Interleukin-6 disrupts blood-testis barrier through inhibiting protein degradation or activating phosphorylated ERK in Sertoli cells

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It has been recently ascribed to several inflammatory cytokines (i.e. TGF-β, TNF-α, and IL-1) a functional role in regulating Sertoli cell blood-testis barrier (BTB) dynamics. In the testis, IL-6 inhibits meiotic DNA synthesis during the seminiferous epithelium cycle, reduces sperm motility and influences the secretion of transferrin and inhibin B by Sertoli cells. Also, it has been shown that IL-6 affects tight junction permeability in Sertoli cells, but, little is known about its role in regulating the BTB. The aim of this study was to investigate the molecular mechanisms by which IL-6 affects BTB dynamics. We show that IL-6 perturbs the integrity of the BTB, and alters the normal localization and steady-state levels of BTB integral membrane proteins. We demonstrated that IL-6 regulates the BTB by inhibiting the degradation of BTB constitutive proteins and activating ERK-MAPK pathways. Our results provide mechanistic insight into the roles of IL-6 in regulating BTB dynamics.

Results

Effects of IL-6 on the assembly and permeability of Sertoli cell TJ in vitro. To determine whether IL-6 can affect BTB, we first investigated the expression level of BTB-constituent proteins exposed to IL-6 2 days at different concentrations (i.e. 0, 20, 50, 100,150 and 200 pg/ml), by using Western blotting assays (Fig. 1a, b). The highest expression level of Occludin and β-Catenin was detected with 50 pg/ml of IL-6 compared to other concentrations used (Fig. 1a, b). Therefore, the effect of 50 pg/ml IL-6 on BTB was most obvious, and then this concentration was used for all the experiments described in this study.
Sertoli cells were cultured at a high density (1.0 × 10^6 cells/cm²) on Matrigel-coated bicameral units, an experimental model that mimics the BTB in vivo. The TER increased steadily to ~57 Ohm.cm² over 3–4 days (Fig. 1c), indicating the assembly of intercellular junctions was complete. Treatment with recombinant rat IL-6 or TGF-β3 on day 3 disrupted the TJ barrier as reflected by a gradual decrease in TER of IL-6/TGF-β3-treated Sertoli cells compared to control-treated cells (Fig. 1c). Herein, TGF-β3 was used as a positive control in TER assay. Effects of IL-6 on the localization of BTB-constituent proteins in cultured Sertoli cells. Immunocytochemistry of Sertoli cells cultured on Matrigel-coated coverslips was performed to investigate the effects of IL-6 on the localization of the TJ and basal ES proteins Occludin and β-Catenin, respectively. In control cells, fluorescent signals for Occludin (Fig. 2a) and β-Catenin (Fig. 2b) were restricted to a finite area. In contrast, in cells treated with IL-6 on day 3 and analyzed after 1 day, the labeling for both proteins was diffuse and no
longer concentrated at cell-cell interfaces, indicating that IL-6 causes mislocalization of BTB-constituent proteins.

Effects of IL-6 on the expression of BTB-constituent proteins in cultured Sertoli cells. We next sought to examine whether there were quantitative changes in the expression of BTB-constituent proteins in Sertoli cells after IL-6 treatment. Immunoblotting revealed that, Occludin was increased at 2-days and β-Catenin was increased at 2 and 3 days at the protein level after treatment with IL-6 compared to controls (Fig. 3a, b). However, real-time RT-PCR analysis revealed the mRNA levels of Occludin and β-Catenin were not affected by IL-6 treatment (Fig. 3c).

IL-6 increases the cellular levels of BTB constituents by delaying protein degradation. To investigate how IL-6 caused BTB-constituent protein levels to increase in light of a leaky Sertoli cell barrier (Fig. 1a, b), the kinetics of Occludin, JAM-a and N-Cadherin degradation were examined using a protein degradation assay (Fig. 4a, b). Briefly, Sertoli cell surface proteins were labeled by biotinylation, and cells were treated with IL-6 or vehicle control. Non-degraded proteins remaining on the cell surface, as well as those that had internalized after IL-6 treatment were affinity purified and detected by immunoblotting. The levels of non-degraded proteins Occludin, JAM-a, and N-Cadherin were significant increases after IL-6 treatment. The level of Occludin was significantly higher in IL-6-treated cells compared to controls.

Figure 2 | IL-6 caused mislocalization of BTB-constituent proteins in Sertoli cells. Sertoli cells (4.0 \( \times \) 10⁴ cells/cm²) were treated with control (con) or 50 pg/ml IL-6 (IL-6) as described in Materials and Methods. Cells were immunostained for Occludin (green; a) or β-Catenin (green; b). Nuclei were visualized with Hoechst 33342 (blue). Scale bar = 50 μm.

