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Loss of Occludin Expression and Impairment of Blood-Testis Barrier Permeability in Rats with Autoimmune Orchitis: Effect of Interleukin 6 on Sertoli Cell Tight Junctions[1]

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ABSTRACT

Inflammation of the male reproductive tract is accepted as being an important etiological factor of infertility. Experimental autoimmune orchitis (EAO) is characterized by interstitial lymphomononuclear cell infiltration and severe damage of seminiferous tubules with germ cells that undergo apoptosis and sloughing. Because the blood-testis barrier (BTB) is relevant for the protection of haploid germ cells against immune attack, the aim of this study was to analyze BTB permeability and the expression of tight junction proteins (occludin, claudin 11, and tight junction protein 1 [TJP1]) in rats during development of autoimmune orchitis. The role of IL6 as modulator of tight junction dynamics was also evaluated because intratesticular content of this cytokine is increased in EAO rats. Orchitis was induced in Sprague-Dawley adult rats by active immunization with testicular homogenate and adjuvants. Control rats (C) were injected with saline solution and adjuvants. Untreated (N) rats were also studied. Concomitant with early signs of germ cell sloughing, a reduced expression of occludin and delocalization of claudin 11 and TJP1 were detected in the testes of rats with EAO compared to C and N groups. The use of tracers showed increased BTB permeability in EAO rats. Intratesticular injection of IL6 induced focal testicular inflammation, which is associated with damaged seminiferous tubules. Rat Sertoli cells cultured in the presence of IL6 exhibited a redistribution of tight junction proteins and reduced transepithelial electrical resistance. These data indicate the possibility that IL6 might be involved in the downregulation of occludin expression and in the modulation of BTB permeability that occur in rats undergoing autoimmune orchitis.

autoimmune orchitis, blood-testis barrier, interleukin 6, testis, tight junction proteins

INTRODUCTION

Infection and inflammation of the male reproductive tract including the testes are widely accepted as important etiological factors of infertility [1]. Although most acute inflammatory disorders of the male genital tract are of infectious origin, a wide spectrum of other etiological factors may induce chronic testicular inflammation characterized by peritubular lymphocytic infiltrates, generally associated with damage of seminiferous epithelium and disruption of spermatogenesis. The most common experimental model for the investigation of autoimmune-based testicular impairment is autoimmune orchitis induced by active immunization with spermatic antigens [2].

Testicular damage in experimental autoimmune orchitis (EAO) is characterized by an increased number of T cells, macrophages, and dendritic cells that infiltrate the interstitium and by increased testicular content of proinflammatory cytokines [3–7]. Seminiferous tubules show apoptotic germ cells and different degrees of cell sloughing within the tubular lumen, with early dysfunction of adhesive mechanisms occurring among Sertoli and Sertoli/germ cells, which results in aspermatogenesis and atrophy [8, 9].

The germinal epithelium of the adult testis is composed of a fixed population of supporting Sertoli cells, a stem cell population of spermatogonia resting on the basal lamina and a mobile population of germ cells in continuous differentiation [10]. Adjacent Sertoli cells are connected by basal ectoplasmic specializations, adherens, and gap and tight junctions that constitute the blood-testis barrier (BTB) [11–13]. The BTB physically divides the seminiferous epithelium into a basal and an adluminal compartment. Spermatogonia and preleptotene spermatocytes are in the basal compartment and leptotene spermatocytes and spermatids are above the BTB, in the adluminal compartment [14]. Therefore, the BTB must disassemble and reassemble periodically to allow preleptotene and leptotene spermatocytes to gain entry into the adluminal compartment for further development [15, 16]. The primary function of the BTB is to create the appropriate microenvironment to control germ cell development and maturation, to protect haploid germ cells from harmful cytotoxic molecules, and to avoid the interaction of autoantigens with interstitial immune cells [17, 18].

Tight junctions are important structural components of the Sertoli cell junctional complex. Tight junctions are regions where plasma membranes of adjacent Sertoli cells form a number of contacts that appear to completely occlude extracellular space, thereby creating an intercellular barrier and intramembrane diffusion fence [19]. Unlike tight junctions in other epithelia, these junctions in the testis have a unique location: close to the basement membrane [20]. The tight junction structure consist of a transmembrane region that includes those molecules that mechanically confer adhesiveness to cells by binding to the same molecule on adjacent cells (occludin, the claudin multigene family, and junctional adhesion molecules) [21–23] and the plaque or peripheral region of the tight junction, consisting of molecules that anchor transmem-

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TABLE 1. Primary antibodies used for Western blot analysis.

Target protein	Working dilution	Animal source	Vendor
Occludin	1:300	Mouse	BD Bioscience
Claudin 11	1:1000	Rabbit	Abcam
TJP1	1:250	Rabbit	Invitrogen
p-MAPK14 (Thr180/Tyr182)	1:200	Mouse	Cell Signaling
MAPK14	1:500	Mouse	Santa Cruz Biotechnology
Actin	1:3000	Rabbit	Sigma-Aldrich

brane proteins to the tight junction structure and link them to the cell cytoskeleton (e.g., tight junction protein 1 [TJP1], formerly known as ZO-1) [24]. Signaling molecules and several transcription factors have also been identified in association with tight junctions [13]. Different factors some of which are cytokines (TNF alpha, TGF beta, and IL1 alpha), growth factors, testosterone and nitric oxide modulate junction dynamics and BTB function [25, 26].

Adherens and tight junction integrity is crucial for normal architecture of the seminiferous epithelium. Because cytoarchitecture is altered in autoimmune orchitis and severe germ cell sloughing occurs, we focused our study on the evaluation of tight junction protein expression and BTB functionality in this experimental model. With the aim of investigating factors that may modulate intercellular junctions in the seminiferous tubules, we also analyzed the in vivo and in vitro action of IL6, which is increased in the testes of rats with EAO, on tight junctions and BTB function.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats aged 50–60 days were kept at 22°C on a 12L:12D schedule and fed standard food pellets and water ad libitum. The use of rats followed National Institute of Health guidelines for care and use of experimental animals and was approved by the local committee (CICUAL, Facultad de Medicina, Universidad de Buenos Aires).

Induction of EAO

Rats in the experimental (E) group were immunized with testicular homogenate (TH) prepared as previously described [27]. Briefly, rats were injected three times with 200 mg of TH/dose/rat at 14-day intervals. TH (0.4 ml) emulsified with complete Freunds adjuvant (CFA) (0.4 ml; Sigma-Aldrich) was injected intradermally in the footpads and at multiple sites near the lymph nodes. The first two immunizations were followed by an intravenous injection of 0.5 ml *Bordetella pertussis* (Bp) (strain 10536; Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by intraperitoneal injection of 5×10^9 microorganisms. Control (C) rats were injected with an emulsion of saline solution and CFA; Bp was used as coadjuvant following the E group schedule. Normal untreated (N), E, and C rats were killed on different days after the first immunization—30 days (end of immunization period), 40 days (period previous to orchitis), 50 days (focal EAO), and 80 days (severe EAO)—and the testes were removed and weighed. One testis was processed for histopathology, and the other was quickly frozen for immunohistochemistry or used for Western blot or BTB permeability assays.

Histopathology

Testes were fixed in Bouin solution and embedded in paraffin. Testicular histopathology was analyzed in transversal sections obtained from the poles and equatorial areas stained with hematoxylin-eosin.

Immunofluorescence

Immunofluorescent analyses were performed on frozen testicular sections (5–7 μm) obtained from four to six animals per group. To study the distribution of occludin, sections were fixed in ethanol for 30 min at 4°C followed by acetone fixation at room temperature (RT) for 5 min. To detect claudin 11 and TJP1, sections were fixed in methanol at −20°C for 15 min. After blocking nonspecific sites with blocking solution (5% normal horse serum in 3% bovine serum albumin [BSA], 5% normal goat serum in 3% BSA, or 5% BSA) at RT for 30 min, sections were incubated with the corresponding primary antibodies—mouse anti-occludin, (1:5; Invitrogen), rabbit anti-claudin 11 (1:50; Santa Cruz Biotechnology), and mouse anti-TJP1-fluorescein isothiocyanate (1:50; Invitrogen)—diluted in blocking solution overnight at 4°C. After three washes with PBS, sections were incubated with the corresponding secondary antibodies—biotinylated horse anti-mouse immunoglobulin G [IgG] (1:50), fluorescein-conjugated goat anti-rabbit IgG (1:50), and biotinylated goat anti-fluorescein (1:100) (all from Vector Laboratories)—for 1 h at RT. To detect occludin and TJP1, immunostaining was amplified with fluorescein-conjugated neutralite avidin (1:200; Southern Biotechnology). For negative controls, the first antibodies were replaced with the corresponding isotype controls at the same concentration as the primary antibodies. After a nuclear staining step with DAPI (4′,6-diamino-2-phenylindole) (Sigma-Aldrich), the sections were mounted in buffered glycerin and observed by Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc.).

Western Blot Analysis

To detect occludin, claudin 11, and TJP1, testes were decapsulated and homogenized in three volumes of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 0.1% SDS, 0.5% sodium desoxycholate, and 1% NP-40) with protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin; Sigma-Aldrich). To detect phospho (p)-MAPK14, the following lysis buffer was used: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM ethylene glycol tetraacetic acid, with protease and phosphatase inhibitors (2 mM sodium fluoride and 50 mM sodium orthovanadate; Sigma-Aldrich). Homogenates were centrifuged at $13\,500 \times g$ for 30 min at 4°C.

