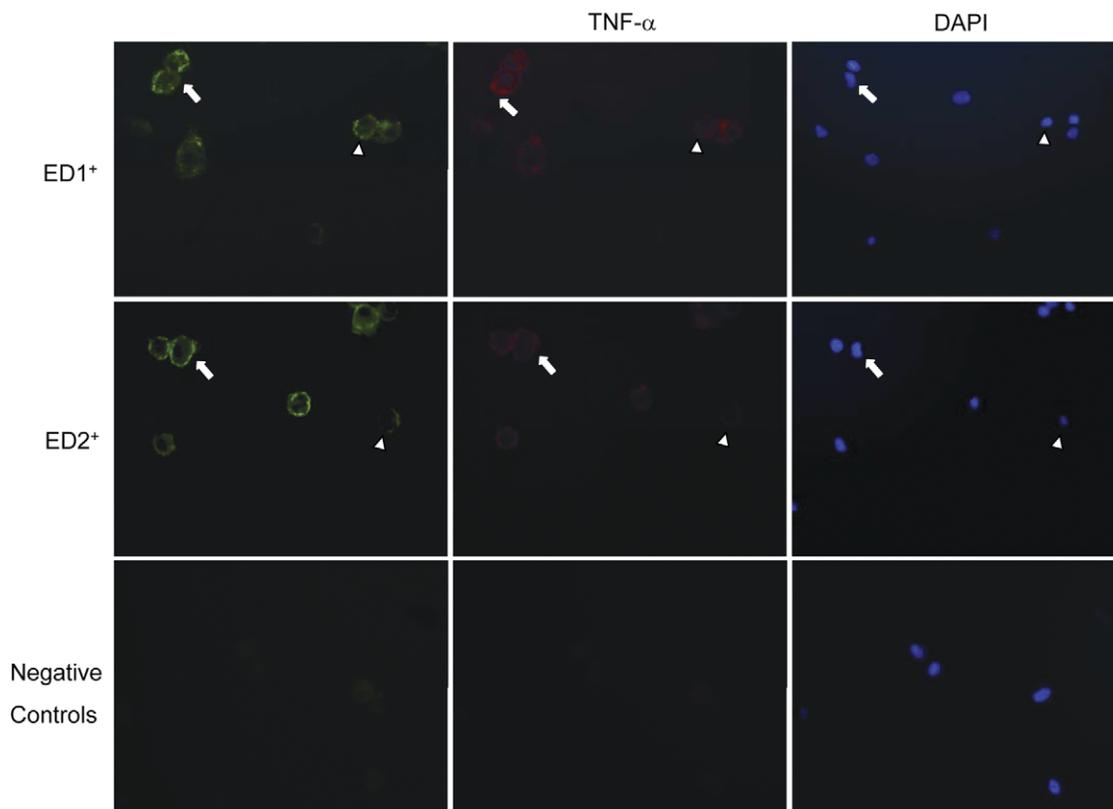
The present results highlight the role of testicular macrophages and TNF- $\alpha$  in the induction of GC apoptosis during the development of autoimmune orchitis.

The fact that CMTM obtained from rats with orchitis induced apoptosis of GCs from EAO rats but not from N rats suggests that the increased expression of cytokine receptors induced by the inflammatory testicular micro-environment during orchitis may sensitize them to the soluble factors present in the conditioned media. In fact, we have previously shown an increased number of GCs expressing TNFR1 and Fas in this model (Suescun *et al.*, 2003; Theas *et al.*, 2003). Importantly, CMTM from EAO but not from C or N rats induced apoptosis of GCs suggesting that only soluble products

released by macrophages obtained from rats with orchitis are able to induce testicular damage.

In the present study, we showed that in rats with severe orchitis TNF- $\alpha$  production by testicular macrophages was higher compared with C rats, however, TNF- $\alpha$  secretion per macrophage was lower. Based on the present and previous data showing that the number of interstitial macrophages increases in the testis of EAO rats (Rival *et al.*, 2008), we postulate that the higher production of TNF- $\alpha$  depends on the increased number of macrophages. The reduced TNF- $\alpha$  secretion per macrophage observed in rats with severe orchitis compared with C might reflect a self control mechanism to adjust the level of TNF- $\alpha$  with the decreased number of target GCs present in the damaged seminiferous tubules. A possible explanation for this phenomenon could be that a different regulation of the TNF- $\alpha$  converting enzyme, which converts membrane bound pro TNF- $\alpha$  to active and soluble TNF- $\alpha$ , might participate in the modulation of TNF- $\alpha$  release, as was demonstrated in other inflammatory models (Colón *et al.*, 2001; Doggrell, 2002).

