

Proteasome activator REG γ promotes inflammation in Leydig cells via I κ B ϵ signaling

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Abstract. The development of testicular inflammation affects the normal male reproductive function. The proteasome activator complex subunit 3 (REG γ) has been suggested to regulate experimental colitis. However, to the best of our knowledge, a potential association between REG γ and testicular inflammation has not been demonstrated. The present study successfully established inflammatory models in C57 mice, primary Leydig cells and the TM3 cell line. It was observed that the absence of REG γ conveyed a significantly protective effect toward testosterone secretion in Leydig cells. REG γ deficiency significantly decreased the expression levels of phosphorylated transcription factor p65 and inflammatory factors in testis tissues, primary Leydig cells and the TM3 cell line. Inflammation also upregulated the expression levels of REG γ . Furthermore, the degradation of the nuclear factor light-chain-enhancer of activated B cells (NF- κ B) inhibitor ϵ (I κ B ϵ) signaling pathway regulated REG γ and NF- κ B expression. Double knockdown of REG γ and I κ B ϵ restored the response in wild-type cells to

LPS-induced inflammation. In summary, these results demonstrated that REG γ regulates NF- κ B activity by specifically degrading I κ B ϵ to regulate inflammation in testicular Leydig cells.

Introduction

Various lifestyle and environmental factors have been associated with an increase in the incidence of male-specific diseases (1), including orchitis, which is characterised by the inflammation of one or both testicles due to local or systemic infection, or non-infectious factors, with infective orchitis being more common (2). The mammalian testis is composed of spermatogenic tubules that primarily consist of spermatogenic epithelium and are separated by stromal layers. The spermatogenic epithelium consists of Sertoli sustentacular cells and spermatogenic cells, while the interstitial space between the seminiferous tubules contains capillaries, capillary lymphatic vessels, nerves and various types of cells, including Leydig cells, fibroblasts, giant cells, mast cells, lymphocytes and eosinophils. Leydig cells are primarily responsible for androgen secretion (3,4).

The development of inflammatory diseases is closely associated with the activation of various signaling pathways including the nuclear factor light-chain-enhancer of activated B cells (NF- κ B) signaling pathway (5,6), which is closely associated with the development of testicular inflammation (7). Phosphorylated transcription factor p65 (p-p65) is an important marker of inflammation and NF- κ B signaling pathway activation (6).

Protein degradation is important for maintaining normal physiological functions and homeostasis (8), as the disruption of this system is a known cause of several diseases. In higher eukaryotes, the majority of proteins (80-90%) are degraded by the proteasome (9,10). The normal function of the proteasome is critical for maintaining relatively stable intracellular activity. The 11S regulatory complex, which includes proteasome activator complex subunit 1, proteasome activator complex subunit 2 and proteasome activator complex subunit 3 (REG γ), has been demonstrated to enhance the proteasomal degradation of substrate proteins and to alter the type and substrate selectivity of enzymes (11). One study has suggested

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Abbreviations: LPS, lipopolysaccharide; ATP, adenosine triphosphate; WT, wild-type; H&E, hematoxylin and eosin; qPCR, quantitative polymerase chain reaction; dd water, double distilled water; dKD, double knockdown

Key words: proteasome activator complex subunit 3, Leydig cells, inflammation, nuclear factor light-chain-enhancer of activated B cells, nuclear factor light-chain-enhancer of activated B cells inhibitor ϵ

that inflammatory stimulation significantly increases REG γ expression in mouse colon epithelial cells and human colon cancer epithelial cells (12). Conversely, intestinal inflammation was decreased in a REG γ -deficient mouse model of inflammatory bowel disease (12). However, the association between REG γ and testicular inflammation remains unclear.

Therefore, the present study aimed to determine the role of REG γ in testicular inflammation using mouse and cell models. It was observed that the knockdown of REG γ resulted in a decreased level of inflammation in the animal and the cell models. Furthermore, REG γ may regulate the NF- κ B signaling pathway via NF- κ B inhibitor (I κ B) ϵ (I κ B ϵ). These data indicate that REG γ may serve as a potential target in the treatment of testis inflammation.