Supernatant proteins were measured using the Bio-Rad DC Protein Assay. Equal amounts of protein (50 μg, or 300 μg to detect p-MAPK14) were diluted 1:1 in SDS-reducing buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), boiled for 5 min, and placed on ice before loading onto the gel. For detection of claudin 11, proteins were diluted in SDS sample buffer without β-mercaptoethanol and not boiled. Samples were resolved in 10% SDS-PAGE except for claudin 11, which was resolved in 15% SDS-PAGE. Proteins were electroblotted at 150 V for 60 min or 100 V for 90 min (claudin 11 and p-MAPK14) to polyvinylidene fluoride membranes (Immobilon P; Millipore Co). The transfer was monitored by Ponceau red staining. Prestained protein standards (Precision Plus Protein Standards; Bio-Rad) with a molecular weight range of 10–250 kDa were used. Membranes were blocked with 5% nonfat dry milk in TBS (10 mM Tris-HCl [pH 7.5], 0.9% NaCl) containing 0.1% Tween 20 for 1 h. Blots were probed overnight with the corresponding primary antibodies (Table 1). As an internal loading control, a rabbit polyclonal anti-actin antibody or a mouse monoclonal anti-MAPK14 antibody was used. Blots were washed and incubated with an appropriate second biotinylated antibody, such as horse anti-mouse IgG or goat anti-rabbit IgG (1:6000; Vector Laboratories). Then the reaction was enhanced with streptavidin-horseradish peroxidase conjugate (Chemicon International Inc.), and the proteins were visualized by enhanced chemiluminescence. Images were captured using the GeneSnap software (7.08.01 version) and were analyzed with Gene Tools software (4.01.02 version) from SynGene (Synoptics Ltd.).

Biotin Tracer Studies

The permeability of the BTB was assessed with a biotin tracer as described previously [28]. Freshly dissected testes (n = 5/group) were injected intratesticularly with 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) freshly dissolved in PBS containing 1 mM $CaCl_2$ whose volume represented 10% testis weight. Testes were then incubated at RT for 30 min, immersed in 4% paraformaldehyde, and embedded in paraffin. For localization of the biotin tracer, testis sections (5 μm thick) obtained from different levels were deparaffinized and hydrated. To avoid nonspecific staining, sections were blocked with 5% nonfat dry milk in PBS containing 0.01% Triton X-100 for 15 min prior to incubation with streptavidin-Alexa Fluor 555 (1:3000; Invitrogen) for 30 min at RT. After nuclear staining with DAPI, sections were mounted in buffered glycerin and observed by Axiophot fluorescent microscope with epi-illumination.

Lanthanum Permeability

Testes (n = 5/group) were perfused through the testicular artery with 2% lanthanum nitrate (Sigma-Aldrich) and 5% glutaraldehyde buffered with 0.1 M

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sodium cacodylate, pH 7.4, for 30 min. Tissue was cut into thin slices and placed in 5% glutaraldehyde buffered for 2 h. Blocks of 1 × 1 mm were postfixed in 1% osmium tetroxide in the same buffer with 1% potassium ferrocyanide as an auxiliary membrane contrast agent, then processed for conventional transmission electron microscopy. Sections (1 μm thick) were stained with toluidine blue for light microscopy and ultrathin sections (about 80 nm thick) were double stained with uranyl acetate and lead citrate and examined by a transmission electron microscope (Zeiss EM-94).

Intratesticular Injection of IL6

To assess the effect of IL6 on testicular histopathology, rats (n = 8/group) were anesthetized, and 1 μg of recombinant rat IL6 (R&D Systems) was injected intratesticularly with a 30-gage needle in a final volume of 100 μl sterile saline solution. Each injection was made into the upper third of the testicular parenchyma. Testes injected with the same volume of saline solution were used as controls. Rats were killed 3 days after the injection, and the testes were removed and immersed in Bouin solution or used to obtain interstitial cells for flow cytometric analysis. After 24 h, the testes were cross-cut at the equatorial area, and each half was processed separately for histopathology. Both halves were analyzed separately in order to discard any traumatic tissue damage induced by the needle. To quantify the number of damaged seminiferous tubules and inflammatory foci, four sections from each half testis per animal were analyzed by light microscopy. The dose of IL6 was selected based on reports of in vivo administration of other cytokines [29, 30].

Isolation of a Cell Fraction from the Testicular Interstitium

Cell fractions obtained from the testicular interstitium of rats injected with saline or IL6 (n = 5/group) were obtained as previously described [3]. Briefly, decapsulated testes were incubated with 0.23 mg/ml type I collagenase (Sigma-Aldrich) at 34°C for 15 min. Then collagenase was inactivated, and the seminiferous tubules were allowed to settle; the supernatant was washed with PBS, and red blood cells were depleted by osmotic lysis with 160 mM NH_4Cl and 170 mM Tris-HCl, pH 7.2. The cells were washed, centrifuged, and counted in a Neubauer chamber by the trypan blue exclusion method.

Flow Cytometric Analysis

Testicular interstitial cells (1×10^6) were stained with CD45-PE-Cy5 antibody (0.072 μg/1×10^6 cells). An appropriate control isotype was used (both purchased from BD Biosciences). A BD FACSCalibur cytometer was used to acquire 150 000 events on the CD45+ cell gate. The absolute number of positive cells per testis was calculated from percentages obtained by cytometric analysis and total number of interstitial cells.

Sertoli Cell Isolation

Sertoli cells were isolated from 20-day-old Sprague-Dawley rats as previously described [31]. Briefly, decapsulated testes were digested with 0.1% collagenase and 0.006% soybean trypsin inhibitor in Hanks balanced salt solution for 5 min at RT. Seminiferous tubules were saved, cut, and treated with 1 M glycine and 2 mM EDTA, pH 7.4, to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at RT to remove germ cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium that consisted of a 1:1 mixture of Ham F12 and Dulbecco modified Eagle medium (F12/DMEM), supplemented with 20 mM HEPES, 100 international units/ml penicillin, 2.5 μg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 μg/ml transferrin, 5 μg/ml insulin, 5 μg/ml vitamin E, and 4 ng/ml hydrocortisone. The day of cell isolation was designated as Day 0.

Culture Conditions

For immunofluorescent studies, Sertoli cells (5 μg DNA/cm^2, corresponding to 0.4×10^6 cells/cm^2) were cultured on glass coverslips coated with 6 μg/cm^2 laminin (Invitrogen) placed on 24-multiwell plates. The cells were cultured at 34°C in a mixture of 5% CO_2:95% air.

In order to study the distribution of occludin and claudin 11, Sertoli cells cultured for 4 days were maintained for 24 h under basal conditions or treated with 5 or 50 ng/ml IL6 (R&D Systems). At the end of the incubation period, cells were fixed in methanol at −20°C for 15 min (claudin 11) or in ethanol at 4°C for 30 min followed by acetone fixation at RT for 5 min (occludin). After permeabilization with 0.1% Triton X-100 in PBS at 4°C for 30 min, immunostaining was performed as described above.

To quantify transepithelial electrical resistance (TER), Sertoli cells were cultured at high cell density (15 μg DNA/cm^2, corresponding to 1.2×10^6 cells/cm^2) on Matrigel-coated (1:6 dilution with F12/DMEM v/v) cell culture inserts (Millicell HA filters, Millipore Co.) placed on 24-multiwell plates. After 2 days in culture, Sertoli cell TER was registered every 24 h until Day 8 in culture. IL6 was added to Sertoli cells on Day 4, by the time the tight junction barrier has formed [32]. Cell cultures incubated for 24 h in the absence of cytokines (Basal), in the presence of variable doses of IL6 (0.1, 0.5, 1, 5, and 50 ng/ml), or in the presence of 100 ng/ml TNF alpha, a cytokine that impairs TER, were used. On Day 5, a medium change to fresh medium without cytokines was performed in cells treated with IL6 or TNF. The effect of an inhibitor of MAPK14 (10 μM SB203580) on Sertoli cells cultured with IL6 was assessed by TER measurement.

TER Measurement

The establishment of tight junctions between Sertoli cells was assessed daily from Day 2 to Day 8 by the measurement of TER across the Sertoli cell monolayer by a Millicell electrical resistance system (Millipore Co.) as described previously [33]. Briefly, a short (~2 sec) 20-μA pulse of current was passed through the epithelial monolayer between 2 silver-silver chloride electrodes, and the electrical resistance was measured. Electrical resistance was then multiplied by the surface area of the filter to yield the area of resistance in ohms·cm^2. The net value of electrical resistance was then computed by subtracting the background, which was determined by Matrigel-coated cell-free chambers. Each time point had quadruplicate bicameral units. This experiment was run four times on different batches of cells.

Cell Proliferation Assay

A cell viability test was performed in Sertoli cells cultured on 96-well plates and treated for 24 h with 5 or 50 ng/ml of IL6. A commercial kit (CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay [MTS]; Promega Corporation) was used.

Statistical Analysis

Western blot results of each group were compared to the corresponding control by ANOVA followed by the Dunnett test. The results from flow cytometry were analyzed by the nonparametric Mann-Whitney test. For evaluation of TER results, statistical analyses were performed by ANOVA using Tukey honestly significant difference test, in which each sample group at a specified time point was compared to other sample groups within the same experiment. Data represent the mean ± SEM. Differences were considered significant when $P < 0.05$.

RESULTS

Histopathology

In the testes of N and C rats (Fig. 1A) as well as E rats killed on Day 30, we observed normal spermatogenesis in all the seminiferous tubules. At 40 days, 10 days after the end of the immunization period, testicular histopathology was not similar in all the rats. Some rats had no lesion, whereas others showed foci of a few seminiferous tubules with germ cells sloughed in the lumen, intermingled with normal tissue (Fig. 1B). Fifty days after the first immunization (focal EAO), testes of EAO rats showed mild interstitial infiltration of lymphomononuclear cells and multiple foci of damaged seminiferous tubules with different degrees of germ cell sloughing (Fig. 1C). From 80 days on (severe EAO), increased interstitial cell infiltrate and severe germ cell sloughing were observed in most seminiferous tubules in which only Sertoli cells and spermatogonia (occasionally some spermatocytes) remained attached to the tubular wall (Fig. 1D).

