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Original Paper

Functional and phenotypical characteristics of testicular macrophages in experimental autoimmune orchitis

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Abstract

Testicular inflammation with compromised fertility can occur despite the fact that the testis is considered an immunoprivileged organ. Testicular macrophages have been described as cells with an immunosuppressor profile, thus contributing to the immunoprivilege of the testis. Experimental autoimmune orchitis (EAO) is a model of organ-specific autoimmunity and testicular inflammation. EAO is characterized by an interstitial inflammatory mononuclear cell infiltration, damage to the seminiferous tubules and germ cell apoptosis. Here we studied the phenotype and functions of testicular macrophages during the development of EAO. By stereological analysis, we detected an increased number of resident (ED2$^+$) and non-resident (ED1$^+$) macrophages in the testicular interstitium of rats with orchitis. We showed that this increase was mainly due to monocyte recruitment. The *in vivo* administration of liposomes containing clodronate in rats undergoing EAO led to a reduction in the number of testicular macrophages, which correlated with a decreased incidence and severity of the testicular damage and suggests a pathogenic role of macrophages in EAO. By immunohistochemistry and flow cytometry we detected an increased number of testicular macrophages expressing MHC class II, CD80 and CD86 costimulatory molecules in rats with orchitis. Also, testicular macrophages from rats with EAO showed a higher production of IFNγ (ELISA). We conclude that testicular macrophages participate in EAO development, and the ED1$^+$ macrophage subset is the main pathogenic subpopulation. They stimulate the immune response through the production of pro-inflammatory cytokines and antigen presentation and thus activation of T cells in the target organ.

Keywords: testicular macrophages; autoimmune orchitis; MHC II molecules; costimulatory molecules; cytokines; testosterone; FSH, LH

Introduction

Testicular macrophages have been implicated in the induction of the immunosuppressor microenvironment [1–3]. However, the behaviour of these cells in normal and inflammatory conditions is insufficiently explored. In the normal rat testis, most (~80%) testicular macrophages express CD163, a marker of tissue resident macrophages [4], recognized by the ED2 antibody. In addition, a minor (~15–20%) proportion express the ED1 antigen, expressed on monocytes or recently arrived macrophages [5–7]. About 50% of the ED2$^+$ subset also reacts with ED1 antibody, suggesting an intermediate stage between the recently arrived ED1$^+$ monocytes and the resident macrophages.

Experimental autoimmune orchitis (EAO) is an organ-specific model of autoimmunity characterized by a testicular interstitial lymphomononuclear infiltration, apoptosis and sloughing of germ cells from damaged seminiferous tubules [8,9]. We previously demonstrated the high production of tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6) [10,11]. The aim of this work was to study the phenotypical characteristics of testicular macrophages in normal and chronically inflamed testes and to evaluate the pathogenic role of these cells in EAO induction.

Materials and methods

Animals

Male Sprague–Dawley rats aged 50–60 days were used and killed according to protocols for animal use, in accordance with NIH guidelines for the care and use of experimental animals.

Immunization schedule

Rats from the experimental (E) group were immunized with testicular homogenate (TH), prepared as previously described [8]. Briefly, adult (50–60 day-old) rats were injected three times with 200 mg w/w TH/dose, emulsified with complete Freund's adjuvant (CFA; F-5881, Sigma Chemical Co., St. Louis, MO, USA). *Bordetella pertussis* (Bp) was used as co-adjuvant. Control (C) rats were injected with an emulsion of saline solution, CFA and Bp following the schedule described above. Normal (N) untreated rats were also studied. The rats were killed at different time periods (7–30, 50–60, 70–100 days) after the first immunization. The testes were removed, weighed and used as described below. Blood was collected and sera were obtained.

Histopathology

The histopathology of the testis was studied in paraffin-embedded Bouin's-fixed sections. To evaluate the extent of germ cell damage that occurs in EAO, we developed a EAO scoring system, determining: (a) the percentage of damaged seminiferous tubules (ST); (b) the degree of the damage (sloughing and/or the presence of degenerating germ cells); and (c) the testicular : body weight ratio (T : Bw) (see Supplementary Material, Figure S1, available at: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2328.html).

Immunohistochemistry

Acetone-fixed frozen testicular sections (6–7 μm) were incubated with 5% BSA followed by avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA, USA). The following primary antibodies were used: ED1 (10 μg/ml; 554 954, BD Pharmingen, San Diego, CA, USA), ED2 (5 μg/ml; 554 900, BD Pharmingen), MHC II (2 μg/ml; 554 929, BD Pharmingen), CD80 (4 μg/ml; sc-1632, Santa Cruz Biotechnology, USA) and CD86 (4 μg/ml; 551 396, BD Pharmingen). A biotinylated rabbit anti-goat IgG (4 μg/ml, BA-5000, Vector Laboratories) or horse anti-mouse IgG (2 μg/ml; BA-2001, Vector Laboratories) were used as secondary antibodies. The reaction was amplified using the Vectastain Elite ABC Kit (PK-6200, Vector Laboratories) and the reaction product visualized by adding diaminobenzidine substrate (SK-4100, Vector Laboratories). For ED1 and ED2 antibodies, positively immunostained cells were counted as previously described [10].

Expression of costimulatory molecules (flow cytometry)

Interstitial testicular cells were obtained as previously described [10]. Cells were incubated with the unlabelled ED1 or ED2 antibodies, followed by a FITC-conjugated anti-mouse IgG (FI-2001, Vector Laboratories) and finally with the following combinations of antibodies: CD45 (559 135, PE-Cy5) plus MHC II (554 929, PE), CD45 plus CD80 (555 014, PE) or CD45 plus CD86 (551 396, PE). The appropriate control isotypes were used. For intracellular labelling (ED1 antibody), the cells were fixed with 2% paraformaldehyde, permeabilized and washed with 0.1% saponin in PBS. BD FACScalibur cytometer was used and ~30 000 events in the $CD45^+$ gate were acquired.

Obtaining testicular macrophage conditioned media (MCM)

Interstitial testicular cells were obtained as previously described [10] and cultured for 2 h to allow the attachment of macrophages to the dishes. Unattached cells were removed by washing with PBS and the attached cells were cultured in M199 plus antibiotic–antimycotic solution (1×; 15 240-096, Gibco, Grand Island, NY, USA). After 20 h culture at 34 °C, supernatants (MCM) were collected. Adherent cells were harvested and counted, while cell viability was assessed by the Trypan blue exclusion method. The purity of the macrophage preparations was 80–90%, as previously described [10].

