

Tumor necrosis factor α reversibly disrupts the blood–testis barrier and impairs Sertoli–germ cell adhesion in the seminiferous epithelium of adult rat testes

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Abstract

The timely restructuring of the blood–testis barrier (BTB) that facilitates the migration of preleptotene and leptotene spermatocytes from the basal to the adluminal compartment in the seminiferous epithelium of adult rat testes, which occurs at late stage VII through early stage VIII of the epithelial cycle, is a crucial cellular event of spermatogenesis. However, the regulation of BTB dynamics at the biochemical level remains elusive. In this study, tumor necrosis factor α (TNF α), a secretory product of Sertoli and germ cells in rat testes, was shown to affect junction dynamics *in vivo*. Following an acute administration of recombinant TNF α directly to adult rat testes *in vivo* at 0.5 and 2 μ g/testis (with a body weight \sim 300 g), this treatment significantly and transiently disrupted the BTB. It also transiently inhibited the steady-state protein levels of occludin, zonula occludens-1, and N-cadherin, but not junction adhesion molecule-A, α -, and β -catenin in testes at the BTB site as illustrated by immunoblottings, immunohistochemistry, electron

microscopy, and fluorescent microscopy. This transient disruption of the BTB integrity induced by TNF α treatment was further demonstrated by a functional test to assess the passage of a fluorescent dye (e.g. fluorescein-5-isothiocyanate) from the systemic circulation to the adluminal compartment. Additionally, both the phosphorylated-Ser/Thr protein kinase activated by MAP kinase kinase (p-p38) and phosphorylated-externally regulated kinase (p-ERK) mitogen-activated protein kinase-signaling pathways were transiently activated. Collectively, these data coupled with the recently published *in vitro* studies have illustrated that the BTB is likely utilizing a novel mechanism in which localized production of TNF α by Sertoli and germ cells into the microenvironment at the basal compartment facilitates the timely restructuring ('opening?') of the BTB during spermatogenesis to facilitate germ cell migration.

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Introduction

In rodent testes, tumor necrosis factor α (TNF α ; M_r 51 kDa consisting of three identical subunits of 17 kDa each) is secreted mostly by germ cells, particularly pachytene spermatocytes and round spermatids, and Sertoli cells and macrophages to some extent; however, its receptors are largely confined to Sertoli cells (De *et al.* 1993, Skinner 1993, Hedger 2002, Hedger & Meinhardt 2003, Siu *et al.* 2003a). TNF α is a multifunctional cytokine in mammalian testes, regulating different cellular processes pertinent to spermatogenesis. These include steroidogenesis, apoptosis by promoting germ cell survival, function of growth factors (e.g. insulin-like growth factor), follicle-stimulating hormone action, Fas-ligand system, Sertoli cell secretory function and energy metabolism, and inflammatory responses, similar to

many of its effects discovered in epithelia from different organs (Mauduit *et al.* 1993, Magueresse–Battistoni *et al.* 1995, Besset *et al.* 1996, Nehar *et al.* 1997, Sigillo *et al.* 1999, Pentikainen *et al.* 2001, Aggarwal 2003, Hedger & Meinhardt 2003, Vilcek 2003, Bornstein *et al.* 2004, Hong *et al.* 2004, Mruk & Cheng 2004, Suominen *et al.* 2004, Xia *et al.* 2005). In addition to these functions that are ascribed to TNF α , an earlier study has shown that this pro-inflammatory cytokine may have another crucial role in testicular function related to cell adhesion in the seminiferous epithelium. For instance, chronic administration of TNF α to adult rats *in vivo* via systemic administration at about $2\text{--}4 \times 10^5$ units/kg body weight (bw) per 24 h was shown to induce germ cell loss from the seminiferous epithelium, which is likely to be via a yet-to-be defined mechanism at the Sertoli–germ cell interface unrelated to inflammatory responses (Mealy *et al.* 1990), but

it was not known at the time if the blood–testis barrier (BTB) was disrupted (Mealy *et al.* 1990).

Recent *in vitro* studies have indeed demonstrated that TNF α is a potential crucial regulator of junction dynamics using Sertoli cells cultured *in vitro*. For instance, treatment of primary rat Sertoli cell cultures during tight junction (TJ) assembly with TNF α was shown to perturb the Sertoli cell TJ-permeability barrier, concomitant with a drop in the steady-state protein level of occlusion produced by Sertoli cells (Siu *et al.* 2003a), suggesting that this cytokine may regulate TJ-barrier function via its effects on the levels of TJ-proteins in the testis. TNF α was also shown to inhibit the expression of claudin-11 by mouse Sertoli cells cultured *in vitro* (Hellani *et al.* 2000). In light of the effects of TNF α on Sertoli cell TJ-barrier function *in vitro*, these earlier findings seem to implicate the potential significance of TNF α in BTB dynamics in the testis, since TJ-barrier function at the BTB is contributed largely by the inter-Sertoli cell TJ. Furthermore, Sertoli and germ cells may secrete this cytokine into the microenvironment at the BTB to facilitate the migration of preleptotene and leptotene spermatocytes at late stage VII through stage VIII of the epithelial cycle in adult rat or mouse testes (Russell 1977). This speculation is not proposed without basis. First, studies in other epithelia have shown that TNF α downregulated occludin in human HT-29/B6 epithelial cells via its effects on the occludin promoter (Mankertz *et al.* 2000). Secondly, TNF α was also shown to induce adhesion disruption in epithelial cells via its disruptive effects on the cadherin/ β -catenin complex and the actin filaments (Tabibzadeh *et al.* 1995). Thirdly, the localization of TNF α in the seminiferous epithelium in adult rat testes was stage-specific, being the highest at stages VI–early VIII (but not late VIII) at the apical ectoplasmic specialization (apical ES) and the BTB sites (Siu *et al.* 2003a). Collectively, these data thus suggest that TNF α may be a crucial regulator of junction dynamics in the seminiferous epithelium *in vivo*.

In this report, we sought to investigate if TNF α produced locally by Sertoli and/or germ cells into the microenvironment in the seminiferous epithelium during spermatogenesis modulated the steady-state levels of TJ- and/or adherens junction (AJ)-proteins at the BTB, thereby regulating the BTB integrity and possibly Sertoli–germ cell adhesion. Since studies using several *in vivo* models of TJ disruption, such as glycerol or cadmium, have shown that a disruption of the TJ-barrier at the BTB can lead to a disruption of Sertoli–germ cell adhesion, these findings illustrate cross-talk between TJ and Sertoli–germ cell AJ in the seminiferous epithelium (Wiebe *et al.* 1986, 2000, Hew *et al.* 1993). Also, it is of interest to investigate if these effects induced by TNF α treatment are reversible. This report represents an initial step to investigate the mechanism utilized by the testis involving cytokines (e.g. TNF α) that regulates BTB dynamics pertinent to germ cell migration and junction restructuring in the seminiferous epithelium during spermatogenesis.

Materials and Methods

Animals

Male Sprague–Dawley rats were purchased from Charles River Laboratories (Kingston, NY, USA). All animals were housed at The Rockefeller University Laboratory Animal Research Center with free access to water and standard chow and under a 12 h light:12 h darkness cycle. The use of animals in this study was approved by the Rockefeller University Animal Care and Use Committee (Protocol numbers: 00111, 03017, and 06018).

Induction of reversible damage to the BTB and Sertoli–germ cell adhesion by TNF α : an *in vivo* model to study BTB dynamics

This study was performed to assess if adult rat testes use TNF α to regulate BTB dynamics *in vivo*. Furthermore, results from this study were also used to assess if local administration of TNF α to the testis of adult rats at a concentration consistent with its physiological level intra-testicularly could become an *in vivo* model to study BTB dynamics. This model apparently should fulfil the following criteria. First, the agent that was used to induce BTB restructuring should preferably be produced locally by Sertoli and/or germ cells in the seminiferous epithelium. Secondly, the agent should be non-cytotoxic to cells in the epithelium at the doses that were effective to induce junction restructuring. Thirdly, the disruptive effects of the compound to the BTB should be reversible. For instance, the disrupted BTB could be resealed once the agent was metabolized or cleared from the host animals. Our postulate is that TNF α is a product of germ and Sertoli cells, and recent *in vitro* studies have shown that at the doses that were effective to induce reversible Sertoli cell TJ-barrier disruption, TNF α was non-cytotoxic to Sertoli cells (Siu *et al.* 2003a). As such, this cytokine is a natural regulator of BTB dynamics *in vivo* and that local administration of this cytokine to adult rat testes can be developed into a useful *in vivo* model to study BTB regulation. In short, groups of rats ($n = 3–5$ for each time point) were treated with recombinant human TNF α (Calbiochem, San Diego, CA, USA) using two dosing regimens of either 0.5 $\mu\text{g}/\text{testis}$ (low dose) or 2 $\mu\text{g}/\text{testis}$ (acute high dose). Recombinant proteins were dissolved in PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 at 22 °C) to a desired volume, so that the right testis of an adult rat (~ 300 g bw) received ~ 150 μl PBS containing either 0.5 or 2 μg TNF α ; the left testis served as a control and received either PBS (vehicle control) or no treatment (time 0). Recombinant protein was administered using a 27 gauge needle as described (Russell *et al.* 1987, Wiebe *et al.* 2000, Lui *et al.* 2003b, Siu *et al.* 2005). Thereafter, rats were killed at different checkpoints (i.e. time point at which a specific cellular event occurs; in groups of three to five rats for each checkpoint) to track the kinetics of changes in the BTB and the status of spermatogenesis: (i) time of TNF α injection (time 0), (ii) time of first appearance of BTB

disruption (~ 7 h–day 3), (iii) time of first appearance of germ cell loss from the seminiferous epithelium (\sim days 3–5), (iv) time of first appearance of BTB recovery (\sim days 5–8), and (v) time of germ cell repopulation in the epithelium (days 14–60). Each experiment was repeated three times. In selected experiments, testes were either immediately placed in Bouin's fixative and processed for paraffin sectioning for hematoxylin–eosin staining, or frozen in liquid nitrogen and stored at -80°C until used for either immunohistochemistry or immunoblottings. Since preliminary experiments have illustrated that in rats treated with 500 ng TNF α /testis, changes detected in the seminiferous epithelium by histological analysis were less pronounced than the high-dose group, results presented in this report were from studies of the high-dose group only. Using paraffin sections stained with hematoxylin–eosin, at least 600 tubules from testes of four rats (~ 150 tubules were randomly selected from each testis) were scored for damage from each treatment group under a light microscope (Olympus BX40). A tubule that displayed significant germ cell loss from the seminiferous epithelium was scored as a damaged tubule, which was manifested by the presence of greater than ten elongating/elongate/round spermatids and/or spermatocytes in the tubule lumen (tubules at late stage VIII of the epithelial cycle that were clearly visible and readily distinguishable were not scored as damaged tubules) or significant thinning of the seminiferous epithelium cell layer by up to 50% versus control tubules. Shrinkage in tubule diameter was also scored by comparing the diameter of each tubule (~ 600 tubules were scored from at least four rats) from treatment groups with control testes. Micrographs were captured using an Olympus DP70 digital camera at 12.5 MPa, and printed on Inkjet paper using an Epson 890 Photo Printer for scoring of damaged tubules.