Changes in the Localization of Tight Junction Proteins in the Testes of Rats with EAO

The localization of integral membrane proteins, occludin and claudin 11, and the tight junction-associated protein TJP1 was evaluated by indirect immunofluorescence in testicular

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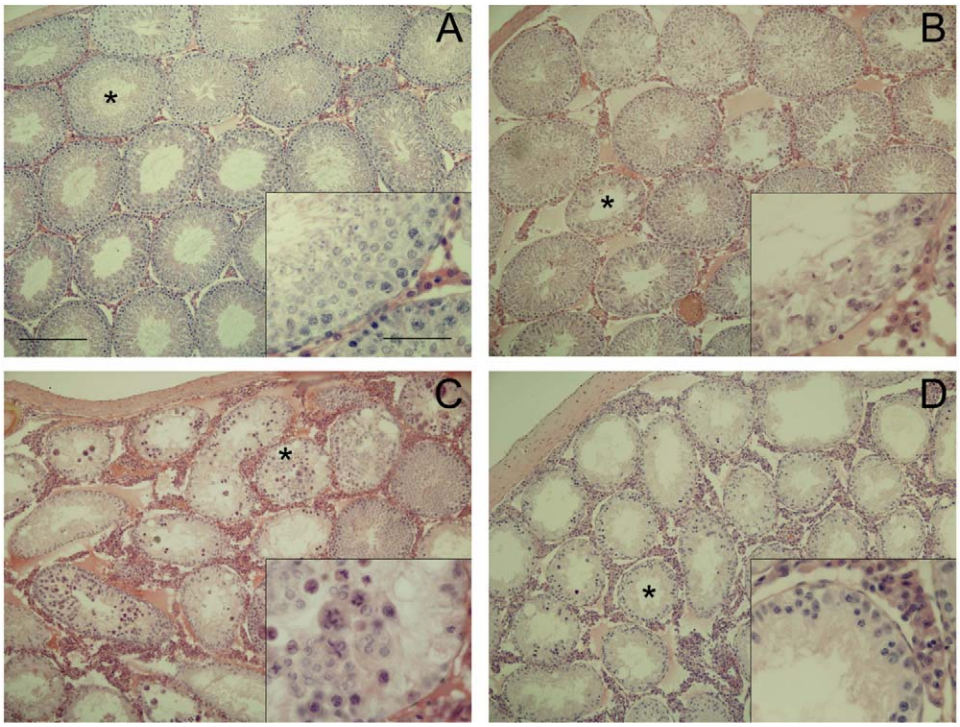

FIG. 1. Histopathology of testes sections. **A**) Normal testicular histology in a control rat. **B**) Few seminiferous tubules with disorganized seminiferous epithelium and mild germ cell sloughing intermingled with normal seminiferous tubules in an E rat killed on Day 40. **C**) Foci of damaged seminiferous tubules with different degrees of germ cell sloughing and mild interstitial cell infiltrate in an E rat killed on Day 50 (focal EAO). **D**) Extensive damage of seminiferous tubules with high degree of germ cell sloughing in an E rat killed on Day 80 (severe EAO). In each microphotograph, the asterisk indicates magnified seminiferous tubule in the insert. Bars = 200 μm and 50 μm (inset).

sections of N, C, and E rats killed 30, 40, 50, and 80 days after the first immunization. In C rats, occludin was localized at the basal compartment of seminiferous tubules with a linear pattern at the basolateral membrane region of adjacent Sertoli cells consistent with its localization at the BTB (Fig. 2A). Claudin 11 localized only at the basal region of seminiferous tubules, at the apical and lateral borders of basal cells, with a thicker immunofluorescent pattern than occludin (Fig. 2D). TJP1 was observed with a thin, lineal, continuous immunofluorescent pattern, defining the apical borders of seminiferous tubule basal cells. It was also localized at elongating spermatids (Fig. 2G). Similar results were observed in N rats (data not shown).

In rats with focal EAO, immunostaining for occludin was more irregular, its intensity decreasing in damaged seminiferous tubules (Fig. 2B). Claudin 11 immunostaining became more diffuse, its immunofluorescent pattern thicker and more intense compared to C rats (Fig. 2E). Occasionally, immunofluorescence was also observed in apical areas. The immunofluorescent pattern of TJP1 was more irregular and discontinuous compared to that observed in C rats (Fig. 2H).

In most seminiferous tubules of rats with severe EAO, occludin immunostaining was not detected (Fig. 2C). Claudin 11 and TJP1 showed a thickened immunofluorescent pattern, distributed in a larger area at the basal region of seminiferous tubules of rats with severe EAO compared to C group rats (Fig. 2, F and I).

A similar immunofluorescent pattern for occludin, claudin 11, and TJP1 was detected in E rats killed at 30 days compared to N and C rats. Similar results were observed in E rats killed on Day 40, except for occludin whose expression was decreased in seminiferous tubules showing some degree of germ cell sloughing (Supplemental Fig. S1; all the Supplemental Data are available online at www.biolreprod.org).

Changes in Tight Junction Protein Expression in the Testes of EAO Rats

The level of occludin, claudin 11, and TJP1 expression was evaluated by Western blot analysis in testicular homogenates from N, C, and E rats killed 30, 40, 50, and 80 days after the first immunization. A significant decrease in occludin expression was observed in the testes of E rats killed on Days 50 (focal EAO) and 80 (severe EAO) compared to C rats (Fig. 3A). No changes in steady-state levels of claudin 11 and TJP1 were detected at any time period studied (Fig. 3, B and C). In the testes of E rats killed on Days 30 and 40 (data not shown), no changes compared to C rats were detected in the expression of all the molecules analyzed. The expression levels observed in C rats were similar to those observed in N rats.

Impairment of BTB Permeability

To assess BTB integrity, we ran a biotin tracer penetration experiment. Figure 4A shows that, in the testes of C rats, biotin tracer was restricted to the interstitial area and basal compartment of seminiferous tubules, around the basal cells. No biotin tracer was detected at the adluminal compartment, illustrating the presence of a functional BTB. In the testes of EAO rats, the biotin tracer was found within the adluminal compartment surrounding some remaining germ cells, indicating loss of BTB function (Fig. 4B).

To confirm the findings described above, we ran lanthanum tracer studies at the ultrastructural level to assess whether the BTB was compromised in EAO rats. In C testes, lanthanum

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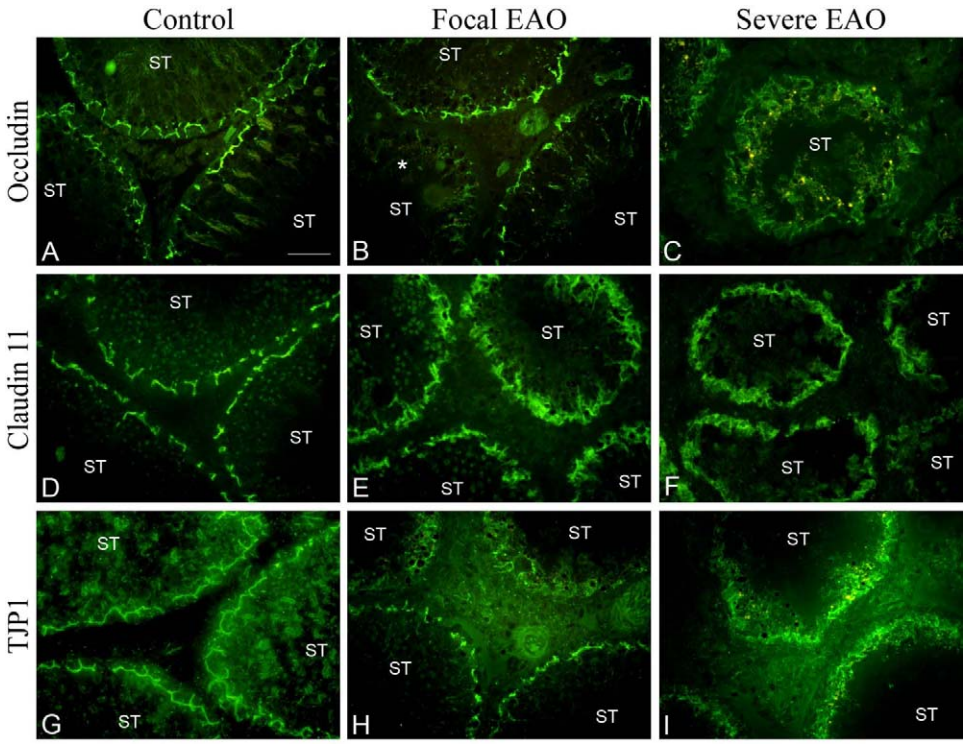

FIG. 2. Expression and distribution of occludin, claudin 11, and TJP1 in testis sections of control and EAO rats. Bright and linear immunofluorescence is detected in the basal area of seminiferous tubules (STs) of control rats (**A**, **D**, and **G**). Damaged STs from a rat with focal EAO show a reduction of occludin expression (asterisk in **B**). Claudin 11 and TJP1 immunofluorescent pattern is irregular and delocalized in rats with focal EAO (**E** and **H**). No expression of occludin was detected in rats with severe EAO (**C**), while claudin 11 (**F**) and TJP1 (**I**) immunofluorescence is distributed over a larger area than in control rats. Bar = 50 μm.

was distributed between peritubular cells, around spermatogonia, and between adjacent Sertoli cells, where it was abruptly halted by the BTB tight junctions. No lanthanum was observed within the adluminal compartment (Fig. 5, A and C). In contrast, in most seminiferous tubules of EAO rats, lanthanum diffused into the adluminal intercellular space between Sertoli cells and damaged germ cells (Fig. 5, B and D).

Increased p-MAPK14 Expression in Testes of Rats with EAO

Lui et al. [34] reported that BTB dynamics are regulated in vivo by the TGF beta3/MAPK14 pathway. Therefore, the expression of p-MAPK14 in the EAO testes was examined. Using Western blot, we detected increased p-MAPK14 expression in testes of E rats killed on Day 80 (severe EAO) compared to C rats (Fig. 6). No changes were detected in rats killed on Day 50 (focal EAO) compared to C rats.