Determination of cytokines by ELISA

Commercial specific ELISA kits for IL-10, IFNγ, GM-CSF and TGFβ were used to quantify the content of these cytokines in testicular MCM. The minimum detectable concentration for each cytokine was IL-10 $<$ 5 pg/ml, IFNγ $<$ 13 pg/ml and TGFβ $<$ 15.6 pg/ml. All procedures followed the manufacturer's instructions.

Depletion of macrophages

In order to deplete macrophages, rats were injected with dichloromethylene biphosphonate (clodronate) containing liposomes. Clodronate- (gift of Roche Diagnostics GmbH, Mannheim, Germany) and PBS-loaded liposomes were prepared as previously described [12]. As preliminary experiments, injecting PBS-liposomes into the testis (via subalbuginea) using a 30 G needle, showed that the injection *per se* induced damage of the testicular parenchyma, all further experiments used •intraperitoneal (i.p.) liposome administration. AQ1

Rats immunized with TH and adjuvants received 2 ml liposomes loaded with clodronate (Clod-Lip, $n = 12$) or PBS (PBS-lip, $n = 15$) i.p. twice weekly (every 2–3 days) from day 0 until euthanization (50–60 days after the first immunization). This dose and frequency of liposome administration have been previously used to fully eliminate blood monocytes [13].

Hormone determinations by radioimmunoassay

Serum LH, FSH and testosterone were measured by radioimmunoassay (RIA) as previously described [14]. The within-assay and interassay coefficients

of variations for both FSH and LH were <8% and <13%, respectively. The minimum detectable concentrations in the rLH, rFSH and testosterone assays were 0.2 ng/ml, 1.0 ng/ml and 12.5 pg/ml, respectively. Testicular homogenates for determination of testosterone content were prepared as previously described [15].

Statistical analysis

Results are expressed as mean ± SEM. Comparisons of groups were assessed using the statistical tests indicated in the legend of each figure. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Histopathology

As described previously [8], a mild infiltration of lymphomononuclear cells with several foci of seminiferous tubules showing germ cell sloughing and degenerating spermatocytes and spermatids were observed during the onset of EAO (50–60 days after the first immunization; Figure 1A). From 70 days on, the inflammatory infiltrate was more abundant and only Sertoli cells and some degenerating germ cells remained in the seminiferous tubules (Figure 1B). In contrast to murine EAO, inflammatory cells were never observed inside the seminiferous tubules. None of the control rats showed pathological alterations of the testis (Figure 1C).

We have previously shown that progression of EAO is associated with infertility and testicular atrophy [8]. Germ cell apoptosis is observed from the EAO onset and an increase in the number of immature germ cells in the epididymal tubules is detected from day 70 on [9]. Thus, in EAO, parameters such as germ cell sloughing and testicular atrophy (associated with the degree of infiltrating interstitial immune cells), were selected to develop a new EAO scoring system, in order to quantify the extent of testicular damage that occurs during EAO development

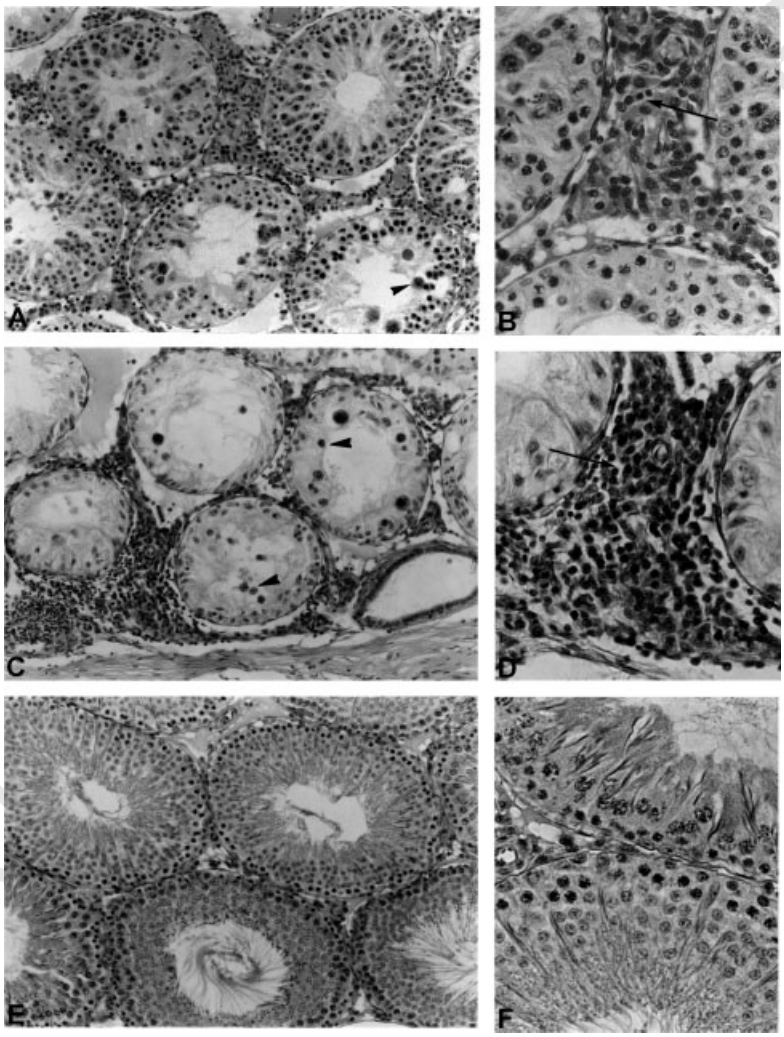

Figure 1. Testicular histopathology. Testis sections from experimental rats with focal (A, B) and severe orchitis (C, D) or from the control group (E, F). Mild (A, B) and severe (C, D) germ cell sloughing and the presence of degenerated germ cells (arrowheads) are shown during onset and advanced EAO. Interstitial inflammatory mononuclear cells (arrow) are abundant in rats with severe orchitis (C, D). Control rats showed normal testicular histology (E, F)

(see Supplementary material, Figure 1S, available at: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2328.html).