Preparation of samples, immunoblot analysis, and semi-quantitative reverse transcriptase (RT)-PCR

Testis samples were prepared in a lysis buffer (50 mM Tris–HCl, 0.15 M NaCl, 1% NP-40 (Nonidet P-40, an octylphenol–ethylene oxide condensate) (v/v), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 1 mM sodium orthovanadate, pH 7.4 at 22°C) as described (Siu *et al.* 2005). Equal amounts of protein (~ 100 μg total protein) from each sample within an experimental group were used for immunoblottings. Protein concentrations in samples were estimated by Coomassie Blue dye-binding assay using BSA as a standard (Bradford 1976). All samples within an experimental group were processed in a single experimental session to eliminate interexperimental variations. Primary antibodies used in this report were obtained commercially. Their sources and the dilutions used for different experiments are listed in Table 1. Total RNA from testicular cells were isolated and RT-PCR was performed as previously described (Siu *et al.* 2003a). Primers used for PCR amplification of different target genes are listed in Table 2.

Immunofluorescent microscopy, immunohistochemistry, and electron microscopy

Immunofluorescent microscopy was performed as described earlier (Siu *et al.* 2003b, 2005) to co-localize occludin/zona occludens-1 (ZO-1), junction adhesion molecule-A (JAM-A)/ZO-1, and N-cadherin/ β -catenin to the seminiferous epithelium at the BTB using the corresponding specific antibodies (see Table 1). Immunohistochemistry was used to visualize the changes in the localization of occludin following TNF α treatment as described earlier (Siu *et al.* 2003a). To eliminate interexperimental variations for studies using fluorescent microscopy or immunohistochemistry, all samples within a treatment group were processed simultaneously by placing two to three cross-sections on a single microscopic slide so that a single investigator could process two to four slides altogether in a single experimental session. Electron microscopy was used as described (Siu *et al.* 2005), which was performed at The Rockefeller University Bio-Imaging Facility to assess damage to the BTB and apical ectoplasmic specialization (ES) in the seminiferous epithelium from adult rats following local administration of TNF α at 2 $\mu\text{g}/\text{testis}$ by day 3.

Assessing the BTB integrity

Earlier studies have shown that a combination of immunoblottings and fluorescent microscopy to co-localize occludin and ZO-1 at the BTB in the rat testes is a reliable approach to monitor BTB integrity (Wong *et al.* 2004, 2005). For instance, using fluorescent microscopy, occludin (e.g. red fluorescence, labeled with Cy3), and ZO-1 (e.g. green fluorescence, labeled with fluorescein-5-isothiocyanate, FITC) were co-localized to the same site in the seminiferous epithelium consistent with their localization at the BTB, forming an almost continuous and superimposable fluorescence ring at the BTB (Wong *et al.* 2004). When the BTB was damaged (e.g. following treatment with CdCl_2), the levels of these two proteins were greatly diminished and the two superimposable fluorescent rings of occludin and ZO-1 became badly disrupted and disorganized at the BTB (Wong *et al.* 2004). These changes were also coupled with a significant decline in the steady-state protein levels of occludin and ZO-1 monitored by immunoblottings and immunohistochemistry. When these techniques were compared with more tedious functional approaches, such as the use of electron microscopy or micropuncture technique to monitor the diffusion of [^{125}I]-BSA from the systemic circulation (administered via the jugular vein) across the BTB to rete testis fluid and seminiferous tubule fluid (Chung *et al.* 2001, Wong *et al.* 2004), it was shown that they could reliably monitor the BTB integrity. As such, the BTB integrity was assessed by immunoblottings, fluorescent microscopy, and immunohistochemistry as reported herein, which was further confirmed by electron microscopy. Additionally, we have also developed an efficient functional approach to monitor the BTB integrity without the need for using a radioactive isotope,

Table 1 The sources of different antibodies and their dilutions used for different studies in this report

	Antibody against target protein	Animal source ^a	Catalog #	Lot #	Use	Working dilution
Vendor						
Santa Cruz	α -Catenin	Rabbit	sc-7894	G3003	IB	1:200
Biotechnologies	β -Catenin	Rabbit	sc-7199	L0203	IB	1:200
(Santa Cruz, CA, USA)	Nectin-3	Goat	sc-14806	K261	IB	1:200
	Actin	Goat	sc-1616	D052	IB	1:1000
	Vimentin	Mouse	sc-6260	D1805	IB	1:200
	TNF α	Goat ^b	sc-1348	J2405	IB	1:200
	N-Cadherin	Rabbit	sc-7939	J1502	IB	1:200
Cell Signaling Technology	p-ERK (Thr ²⁰² /Tyr ²⁰⁴)	Rabbit	9101S	13	IB	1:1000
(Beverly, CA, USA)	ERK	Rabbit	9102	10	IB	1:1000
	p-p38 MAPK (Thr ¹⁸⁰ /Tyr ¹⁸²)	Rabbit	9211	10	IB	1:1000
	p38 MAPK	Rabbit	9212	3	IB	1:1000
	JNK	Rabbit	9252	4	IB	1:200
	p-JNK	Mouse	9255	10	IB	1:200
Zymed Laboratories Inc.	Occludin	Rabbit	71-1500	30979485	IBIF, IHC	1:200
(South San Francisco, CA, USA)	JAM-A	Rabbit	36-1700	30979650	IB, IF	1:250
	ZO-1	Rabbit	61-7300	50799336	IB	1:150
	ZO-1	Mouse	33-9100	40890448	IF	1:100
	N-Cadherin	Mouse	33-3900	50393487	IF	1:100

^aAll antibodies purchased were polyclonal antibodies except for those prepared in mice, which were monoclonal antibodies.

^bThis anti-TNF α antibody was raised in goat against a fragment of the mouse TNF α from its C-terminus, and it cross-reacted with TNF α of mouse, rat, and human origin by IB as indicated by the vendor. Furthermore, homology search between the primary sequence of the human and rat TNF α proteins using BLAST at GenBank has indicated that they shared ~80% identity. IB, immunoblotting; IF, immunofluorescent microscopy; IHC, immunohistochemistry.

such as [¹²⁵I]-BSA. In this approach, the diffusion of a fluorescent dye (e.g. FITC) from the systemic circulation to the seminiferous epithelium in treatment groups versus control rats was monitored by fluorescent microscopy. In short, rats following recombinant TNF α treatment (at 2 μ g/testis) on days 3 and 10, normal rats (either vehicle control or no treatment, Ctrl), and rats receiving CdCl₂ (3 mg/kg bw, i.p., on day 3, which is known to disrupt the BTB, to serve as a positive control (Setchell & Waites 1970, Hew *et al.* 1993, Wong *et al.* 2004)) were anesthetized with ketamine HCl (~40–60 mg/kg bw, via i.m.) with xylazine (~10–13 mg/kg, i.m.) as an analgesia. An incision of about 0.5 cm was made with an Iris scissors over the jugular vein to expose the vessel. Each rat (with two rats for each treatment group including controls) received an infusion of FITC (isomer I, C₂₁H₁₁NO₅S, *M_r* 389.39) (AnaSpec, Inc., San Jose, CA,

USA) in a final volume of ~200 μ l (containing ~0.2 mg dissolved in 10% dimethyl sulphoxide /90% PBS, v/v) via the jugular vein using a 27 gauge needle. The surgical area was then stitched with nylon suture black monofilament (Ethicon, Inc., Somerville, NJ, USA), cleansed with 70% ethanol, and the rats allowed to recover. About 90 min thereafter, rats were killed by carbon dioxide asphyxiation, testes were immediately removed, frozen in liquid nitrogen, and frozen sections of ~8 μ m were cut in a cryostat and examined by an Olympus BX40 microscope equipped with fluorescence optics and an Olympus DP-70 digital camera. The presence of FITC (green fluorescence) in the seminiferous epithelium in treatment groups versus negative (normal rats) and positive (CdCl₂ treatment) controls were photographed using the built-in digital camera and images acquired with the QCapture Suite (version 2.60, Quantitative Imaging Corp., Burnaby, BC,

Table 2 The nucleotide sequences for primers used for RT-PCR to identify different target genes

Target gene	Primer sequence	Orientation	Position	Length (bp)	GenBank accession # or reference
TNFR1	5'-GGGATTCAGCTCCTGTCAAA-3'	Sense	983–1002	488	M63122
	5'-CAGCATGCTGTAATGAGCCT-3'	Antisense	1451–1470		
TNFR2	5'-GTTCTCTGACACCACATCATCC-3'	Sense	579–600	456	AF498039
	5'-GTCAATAGGTGCTGCTGTTCAA-3'	Antisense	1013–1034		
S-16	5'-TCCGCTGCAGTCCGTT-CAAGTCTT-3'	Sense	15–38	385	Chan <i>et al.</i> (1990)
	5'-GCCAAACTCTTGATTCCG-CAGCG-3'	Antisense	376–399		

Canada) software and examined in Adobe PhotoShop (version 7.0). To eliminate interexperimental variations, all cross-sections (about two to three sections per glass slide) from both treatment and control groups were analyzed simultaneously in a single experimental session. Fluorescent images were randomly selected from five different areas per section, photographed, and printed using an Epson 875EX Inkjet printer for examination and scoring. At least 50 tubules from each testis of different treatment groups (e.g. TNF α and CdCl₂) versus controls (normal testes and testes receiving vehicle) were randomly selected and a total of 100 tubules from two testes of different rats were scored. The distance of the fluorescence (FITC) diffused from the BTB site (D_{FITC}) versus the radius of the seminiferous tubule (D_{radius}) were obtained for each tubule in treatment groups and controls, which served as an index to assess the BTB integrity.