IL6 Induces Histopathological Alterations and Changes in Tight Junction Protein Expression

When IL6 was administered intratesticularly to adult rats (1 μg/testis), histopathological changes were detected 3 days after the injection. Testes of rats injected with IL6 showed foci of few seminiferous tubules with different degrees of germ cell sloughing as well as interstitial lymphomononuclear cell infiltrates close to the damaged tubules (Fig. 7, B and D). Some rats injected with saline showed a few damaged seminiferous tubules near the site of the injection but not in other areas (Fig. 7, A and C). In contrast, damage of IL6-injected rats was observed both near and distant from the injection site. A higher number of inflammatory foci (IL6 versus saline: 2.8 ± 1.5 versus 0.5 ± 0.1, $P = 0.04$) and a higher number of damaged seminiferous tubules/focus (IL6 versus saline: 3.6 ± 1.1 versus 1.2 ± 0.4, $P = 0.03$) were observed in the testes of rats injected with IL6 compared to saline.

To determine the number of interstitial leukocytes in the testis injected with IL6, a flow cytometric analysis was performed using the CD45 marker. A significant increase in the absolute number of CD45+ cells was observed in testes injected with IL6 compared to those injected with saline (Fig. 8).

The results of immunofluorescence showed reduction of occludin expression (Fig. 9, A and B) and delocalization of claudin 11 (Fig. 9, E and F) in damaged seminiferous tubules of rats injected with IL6. A normal immunofluorescent pattern of these proteins was observed at seminiferous tubules of rats injected with saline (Fig. 9, C, D, G, and H).

IL6 Induces Structural and Functional Alterations of Sertoli Cell Tight Junction Barrier In Vitro

Because IL6 induces interstitial inflammation and germ cell sloughing, we investigated its effect on the integrity of the Sertoli cell tight junction barrier in vitro. As a functional parameter of BTB integrity, we quantified TER across the Sertoli cell epithelium in the presence of different concentrations of IL6. On Day 4, when Sertoli cells completed the tight junction barrier assembly, IL6 was added to the culture medium for 24 h. TER was assessed daily from the second day of culture until Day 8.

The addition of recombinant IL6 at 5 and 50 ng/ml to Sertoli cells cultured in vitro on Matrigel-coated bicameral units

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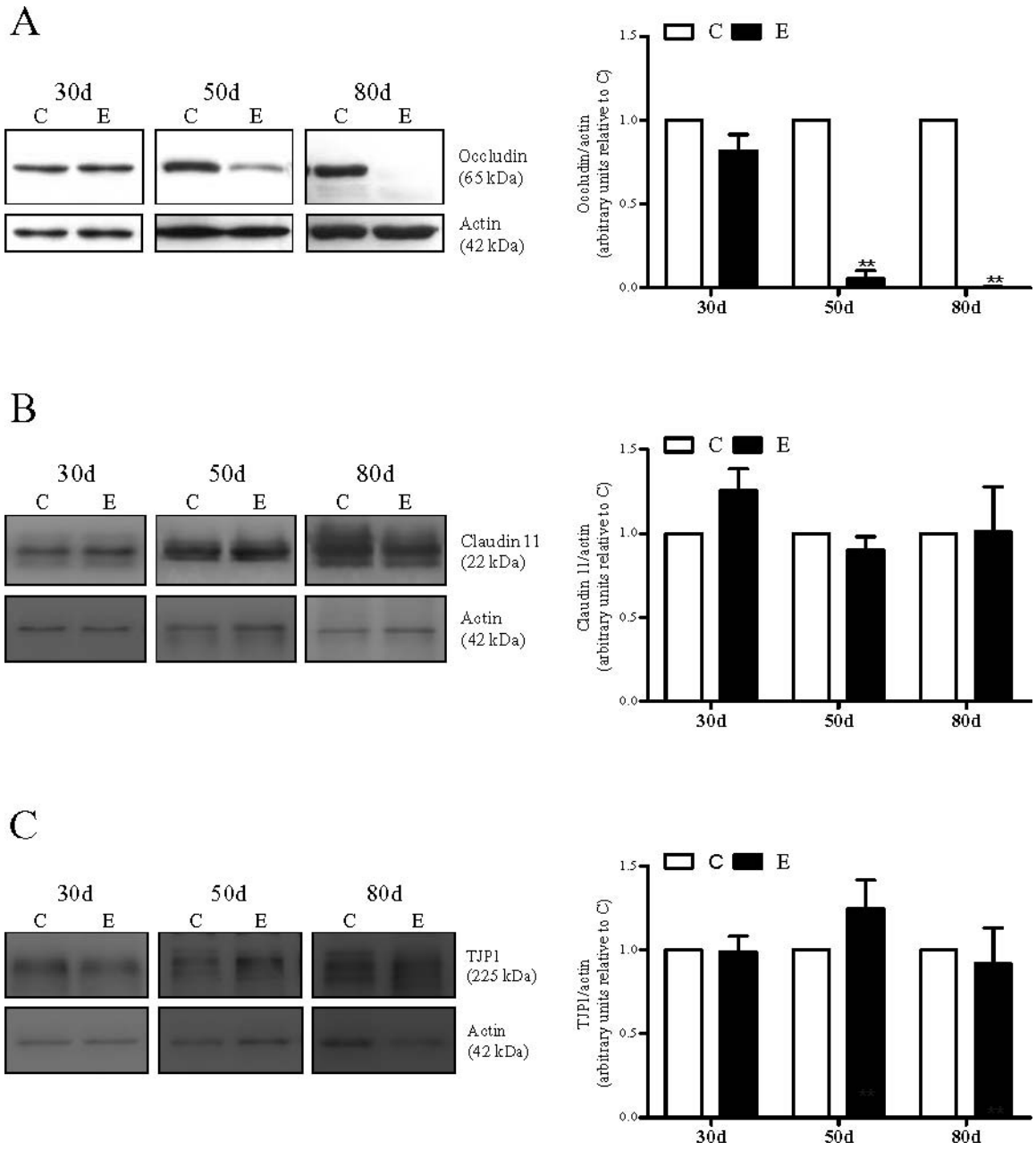

FIG. 3. Changes in steady-state levels of tight junction proteins in testis of rats with EAO (Western blot). A significant decrease in occludin expression was observed in experimental (E) rats killed on Days (d) 50 and 80 (**A**). No changes in claudin 11 and TJP1 levels were detected at any time period studied (**B** and **C**). Data from E groups were compared to data from control (C) rats arbitrarily set at 1. Each bar represents the mean ± SEM of five animals; $^{**}P < 0.01$ versus C.

disrupted the Sertoli cell tight junction barrier, as demonstrated by a significant decline in TER when compared to basal conditions (Fig. 10). As a positive control, we used TNF alpha, known to affect the assembly of Sertoli cell tight junctions in vitro [35]. The decrease in TER induced by both cytokines was reversible because when cytokines were removed at Day 5, TER returned to values similar to those found under basal conditions (Fig. 10). A dose-dependency of TER in response to variable doses of IL6 was observed (Supplemental Fig. S2).

We next evaluated the effects of IL6 exposure to Sertoli cells on localization of tight junction proteins. Immunofluorescent staining demonstrated that occludin and claudin 11 were present at the zone of contact between adjacent cells in a linear and continuous pattern that delineated cell borders under basal conditions (Fig. 11, A:a–c and B:a–c). Addition of IL6 induced redistribution of both proteins because immunofluorescence for occludin became discontinuous and was localized at punctual zones of cell borders (Fig. 11A:d–f); claudin 11 was redistributed from the cell surface into the cytoplasm (Fig. 11B:d–f). In order to evaluate if IL6 affected Sertoli cell viability, an MTS assay was performed. Viability of Sertoli cells cultured in the presence of IL6 was similar to that of cells cultured under basal conditions considered as a 100% (5 ng/ml IL6: 96% ± 3%; 50 ng/ml IL6: 107% ± 5%).

Combined treatment of IL6 with a specific inhibitor of the MAPK14 pathway (10 μM SB203580) showed a decrease in the action of IL6, suggesting the participation of this signaling

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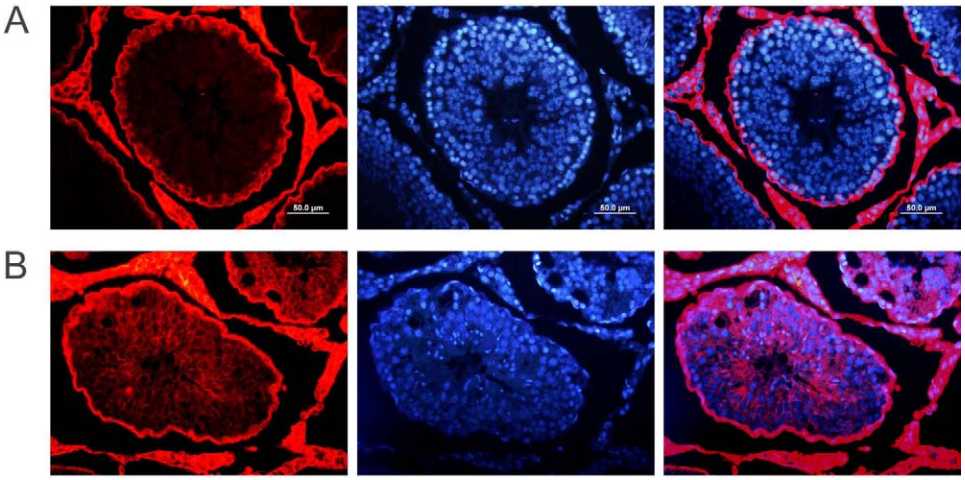

FIG. 4. Biotin tracer experiments to evaluate BTB permeability. Biotin (red) is observed only in the basal area of seminiferous tubules of a control rat (**A**) whereas in a rat with focal EAO (50 days, **B**) the tracer is also found in the adluminal compartment of seminiferous tubules. Cell nuclei are stained with DAPI (middle column). Merged images are shown in the right column. Bar = 50 μm.

pathway in IL6 effect on Sertoli cell tight junction barrier (Fig. 12).