Testicular macrophages are increased in number

By immunohistochemistry we identified the recently arrived ED1$^+$ monocytes/macrophages and the ED2$^+$ resident macrophages in the normal and inflamed testis. We quantified the number of testicular macrophages during the immunization period (7–30 days), disease onset (50–60 days) and the severe testicular damage period (70–100 days); 7–30 days after the first immunization, when the experimental rats showed no histopathological alterations of the testis, the number of testicular macrophages (total and from each subset) were similar between the control and experimental groups (Table 1). However, an increased number of total testicular macrophages was observed in control rats compared to normal rats, due to an increase in the three subpopulations analysed, the ED1$^+$ ED2$^-$ subset being the main contributor (4.7-, 2.9- and 1.9-fold increase of control versus normal for ED1$^+$ ED2$^-$, ED1$^+$ ED2$^+$ and ED2$^+$ ED1$^-$, respectively; Table 1). Based on the wide variability of the EAO score observed in experimental rats during the disease onset (50–60 days), the number of testicular macrophages was analysed separately in rats with an EAO score of 1–4 or 5–7. An association between the degree of testicular damage and the number of testicular macrophages was observed. Rats with mild orchitis (EAO score 1–4) were found to have a number of testicular macrophages similar to control rats, while an increased number of these cells was detected in experimental rats with an EAO score of 5–7 (Table 1). The increase was due to a higher number of double-positive macrophages. In severe EAO (70–100 days), an increased number of total as well as double-positive macrophages compared to control rats was also observed (Table 1).

Expression of costimulatory molecules by testicular macrophages

By immunohistochemistry we detected MHC class II$^+$ cells in the interstitium of control and experimental rats. A large number of MHC class II$^+$ cells, probably macrophages and/or dendritic cells, was observed in the interstitium of rats with orchitis, being mainly concentrated in granulomae (Figure 2). Similarly, cells expressing CD80 and CD86 costimulatory molecules showed an analogous localization and morphology (data not shown).

The expression of MHC class II and costimulatory molecules in the testicular macrophages was further analysed by flow cytometry. The common leukocyte marker, CD45, was used to identify the whole population of leukocytes in the testicular interstitium (Figure 3). An increased number of CD45$^+$ cells was detected in the testes of experimental rats compared to control and normal groups (see Supplementary Material, Table 1S, available at: htttp//www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2328.html), reflecting the increased influx of leukocytes in the inflamed testis in EAO. Pooled results from the 50–60 and 70–100 day groups are shown, since a similar pattern of costimulatory and MHC class II molecules expression was observed. An increased number of ED1$^+$ testicular macrophages expressing MHC Class II, CD80 and CD86 was observed in rats with EAO compared to normal and control rats (Figure 4A), while the percentage of positive macrophages was comparable (see Supplementary Material, Table 1S). Similar results were obtained for ED2$^+$ testicular macrophages expressing MHC Class II and CD80 molecules, although the number (Figure 4B) and the percentage (Table 1S) were much lower compared to ED1$^+$ testicular macrophages.

Only ED1$^+$ testicular macrophages from rats with EAO showed an increased MHC Class II expression compared to normal rats (Figure 4C), while the mean

Table 1. Number ($\times 10^6$) of testicular macrophages per testis

	Days	ED1$^+$ ED2$^-$	ED1$^+$ ED2$^-$	ED2$^+$ ED1$^-$	Total
Normal	—	0.64 ± 0.32	3.76 ± 0.26	3.56 ± 0.34	7.46 ± 1.13
Control	7–30	3.00 ± 0.51	10.75 ± 1.80	6.61 ± 1.39	19.60 ± 1.59
Experimental		3.87 ± 0.80[a]	11.95 ± 1.13[a]	6.78 ± 1.21[b]	21.63 ± 1.89[a]
Control	50–60	3.54 ± 0.40	10.21 ± 1.67	5.56 ± 0.74	19.31 ± 1.54
Experimental					
EAO score 1–4		2.40 ± 0.49	14.37 ± 0.94	4.87 ± 1.12	20.84 ± 0.78
EAO score 5–7		5.22 ± 0.95	20.51 ± 1.34[c]	7.55 ± 0.92	33.28 ± 2.31[c]
Control	70–100	5.10 ± 0.85	9.66 ± 1.89	9.47 ± 1.46	21.76 ± 2.50
Experimental		5.31 ± 1.07	20.45 ± 1.90[d]	6.22 ± 0.96	31.98 ± 0.76[d]

Number of labelled macrophages was estimated by ED1 and ED2 immunohistochemistry in testis sections of rats from normal, control and experimental groups sacrificed at different time periods after the first immunization. Total number of macrophages was quantified using ED1 and ED2 antibodies simultaneously. Data of the different subsets were obtained as follows: number of ED1$^+$ ED2$^-$ cells = total − ED2$^+$ cells; number of ED2$^+$ ED1$^-$ cells = total − ED1$^+$ cells; ED1$^+$ ED2$^+$ = ED1$^+$ cells − ED1$^+$ ED2$^-$ cells. Data are expressed as mean ± SEM. n = 5–8 rats/group/period of time.

[a] $p < 0.05$ versus respective subpopulation from N group.

[b] $p = 0.05$ versus respective subpopulation from N group.

[c] $p < 0.05$ versus respective subpopulation from C group (50–60 days).

[d] $p < 0.05$ versus respective subpopulation from C group (70–100 days; Mann–Whitney test).