Assessing endogenous TNF α levels in adult rat testes

An efficient solid-phase immunoblot-based assay was developed and used to estimate the endogenous level of TNF α in adult rat testes as follows. In brief, different amounts of recombinant human TNF α (Calbiochem) protein at 1, 3, 5, 10, and 15 ng were resolved by SDS-PAGE on a 12.5% T SDS-polyacrylamide gel under reducing conditions with ~300 μ g total protein from spent media of Sertoli and germ cells, and lysates of Sertoli cells, germ cells and testes. Each sample was run in duplicates. To eliminate interexperimental variations, all unknowns and standards were resolved onto a single SDS-polyacrylamide gel with one set of samples, and each experimental session routinely consisted of two gels that were processed in the same SDS-PAGE tank. Following electrophoretic transfer of these proteins onto nitrocellulose membranes, the blots were immunostained with an anti-TNF α antibody against mouse TNF α , which is known to cross-react with the human and rat TNF α (see Table 1). The resultant blots were then densitometrically scanned at 610 nm using SigmaGel software (version 1.05; SPSS, Inc., Chicago, IL, USA), and the area of the protein peak corresponding to TNF α for both standards (i.e. recombinant TNF α), and lysates of testicular cells and testes were obtained. Data were analyzed using GraFit Data Analysis Software (version 5, Erithacus Software Ltd, Horley, Surrey, UK) using linear regression by plotting the mean area for each of the standards on the y -axis against TNF α (ng) on the x -axis, and the levels of TNF α in unknowns were interpolated from the resultant curve.

Statistical analysis

Experiments reported herein were repeated at least three times unless otherwise specified, including all studies using immunohistochemistry, fluorescent microscopy, and immunoblottings. In most morphology studies, experiments were conducted by two different investigators in the laboratory using different sets of treated samples versus controls over a 2-year period. Data shown herein are

representative results of these analyses. Multiple comparisons were performed using ANOVA, followed by Tukey's honestly significant test to compare selected pairs of samples within an experimental group. Statistical analysis was performed using the GB-STAT statistical analysis software package (version 7, Dynamic Microsystems, Inc., Silver Spring, MD, USA).

Results

TNF α regulates BTB dynamics in adult rat testes

When recombinant TNF α was administered to testes of adult rats at 500 ng/testis, a loss of occludin from the BTB was detected by fluorescent microscopy and immunoblottings (data not shown). This loss became much more pronounced when an acute dose at 2 μ g TNF α was administered per testis (see Fig. 1). As shown in Fig. 1, occludin (a TJ-integral membrane protein) and ZO-1 (a TJ-associated adaptor) were co-localized to the same site at the basal compartment in normal rat testes or testes from rats treated with vehicle only (see Fig. 1A–D) consistent with their localization at the BTB and that these proteins formed an almost continuous belt at the BTB surrounding the entire seminiferous tubule (see Fig. 1A–D). However, both TJ-proteins were greatly reduced by day 3 following TNF α treatment with diminished fluorescence (Fig. 1E–H vs A–D). However, this effect apparently was limited to occludin and ZO-1, since the localization pattern and the fluorescence of JAM-A (also a TJ-integral membrane protein) was not perturbed (Fig. 1I–P vs A–H). The TNF α -induced damage to the BTB was further examined by electron microscopy. As shown in Fig. 2A and B, the actin filament bundles and the associated endoplasmic reticulum (ER) at the basal ES that co-exists with TJs at the BTB became severely disorganized and defragmented (see black arrowheads in the boxed area in Fig. 2B) by day 3 after TNF α treatment at a dose of 2 μ g/testis when compared with normal testes shown in Fig. 2A (see the boxed area showing the typical ultrastructure of the BTB in normal adult rat testes). While TJs in this area (see black arrowheads in Fig. 2B) remained largely unaffected even though the co-existing basal ES was badly damaged, TJs that were found further up of the BTB became severely damaged as manifested by a significant increase in the intercellular space between the two adjacent Sertoli cells and the loss of actin filament bundles and cisternae of ER typical of basal ES (see asterisks in the boxed area in Fig. 2B versus the boxed area in Fig. 2A). Interestingly, this TNF α treatment also induced disruption and/or disorganization of the actin filament bundles and the cisternae of ER at the apical ES (Fig. 2D, E vs C; Fig. 2C is from a normal rat testis showing the typical ultrastructural features of apical ES in which the actin filament bundles were sandwiched between the cisternae of ER and the Sertoli cell plasma membrane, which was restricted only to the Sertoli cell side and not found in elongating spermatids. In the basal

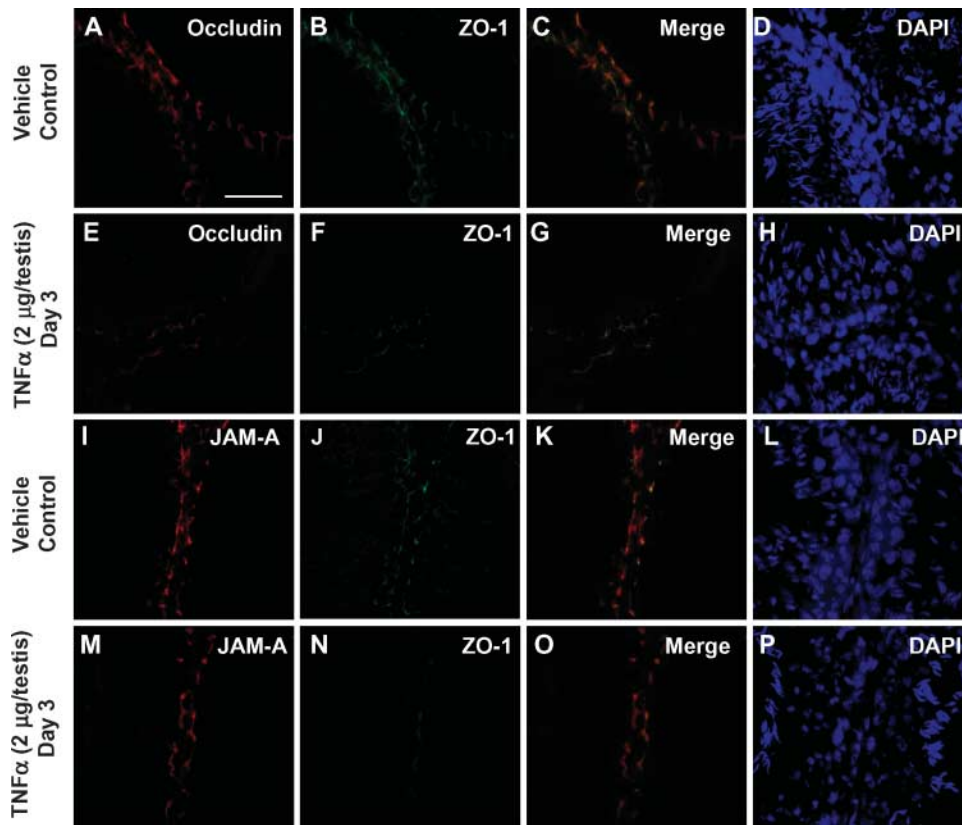


Figure 1 (A)–(P) A study to assess the tumor necrosis factor (TNF) α -induced BTB damage by immunofluorescent microscopy. Fluorescent micrographs using cross-sections of frozen testes from rats received vehicle control (A)–(D), (I)–(L), or TNF α (2 μ g/testis) (E)–(H), (M)–(P). Rats were killed on day 3 in the treatment groups. Red fluorescence (Cy3) represents occludin, (A) and (E), and JAM-A, (I) and (M), whilst the green fluorescence (FITC) represents ZO-1, (B), (F), (J), and (N). The merged micrographs (C), (G), (K), and (O) illustrate the co-localization of occludin and ZO-1 (A)–(H) or JAM-A and ZO-1 (I)–(P) to the same site in the basal compartment, consistent with their localization at the BTB, which appears as orange fluorescence. (D), (H), (L), and (P) are 4'-6-diamidino-2-phenylindole stained for nuclei. In testes receiving vehicle control, occludin, ZO-1, and JAM-1 were found at the BTB forming a continuous fluorescent belt at the basal compartment, (A), (B) and (I), (J). On day 3, the occludin and ZO-1 (E), (F) vs (A), (B); (N) vs (J), but not JAM-A (M) vs (I) fluorescence was greatly reduced. Bar in A = 80 μ m, which also applies to (B)–(P). These fluorescent micrographs are the representative result of one experiment, two additional experiments using sections from different animals yielded similar results.