DISCUSSION

Testes of rats undergoing autoimmune orchitis present foci of seminiferous tubules with germ cell sloughing and apoptosis. The present results show that, in association with seminiferous tubule damage, changes in tight junction molecule expression and impairment of BTB function occur. Tight junction proteins of N and C rats localize at the basal compartment of seminiferous tubules where the BTB is normally formed, in accordance to the localization described by others [21, 36, 37]. TJP1 also localize in elongating spermatids, as previously reported in mice by Byers et al. [38].

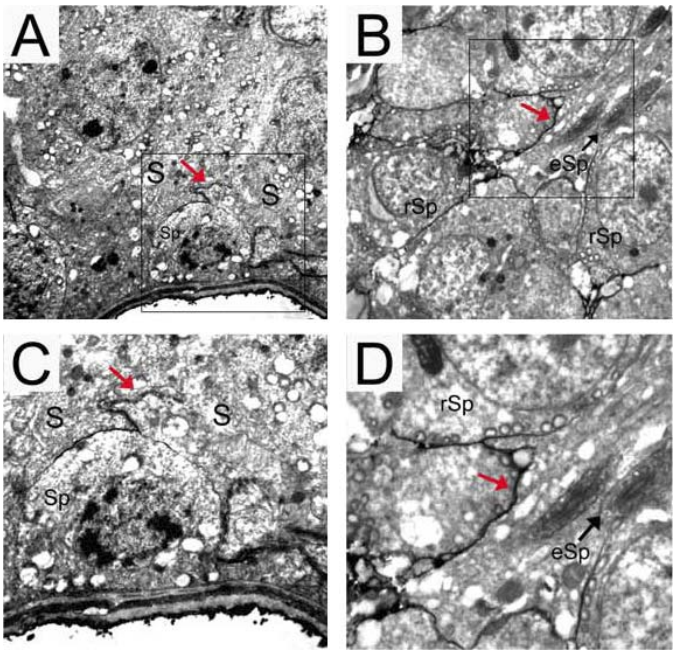

FIG. 5. Electron micrographs of lanthanum tracer studies. Lanthanum is observed around spermatogonia (Sp), and its diffusion is restricted at the level of the BTB (red arrow) between two adjacent Sertoli cells (S) in a control rat (**A** and **C**). In a rat with focal EAO (50 days, **B** and **D**), lanthanum is observed around germ cells of the adluminal compartment (red arrow). Round spermatid, rSp; elongated spermatid, eSp. **C** and **D** represent magnified areas indicated in **A** and **B**, respectively. Magnification ×4400 (**A**, **B**) and ×8800 (**C**, **D**).

Changes in tight junction protein expression observed in E rats are concomitant with histopathological lesions of seminiferous tubules. In fact, in testes of E rats killed on Day 40, when the lesion is limited to a few tubules, we observed reduced expression of occludin in those seminiferous tubules that showed some degree of germ cell sloughing. In contrast, adjacent seminiferous tubules with normal spermatogenesis showed a normal pattern of occludin expression. By Western blot analysis, we detected no changes in occludin expression in testes of these animals because only a few foci of seminiferous tubules were damaged. The use of laser microcapture methodology followed by real-time PCR would enable us to confirm reduction of occludin expression in identified altered seminiferous tubules.

In testes of rats with focal and severe EAO (50 and 80 days, respectively) we observed a significant decrease in occludin expression by immunofluorescence and Western blot analysis. Similar results were described in seminiferous tubules damaged by the action of toxicants such as cadmium chloride, vasectomy, or heat stress [39–41].

In contrast with N and C rats, a more diffuse localization and a higher intensity of claudin 11 immunofluorescence was observed at the basal compartment of seminiferous tubules in rats with focal EAO. In severe EAO, this protein had the same localization but was distributed over a larger area. However, Western blot analysis revealed no differences in protein expression.

The delocalization of claudin 11 observed in EAO rats suggests that this protein is not properly assembled in tight junction complexes. Several studies have demonstrated that endocytosis is a novel mechanism utilized by an epithelium to rapidly impair cell junction dynamics [42–44]. Cytokines such as TNF alpha and TGF betas can alter testicular tight junction function by accelerating endocytosis of integral membrane proteins [45]. Other authors report that chemokine CCL2 induces remodeling of brain endothelial tight junctions through internalization and recycling of occludin and claudin 5 [46]. Previous results of increased testicular content of CCL2 and TNF alpha [47, 48] and present observations of frequent endocytic vesicles in seminiferous tubule cells of rats with

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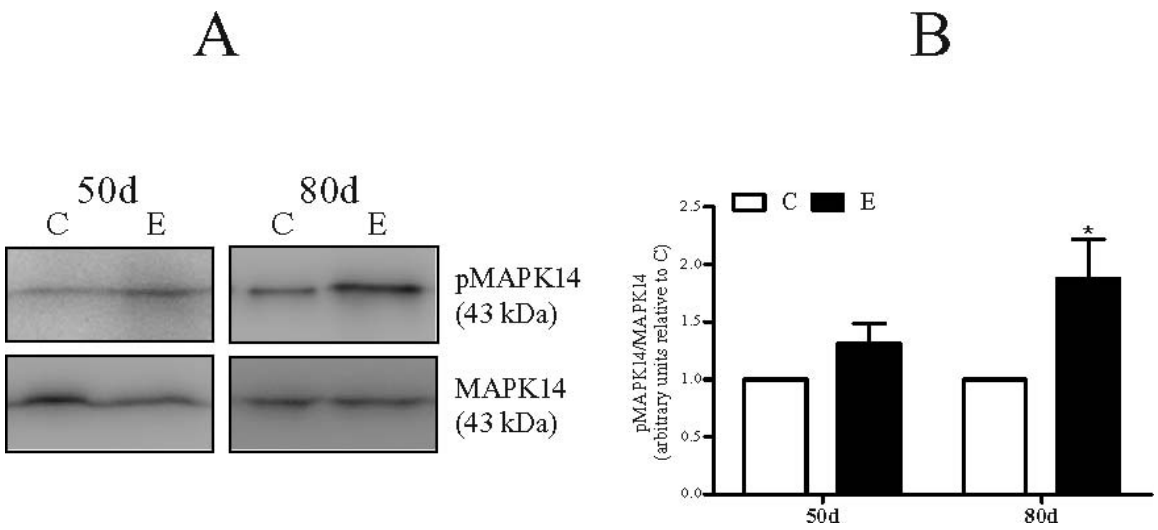

FIG. 6. Activation of MAPK14 signaling pathway. **A**) Representative Western blot showing increased p-MAPK14 expression in the testis of an experimental (E) rat killed on Day 80 (d) compared to control rat (C). **B**) Densitometric analysis of p-MAPK14/total MAPK14. Data from E groups were compared to data from C groups arbitrarily set at 1. Each bar represents the mean ± SEM of five animals; $*P < 0.05$ versus C.

EAO suggest that chemokines and cytokines may modulate claudin 11 internalization in EAO rats.

Using biotin and lanthanum nitrate, we showed impairment of BTB function in rats with autoimmune orchitis because both permeability tracers were detected in the adluminal compartment of seminiferous tubules in contrast with N and C rats that showed a basal localization. Pelletier [16], in a study of the ultrastructure of the BTB by freeze-fracture technique, described that particulate elements in junctions form either a short fibril confined to a focal tight junction called macula occludens, which is readily bypassed by interstitial fluids, or a continuous fibril, called zonula occludens, which completely encircles the body of the Sertoli cell and blocks the passage of vascularly infused permeability tracers. Our results showed that in testis of EAO rats a significant reduction of occludin expression and delocalization of claudin 11 occur in seminiferous tubules concomitant with impairment of BTB permeability. Therefore, we may speculate that in EAO, tight junction proteins are mainly involved in macula occludens structures whereas zonula occludens assembly is impaired.

In focal EAO, an irregular and discontinuous TJP1 immunofluorescent pattern was observed at the seminiferous

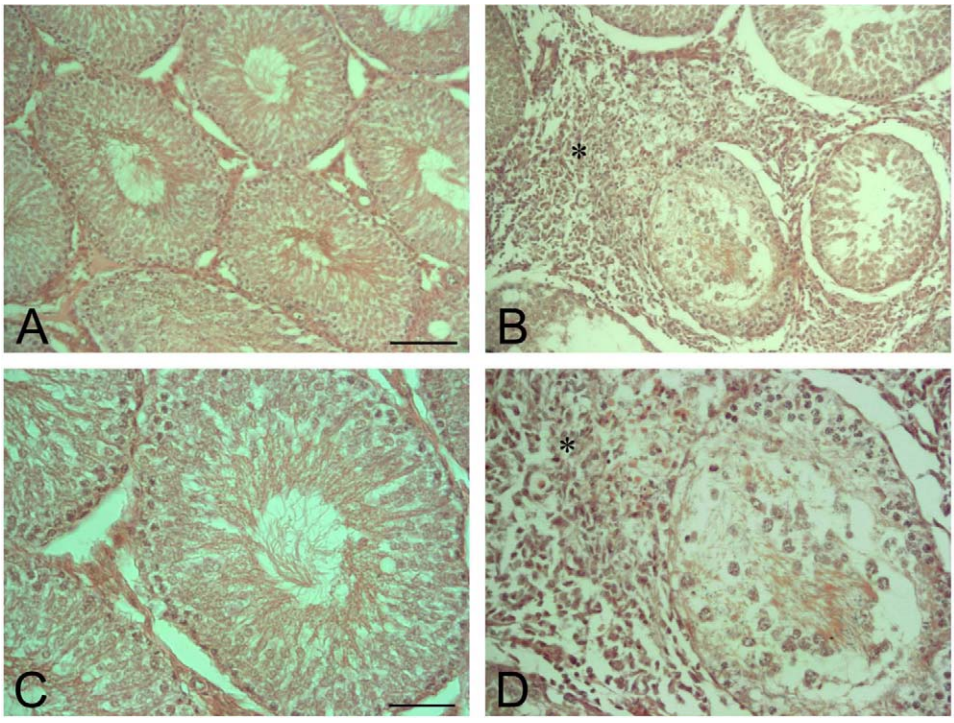

FIG. 7. Testes sections of normal rats injected with saline (**A** and **C**) or with IL6 (**B** and **D**). **A** and **C**) Normal testicular histopathology. **B** and **D**) Large interstitial area with inflammatory cell infiltrates (asterisk) intermingled with seminiferous tubules showing germ cell sloughing. Bar = 100 μm (**A**, **B**) or 50 μm (**C**, **D**).