Materials and methods

Experimental mice. The 6-week-old C57BL/6 wild-type (WT) (REG $\gamma^{+/+}$) and REG γ -deficient (REG $\gamma^{-/-}$) male mice were obtained from the Minhang Laboratory Animal Center of East China Normal University (Shanghai, China) and housed under specific pathogen-free conditions at 21 \pm 2°C and 55 \pm 10% humidity under a 12 h light/dark cycle, and fed normal chow with *ad libitum* access to water. The C57BL/6 REG $\gamma^{-/-}$ mice were originally acquired from Dr John J. Monaco (University of Cincinnati College of Medicine, Cincinnati, OH, USA) (11-12). A total of 36 REG $\gamma^{+/+}$ mice and 24 REG $\gamma^{-/-}$ mice were used for the current study.

Cell culture and expression constructs. Primary Leydig cells were collected from mouse testes. TM3 cells were purchased from the Cell Bank of Type Culture Collection Chinese Academy of Sciences (Shanghai, China; cat. no. GNM24). The TM3 cell line is a mouse epithelial Leydig cell line. Primary Leydig cells and the TM3 cell line were grown in Dulbecco's modified Eagle's medium/F-12 nutrient mixture (DMEM/F-12) supplemented with 5% fetal bovine serum, 2.5% horse bovine serum (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), L-glutamine (150 mg/l), NaHCO₃ (1.5 g/l), penicillin (100 U/ml) and streptomycin (100 μ g/ml). Cells were cultured in a 37°C incubator with 5% CO₂ and 95% air.

Animal and cell inflammation models. Based on preliminary experiments, 12 REG $\gamma^{+/+}$ mice were selected and 6 of them were injected intraperitoneally with LPS (20 mg/kg body weight) dissolved in double distilled (dd) water for 6 h. As a control, 6 mice were injected intraperitoneally with equal volumes of dd water for 6 h.

The optimal conditions for creating an inflammatory cell model were explored. Concentration (0, 1, 5 or 10 mg/ml) and time gradients (0, 10, 20, 40 or 60 min) were established to determine the optimal model conditions. TM3 cells were treated for 10 min with LPS at concentrations of 0, 1, 5 or 10 mg/ml. The optimal concentration of LPS was then selected to treat the cells for different times (0, 10, 20, 40 or 60 min) in order to select the optimal treatment time.

Primary Leydig cells. Upon LPS treatment, mice were sacrificed by CO₂ anesthesia (SMQ-II; Tianhuan Technology, Co., Ltd., Shanghai, China; final CO₂ concentration, 80-100%).

The displacement rates were between 10-30% of the chamber volume per minute (cv/min), and death was confirmed by observation of loss of respiration, heart beat and reflexes. Then, cervical dislocation was performed as a secondary physical method of sacrifice.

The testes were then collected from the mice in a sterile hood. Sterile tweezers were used to remove the testicular capsule. The tissue was digested in 0.25% collagenase at 37°C for 10 min (1 ml per testis). Next, the tissue suspension was filtered with a 40 μ m filter. Following centrifugation at 200 x g and 4°C for 5 min, the cell suspension was placed in culture dishes and cultured in an incubator for 4 h. The adherent cells that were attached to the dishes were considered to be primary Leydig cells. The purity of the Leydig cells, as assessed by immunocytochemical staining for 3 β -hydroxysteroid dehydrogenase.

For immunocytochemical staining, after the cells adhered to the slip, the glass slips were washed three times with 1X PBS, fixed with 4% paraformaldehyde at room temperature for 20 min and again washed three times with PBS. Then the glass slips were put in 0.5% Triton X-100 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 20 min and washed with PBS. Then, the slips were put in 3% H₂O₂ at room temperature for 15 min, washed with PBS and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. The glass slips were then treated with anti-3 β -hydroxysteroid dehydrogenase primary antibodies (cat. no. sc-515120; 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C, washed with PBS, treated with horseradish peroxidase-conjugated secondary antibodies (cat. no. K5007; 1:200; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at room temperature for 1 h and again washed with PBS. Next, the slips were washed with PBS, stained with 0.5 μ g/ml DAPI for 3 min at room temperature and washed with PBS. Then, the glass slips were observed by fluorescence microscopy at a magnification of x600. In each field of vision, the total number of cells and positive cell were counted, positive cells accounted for >90% of total cells.