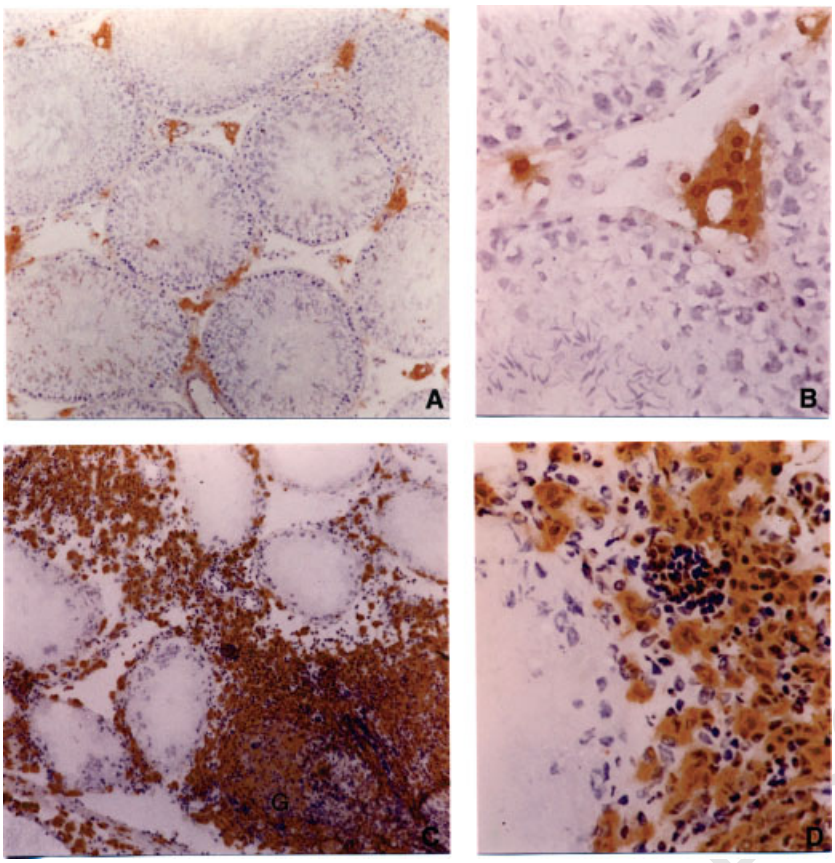

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Figure 2. Immunoperoxidase for MHC Class II molecules in testicular sections of rats from control (A, B) and experimental (C, D) groups. Positive cells are mainly located in the interstitium, at perivascular areas (A, B). In rats with orchitis (C, D) MHC^+ cells are observed in large numbers in peritubular areas and also concentrated in the granulomae (G)

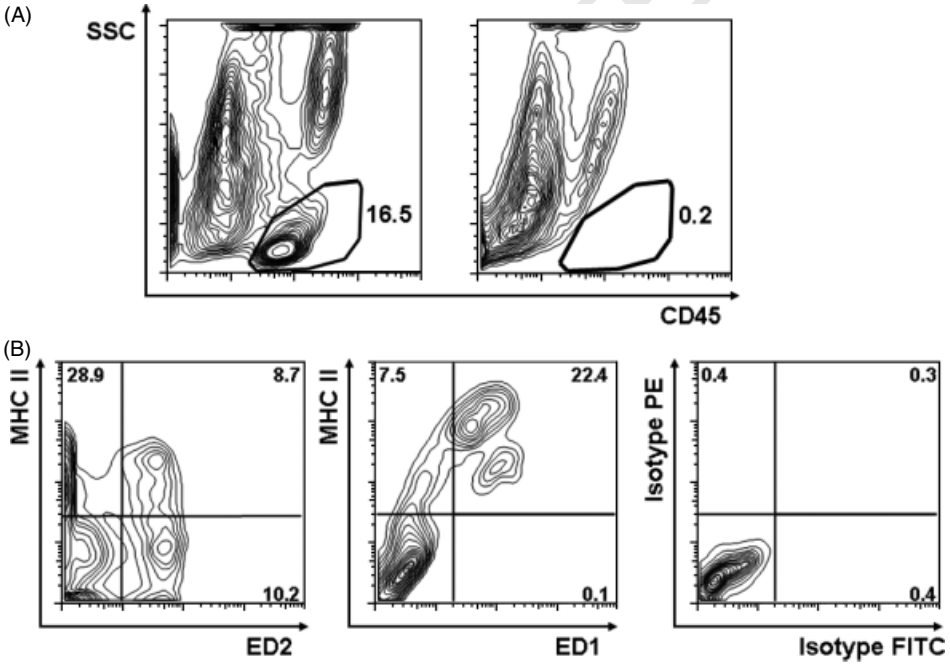

Figure 3. Representative flow cytometry contour plots of the testicular interstitial cells obtained from rats with orchitis. (A) An electronic gate was drawn selecting the total leukocyte population ($CD45^+$ cells, left plot) based on the isotype control IgG (right plot). Numbers are the percentages of $CD45^+$ cells. (B) The expression of MHC class II (left and middle plots) and costimulatory molecules (not shown) in the $ED2^+$ (left plot) and $ED1^+$ (middle plot) macrophages was analysed in the $CD45^+$ gate. Quadrants were set based on the isotypes control IgG (right plot). The percentages of cells in the different quadrants are indicated