ES, this feature was found in both sides of the two Sertoli cells, see Fig. 2C vs A). The apical ES is a testis-specific, cell–cell actin-based hybrid AJ and focal contact junction type (Siu & Cheng 2004) as seen in Fig. 2D and E between an elongating spermatid and Sertoli cell in the seminiferous epithelium.

TNF α affects Sertoli–germ cell adhesion in the seminiferous epithelium

The status of spermatogenesis in the TNF α -treated animals was further examined using hematoxylin–eosin-stained paraffin sections at selected time points and compared with controls (rats received either vehicle control or no treatment) as shown in Fig. 3. It was noted that by day 3 after TNF α

(2 μ g/testis) treatment, about 15% of the tubules scored from a total of 600 tubules from four different rats displayed signs of damage as manifested by the appearance of spermatids (elongating and round spermatids) and spermatocytes in the tubule lumen and the thinning of the cell layer in the seminiferous epithelium (Fig. 3A vs B–D). By day 5, over 30% of the tubules were damaged (Fig. 3E–G), which was accompanied by a significant shrinkage in tubular diameter (Fig. 3G). Thus, these data (Fig. 3B–F vs A) are in agreement with results of the electron microscopy that have illustrated a disruption of the apical ES (see Fig. 2D and E vs C), suggesting that TNF α treatment perturbed Sertoli–germ cell adhesion in the seminiferous epithelium in particular at the apical ES. Since some spermatocytes and early round spermatids were also depleted from the epithelium (see Fig. 3C and D)

and the anchoring junctions between these germ cells and Sertoli cells are largely desmosome-like junctions without apical ES, this suggests that TNF α may have a generalized effect on Sertoli–germ cell adhesion. However, the fact that spermatogenesis could be recovered thereafter and the amount of TNF α administered was within the physiological range (see below), suggests that these effects are not the result of cytotoxicity.

Occludin is one of the target proteins of TNF α in the seminiferous epithelium

To further verify the results of fluorescent microscopy shown in Fig. 1 that occludin is likely a target protein of TNF α -induced BTB damage, immunohistochemistry was performed. It was noted that in normal adult rat testes, occludin was restricted to the basal compartment consistent with its localization at the BTB in almost all tubules except for stage VIII tubule (see reddish-brown precipitates in Fig. 4A and B) at the time when preleptotene and leptotene spermatocytes must traverse the BTB, entering the adluminal compartment for further development (Russell 1977). Results reported in Fig. 4 also illustrate that the localization of occludin in the seminiferous epithelium is stage-specific, being the lowest at stage VIII tubules in adult rat testes. Following TNF α treatment (2 μ g/testis) on day 3, the amount of immunoreactive occludin at the BTB was greatly diminished (Fig. 4C and D vs A and B). Figure 4E was a control staining using normal rabbit IgG in substitution of the primary antibody. Immunoblottings were subsequently used to validate changes in the occludin protein level and the possible downstream signaling pathways utilized by TNF α to perturb BTB integrity and Sertoli–germ cell adhesion in the seminiferous epithelium. As shown in Fig. 5A (upper panel), the TNF α -induced BTB damage was associated with a transient loss of occludin, ZO-1, and N-cadherin, but not JAM-A, claudin-11, α -, and β -catenin (Fig. 5A, B-a, B-b), consistent with results shown in Fig. 1–3. Since earlier studies have shown that transforming growth factor β 3 (TGF- β 3), another crucial regulator of BTB dynamics in the testis, modulates BTB dynamics via the Ser/Thr protein kinase (p38) activated by mitogen-activated protein kinase (MAPK)-signaling pathway (Lui *et al.* 2003b, Wong *et al.* 2004), but TGF- β 3 can also limit its effects on Sertoli–germ cell adhesion via the externally regulated kinase (ERK) MAPK-signaling pathway (Xia & Cheng 2005), we thus sought to examine if TNF α is utilizing similar mechanism. Indeed, the TNF α -induced BTB damage and Sertoli–germ cell adhesion disruption were associated with a transient induction in phosphorylated (p)-p38 MAPK and phosphorylated (p)-ERK, but not phosphorylated (p)-C-Jun N-terminal protein kinase (JNK) (Fig. 5A, middle panel and B-c). The lower panel in Fig. 5A shows the steady-state protein levels of vimentin and β -actin, which served as loading controls.

TNF α -induced BTB damage and Sertoli–germ cell AJ disruption is reversible

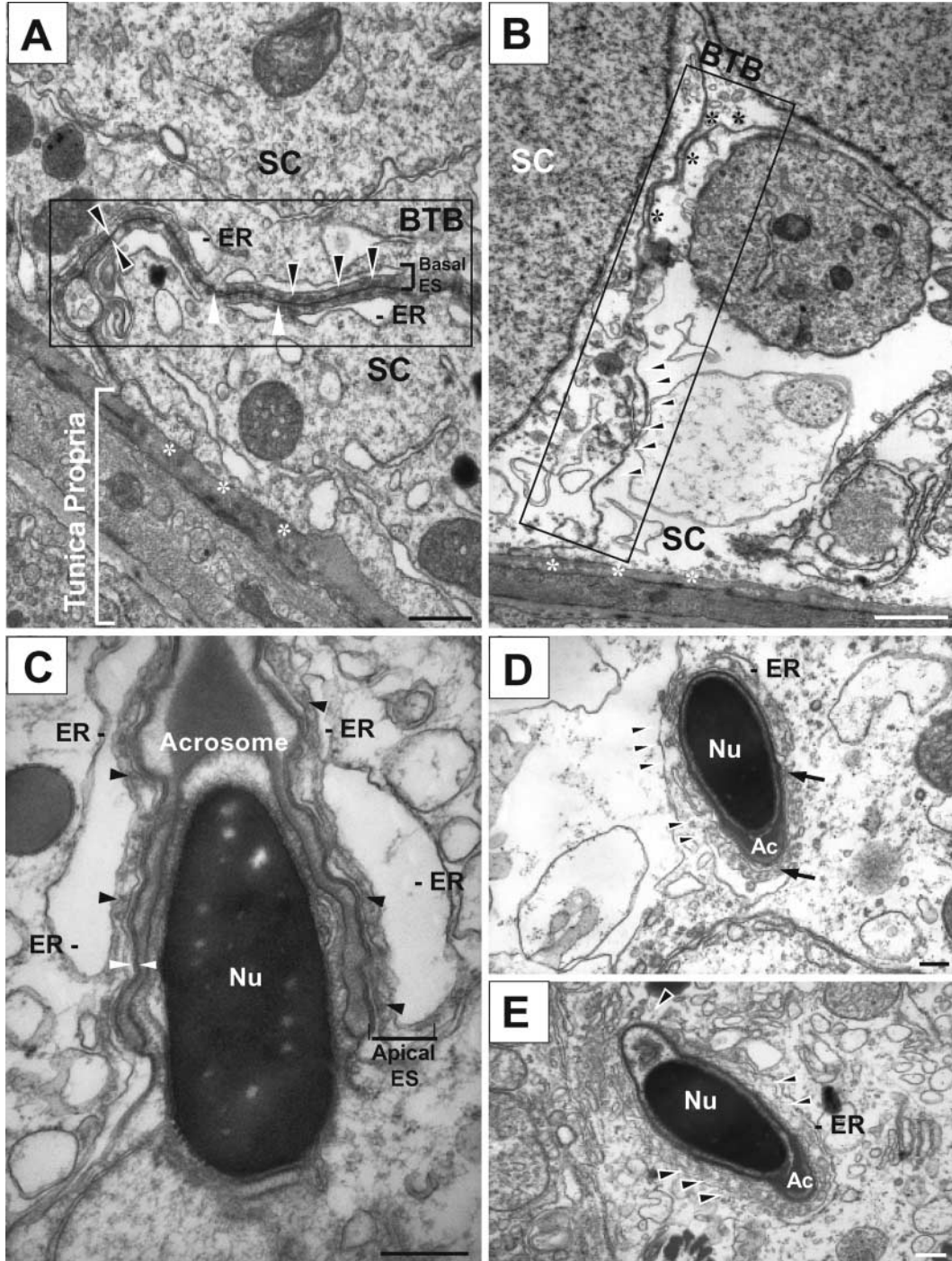
Since immunoblot studies have suggested that the significant decline of occludin, ZO-1, and N-cadherin in the testis following TNF α treatment (2 μ g/testis) was reversible, as these proteins rebound by day 8 (see Fig. 5), we sought to examine if their losses at the BTB were indeed transient and reversible. Consistent with the immunoblotting data shown in Fig. 5, a significant loss of fluorescence of occludin and ZO-1 (Fig. 6A, e–h vs a–d) was detected at the BTB site in the seminiferous epithelium as early as 7-h post-TNF α treatment. Yet this loss fully recovered by day 8, making the fluorescent staining almost indistinguishable from normal testes or testes receiving vehicle control (Fig. 6A, i–l vs a–d and e–h). Similar results were obtained where the loss of N-cadherin from the BTB was also transient as shown in Fig. 6A (q–t vs m–p and u–x). These results seemingly suggest that if TNF α can indeed be produced in the microenvironment at the site of BTB, where preleptotene and leptotene spermatocytes are traversing the BTB, this cytokine likely disrupts the BTB transiently to facilitate germ cell migration. Indeed, consistent with an earlier report (De Cesaris *et al.* 1999), both the p55 TNF α receptor (TNFR1) and p75 TNF α receptor (TNFR2) were shown to be predominantly expressed by Sertoli cells (Fig. 6B). Since TNF α is a product of both germ and Sertoli cells (De *et al.* 1993, Siu *et al.* 2003a), it is likely that these testicular cells release TNF α into the microenvironment to facilitate germ cell migration at late stages VII and VIII of the epithelial cycle by temporarily reducing the occludin, ZO-1, and N-cadherin levels, thereby transiently ‘opening’ the BTB. Using a solid-phase immunoblot-based assay (Fig. 6C), it was shown that germ cells appeared to contribute most of the TNF α in the seminiferous epithelium. The level of TNF α produced by Sertoli cells might be too low to be detected by this method because of its sensitivity, which is \sim 3 ng (see Fig. 6C) and the loading capacity of an SDS-slab gel (\sim 300 μ g protein per lane). Nonetheless, using this solid-phase immunoblot-based assay as described in Materials and Methods, it was estimated that an adult rat testis contained \sim 0.5 \pm 0.15 μ g TNF α ($n=6$). This also illustrates that the amount of TNF α that was administered per testis at 2 μ g as reported here is still the likely physiological range in the microenvironment in the testis.