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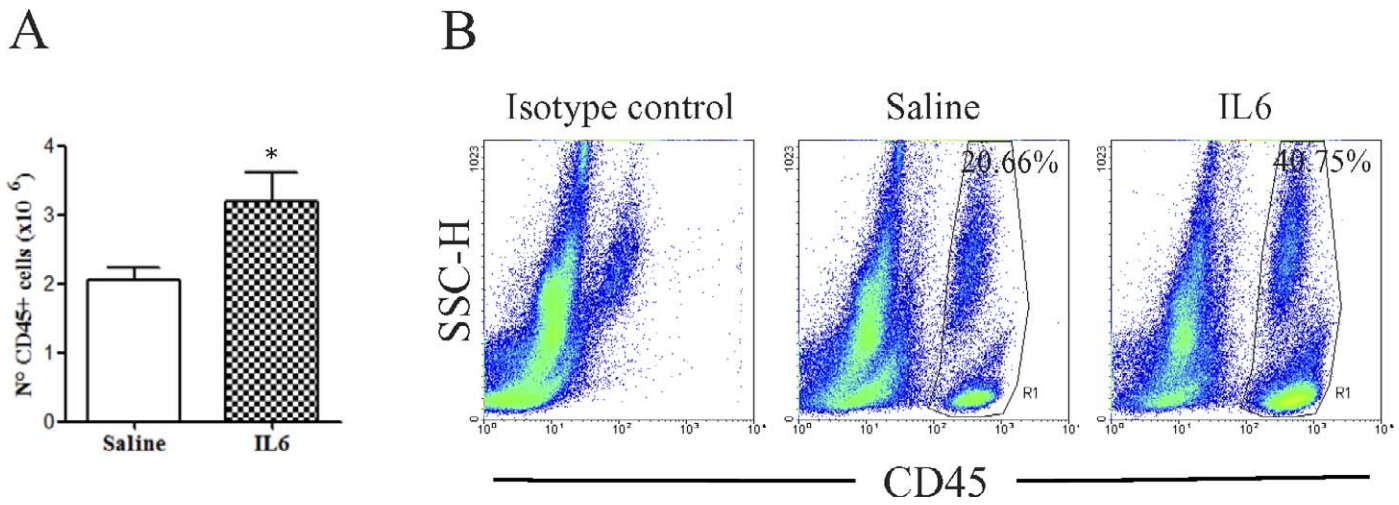

FIG. 8. Testicular interstitial cells were stained for CD45 and analyzed by flow cytometry. **A**) An increase in the absolute number of CD45+ cells is observed in testes of rats intratesticularly injected with IL6 compared to rats injected with saline. **B**) Representative density plots showing CD45 expression. A gate was drawn selecting the total leukocyte population (CD45+ cells, R1) based on the isotype control. The percentages of cells in R1 are indicated; n = 5 rats/group; $*P < 0.05$ versus saline.

tubule basal compartment. Although its localization was the same, TJP1 was distributed over a larger area in rats with severe EAO. These alterations may be a result of the concomitant reduced expression of occludin and gap junction protein alpha 1 [9] because both proteins associate with TJP1 under normal conditions [49].

Based on the results of Chung et al. [50] who highlighted the importance of occludin for normal BTB function, we speculate that increased BTB permeability in EAO rats is dependent on the reduced expression of occludin. Those authors injected rats with a 22-amino acid synthetic peptide corresponding to the second extracellular loop of occludin and induced a reversible infertility via BTB disruption.

Cytokines, mainly TNF alpha and TGF beta3 produced by Sertoli and/or germ cells [44], have been described to contribute to restructuring and/or opening of the BTB, facilitating the transit of preleptotene/leptotene spermatocytes at the BTB. IL6 secreted by Sertoli cells [51] could also play a role in the physiological remodeling of BTB in normal conditions. The integrity and function of tight junctions may be altered by cytokines through endocytic mechanisms or by modulation of protein expression. Reports showed that hepatocyte growth factor is also involved in BTB regulation, reducing occludin levels [52, 53].

During EAO development in rats, proinflammatory cytokines, mainly TNF alpha and IL6, increase in the testicular microenvironment [48, 51]. Our results now show that intratesticular injection of IL6 induced focal inflammatory cell infiltration in the interstitium and germ cell sloughing in adjacent seminiferous tubules. These effects may be due to the

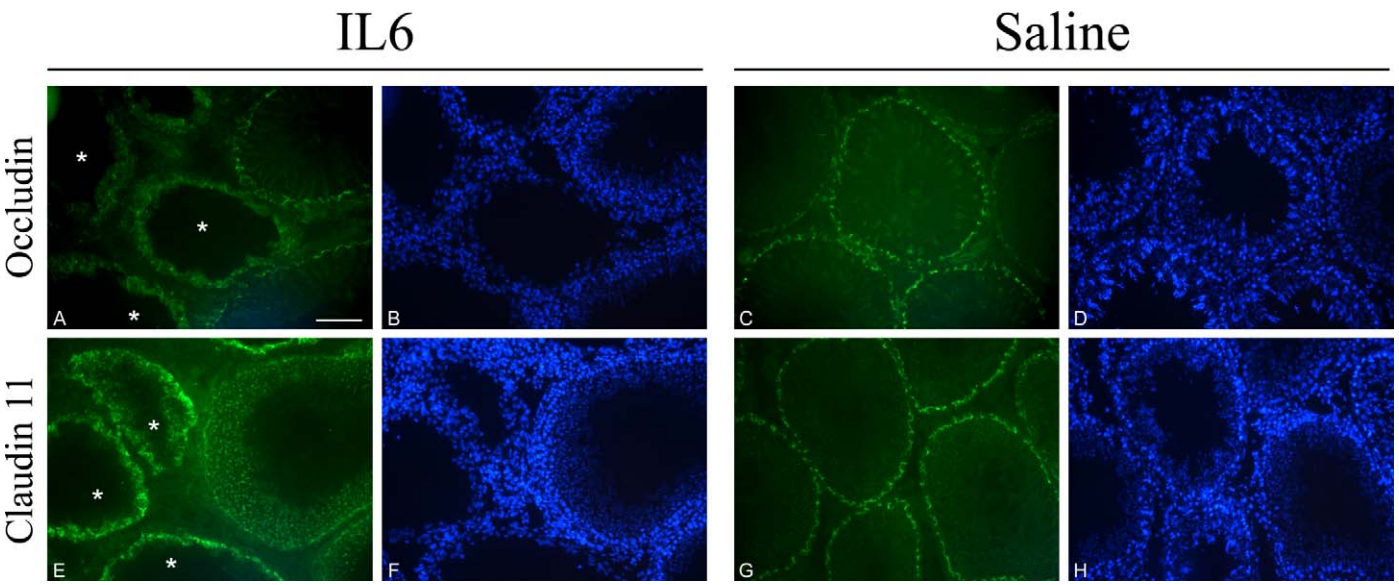

FIG. 9. Immunofluorescence to detect occludin and claudin 11 expression in rats injected with IL6 or saline. In rats injected with IL6, a reduction in occludin expression (**A**) and brighter immunostaining distributed over a larger area for claudin 11 (**E**) are observed in damaged seminiferous tubules (asterisks) in contrast to a normal linear immunofluorescent pattern observed in seminiferous tubules with normal spermatogenesis. In rats injected with saline, occludin (**C**) and claudin 11 (**G**) localize to the basal compartment of seminiferous tubules in a linear and continuous immunofluorescent pattern. **B**, **D**, **F**, and **H**) Nuclear staining with DAPI. Bar = 100 μm.

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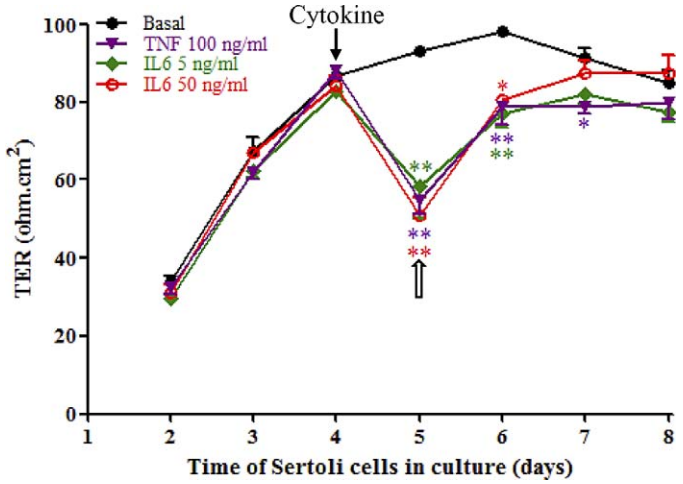

FIG. 10. Transepithelial electrical resistance (TER) across Sertoli cells in culture exposed to 5 or 50 ng/ml IL6 for 24 h was shown to decline, indicating disruption of the tight junctional complexes between Sertoli cells. White arrow indicates that the medium with cytokine was replaced by medium alone. TNF alpha at 100 ng/ml was used as a positive control. Each data point represents the mean ± SEM of quadruplicate cultures from a representative experiment out of four; $*P < 0.05$; $**P < 0.01$ versus basal.