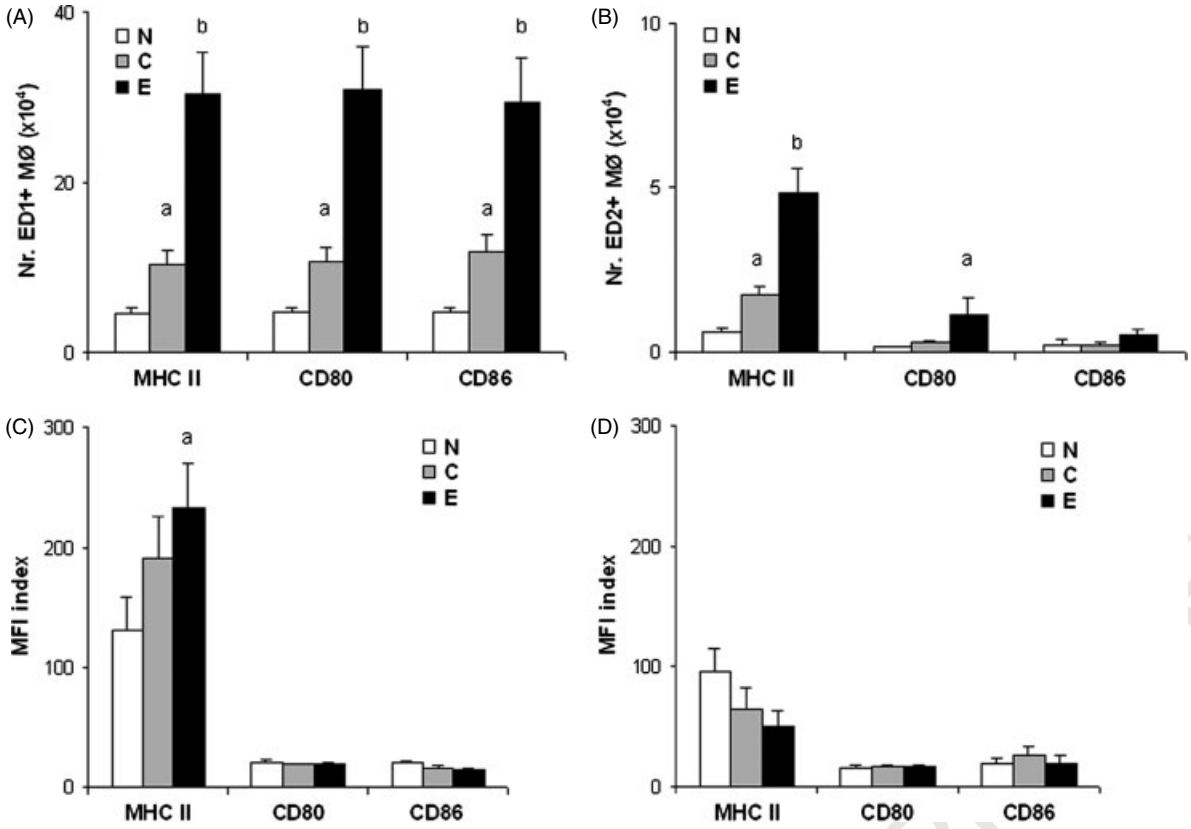

Figure 4. Expression of MHC Class II and costimulatory molecules by flow cytometry in the testis. Absolute number (Nr., $\times 10^4$) of ED1$^+$ (A) and ED2$^+$ (B) testicular macrophages expressing MHC class II, CD80 and CD86 from rats of normal (N), control (C) and experimental (E) groups. Note the different scales of the *y* axes in plots (A) and (B). Mean fluorescence intensity (MFI) for the expression of MHC Class II, CD80 and CD86 molecules on ED1$^+$ (C) and ED2$^+$ (D) macrophages of N, C and E rats. MFI index was calculated as MFI of positive population/MFI of negative population. Data are expressed as mean ± SEM. $n = 7$ rats/group. (A) $p < 0.05$ versus respective N; (B) $p < 0.01$ versus respective N and C (Student's *t*-test for absolute numbers and median test for MFI index)

fluorescence intensity for MHC Class II, CD80 and CD86 molecules were similar in ED2$^+$ macrophages from the testes of normal, control and experimental rats (Figure 4C, D). In addition, ED1$^+$ macrophages showed a higher expression of MHC class II molecules compared to ED2$^+$ cells in the testes from control and experimental rats (Figure 4C, D).

Production of cytokines by testicular macrophages

The production of IFNγ, GM-CSF, TGFβ and IL-10 by testicular macrophages was measured in the MCM from normal, control and experimental rats by ELISA. We observed that testicular macrophages obtained from rats of the experimental group produced higher levels of IFNγ compared to control rats (Figure 5). The concentration of GM-CSF and IL-10 was similar in the MCM obtained from control and experimental rats (data not shown) and TGFβ was undetectable in all the samples analysed.

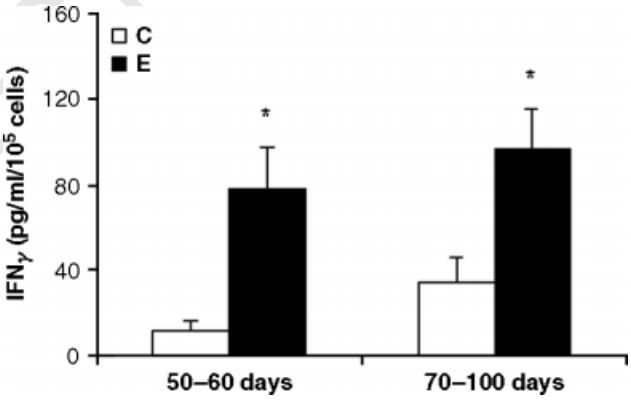

Figure 5. Concentration of IFNγ (pg/ml/10^5 cells) in the conditioned medium of testicular macrophages obtained from rats of the control (C) and experimental (E) groups. Data are expressed as mean ± SEM. $n = 4$–5 rats/group. $^*p < 0.05$ versus respective control (Kruskal–Wallis ANOVA)

Macrophages are required for EAO development

In order to evaluate the role of macrophages in EAO, we depleted circulating monocytes via i.p. injection of clodronate-containing liposomes (Clod-lip). As shown in Table 2 and Figure 6, 2.3- and 2.6-fold reductions in the number of testicular ED1$^+$ and ED2$^+$ cells, respectively, was observed after Clod-lip administration. However, while the number of ED2$^+$ macrophages in the testis of Clod-lip-treated rats was similar to that of normal rats, the number of ED1$^+$ cells remained higher compared to control

Table 2. Number ($\times 10^6$) of testicular macrophages per testis

Group	ED1$^+$ macrophages	ED2$^+$ macrophages
Normal	4.40 ± 0.49	7.32 ± 0.57
Control (50–60 days)	13.76 ± 1.58	18.45 ± 1.53
PBS-lip	25.44 ± 2.33	20.44 ± 2.64
Clod-lip	10.96 ± 0.93*	7.96 ± 0.74*

The number of ED1$^+$ and ED2$^+$ macrophages was quantified by immunohistochemistry on testis sections of rats from the normal and control groups and from rats immunized with TH and adjuvants and injected with liposomes containing PBS (PBS-lip) or clodronate (Clod-lip), sacrificed 50–60 days after the first immunization. Data are expressed as mean ± SEM. $n = 5$–10 rats/group.
* $p < 0.05$ versus respective PBS-lip group (Mann–Whitney test).

rats (Table 2). The histopathological results showed that all rats from the experimental group treated with PBS-lip developed orchitis and that 73% also showed severe testicular damage (EAO score ≥4; Figure 7). In contrast, most of the rats (83%) injected with Clod-lip presented a mild presence (EAO score <4) or absence of testicular damage (Figure 7). In addition, rats from the Clod-lip group showed a significant reduction (2.2-fold) in the EAO score compared to PBS-lip rats (Figure 7). Moreover, we analysed whether Clod-lip treatment also induces depletion of testicular dendritic cells (DCs). We previously reported [24] an increased number of OX62$^+$ DC in the testes of rats with EAO compared to control rats [number ($\times 10^6$) of OX62$^+$ DCs/testis: normal, 4.47 ± 1.0; control, 4.67 ± 0.4; experimental, 10.8 ± 2.4]. Using the same methodology, we observed that rats injected with Clod-lip showed a similar number of OX62$^+$ DCs [6.49 ± 0.70 ($\times 10^6$)/testis] compared to normal and control rats (Clod-lip versus normal and control, $p > 0.05$, Kruskal–Wallis ANOVA).