Functional test for assessing the BTB integrity following TNF α treatment

The BTB integrity was further assessed by a functional test by monitoring the diffusion of FITC (Mr 389) from the systemic circulation to the seminiferous epithelium as described in Materials and Methods. It was shown that treatment of testes with TNF α at 2 μ g/testis indeed induced reversible damage to the BTB (Fig. 7E, G vs H, I, and A–C) with CdCl₂ that

served as a positive control (Fig. 7D and E). For instance, FITC was shown to diffuse into the adluminal compartment by day 3 following TNF α treatment, away from the BTB, which is in sharp contrast to control testes in which the FITC

fluorescence was limited to the BTB (Fig. 7F, G vs A–C). However, this transient loss of BTB integrity was reversed by day 10 (Fig. 7H–J) versus controls (Fig. 7A–C, J), consistent with the data shown in Fig. 6.



Discussion

TNF α affects BTB dynamics in the seminiferous epithelium of adult rat testes

Earlier *in vitro* and *in vivo* studies have implicated the role of cytokines (e.g. TNF α and TGF- β 3) (Hellani *et al.* 2000, Lui *et al.* 2003a, 2003b, Wong *et al.* 2004, Xia & Cheng 2005) in Sertoli cell TJ-dynamics, since they were shown to regulate the steady-state levels of TJ proteins, such as claudins, occludins, and ZO-1, at the BTB site in adult rat testes. However, these earlier reports were based on studies using *in vivo* models, where animals were treated with environmental toxicants (e.g. CdCl₂) to induce rapid BTB restructuring or they were *in vitro* in nature using primary Sertoli cell cultures to induce TJ-barrier assembly, so that changes in different target proteins and their regulation can be monitored and assessed. As such, the physiological relevance of earlier studies that implicate TNF α as a potential regulator of BTB dynamics during spermatogenesis *in vivo* remains to be established.

We report herein that local administration of TNF α to adult rat testes at a concentration approximately fourfold of the endogenous TNF α level in the testis indeed regulated the steady-state levels of several proteins at the BTB in adult rat testes including occludin, ZO-1, and N-cadherin, inducing transient disruption of the BTB integrity. Collectively, the data presented in this report have helped to formulate a working hypothesis (see Fig. 8) suggesting that at the time preleptotene and leptotene spermatocytes are traversing the BTB at stages VII–VIII of the epithelial cycle, these spermatocytes likely coordinate with Sertoli cells to secrete TNF α to the microenvironment at the BTB in the seminiferous epithelium. TNF α exerts its effects via either one or both of its receptors, TNFR1 and TNFR2, residing on Sertoli cells. This, in turn, activates the p38 MAPK-signaling pathway downstream to ‘open’ (or disassemble?) the BTB to facilitate preleptotene/leptotene spermatocyte movement across the barrier, such as by reducing the steady-state levels of occludin and ZO-1 at the BTB. Since this effect is transient, the temporarily ‘opened’ BTB can be rapidly ‘resealed’ to maintain the immunological barrier function of the BTB as illustrated in the results reported

herein. TNF α can also exert its effects on apical ES via an activation of the ERK-signaling pathway, plausibly facilitating the release of spermatids at spermiation, which also takes place at stage VIII of the epithelial cycle. As such, TNF α is working in concert with other cytokines (e.g. TGF- β 3) to serve a dual function in the seminiferous epithelium by facilitating spermiation and preleptotene/leptotene spermatocyte movement across the BTB at stage VIII of the epithelial cycle. This possibility is strengthened by a recent immunohistochemistry study that has localized TNF α to the basal compartment of the seminiferous epithelium consistent with its localization at the BTB, and to the apical ES predominantly at stages VII through early stage VIII of the epithelial cycle prior to spermiation in adult rat testes (Siu *et al.* 2003a). We have attempted to perform a similar immunohistochemistry study with the available anti-TNFR1 and anti-TNFR2 receptor antibodies from two different vendors, but without success because of the low antibody titer and specificity issue. However, an earlier study using an experimental autoimmune orchitis model has illustrated the presence of TNFR1 in adult rat testes by immunohistochemistry, but it was not reported if TNFR1 was expressed stage specifically (Suescun *et al.* 2003). Undoubtedly, a detailed study to localize TNFR1 and TNFR2 in normal testes and their changes during TNF α -induced seminiferous tubular damage should be conducted in future studies.

It must be noted that the hypothesis outlined in Fig. 8 should be viewed as a working model, since much research is needed to validate different possibilities. It must also be understood that the hypothesis depicted in Fig. 8 will be rapidly updated as more data become available in the field. This hypothesis somehow serves as a framework upon which functional experiments can be designed. In this context, it is of interest to note that while acute administration of TNF α disrupts the BTB integrity in virtually all the tubules examined, which is consistent with the immunoblotting data, its effects in inducing germ cell loss from the epithelium are limited to only ~30% of the tubules scored by day 5 after its administration in three separate experiments, suggesting that other cytokines and/or proteins/peptides/factors are also involved in regulating Sertoli–germ cell adhesion in the seminiferous epithelium. Alternatively, TNF α may affect only

Figure 2 (A)–(E) A study by electron microscopy assessing damage to the BTB and apical ES following TNF α treatment. EM micrographs of testes from rats that received vehicle control. The BTB is shown in the boxed area in (A), which is typified by the presence of basal ES (manifested by the presence of actin filament bundles (see black arrowheads) sandwiched between the endoplasmic reticulum (ER) and Sertoli cell (SC) plasma membrane found on both sides of the two apposing SC (see apposing black arrowheads) that co-exist with tight junctions (TJ, see white arrowheads)). The seminiferous epithelium composed of Sertoli and germ cells rests on the basement membrane (asterisks), which is part of the tunica propria. On day 3 after TNF α treatment (2 μ g/testis), the BTB was damaged (B), which was manifested by the disappearance of the basal ES where the actin filaments apparently were disintegrated and the ER also became disorganized (see black arrowheads in (B) vs (A)). The TJ apparently remained largely intact near the basement membrane (white asterisks); however, the TJ seen was broken since the intercellular space was significantly widened between the two adjacent Sertoli cell plasma membranes (see black asterisks in (B)). (C) EM micrograph illustrating the apical ES found between a developing spermatid (Nu, nucleus) and SC, which is typified by the presence of actin filament bundles (black arrowheads) sandwiched between ER and the SC plasma membrane (the two opposing white arrowheads represent the apposing SC and spermatid plasma membranes). However, on day 3 after TNF α treatment (2 μ g/testis), the apical ES found between an elongating spermatid and Sertoli cells as shown in (D) and (E) also became damaged, as manifested by the disintegration (and disappearance) of actin filament bundles (see black arrowheads) and the loss of ER (see (D), (E) vs (C)) except that in some areas, the actin filament bundles were still seen (see arrowheads in (D)). Ac, acrosome. Bar in A = 0.5 μ m; B = 1 μ m; C = 0.2 μ m, which applies to (D) and (E). These micrographs are the representative result of one experiment with two rats for each time point including controls.

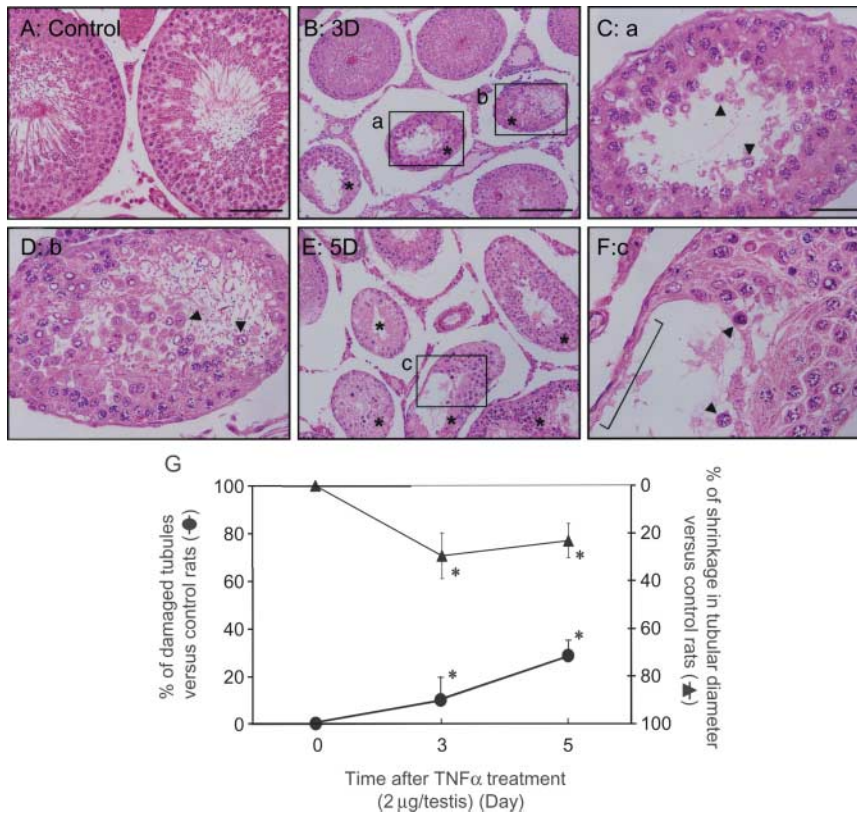


Figure 3 (A)–(G) A study to assess the effects of TNF α treatment (2 μ g/testis) on the status of spermatogenesis in adult rat testes. (A)–(F) Micrographs of normal rat testes (A), rat testes after treatment with TNF α on day 3 (B), (C) and (D), and day 5 (E) and (F). The boxed areas (a, b) shown in (B) were magnified and shown as (C) and (D) respectively. The boxed area (c) in (E) was also magnified and shown as (F). Asterisk (*) indicates representative damaged tubules with either spermatids or spermatocytes found in the tubule lumen (see arrowheads in (C), (D) and (F)) or significant thinning in the seminiferous epithelium (see bracket in (F)). Bar in A=60 μ m; B=120 μ m, which applies to (E); C=30 μ m, which applies to (D) and (F). These micrographs are the representative results of one experiment, three other experiments using different cross-sections of testes yielded similar observations. (G) This bar graph summarizes results of analysis by scoring the damaged tubules and changes in tubule diameter following treatment of rats with TNF α versus controls ($n=3$ –5 rats for each time point). *Significantly different; $P<0.05$.