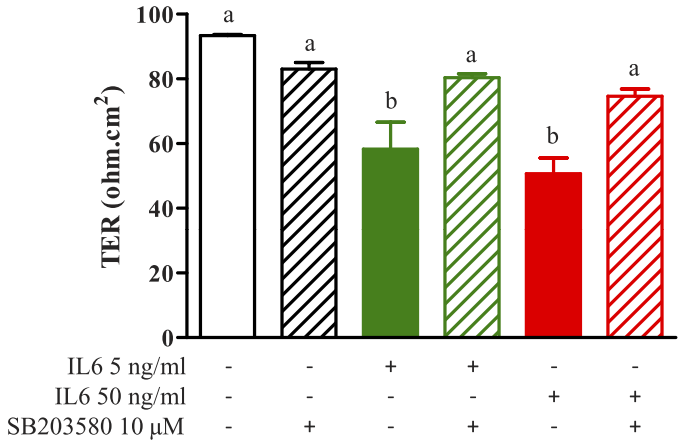

FIG. 12. Transepithelial electrical resistance (TER) across Sertoli cells in culture exposed to 5 or 50 ng/ml IL6 for 24 h in the presence or absence of SB203580, an inhibitor of the MAPK14 pathway. The addition of SB203580 blocked the effect of IL6 on TER. Each bar represents the mean ± SEM of triplicate cell cultures. Values with different letters differ significantly ($P < 0.05$).

inflammatory role of IL6 as shown in other models [54, 55] and to the proapoptotic role of IL6 on germ cells as we showed previously [51]. Also, damaged seminiferous tubules exhibited alterations in tight junction protein expression similar to those observed in EAO rats. In turn, by in vitro experiments, we demonstrated the ability of IL6 to modify distribution of Sertoli cell tight junction proteins and to perturb the Sertoli cell tight junction barrier, reducing TER across the cell epithelium. It is possible that IL6, aside from its action on BTB, may also increase endothelial permeability through changes in the distribution of endothelial tight junction proteins as demonstrated in vitro by Desai et al. [56], facilitating inflammatory cell extravasation. Collectively, these results highlight the role of IL6 in recruiting immune cells to the testicular interstitium and altering the normal structure and function of BTB, facilitating the development of EAO. In a transgenic mouse model in which astrocytes express IL6, central nervous system injury associates with blood-brain barrier breakdown [57, 58].

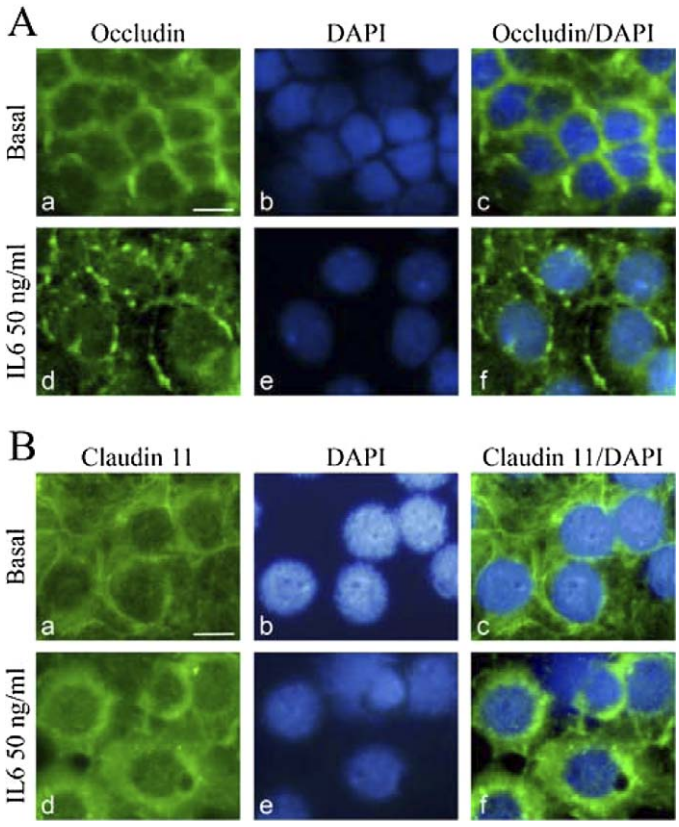

FIG. 11. Effect of IL6 on distribution of occludin (**A**) and claudin 11 (**B**) in Sertoli cell cultures. In the presence of IL6, the immunofluorescent pattern for occludin becomes discontinuous (**A**:**d**) whereas a diffuse pattern is observed for claudin 11 (**B**:**d**). Bar = 10 μm.

Other cytokines such as TNF alpha, TGF beta2, and TGF beta3 are known to regulate the dynamics of Sertoli cell tight junctions through the MAPK14 pathway [29, 39, 59]. The present results also show that BTB impairment is associated with increased p-MAPK14 expression in testes of rats with severe orchitis when germ cell apoptosis is increased. This finding tallies with Johnson et al. [60], who described activation of the MAPK14 signaling pathway during germ cell apoptosis. Because inflammatory cells also express p-MAPK14 [61], the increase in this kinase might not be related to BTB impairment. However, the present in vitro experiments show that IL6 is able to impair the Sertoli cell tight junction barrier via the MAPK14 signaling pathway.

Also, reactive oxygen species such as nitric oxide, produced by nitric oxide synthase, have been reported as regulators of tight junction dynamics in the testis [62]. We have shown that in rats with EAO, an increase in the testicular content of nitric oxide is concomitant with elevated nitric oxide synthase activity [63].

The BTB is composed of coexisting tight junctions, anchoring junctions, and gap junctions. Because tight junctions are closely associated with adherens junctions functionally, spatially, and biochemically, disruption of tight junctions would also induce adherens junction dissociation and germ cell detachment from the epithelium. Studies using cadmium chloride and glycerol have shown that these toxicants can irreversibly perturb tight junctions that can lead, in turn, to anchoring junction damage, thereby dislodging germ cells from

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the epithelium [64]. Blockage of testicular connexins by panconnexin peptide treatment decreases occludin expression, while upregulating cadherin 2 expression [65]. Similarly, in EAO rats, downregulation of occludin expression is accompanied by increased cadherin 2 expression and a decrease in the content of testicular gap junction protein alpha 1 [9].

The main feature of testicular damage in autoimmune orchitis is germ cell sloughing. Concomitant with alterations of adherens junction protein expression that we previously described [9], changes in tight junction protein expression, and increased BTB permeability occurred in this experimental model of testicular autoimmunity. Results of our in vivo and in vitro experiments with IL6 suggest that this cytokine (whose testicular content is increased in EAO) is one factor, among others, involved in the impairment of BTB integrity and function in autoimmune orchitis. Although the proinflammatory role of IL6 has been reported in many autoimmune diseases, the action of this cytokine on intercellular junctions of the seminiferous epithelium has not been previously described. Knowledge of intercellular cell junction protein expression and function and their regulatory factors is relevant for understanding chronic inflammation and other testicular pathologies involving germ cell sloughing.