Serum testosterone (T), FSH and LH and testicular T content in macrophage-depleted rats

Since testicular macrophages and Leydig cells are known to regulate each other, we investigated how macrophages from rats with EAO influence Leydig cell function. Rats immunized with TH and adjuvants

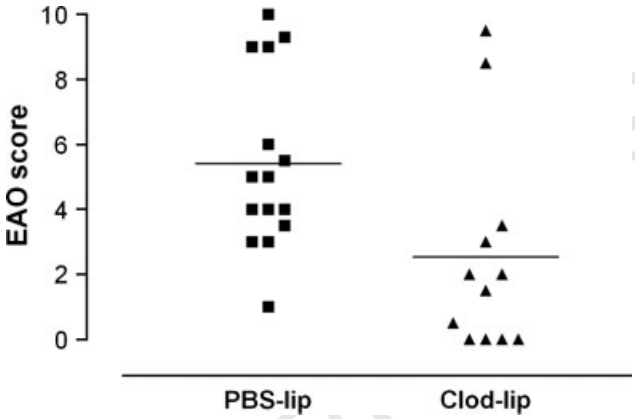

Figure 7. EAO score of testes from rats immunized with TH and adjuvants and injected with PBS (PBS-lip) or clodronate liposomes (Clod-lip) and sacrificed 50–60 days after the first immunization. Horizontal lines represent the mean. Each symbol represents a single rat. EAO score (mean ± SEM): PBS-lip, 5.4 ± 0.7; Clod-lip, 2.5 ± 0.9**; **$p = 0.006$ (Mann–Whitney test). $n = 12$–15 rats/group

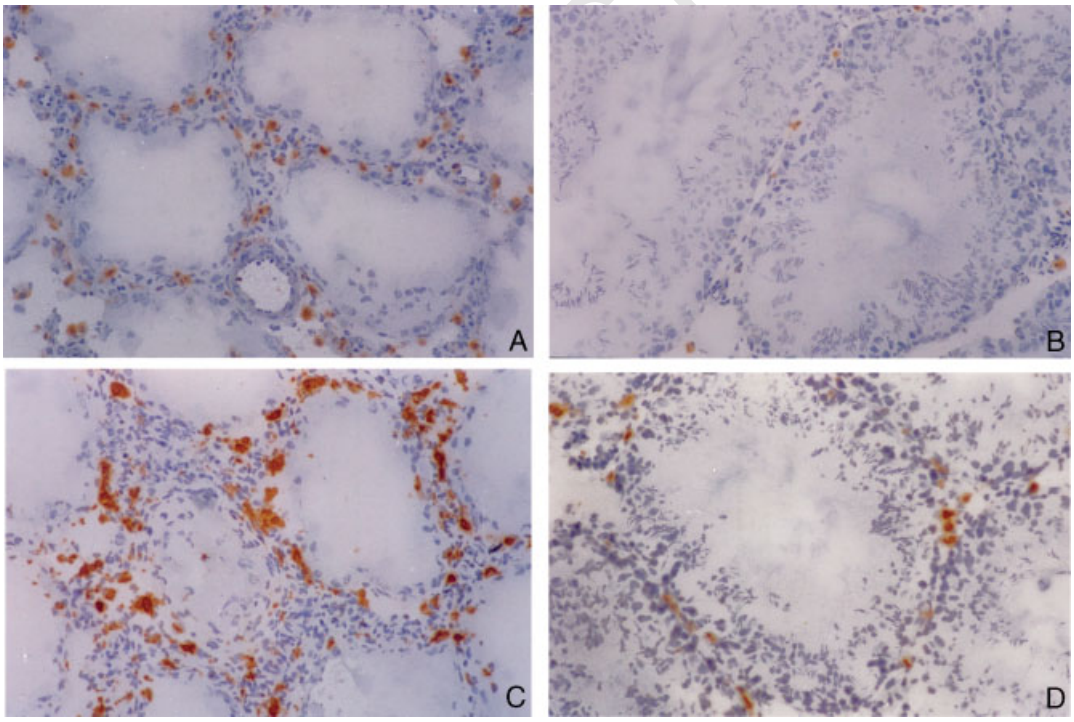

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Figure 6. Immunoperoxidase. Localization of ED1$^+$ (A, B) and ED2$^+$ (C, D) testicular macrophages in testicular sections from rats immunized with TH and adjuvants and injected with PBS (A, C) or clodronate (B, D) containing liposomes. Note the severe atrophy of the seminiferous tubules of the testis from rats with orchitis (A, C)

Table 3. Serum testosterone (T), FSH and LH levels and testicular T content in rats immunized with TH and adjuvants and injected with PBS or clodronate containing liposomes

Group (units)	Serum T (ng/ml)	Intratesticular T content (ng/testis)	Serum FSH (ng/ml)	Serum LH (ng/ml)
PBS-lip	3.0 ± 0.5	277.0 ± 24.9	11.8 ± 3.0	3.4 ± 0.7*
Clod-lip	2.6 ± 0.6	131.3 ± 28.2*	2.8 ± 0.1*	1.1 ± 0.3*

Hormone levels were measured by RIA in the sera and testes of rats immunized with TH and adjuvants and injected with liposomes containing PBS (PBS-lip) or clodronate (Clod-lip). Data are expressed as mean ± SEM. $n = 5–8$ rats/group. $^*p < 0.05$ versus respective PBS-lip group (Mann–Whitney test).

and injected with either Clod-lip or PBS-lip were analysed for serum testosterone, FSH and LH levels. Rats in the PBS-lip and Clod-lip groups showed similar levels of serum testosterone (Table 3), comparable to those previously observed in normal, control and experimental rats in the same experimental model [15]. However, a 1.9-fold increase in T content was observed in rats from the PBS-lip group compared to rats from the Clod-lip group. Consistent with our previous data showing a four-fold increase in serum FSH levels in rats with EAO compared to control group [15], rats immunized with TH and adjuvants and injected with PBS-lip exhibited higher FSH levels compared to rats from the Clod-lip group (Table 3).