‘selected’ tubules stage specifically. As such, future studies should include a detailed morphometric analysis to determine whether the TNF α -induced seminiferous tubule damage is stage-specific and if Sertoli cells are more susceptible to the TNF α -induced damage at stages VII–VIII of the epithelial cycle. Also, a testis-specific knockout of TNF α study in mice will address some important issues pertinent to the precise role of TNF α in junction dynamics.

Additionally, it is of interest to note that Russell & Peterson (1985) and Russell (1993) have suggested that preleptotene spermatocytes were assimilated into the adluminal compartment by initially constructing new TJs below these cells, to be followed by a dissolution of the ‘original’ TJs above them at stages VII–VIII in the ‘zipper theory’. It seems that TNF α (and other cytokines) plays a role in disrupting the ‘original’ TJs, yet it has no effects on the ‘newly formed’ TJs. It can be

seen that TNF α exerts its effects only locally by limiting its effects to a specific microenvironment. Alternatively, the ‘newly formed’ TJs are being protected from TNF α via a yet-to-be defined mechanism. These possibilities must be carefully evaluated in future studies.

Issue on changes in target protein levels during TNF α -induced BTB restructuring

During the TNF α -induced restructuring in the seminiferous epithelium, we have detected changes in the steady-state levels of several target proteins, such as a transient but significant reduction of occludin, ZO-1, and N-cadherin, but not JAM-A, claudin-11, α -, and β -catenin using lysates from these testes. Since the cellular composition in the seminiferous epithelium underwent extensive changes because of the

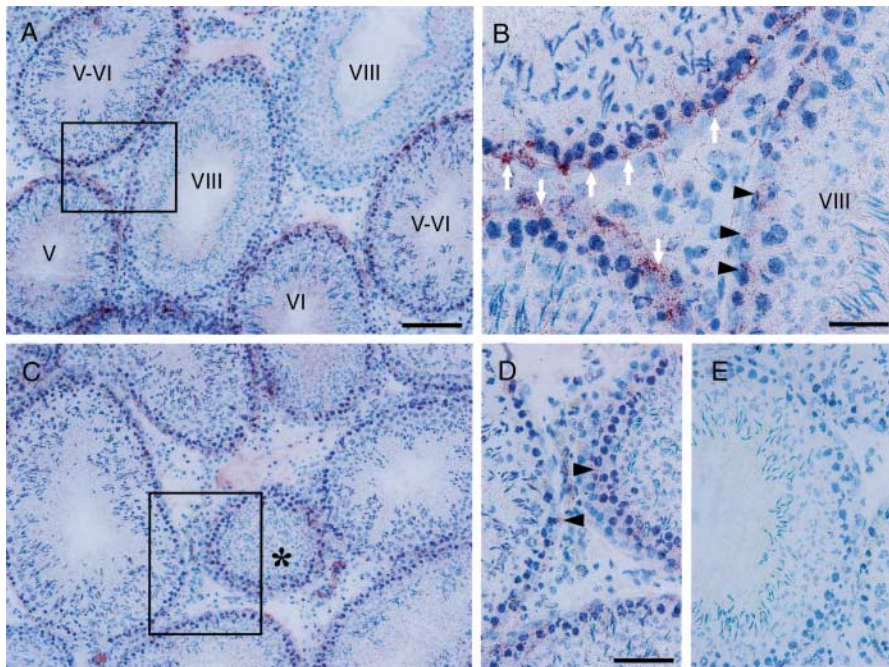


Figure 4 (A)–(E) A study by immunohistochemistry to assess the disappearance of occludin from the BTB site following TNF α treatment (2 μ g/testis). Micrographs using frozen sections of testes for immunohistochemistry to localize occludin in the seminiferous epithelium. In control (normal rat) testes, occludin appears as reddish-brown precipitates confined largely to the basal compartment, consistent with its localization at the BTB (see also Fig. 2). The boxed area in (A) was magnified in (B) and immunoreactive occludin (see white arrows) forms an almost continuous ring at the BTB surrounding the entire tubule at the basal compartment except at stage VIII (see black arrowheads) when preleptotene and leptotene spermatocytes were known to traverse the BTB (Russell 1977). However, on day 3 after TNF α treatment, there was a loss of occludin in the seminiferous epithelium (C), as manifested by the disappearance of occludin from the BTB (see (D), which is the magnified view of the boxed area shown in (C), the arrowheads illustrate the drastic reduction of occludin at the BTB site). (E) is a control in which the primary anti-occludin antibody was substituted by normal rabbit IgG. These immunohistochemistry data are derived from a single experiment, two additional experiments using testes from different animals yielded similar observations. Bar in A = 60 μ m, which applies to C; B = 15 μ m; D = 30 μ m, which applies to E.

TNF α -induced germ cell loss and BTB restructuring, the relative contribution of proteins by Sertoli and germ cells in the lysates would be altered in the time course experiments, such as reported in Fig. 5, negating the significance, perhaps the validity, of these findings. We offer the following explanations to address this important issue.

First, it must be noted that the target proteins that displayed changes in their steady-state levels, such as occludin and ZO-1, were restricted exclusively to Sertoli cells in the seminiferous epithelium. In testis samples that were being analyzed by 7 h–3 days, the lysates contributed to a wider margin by Sertoli cells since germ cells began to deplete from the epithelium at this time, thus one would expect a surge in occludin and ZO-1. Instead, a significant decline was detected. This, in turn, suggests that the ‘actual’ loss of occludin and ZO-1 could have been more significant. Yet, by day 8, the levels of occludin bounced back, this also suggests that there was an enhanced production of occludin and ZO-1 by Sertoli cells at this time. We did not attempt to

correlate the changes in target protein levels and the alteration of cellular composition since the present technology could not allow us to do so as yet. However, it is obvious that by 7 h–3 days following TNF α treatment, when germ cells were depleting from the epithelium, the relative contribution of Sertoli and germ cells in the lysates being analyzed was not different from rats at time 0 (or other normal rat testes), since many of the dislodged germ cells were still present in the tubule lumen. This logic is applicable to the changes that were detected in p-ERK1/2 and p-p38 MAPK; it also applies to other target proteins, such as N-cadherin and catenins, when they are products of both Sertoli and germ cells at least in these early time points.

Secondly, because of the issue mentioned above, it is crucial that any changes in a target protein should be confirmed with other approaches besides immunoblotting. For instance, the transient loss of occludin during TNF α -induced restructuring in the seminiferous epithelium was confirmed by immunohistochemistry, immunofluorescent microscopy, and