Figure 3 | IL-6 increased the steady-state levels of BTB-constituent proteins in vitro. (a) Western blot analysis of BTB-constituent proteins expression after treated with vehicle control (con) or 50 pg/ml IL-6 for increasing periods of time. β-Actin served as the loading control. All gels had been run under the same experimental conditions. (b) Bar plots summarizing relative Occludin and β-Catenin results from several independent experiments after normalizing each data point against its corresponding actin time point and then against its corresponding control at 0D. Control in both experimental groups was arbitrarily set at 1. Data points represent median ± AD (n = 3). *P < 0.05, vs. control. (c) The mRNA levels of BTB components after treatment with control (con) or IL-6 for increasing periods of time. Data represent median ± AD (n = 3).
over 1 hour, while the levels JAM-a, and N-Cadherin were up-regulated in IL-6-treated cells over 3 hours (Fig. 4a, b). These results indicated that IL-6 delayed the degradation of these proteins.

**IL-6 regulates ES dynamics through the ERK-MAPK pathway.** To gain insight on the molecular mechanisms that contribute to the actions of IL-6 on the BTB, signaling pathways known to be important for activity of the cytokine were studied in Sertoli cells by immunoblotting. IL-6 triggers two main signaling cascades; the SHP-2/ERK-MAPK pathway and the JAK/STAT pathway. Sertoli cells were treated with IL-6 in vitro, and the time course of ERK1/2 activation was examined (Fig. 5a, b). The levels of phosphorylated proteins increased from 45 min to 2 h (Fig. 5a, b). To determine whether ERK1/2 phosphorylation affects the level of BTB-constituent proteins, cells were pre-incubated with the MEK1/2-specific inhibitor U0126 (10 µM) 30 min prior to treatment with IL-6 to block ERK1/2 phosphorylation (Fig. 5c). The levels of BTB-constituent proteins were examined after 1 day of IL-6 treatment, and this revealed that IL-6 could perturb Sertoli cell BTB integrity during testicular infection and inflammation.

When spermatozoa are released from the seminiferous epithelium, the BTB undergoes restructuring at late stage VIII to facilitate the transit of preleptotene/leptotene spermatocytes from the basal to the apical compartment. IL-6 production under physiological conditions is lowest at stages VII-VIII of the seminiferous epithelium cycle. However, testicular IL-6 levels are up-regulated in the setting of injury and inflammation. Herein, IL-6 could affect the contractility of BTB by changing the localization and amount of

![Figure 4](image-url)
BTB-constituent proteins, while the mRNA levels of BTB-constituent proteins were not changed. Moreover, a protein degradation assay revealed the kinetics of Occludin, JAM-a, and N-Cadherin degradation were delayed after IL-6 treatment, leading to their accumulation in Sertoli cells. Our data confirmed previous results showing that IL-6 can affect TJ permeability in Sertoli cells. Also, in other epithelial cells the effect of IL-6 on TJ permeability was recently reported.

Previous studies showed that ERK is a crucial regulator of junction restructuring in the seminiferous epithelium, effects that are mediated by its downstream actions on proteases and protease inhibitors as well as actin dynamics at the apical ES. Herein, IL-6 can stimulate p-ERK1/2 expression in Sertoli cells. Moreover, blocking the ERK-MAPK pathway led to restoration of the ES protein β-Catenin, but had no effect on the TJ protein Occludin. Furthermore, after blocking the ERK-MAPK pathway, Sertoli cell BTB permeability had a less disrupt. Thus, IL-6 could disassemble the ES permeability barrier via ERK-MAPK signaling pathway. Because ERK is considered as a key player in modulating cell and motility in different epithelia, it is plausible that an altered p-ERK1/2 expression may influence the homeostasis of Sertoli cells and thus the integrity of BTB.

Since overexpression of IL-6 could disrupt the integrity of the Sertoli cell BTB (including our results) and ultimately impair