Discussion

Macrophages constitute the main subpopulation of leukocytes in the testis of rats, mice and humans [16]. However, little is known about the role of macrophages in testicular inflammation/infection. In rats, the subpopulations of resident ($ED2^+$) and non-resident ($ED1^+$) testicular macrophages have been described previously [6]. This heterogeneity in the phenotype has been correlated with functional differences: the expression of several pro-inflammatory molecules was detected mainly in $ED1^+$ testicular macrophages [7,11,17,18]. In this context, $ED2^+$ testicular macrophages could be termed M2 macrophages with an immunosuppressive profile, while the $ED1^+$ subset is comparable to the pro-inflammatory M1 macrophages [19]. These authors highlighted the plasticity and functional polarization of macrophages, which crystallizes a continuum of diverse developmental stages from M1 mononuclear phagocytes to the various M2 macrophages.

Thus, we considered it interesting to analyse the number of macrophages from each subpopulation present in the testis during the development of EAO. We showed that the number of total testicular macrophages (ED1 + ED2) increased when rats immunized with TH and adjuvants developed testicular damage. This association suggests that testicular macrophages play a pathogenic role in EAO by inducing, directly or indirectly, germ cell apoptosis and sloughing from the germinal epithelium. The double positive ($ED1^+$ $ED2^+$) cells were the main subpopulation contributing to the increase in the total number of testicular macrophages. It has been postulated that this double-positive subset represents a transition stage between the $ED1^+$ $ED2^-$ monocytes/macrophages recently arrived from the circulation and the $ED2^+$ $ED1^-$ resident macrophages [6]. Therefore, it could be expected that the high number of the double-positive subset observed during multifocal EAO would lead to an increased number of $ED2^+$ cells in the next period of time studied (70–100 days). However, we showed that during severe EAO, an increased number of double-positive macrophages also occurred, with no concomitant increase in the $ED2^+$ resident macrophage subset. These findings led us to speculate that the $ED1^+$ $ED2^+$ macrophages remained in an 'undifferentiated' stage, closer to the profile of their $ED1^+$ $ED2^-$ precursors, probably under the influence of pro-inflammatory factors such as TNFα, MCP-1 and IL-6 that are found at higher concentrations in the testis of rats with orchitis [10,11,17]. Accordingly, in control rats sacrificed 70–100 days after the first immunization, the number of $ED2^+$ $ED1^-$ resident macrophages was increased compared to the 50–60 day period, probably via the differentiation of the high number of the double-positive subset observed in the latter period.

Interestingly, the activation of toll-like receptors (TLRs) by adjuvants induced an increase in the total number of testicular macrophages (control versus normal rats), where the $ED1^+$ $ED2^-$ subset was the main contributor. Similarly, Gerdprasert *et al* [20] showed that after the systemic administration of LPS, an increase in the number of the $ED1^+$ $ED2^-$ subpopulation was also detected. To further determine the role of testicular macrophages in the development of EAO, rats were injected with clodronate-containing liposomes. We observed a 2.3- and 2.6-fold reduction in the number of $ED1^+$ and $ED2^+$ testicular macrophages, respectively. The number of $ED2^+$ macrophages returned to values detected in the normal testis, while the number of $ED1^+$ testicular macrophages resembled those found in control rats. Since we cannot directly eliminate the testicular $ED2^+$ macrophages because the liposomes are not able to cross the endothelium [21], we can assume that the increased number of $ED2^+$ macrophages detected in EAO is due to the recruitment of $ED1^+$ monocytes from the circulation, and not as the result of the proliferation of resident macrophages, as was found for the normal testis [22]. Results showed that the depletion of macrophages significantly reduced the incidence and severity of EAO. Concomitantly, a decrease in the total number of testicular macrophages was observed. Taken together, these results suggest that testicular macrophages are required for the induction of EAO.

Hormone profiles of rats injected with PBS or clodronate liposomes confirm previous results obtained

for EAO and control rats [15], supporting the testicular histopathological observations. The fold increase in serum FSH, LH levels and testicular testosterone content observed in orchitic rats from the PBS-lip group compared to the Clod-lip group is similar to those previously detected in rats with EAO compared to controls [15]. We previously demonstrated [23] that factors secreted by testicular macrophages of rats with EAO are able to modulate Leydig cell steroidogenesis.

We recently showed an increase in the number of dendritic cells in the testis of rats with orchitis, suggesting that these cells are involved in the pathogenesis of EAO [24]. The fact that the administration of Clod-lip also prevented the increase in the number of testicular dendritic cells in rats immunized with testicular homogenate and adjuvants have hindered us in elucidating whether both subpopulations of APC (macrophages/DCs) are playing a central role in the development of EAO.

APCs, such as macrophages and dendritic cells, present self- and foreign peptides to $CD4^+$ T cells in the context of MHC Class II molecules and costimulatory signals. Sainio-Pollanen *et al* [25] showed no expression in normal murine testis of the costimulatory molecules CD80 and CD86. Therefore, a possible mechanism explaining testicular immunoprivilege was that T cells become anergic after an encounter with testicular APC lacking the second costimulatory signal. Our results indicate that the hypothesis of clonal anergy should be revisited and further functional studies done. In rats with EAO, we detected a higher number of macrophages expressing MHC Class II, CD80 and CD86 molecules. These results, together with our previous findings [26] showing an increased number of $CD4^+$ T cells in the testis of EAO rats, suggest the local antigen presentation and activation of the antigen specific T cells. In addition, the different expression pattern of MHC Class II and costimulatory molecules supports a distinct role for $ED1^+$ and $ED2^+$ macrophages, suggesting a proinflammatory profile for the $ED1^+$ macrophages.

It has been shown that certain APC subsets secrete IFNγ [27,28]. We detected a higher production of IFNγ by testicular macrophages from rats with orchitis compared to control rats, suggesting an activated status for these cells. IFNγ is able to tightly control leukocyte recruitment through modulation of chemokine production [29] and is a classic stimulus for APCs, increasing MHC Class II expression levels [30]. However, in spite of the higher content of IFNγ in the testes of rats with EAO, MHC Class II expression on testicular macrophages was not increased compared to control rats. We cannot rule out the possibility that testicular macrophages lack the expression of IFNγ receptors, or that they might have a different MHC Class II gene regulation compared to classic APCs. Our *in vivo* results suggest an active role of these cells in antigen presentation and activation of T lymphocytes, promoting the induction and maintenance of a testicular chronic inflammation.