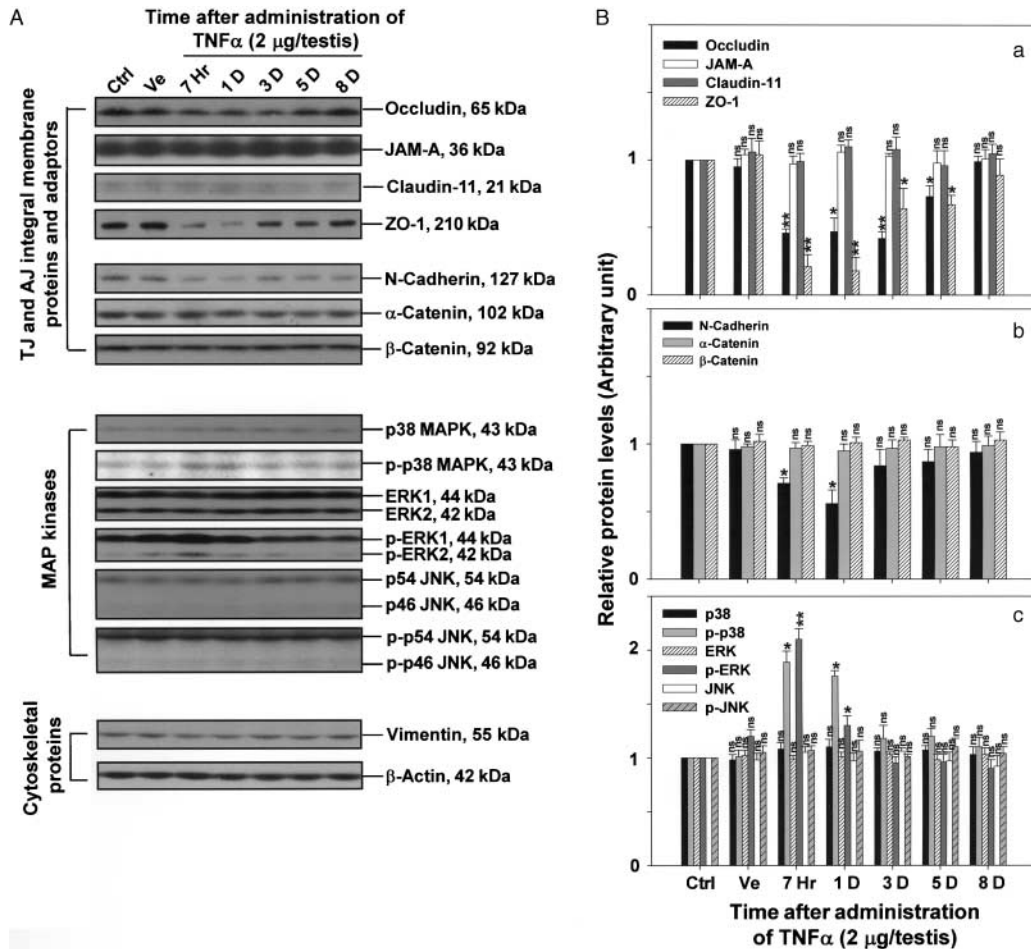


Figure 5 (A) and (B) A study to assess the steady-state levels of different target proteins during TNF α -induced BTB restructuring in adult rat testes. Rats were treated with recombinant TNF α (2 μ g/testis) via local administration as described in Materials and Methods. (A) Lysates were prepared for immunoblottings to quantify the levels of different TJ- and AJ-proteins and their adaptors (top panel; a and b in (B)), MAP kinases (middle panel; c in (B)), and cytoskeletal proteins (bottom panel). (B) Histograms using data, such as those shown in (A), densitometrically scanned and normalized against β -actin, plotted where the target protein levels in normal testes (Ctrl) were arbitrarily set at 1 with $n=3-4$ for each sample from at least two sets of experiments. Ve, rats treated with vehicle control and killed on day 3; Hr, hour; D, day. n.s., not significantly different by ANOVA; * $P<0.05$; $^{\dagger}P<0.01$.

a functional test to assess BTB integrity. Collectively, these multiple approaches can accurately assess changes of selected target proteins during TNF α -induced damage to the BTB and the seminiferous epithelium.

Mechanism of action of TNF α in affecting BTB dynamics

It seems that one of the primary targets of TNF α at the BTB is the TJ integral membrane proteins: occludin, claudin-11, and their adaptor ZO-1. For instance, studies using Sertoli cell cultures have shown that TNF α can inhibit claudin-11 (Hellani *et al.* 2000) and occludin (Siu *et al.* 2003a) production by Sertoli cells. A transient loss of these proteins plus cadherins at the BTB can likely render a temporal and spatial disruption of the BTB as illustrated by electron

microscopy. Interestingly, JAM-A and catenins were not perturbed by TNF α . Furthermore, a recent study has shown that catenins are crucial adaptors that cross-link the occludin/ZO-1 and the cadherin/catenin protein complexes at the BTB via the catenin-ZO-1 interaction to reinforce the BTB integrity (Yan & Cheng 2005). It is plausible that the remaining JAM-A/ZO-1/catenin protein complex, which is largely unaffected by TNF α , can temporarily safeguard the BTB, such as its immunological barrier function and polarity function, while the occludin-ZO-1/cadherin-cadherin protein complex is transiently disrupted so as to facilitate germ cell migration. It is conceivable that another yet-to-be defined mechanism is in place at the BTB to perturb the JAM-A/ZO-1/catenin protein complex, which is working in concert with the occludin/ZO-1 and cadherin/catenin

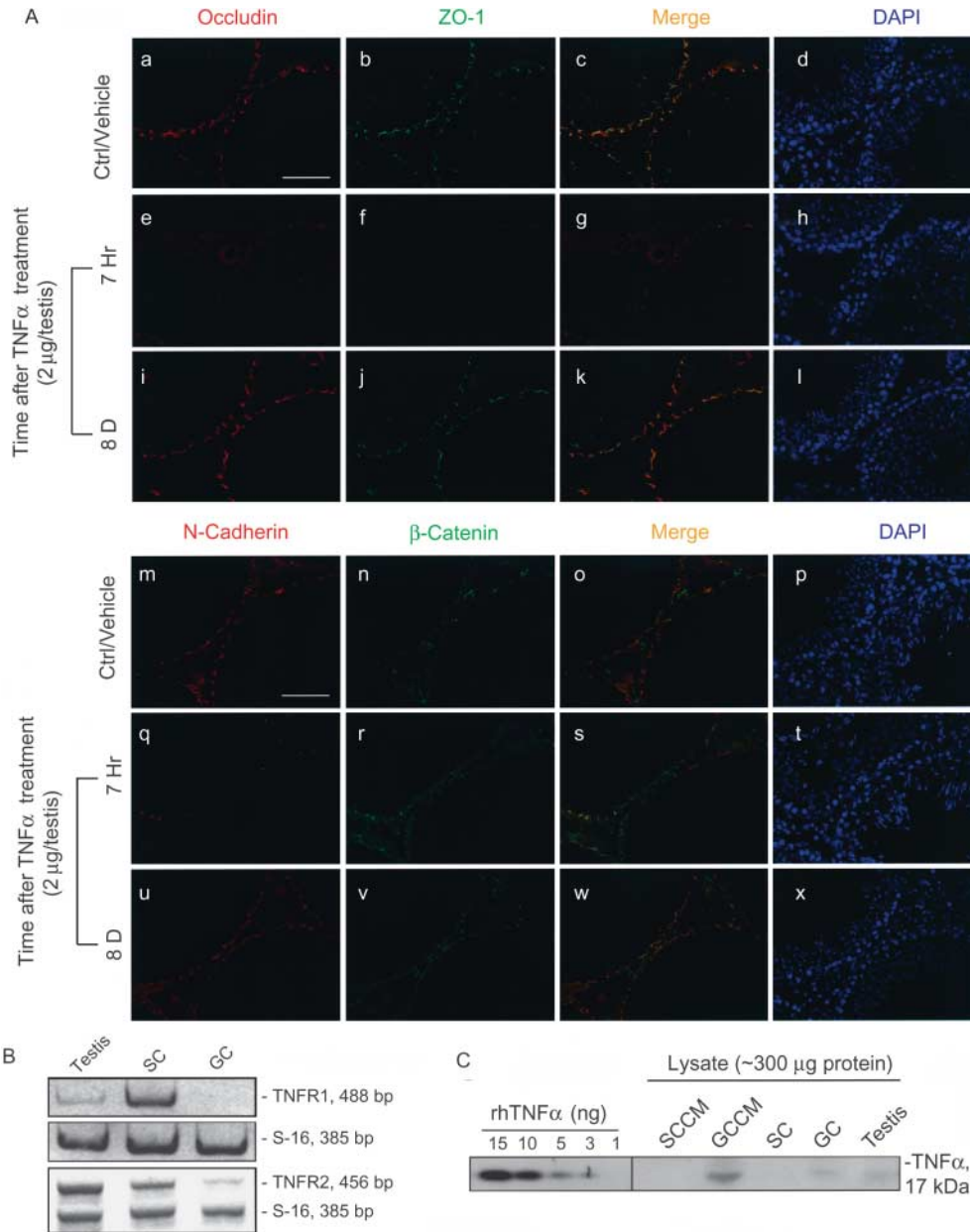


Figure 6 (A)–(C) A study to assess whether the disruptive effect of TNF α on the BTB integrity is reversible and the functional implication of TNF α in the microenvironment of the seminiferous epithelium *in vivo*. (A) To assess if the TNF α -induced BTB disruption is reversible, fluorescent microscopy was used to assess BTB integrity by co-localizing occludin (a, e, and i) and ZO-1 (b, f, and j), as well as N-cadherin (m, q, and u) and β -catenin (n, r, and v) in the testis. Consistent with the immunoblotting data shown in Fig. 5, there was a transient loss of occludin/ZO-1 (e and f) and N-cadherin (q) from the seminiferous epithelium at the BTB site by 7 h after TNF- α treatment, since on day 8, the levels of fluorescence of occludin (i vs e), ZO-1 (j vs f) and N-cadherin (u vs q) were all recovered, making them almost indistinguishable from control testes (or testes treated with vehicle control). These are representative fluorescent micrographs from an experiment, two additional experiments using different testes sections yielded similar results. Bar in a and m = 80 μ m, which applies to b–l and n–x respectively. (B) A study using RT-PCR that illustrates the p55 TNF α receptor (TNFR1) was exclusively expressed by Sertoli cells (SC) whereas the p75 TNF α receptor (TNFR2) was also expressed predominantly by SC. (C) This is the representative result of a study to assess the levels of TNF α in lysates of testicular cells and testes, and spent media versus recombinant TNF α that served as standards (see Materials and Methods for details of this TNF α solid-phase immunoblot-based assay) SCCM, Sertoli cell-enriched culture media; GCCM, germ cell-enriched culture media; SC, Sertoli cells; GC, germ cells.