Knockdown of REG γ . Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) was used to knock down REG γ and I κ B ϵ expression in TM3 cells using small hairpin (sh)RNA: shREG γ and shI κ B ϵ . TM3 control (shN) and REG γ -knockdown (shR) cells were generated by retroviral shRNA plasmids specific for REG γ or control vectors, respectively, from OriGene Technologies, Inc. (Rockville, MD, USA). The plasmids were provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). A total of 1 μ g/ μ l pshRNAI κ B ϵ or pshRNAREG γ 1 vectors were incubated with TM3 cells for 12 h to obtain shN or shR cells, respectively. Then, the expression levels of REG γ in the shN and shR groups were detected by western blot analysis.

Western blot analysis. Following treatment, Leydig and TM3 cells were lysed in 1X loading buffer (5X loading buffer: 250 mM Tris-HCl pH 6.8, 10% SDS, 10% bromophenol blue, 50% glycerin and 5% β -5 Mercaptoethanol) for total protein collection. Protein concentrations were determined using the bicinchoninic acid method. The buffer, including all the extracted proteins, was then heated at 100°C for 30 min. Protein samples (50 μ g/lane) were subsequently separated using SDS-PAGE

on 11% gels and transferred onto nitrocellulose membranes. Upon blocking with 7% BSA dissolve in PBS at room temperature for 1 h, the membranes were incubated with primary antibodies (1:1,000) at 4°C for 12 h and then with IRDye® 800CW-conjugated donkey anti-rabbit secondary antibodies (cat. no. 925-32213; 1:5,000; LI-COR Biosciences, Lincoln, NE, USA). β -actin (1:5,000) was used as a loading control. Finally, the membranes were scanned using an Odyssey imaging system (LI-COR Biosciences). Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used for the densitometric analysis. The following antibodies were employed: Anti-I κ B ϵ (cat. no. sc-7155; Santa Cruz Biotechnology, Inc.), anti-I κ B α (cat. no. 4812), anti-I κ B β (cat. no. 94101; both Cell Signaling Technology, Inc., Danvers, MA, USA), anti- β -actin (cat. no. a2228; Sigma-Aldrich; Merck KGaA) and anti-p-p65 (cat. no. ab16502; Abcam, Cambridge, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and reverse transcribed into complementary DNA using the Mx3005P qPCR system (Agilent Technologies, Inc.) under the following thermocycling conditions: Denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55–58°C for 30 sec, 72°C for 30 sec and 70°C for 1 min, and a final step at 4°C for 10 min. Each PCR mixture contained 10 μ l SYBR-Green Premix Ex Taq polymerase (Takara Biotechnology Co., Ltd., Dalian, China). For each sample, the mRNA expression levels of the genes of interest were normalized to those of GAPDH. The $2^{-\Delta\Delta C_q}$ method was used for quantification (13). The primers used for the qPCR were as follows: Tumor necrosis factor- α (TNF- α) forward (F), 5'-GACGTGGAAGTGGCAGAAGAC-3' and reverse (R), 5'-TCGCACAAGCAGGAATGAGA-3'; interleukin (IL)-6 F, 5'-CCACGGCCTTCCCTACTTC-3' and IL-6 R, 5'-CTG TTGGGAGTGGTATCCTCTGT-3'; IL-1 β F, 5'-GATGATAAC CTGCTGGTGTGTGA-3' and R, 5'-GTTGTTCATCTCGGA GCCTGTAG-3'; and GAPDH F, 5'-AGGTCGGTGTGAACG GATTTG-3' and R, 5'-GGGGTCGTTGATGGCAACA-3'.