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Supplementary material

Supplementary material may be found at the web address: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2328.html

References

1. Bergh A, Damber JE, van Rooijen N. The human chorionic gonadotrophin-induced inflammation-like response is enhanced in macrophage depleted rat testes. *J Endocrinol* 1993;**136:**415–420.
2. Hales DB, Diemer T, Hales KH. Role of cytokines in testicular function. *Endocrine* 1999;**10:**201–217.
3. O'Bryan MK, Schlatt S, Phillips DJ, de Kretser DM, Hedger MP. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels *in vivo*. *Endocrinology* 2000;**141:**238–246.
4. van den Berg TK, Dopp EA, Dijkstra CD. Rat macrophages: membrane glycoprotein differentiation and function. *Immunol Rev* 2001;**184:**45–57.
5. Hedger MP. Macrophages and the immune responsiveness of the testis. *J Reprod Immunol* 2002;**57:**19–34.
6. Wang J, Wreford NGM, Lan HY, Atkins R, Hedger MP. Leukocyte populations of the adult rat testis following removal of the Leydig cells by treatment with ethane dimethane sulfonate and subcutaneous testosterone implants. *Biol Reprod* 1994;**51:**551–561.
7. Gerdprasert O, O'Bryan MK, Nikolic-Paterson DJ, Sebire K, de Kretser DM, Hedger MP. Expression of monocyte chemoattractant protein-1 and macrophage colony-stimulating factor in normal and inflamed rat testis. *Mol Hum Reprod* 2002;**8:**518–524.
8. Doncel GF, Di Paola JA, Lustig L. Sequential study of the histopathology and cellular and humoral immune response during the development of an autoimmune orchitis in Wistar rats. *Am J Reprod Immunol* 1989;**20:**44–51.
9. Theas S, Rival C, Lustig L. Germ cell apoptosis in autoimmune orchitis involvement of the Fas–Fas L system. *Am J Reprod Immunol* 2003;**50:**166–176.
10. Suescun MO, Rival C, Theas MS, Calandra RS, Lustig L. Involvement of tumor necrosis factor-alpha in the pathogenesis of autoimmune orchitis in rats. *Biol Reprod* 2003;**68:**2114–2121.
11. Rival C, Theas MS, Guazzone VA, Lustig L. Interleukin-6 and IL-6 receptor cell expression in testis of rats with autoimmune orchitis. *J Reprod Immunol* 2006;**70:**43–58.
12. van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994;**174:**83–93.
13. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* 2006;**211:**609–618.
14. Suescun MO, Gonzalez SI, Chiauzzi VA, Calandra RS. Effects of induced hypo-prolactinemia on testicular function during gonadal maturation in the rat. *J Androl* 1985;**6:**77–82.
15. Suescun MO, Calandra RS, Lustig L. Alterations of testicular function after induced autoimmune orchitis. *J Androl* 1994;**15:**442–448.
16. Fijak M, Meinhardt A. The testis immune privilege. *Immunol Rev* 2006;**213:**66–81.
17. Guazzone VA, Rival C, Denduchis B, Lustig L. Monocyte chemoattractant protein-1 (MCP-1/CCL2) in experimental autoimmune orchitis. *J Reprod Immunol* 2003;**60:**143–157.

18. O'Bryan MK, Gerdprasert O, Nikolic-Paterson DJ, Meinhardt A, Muir JA, Foulds LM, *et al*. Cytokines profile in the testes of rats treated with lipopolysaccharide revealed localized suppression of inflammatory responses. *Am J Physiol Regul Integr Comp Physiol* 2005;**288:**R1744–1755.
19. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004;**25:**677–686.
20. Gerdprasert O, O'Bryan MK, Muir JA, Caldwell AM, Schlatt S, de Kretser DM, *et al*. The response of testicular leukocytes to lipopolysaccharide-induced inflammation: further evidence for heterogeneity of the testicular macrophage population. *Cell Tissue Res* 2002;**308:**277–285.
21. van Amerongen MJ, Harmsen MC, van Rooijen N, Petersen AH, van Luyn MJA. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am J Pathol* 2007;**170:**818–829.
22. Schlatt S, de Kretser DM, Hedger MP. Mitosis of resident macrophages in the adult rat testis. *J Reprod Fertil* 1999;**116:**223–228.
23. Suescun MO, Calandra RS, Lustig L. Effect of testicular macrophage conditioned media from rats with autoimmune orchitis on Leydig cell function. *Am J Reprod Immunol* 2000;**43:**116–123.
24. Rival C, Lustig L, Iosub R, Guazzone VA, Schneider E, Meinhardt A, *et al*. Identification of a dendritic cell population in normal testis and in chronically inflamed testis of rats with autoimmune orchitis. *Cell Tissue Res* 2006;**324:**311–318.
25. Sainio-Pollanen S, Saari T, Simell O, Pollanen P. CD28–CD80/CD86 interactions in testicular immunoregulation. *J Reprod Immunol* 1996;**31:**145–163.
26. Lustig L, Lourtau L, Perez RP, Doncel GF. Phenotypic characterization of lymphocytic cell infiltrates into the testes of rats undergoing autoimmune orchitis. *Int J Androl* 1993;**16:**279–284.
27. Frucht DM, Fukao T, Bogdan C, Schindler H, O'Shea JJ, Koyasu S. IFNγ production by antigen-presenting cells: mechanisms emerge. *Trends Immunol* 2001;**22:**556–560.
28. Bastos KR, Barboza R, Sardinha L, Russo M, Alvarez JM, Lima MR. Role of endogenous IFNγ in macrophage programming induced by IL-12 and IL-18. *J Interferon Cytokine Res* 2007;**27:**399–410.
29. McLoughlin RM, Witowski J, Robson RL, Wilkinson TS, Hurst SM, Williams AS, *et al*. Interplay between IFNγ and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J Clin Invest* 2003;**112:**598–607.
30. Dijkmans R, Billiau A. Interferon-γ: a master key in the immune system. *Curr Opinion Immunol* 1988;**114:**307–323.

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