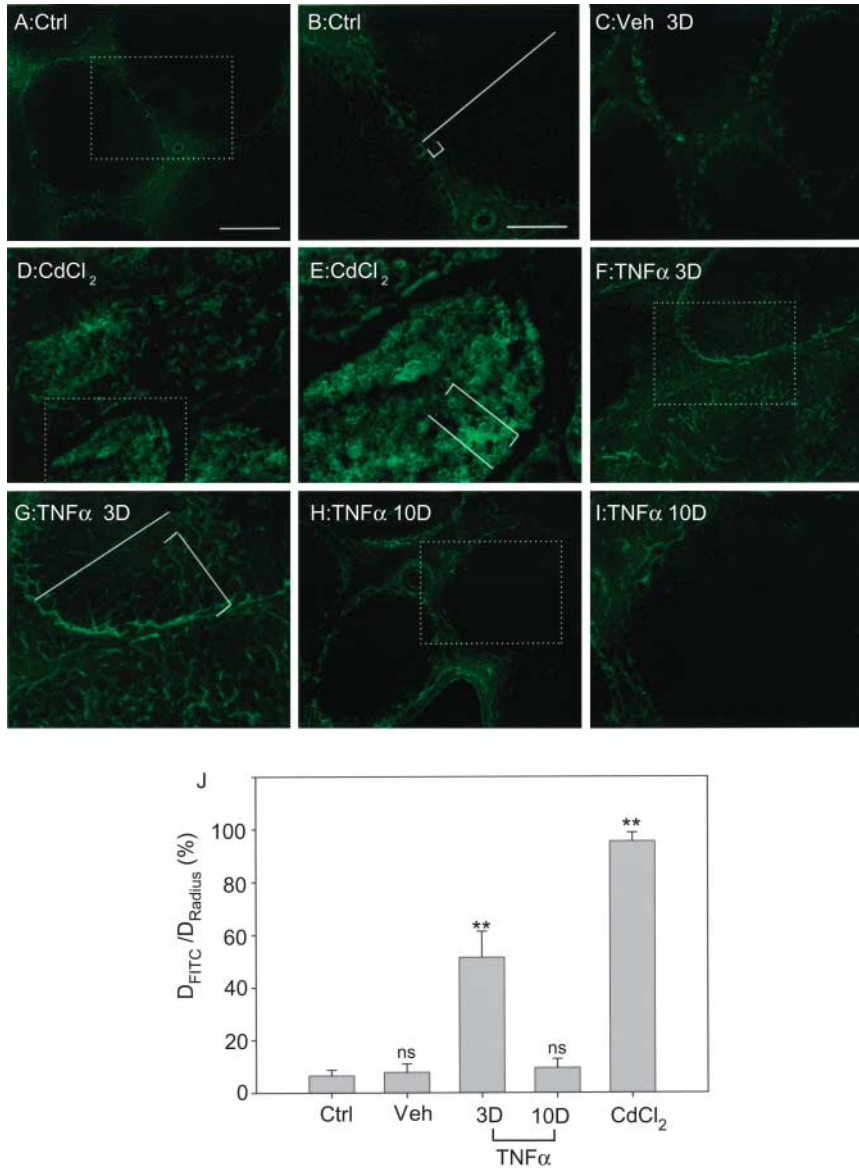


Figure 7 (A)–(I) A functional study to assess the BTB integrity following TNF α treatment in adult rats. Adult rats ($n=2$ for each time point in each treatment group; for controls, $n=3$) were treated with either TNF α (2 $\mu\text{g}/\text{testis}$ via intratesticular injection; terminated on day 3 and 10) or CdCl₂ (3 mg/kg bw, i.p., terminated on day 5, positive control) and controls (normal testes, and testes treated with PBS terminated on day 3). The integrity of the BTB was then assessed by monitoring the diffusion of FITC across the barrier from the systemic circulation as shown in (A)–(C) (control groups) versus CdCl₂ treatment (D)–(E) on day 5 (positive control), and TNF α treatment terminated on day 3 (F)–(G) and day 10 (H)–(I). These analyses were summarized in (J), which illustrates the distance of the FITC fluorescent staining from the basement membrane (D_{FITC}) (see white bracket in (B), (E), and (G)) versus the radius of a seminiferous tubule (D_{radius}) as annotated by the white-line in (B), (E) and (G). In control groups (A)–(C), FITC was confined at or near the basement membrane. In the positive control group in which rats were treated with CdCl₂ to disrupt the BTB for 5 days, FITC was found in the entire epithelium, and in the TNF α -treated group, FITC was also detected in the epithelium away from the basement membrane by day 3 (F), (G), but this FITC movement into the epithelium was not significantly different by day 10 (H), (I) versus controls ((H), (I) vs (A)–(C)). The boxed areas shown in (A), (D), (F), and (H) were magnified and shown in (B), (E), (G), and (I) respectively. Bar in A = 60 μm , which applies to (C), (D), (F), and (H); bar in B = 60 μm , which applies to (E), (G), and (I). n.s., not significantly different by one-way ANOVA; ** $P < 0.01$.

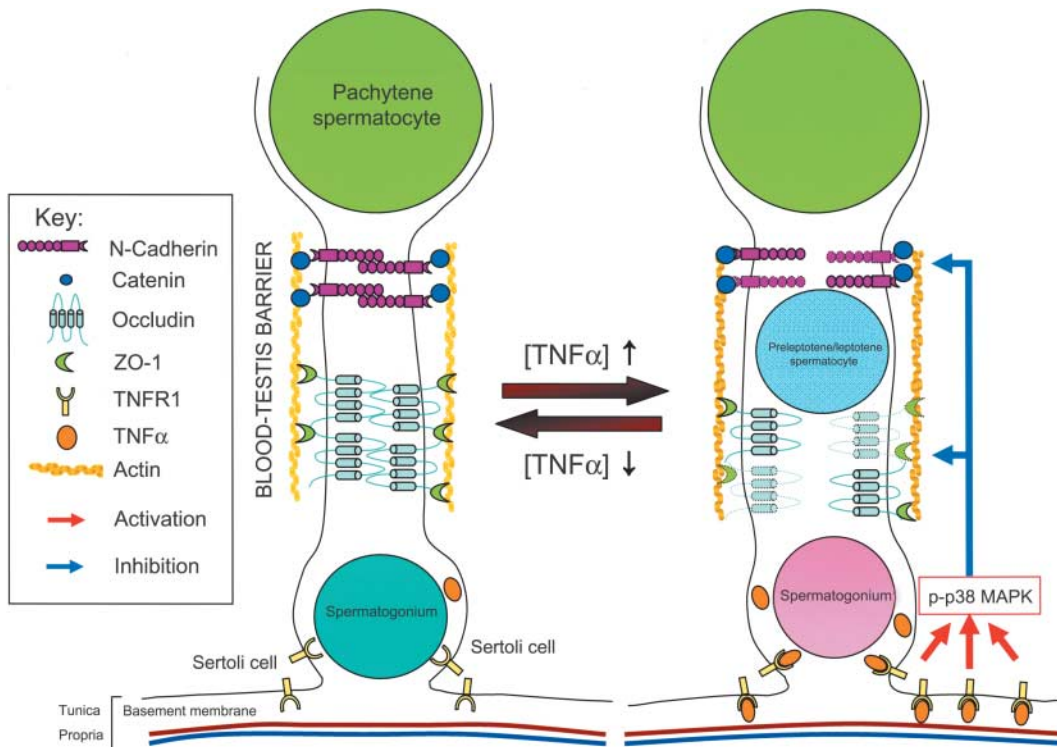


Figure 8 A schematic drawing illustrating the possible mechanism utilized by TNF α to reversibly perturb the BTB integrity facilitating germ cell migration. The left panel depicts the BTB at a 'closed' state when a relatively low level of TNF α is found in the microenvironment at stages other than VII and VIII. At late stages VII through VIII of the epithelial cycle, both Sertoli and germ cells release TNF α into the microenvironment (see right panel), this triggers an activation of p-38 MAPK. This, in turn, lowers the steady-state levels of occludins and cadherins (proteins in broken lines represent 'disintegrated' or 'internalized' BTB-integral membrane proteins), 'opening' the BTB to facilitate the migration of preleptotene and leptotene spermatocytes across the BTB while differentiating into pachytene spermatocytes. Thereafter, the production of TNF α by Sertoli and germ cells is reduced, favoring the 'closing' of the BTB at stages IX through early VII of the epithelial cycle.

protein complexes to regulate BTB dynamics. It is obvious that these events must be precisely coordinated via intriguing crosstalk between different protein complexes at the BTB, possibly kinases and phosphatases (Xia *et al.* 2005) as well as different cytoskeletons (Vogl 1989, Vogl *et al.* 1993, Green *et al.* 2005). Yet, there is another possible mechanism, which can also be utilized by TNF α to affect BTB dynamics. For instance, a recent study has shown that TNF α is capable of promoting the activation of pro-matrix metalloproteinase-9 (MMP-9) to MMP-9 (the activated form of MMP-9) in Sertoli cells (Siu *et al.* 2003a), which is known to cleave type IV collagen (Collier *et al.* 1988). Since type IV collagen is a major component of the basement membrane in the seminiferous epithelium adjacent to the BTB (Dym 1994, Siu & Cheng 2004), and that fragments of collagens are known to act as biologically active peptides, such as inducing disassembly of anchoring junctions (e.g. focal contacts) via their effects on focal adhesion kinase (Carragher *et al.* 1999); it is likely that TNF α can use this mechanism to regulate the steady-state level of type IV collagen in the basement

membrane. The net result of such interactions can affect the BTB integrity, since a disruption of collagen function can perturb the Sertoli cell TJ-permeability barrier function (Siu *et al.* 2003a) and Sertoli-germ cell adhesion (Lustig *et al.* 1978), possibly by perturbing the scaffolding function of collagen in the basement membrane. Work is now in progress to investigate this possibility.

Additionally, a recent study has shown that TNF α also induces nitric oxide and inosine production by Sertoli cells, protecting these cells against hydrogen peroxide-induced damage via the ERK1/2 signaling pathway (Souza *et al.* 2005). Recent studies have also shown that nitric oxide regulates Sertoli cell TJ-barrier function via the cGMP/PKG signaling pathway (Lee & Cheng 2003). Collectively, these data illustrate that TNF α can exert its effects via multiple pathways to affect BTB dynamics and Sertoli-germ cell adhesion in the testis.

In summary, we have reported some interesting effects of TNF α in BTB dynamics in adult rat testes. TNF α is likely a key player in promoting transient disassembly of

the BTB *in vivo* to facilitate the migration of preleptotene and leptotene spermatocytes across the barrier at stage VIII of the epithelial cycle. Furthermore, results of this study have also illustrated that local administration of TNF α to adult rat testes to induce junction restructuring in the seminiferous epithelium can be an *in vivo* model to study BTB dynamics, since this cytokine is produced locally by Sertoli and germ cells in the testis, and its effects on the BTB and the seminiferous epithelium are reversible.

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