Immunohistochemistry. Tissues were fixed with Bouin's solution (Sigma-Aldrich; Merck KGaA) overnight at room temperature. The tissues were transferred to 70% ethanol for 48 h prior to treatment with a solution of lithium carbonate (1.25%). Then the samples were dehydrated through a graded series of ethanol and embedded in paraffin. The inflamed testes were sectioned into 5- μ m-thick slices, and stained with 5% hematoxylin at room temperature for 10 min and 0.5% eosin for at room temperature 3 min to observe structural changes. Other slices were subjected to immunohistochemical (IHC) analysis to observe specific expression patterns of REG γ , I κ B ϵ and p-p65 in testicular tissue upon LPS treatment. IHC was performed using the Histostain-Plus (DAB) kit (PH0723; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The slices were stained with anti-REG γ , anti-I κ B ϵ and anti-p-p65 antibodies (dilution, 1:100) overnight at 4°C and observed under a light microscope (Eclipse E100; Nikon Corporation, Tokyo, Japan) to evaluate histological changes. The following antibodies were employed:

Anti-I κ B ϵ (cat. no. sc-7155; Santa Cruz Biotechnology, Inc.) and anti-p-p65 (cat. no. ab16502; Abcam). The slides were observed by a fluorescence microscope at a magnification of x200.

Detection of cytokines. Venous blood was collected from the tail vein of mice, coagulated for 2 h at room temperature and then centrifuged at 1,800 x g for 15 min at 4°C (Centrifuge 5408R; Eppendorf, Hamburg, Germany). The upper serum layer was collected to determine the testosterone levels using the F-TESTO ELISA kit (cat. no. JL10895; Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol.

Flow cytometry. Leydig and TM3 cells were stained with fluorescein isothiocyanate and propidium iodide, and then analysed with a BD™ LSR II flow cytometer (all BD Biosciences, San Jose, CA, USA) using FlowJo software (version 10; FlowJo LLC, Ashland, OR, USA).

Cycloheximide treatment. TM3 shN and shR cells were seeded into a six-well plate at a density of 1x10⁶ cells/well. Cells were then treated with 50 μ g/ml cycloheximide (Sigma-Aldrich; Merck KGaA) for 0, 20, 40 and 60 min. For this treatment, cells were culture in DMEM/F-12 supplemented with 5% fetal bovine serum, 2.5% horse bovine serum, L-glutamine (150 mg/l), NaHCO₃ (1.5 g/l), penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C with 5% CO₂.

Statistical analysis. All data analyses were conducted using the Student's t-test or one-way analysis of variance followed by a Fisher's Least Significant Difference post-hoc test for multiple comparisons with SPSS v12.0 software (SPSS, Inc., Chicago, IL, USA). Values are presented as the mean \pm standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Validation of inflammation models. Testes were collected from C57BL/6 WT mice upon LPS treatment for hematoxylin and eosin (H&E) staining and western blot analysis. The results of the western blot analysis indicated that the p-p65 levels were significantly increased in the inflammatory group (Fig. 1A). H&E staining revealed that the testicular structure of the inflammatory group was damaged compared with the control group (Fig. 1B). These results confirmed the successful establishment of a mouse model of testicular inflammation. The western blot analysis results of primary Leydig cells demonstrated that the p-p65 expression levels in the Leydig cells in the inflammation group were increased compared with those in the control group (Fig. 1C). For the TM3 cell line, the inflammation was most pronounced in cells treated with LPS at a concentration of 5 mg/ml (Fig. 1D) for 10 min (Fig. 1E).