The facts that TNF- $\alpha$  neutralization reverted the apoptotic effect of the CMTM and that this cytokine induced apoptosis of GCs *in vitro* suggest that the TNF- $\alpha$  released by testicular macrophages may be an important factor responsible for GC apoptosis in severe autoimmune orchitis. Also, in an *in vitro* system of tissue damage, neutralization of TNF- $\alpha$  prevented the apoptosis induced by activated macrophages (Satsu *et al.*, 2006). Although



**Figure 5:** TNF- $\alpha$  expression in ED1<sup>+</sup> and ED2<sup>+</sup> testicular macrophages isolated from a rat with severe orchitis. Secondary antibodies conjugated with fluorescein isothiocyanate or rhodamine were used to detect ED1<sup>+</sup> and ED2<sup>+</sup> macrophages or TNF- $\alpha$ <sup>+</sup>, respectively. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). In negative controls, the first antibody was omitted. Arrows indicate double positive cells. Arrow heads indicate macrophages that do not express TNF- $\alpha$ ,  $\times 375$ .

we showed an apoptotic effect of TNF- $\alpha$  on GCs of rats undergoing EAO, Pentikäinen *et al.* (2001) demonstrated that this cytokine prevents human GC apoptosis induced *in vitro* by serum deprivation. This discrepancy may be explained by the different experimental conditions and species studied. For instance, Riccioli *et al.* (2000) postulated that TNF- $\alpha$  may act as a survival factor at physiologically low concentrations present in the normal mice testis and as a proapoptotic cytokine during inflammatory disease. In accordance, the high levels of TNF- $\alpha$  induced by the intratesticular injection of this cytokine in rats or those present in the testis of rats treated with MEHP (a toxicant of Sertoli cells) are associated with the disruption of spermatogenesis and loss of GCs (Li *et al.*, 2006a; Yao *et al.*, 2007). Also, in another model of testicular chronic inflammation induced by the expression of p450 aromatase, an increased number of testicular macrophages expressing TNF- $\alpha$  was associated with spermatogenic disruption and GC sloughing (Li *et al.*, 2006b).

We have previously shown that the number of apoptotic GCs significantly increases from focal to severe orchitis (Theas *et al.*, 2003). As testicular TNF- $\alpha$  content is similar in both EAO stages, we speculate that the increased expression of TNFR1 during severe orchitis (Suescun *et al.*, 2003) renders GCs more sensitive to TNF- $\alpha$  effect. Other soluble factors may be responsible for GC apoptosis during focal orchitis. NO and IFN- $\gamma$  that have been reported to act as effectors of cell death (Chen *et al.*, 2002; Liversidge *et al.*, 2002) may participate as potential inducers of apoptosis in this model since we observed a high content of these factors in the CMTM from rats with EAO (Jarazo-Dietrich, 2006, personal communication; Rival *et al.*, 2006, 2008). As a synergistic effect of TNF- $\alpha$  with NO on apoptosis has been reported (Boyle *et al.*, 2003), we propose that during focal orchitis these two factors may act together to induce GC damage. In fact, it has been postulated that in the testis TNF- $\alpha$  induces apoptosis of GCs indirectly through NO produced by Sertoli cells (Stéphan *et al.*, 1995; de Souza *et al.*, 2005). The *in vivo* experiments of Yule and Tung (1993) and Itoh *et al.* (1998) showed the partial abrogation of orchitis by TNF- $\alpha$  or IFN- $\gamma$  neutralization, suggesting that *in vivo* a redundant action of proinflammatory cytokines is needed in order to induce GC damage. In fact, we demonstrated that IL-6 is able to induce apoptosis of GCs (Rival *et al.*, 2006). Therefore, TNF- $\alpha$  may be one of the major factors involved in GC apoptosis during severe orchitis but it is not the only one. Moreover, as TNF- $\alpha$  has a main role on GC apoptosis in severe orchitis (late phase of the disease) we speculate that this effect is dependent on the changes induced in the testicular micro-environment by other cytokines or factors during the onset of orchitis.