Figure 5 | IL-6 regulated the dynamics of ES through the ERK-MAPK pathway. (a) Western blot analysis of ERK and p-ERK levels after 50 pg/ml IL-6 treatment at different time points. All gels had been run under the same experimental conditions. (b) Bar plots summarizing relative ERK activation results from several independent experiments after normalizing each data point against its corresponding total ERK time point and then against its corresponding control at 0 min. Control in both experimental groups was arbitrarily set at 1. Data points represent median ± AD (n = 3). *P < 0.05, vs. control. (c) The MEK1/2-specific inhibitor 10 μM U0126 blocked the ERK-MAPK pathway, + , Present; –, absent. All gels had been run under the same experimental conditions. (d) Western blot analysis of BTB-constituent proteins after treatment with 10 μM U0126 prior to 50 pg/ml IL-6. β-Actin served as the loading control. All gels had been run under the same experimental conditions. (e) Bar plots summarizing relative Occludin and β-Catenin results from several independent experiments after normalizing each data point against its corresponding actin time point and then against its corresponding control at 0D. Control in both experimental groups was arbitrarily set at 1. Data represent the median ± AD of three independent experiments performed in triplicate. *P < 0.05, compared with absence of U0126. (f) TER across Sertoli cells in culture exposed to 50 pg/ml IL6 for 24 h in the presence or absence of 10 μM U0126. The addition of U0126 partly blocked the effect of IL6 on TER. Data represent median ± AD (n = 3). *P < 0.05, compared with absence of U0126. +, Present; –, absent.
IL-6 treatment. Rat Sertoli cells isolated and cultured for 3–4 days were treated at different time points with 50 pg/ml recombinant IL-6 (R&D Systems, Minneapolis, MN, USA; stock dissolved in PBS, pH 7.4, containing 0.1%BSA, w/v) or control (PBS, pH 7.4, containing 0.1%BSA, w/v) diluted in DMEM/F12.

Trans epithelial electrical resistance (TER). Freshly isolated Sertoli cells were cultured at high density (1.0 × 10^5 cells/cm²) to promote Tj formation that was quantified using a Millicell Electrical resistance system (Millipore Corp, USA) as described previously48,49. The first TER measurement across the Sertoli cell epithelium was obtained by measurement of cell-free Matrigel-coated bicameral units. A functional barrier was established between adjacent Sertoli cells26,45–47.

Immunofluorescence analysis. Cells were fixed for 20 min in 4% (w/v) paraformaldehyde, washed in PBS for 10 min three times, permeabilized with 0.25% Triton and 0.1% (w/v) Tween in PBS for 10 min, blocked with 1% BSA in PBS for 1 h. After overnight incubation at 4°C with anti-Occludin or anti-b-Catenin antibody (1:50 dilution in blocking buffer), cells were washed then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution) for 30 min at room temperature. Nuclei were stained with Hoechst 33342 (Sigma, USA) for 2 min at room temperature. Fluorescent signals were acquired with an Nikon epifluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Protein degradation assay. A Protein degradation assay was performed as described previously50 with minor modifications. Briefly, Sertoli cells were seeded at 0.5 × 10^5 cells/cm² on Matrigel-coated dishes. After 4 days the cells were washed twice with ice-cold PBS and surface proteins were biotinylated with 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce, USA) in PBS, pH 7.4, 1 mM CaCl₂ and 0.7 mM MgCl₂, and the reaction was quenched in the same buffer containing 50 mM Tris for 15 min at 4°C. Cells were then washed twice with ice-cold PBS and incubated in DMEM/F12 with (test) or without (control) 50 ng/ml recombinant IL-6 for various time points. This was performed at 35°C to allow internalization of cell surface biotinylated proteins, since endocytosis does not occur at 4°C. Cells were then washed twice with ice-cold PBS and harvested in 50 mM Tris, pH 7.4, 150 mMNaCl, 2 mM EDTA, and the protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) or a Protein Assay Coomassie Plus (Pierce, Rockford, IL). Protein samples (50 μg) were then separated by 12% (w/v) SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblotting using an anti-α-tubulin or anti-actin (Sigma, USA) or a goat anti-rabbit IgG secondary antibody (1:10,000), followed by a donkey anti-goat IgG secondary antibody (1:10,000) and chemiluminescence detection (Enzyme Research, South Bend, IN). Band intensities were measured using ImageJ (National Institute of Health, USA). Protein degradation was calculated by the ratio of test/control protein expression for each protein evaluated. A non-parametric Kruskal-Wallis test was performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA) to evaluate differences among variables. P values < 0.05 were considered statistically significant.

Statistical analysis. All experiments in this study were repeated at least three times. Data were expressed as median ± AD. The non-parametric Kruskal-Wallis test was performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA) to evaluate differences among variables. P values < 0.05 were considered statistically significant.
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**Author contributions**

F.S. initiated the project, conceived and designed the experiments; H.Z. Y.Y. and Z.L. were involved in preparation and isolation of Rat Sertoli cells; H.Z. and G.W. performed Western blotting; H.Z. and L.L. performed Real-Time qPCR; H.Z. carried out data analysis interpretation. All authors discussed the results and commented on the manuscript.

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