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REFERENCES

1. Schuppe HC, Meinhardt A, Allam JP, Bergmann M, Weidner W, Haidl G. Chronic orchitis: a neglected cause of male infertility? Andrologia 2008; 40:84–91.
2. Tung KS, Yule TD, Mahi-Brown CA, Listrom MB. Distribution of histopathology and Ia positive cells in actively induced and passively transferred experimental autoimmune orchitis. J Immunol 1987; 138: 752–759.
3. Jacobo P, Guazzone VA, Jarazo-Dietrich S, Theas MS, Lustig L. Differential changes in CD4+ and CD8+ effector and regulatory T lymphocyte subsets in the testis of rats undergoing autoimmune orchitis. J Reprod Immunol 2009; 81:44–54.
4. Jacobo P, Pérez CV, Theas MS, Guazzone VA, Lustig L. CD4+ and CD8+ T cells producing Th1 and Th17 cytokines are involved in the pathogenesis of autoimmune orchitis. Reproduction 2011; 141:249–258.
5. Rival C, Theas MS, Suescun MO, Jacobo P, Guazzone V, van Rooijen N, Lustig L. Functional and phenotypic characteristics of testicular macrophages in experimental autoimmune orchitis. J Pathol 2008; 215: 108–117.
6. Rival C, Lustig L, Iosub R, Guazzone VA, Schneider E, Meinhardt A, Fijak M. 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.
7. Guazzone VA, Jacobo P, Theas MS, Lustig L. Cytokines and chemokines in testicular inflammation: a brief review. Microsc Res Tech 2009; 72: 620–628.
8. Theas S, Rival C, Lustig L. Germ cell apoptosis in autoimmune orchitis: involvement of the Fas-FasL system. Am J Reprod Immunol 2003; 50: 166–176.
9. Pérez C, Sobarzo C, Jacobo P, Jarazo Dietrich S, Theas M, Denduchis B, Lustig L. Impaired expression and distribution of adherens and gap junction proteins in the seminiferous tubules of rats undergoing autoimmune orchitis. Int J Androl 2011; 34:e566–e577.
10. Dym M, Fawcett DW. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol Reprod 1970; 3:308–326.
11. Russell L. Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am J Anat 1977; 148:313–328.
12. Kopera IA, Bilinska B, Cheng CY, Mruk DD. Sertoli-germ cell junctions in the testis: a review of recent data. Philos Trans R Soc Lond B Biol Sci 2010; 365:1593–1605.
13. Mruk DD, Cheng CY. Tight junctions in the testis: new perspectives. Philos Trans R Soc Lond B Biol Sci 2010; 365:1621–1635.
14. Morrow CM, Mruk D, Cheng CY, Hess RA. Claudin and occludin expression and function in the seminiferous epithelium. Philos Trans R Soc Lond B Biol Sci 2010; 365:1679–1696.
15. Cheng CY, Mruk DD. Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiol Rev 2002; 82:825–874.
16. Pelletier RM. The blood-testis barrier: the junctional permeability, the proteins and the lipids. Prog Histochem Cytochem 2011; 46:49–127.
17. Lustig L, Tung KSK. Orchitis and male infertility. In: Rose NR, and Mackay IR (eds.), The Autoimmune Diseases. St. Louis: Elsevier Academic Press; 2006: 841–848.
18. Mital P, Hinton BT, Dufour JM. The blood-testis and blood-epididymis barriers are more than just their tight junctions. Biol Reprod 2011; 84: 851–858.
19. Fink C, Weigel R, Hembes T, Lauke-Wettwer H, Kliesch S, Bergmann M, Brehm RH. Altered expression of ZO-1 and ZO-2 in Sertoli cells and loss of blood-testis barrier integrity in testicular carcinoma in situ. Neoplasia 2006; 8:1019–1027.
20. Mruk DD, Cheng CY. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr Rev 2004; 25:747–806.
21. Moroi S, Saitou M, Fujimoto K, Sakakibara A, Furuse M, Yoshida O, Tsukita S. Occludin is concentrated at tight junctions of mouse/rat but not human/guinea pig Sertoli cells in testes. Am J Physiol 1998; 274: C1708–C1717.
22. Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. Structure and function of claudins. Biochim Biophys Acta 2008; 1778: 631–645.
23. Mandell KJ, Parkos CA. The JAM family of proteins. Adv Drug Deliv Rev 2005; 57:857–867.
24. González-Mariscal L, Betanzos A, Avila-Flores A. MAGUK proteins: structure and role in the tight junction. Semin Cell Dev Biol 2000; 11: 315–324.
25. Lee NP, Cheng CY. Nitric oxide and cyclic nucleotides: their roles in junction dynamics and spermatogenesis. Oxid Med Cell Longev 2008; 1: 25–32.
26. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Mol Cell Endocrinol 2010; 315:49–56.
27. Doncel GF, Di Paola J, 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.
28. Meng J, Holdcraft RW, Shima JE, Griswold MD, Braun RE. Androgens regulate the permeability of the blood-testis barrier. Proc Natl Acad Sci U S A 2005; 102:16696–16700.
29. Li MW, Xia W, Mruk DD, Wang CQ, Yan HH, Siu MK, Lui WY, Lee WM, Cheng CY. Tumor necrosis factor {alpha} reversibly disrupts the blood-testis barrier and impairs Sertoli-germ cell adhesion in the seminiferous epithelium of adult rat testes. J Endocrinol 2006; 190: 313–329.
30. Sarkar O, Mathur PP, Cheng CY, Mruk DD. Interleukin 1 alpha (IL1A) is a novel regulator of the blood-testis barrier in the rat. Biol Reprod 2008; 78:445–454.
31. Meroni SB, Riera MF, Pellizzari EH, Cigorraga SB. Regulation of rat Sertoli cell function by FSH: possible role of phosphatidylinositol 3-kinase/protein kinase B pathway. J Endocrinol 2002; 174:195–204.
32. Lui WY, Lee WM, Cheng CY. Transforming growth factor-beta3 perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated via its effects on occludin, zonula occludens-1, and claudin-11. Endocrinology 2001; 142:1865–1877.
33. Grima J, Wong CC, Zhu LJ, Zong SD, Cheng CY. Testin secreted by Sertoli cells is associated with the cell surface, and its expression correlates with the disruption of Sertoli-germ cell junctions but not the inter-Sertoli tight junction. J Biol Chem 1998; 273:21040–21053.
34. Lui WY, Wong CH, Mruk DD, Cheng CY. TGF-beta3 regulates the blood-testis barrier dynamics via the p38 mitogen activated protein (MAP) kinase pathway: an in vivo study. Endocrinology 2003; 144:1139–1142.
35. Siu MK, Lee WM, Cheng CY. The interplay of collagen IV, tumor necrosis factor-alpha, gelatinase B (matrix metalloprotease-9), and tissue inhibitor of metalloproteases-1 in the basal lamina regulates Sertoli cell-tight junction dynamics in the rat testis. Endocrinology 2003; 144: 371–387.
36. Morita K, Sasaki H, Fujimoto K, Furuse M, Tsukita S. Claudin-11/OSP-

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based tight junctions of myelin sheaths in brain and Sertoli cells in testis. J Cell Biol 1999; 145:579–588.
37. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 1986; 103:755–766.
38. Byers S, Graham R, Dai HN, Hoxter B. Development of Sertoli cell junctional specializations and the distribution of the tight-junction-associated protein ZO-1 in the mouse testis. Am J Anat 1991; 191:35–47.
39. Wong CH, Mruk DD, Lui WY, Cheng CY. Regulation of blood-testis barrier dynamics: an in vivo study. J Cell Sci 2004; 117:783–798.
40. Contuk G, Orun O, Demiralp-Ekşioğlu E, Ercan F. Morphological alterations and distribution of occludin in rat testes after bilateral vasectomy. Acta Histochem 2012; 114:244–251.
41. Cai H, Ren Y, Li XX, Yang JL, Zhang CP, Chen M, Fan CH, Hu XQ, Hu ZY, Gao F, Liu YX. Scrotal heat stress causes a transient alteration in tight junctions and induction of TGF-β expression. Int J Androl 2011; 34: 352–362.
42. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of the apical junctional complex: mechanisms and possible roles in regulation of epithelial barriers. Bioessays 2005; 27:356–365.
43. Morimoto S, Nishimura N, Terai T, Manabe S, Yamamoto Y, Shinahara W, Miyake H, Tashiro S, Shimada M, Sasaki T. Rab13 mediates the continuous endocytic recycling of occludin to the cell surface. J Biol Chem 2005; 280:2220–2228.
44. Xia W, Wong EW, Mruk DD, Cheng CY. TGF-beta3 and TNFalpha perturb blood-testis barrier (BTB) dynamics by accelerating the clathrin-mediated endocytosis of integral membrane proteins: a new concept of BTB regulation during spermatogenesis. Dev Biol 2009; 327:48–61.
45. Li MWM, Mruk DD, Lee WM, Cheng CY. Cytokines and junction restructuring events during spermatogenesis in the testis: an emerging concept of regulation. Cytokine Growth Factor Rev 2009; 20:329–338.
46. Stamatovic SM, Keep RF, Wang MM, Jankovic I, Andjelkovic AV. Caveolae-mediated internalization of occludin and claudin-5 during CCL2-induced tight junction remodeling in brain endothelial cells. J Biol Chem 2009; 284:19053–19066.
47. 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.
48. Suescun MO, Rival C, Theas MS, Calandra R, Lustig L. Involvement of tumor necrosis factor-alpha in the pathogenesis of autoimmune orchitis in rats. Biol Reprod 2003; 68:2114–2121.
49. Giepmans BN, Moolenaar WH. The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol 1998; 8:931–934.
50. Chung NP, Mruk D, Mo MY, Lee WM, Cheng CY. A 22-amino acid synthetic peptide corresponding to the second extracellular loop of rat occludin perturbs the blood-testis barrier and disrupts spermatogenesis reversibly in vivo. Biol Reprod 2001; 65:1340–1351.
51. 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.
52. Catizone A, Ricci G, Galdieri M. Hepatocyte growth factor modulates Sertoli-Sertoli tight junction dynamics. J Cell Physiol 2008; 216:253–260.
53. Catizone A, Ricci G, Caruso M, Ferranti F, Canipari R, Galdieri M. Hepatocyte growth factor (HGF) regulates blood-testis barrier (BTB) in adult rats. Mol Cell Endocrinol 2012; 348:135–146.
54. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol 2003; 149:1–38.
55. Hashizume M, Mihara M. The roles of interleukin-6 in the pathogenesis of rheumatoid arthritis. Arthritis 2011; 2011:765624.
56. Desai TR, Leeper NJ, Hynes KL, Gewertz BL. Interleukin-6 causes endothelial barrier dysfunction via the protein kinase C pathway. J Surg Res 2002; 104:118–123.
57. Brett FM, Mizisin AP, Powell HC, Campbell IL. Evolution of neuropathologic abnormalities associated with blood-brain barrier breakdown in transgenic mice expressing interleukin-6 in astrocytes. J Neuropathol Exp Neurol 1995; 54:766–775.
58. Quintana A, Müller M, Frausto RF, Ramos R, Getts DR, Sanz E, Hofer MJ, Krauthausen M, King NJ, Hidalgo J, Campbell IL. Site-specific production of IL-6 in the central nervous system retargets and enhances the inflammatory response in experimental autoimmune encephalomyelitis. J Immunol 2009; 183:2079–2088.
59. Lui WY, Lee WM, Cheng CY. Transforming growth factor beta3 regulates the dynamics of Sertoli cell tight junctions via the p38 mitogen-activated protein kinase pathway. Biol Reprod 2003; 68:1597–1612.
60. Johnson C, Jia Y, Wang C, Lue YH, Swerdloff RS, Zhang XS, Hu ZY, Li YC, Liu YX, Hikim AP. Role of caspase 2 in apoptotic signaling in primate and murine germ cells. Biol Reprod 2008; 79:806–814.
61. Dodeller F, Schulze-Koops H. The p38 mitogen-activated protein kinase signaling cascade in CD4 T cells. Arthritis Res Ther 2006; 8:205.
62. Lee NP, Cheng CY. Regulation of Sertoli cell tight junction dynamics in the rat testis via the nitric oxide synthase/soluble guanylate cyclase/3′,5′-cyclic guanosine monophosphate/protein kinase G signaling pathway: an in vitro study. Endocrinology 2003; 144:3114–3129.
63. Jarazo Dietrich S, Jacobo P, Pérez CV, Guazzone VA, Lustig L, Theas MS. Up regulation of nitric oxide synthase-nitric oxide system in the testis of rats undergoing autoimmune orchitis. Immunobiology 2012; 217: 778–787.
64. Yan HH, Mruk DD, Lee WM, Cheng CY. Cross-talk between tight and anchoring junctions-lesson from the testis. In: Cheng CY (ed.), Molecular Mechanisms in Spermatogenesis. New York: Landes Bioscience and Springer Science; 2008: 234–254.
65. Lee NP, Leung KW, Wo JY, Tam PC, Yeung WS, Luk JM. Blockage of testicular connexins induced apoptosis in rat seminiferous epithelium. Apoptosis 2006; 11:1215–1229.

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