It has been suggested that resident macrophages (ED2<sup>+</sup>) exhibit an immunosuppressive profile whereas non-resident macrophages (ED1<sup>+</sup>) preserve the pro-inflammatory status of circulating monocytes and may have different functional roles within the testis (Gerdprasert *et al.*, 2002; Guazzone *et al.*, 2003; Rival *et al.*, 2006). Unexpectedly, we observed TNF- $\alpha$  expression in ED2<sup>+</sup> testicular macrophages. As ED2 antiserum identifies both resident macrophages (ED2<sup>+</sup>) and the double positive macrophage subset (ED1<sup>+</sup> ED2<sup>+</sup>) which is the main cell population responsible for the increased number of interstitial macrophages in EAO (Rival *et al.*, 2008), TNF- $\alpha$  might be

expressed in this subset, that represents an intermediate differentiation stage between proinflammatory infiltrating monocytes (ED1<sup>+</sup>) and resident immunosuppressor macrophages (ED2<sup>+</sup>).

TNF- $\alpha$  signalling to induce cell death through the TNFR1 death receptor has been well characterized. Upon binding its receptor, TNF- $\alpha$  activates initiator caspase 8 that induces downstream effector procaspases to kill the cell (Zimmermann *et al.*, 2001). Apoptosis may be triggered by TNFR1-induced proteins that activate the mitochondrial pathway such as Bid and Bax (Pei *et al.*, 2007). In EAO, we have previously demonstrated caspase 8 activation and also Bax translocation to mitochondria, suggesting that TNF- $\alpha$  may activate these apoptotic pathways upon binding to TNFR1 in GCs (Theas *et al.*, 2006). In fact, Chen *et al.* (2003) reported that the supernatant of activated macrophages containing TNF- $\alpha$  and IFN- $\gamma$  induces caspase 8 activation, Bax expression and apoptosis of glioma cells.

The efficiency of anti-TNF- $\alpha$  treatment in EAO remains controversial. Initially, Teuscher *et al.*, (1990) demonstrated that *i.v.* administration of anti-TNF- $\alpha$  IgG in mice actively immunized with spermatogenic antigens and adjuvants did not influence the pathogenesis of orchitis. However, these authors were unable to maintain a continued presence of anti-TNF- $\alpha$  IgG to cover the full period between the challenge and sacrifice of the animals. On the other hand, Yule and Tung (1993) defined TNF- $\alpha$  as an important cytokine in the pathogenesis of autoimmune orchitis developed in mice by adoptive transfer with T cells since *i.p.* neutralization of the cytokine reduced the incidence and severity of the disease. In human testicular pathology (Sertoli cell-only syndrome and GC arrest), an increase in the number of macrophages expressing TNF- $\alpha$  and other proinflammatory cytokines (IL-6, IL-1) has been reported (Frungeri *et al.*, 2002). Also, a role for TNF- $\alpha$  as a paracrine factor modulating human peritubular cell function has been recently established (Schell *et al.*, 2008). These results indicate a potential role for TNF- $\alpha$  in controlling human testicular function in health and disease. Researches based on models of autoimmune orchitis are useful to study the interactions among immune and GCs and to identify the mediators involved in GC damage during a chronic inflammation of the testis. We postulate that in our model of orchitis it is the intratesticular, and not the systemic, TNF- $\alpha$  cytokine that induces testicular damage, since we were unable to detect it in the serum of EAO rats (Suescun *et al.*, 2003). In conclusion, we demonstrated in autoimmune orchitis an increase in TNF- $\alpha$  secretion by testicular macrophages and the local effect of this cytokine in GC apoptosis. Since TNF- $\alpha$  neutralization abrogated this phenomenon, this cytokine might be a target for specific therapy preventing testicular autoimmune reactions. The beneficial effect of therapy with TNF- $\alpha$  antagonist (Etanercept and Infliximab) in other human autoimmune diseases has been well defined (Scallon *et al.*, 2002).

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