Inflammation affects the function of Leydig cells. In addition, the ELISA results demonstrated that the serum testosterone levels in the inflammatory group were decreased compared with in the control group (Fig. 1F). Leydig cells are the

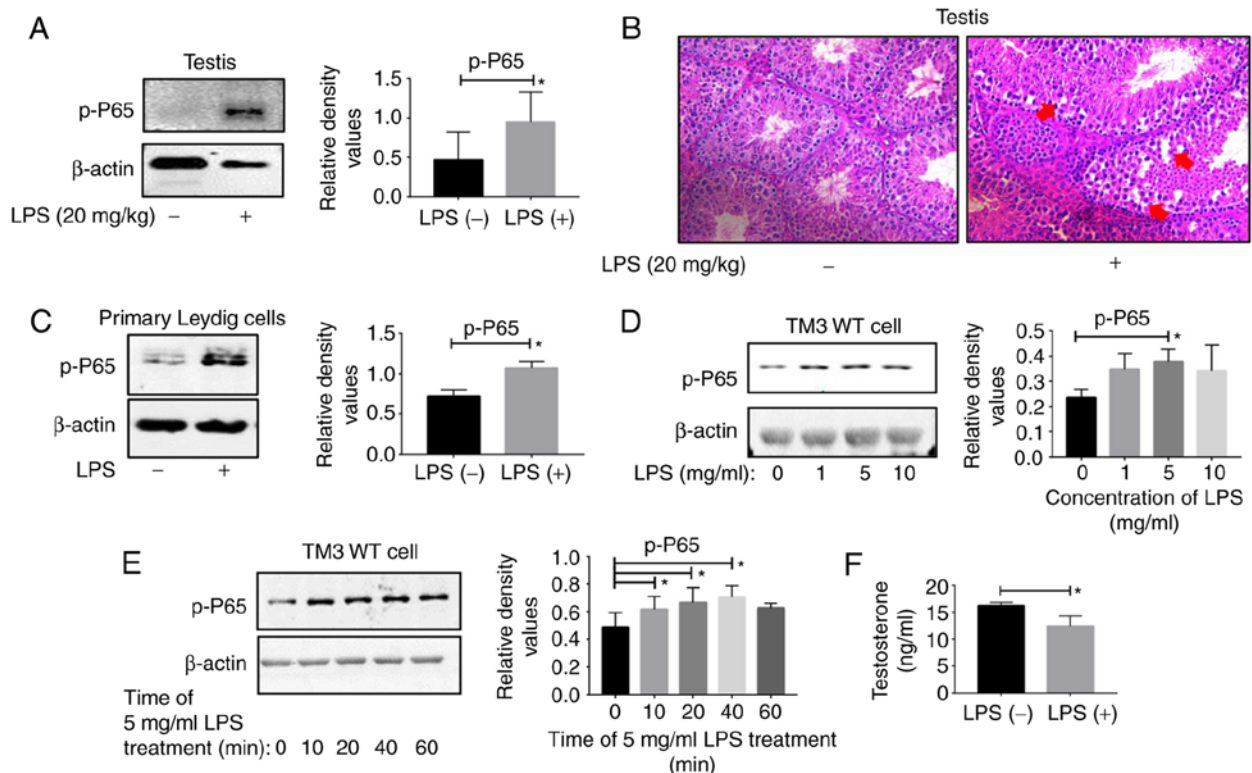


Figure 1. LPS induces testicular inflammation in mice. Testis tissues were collected from two groups of WT C57 male mice treated with double distilled water and LPS (20 mg/kg) for 6 h. (A) Proteins collected from testis tissues were subjected to western blot analysis. (B) The testis tissues of the two groups were subjected to hematoxylin and eosin staining. The damaged testis tissues are highlighted by the red arrows. Magnification, x20. (C) Leydig cells were extracted from the testis tissues of the two groups, and proteins were collected from Leydig cells for western blot analysis. (D) TM3 WT cells were treated with different concentrations of LPS (0, 1, 5 and 10 mg/ml) and analyzed by western blot analysis. (E) TM3 WT cells were treated with LPS at 5 mg/ml for different times (0, 10, 20, 40 and 60 min) and subjected to western blot analysis. (F) The serum testosterone levels of the two groups were determined with an ELISA kit. * $P < 0.05$. Representative data from 3 replicates are presented. WT, wild-type; LPS, lipopolysaccharide; p, phosphorylated; p65, transcription factor p65.

primary cells that secrete testosterone, which indicated that the function of these cells was impaired.

REG γ deficiency decreases LPS-induced inflammation in vivo. Control and inflammatory groups were established in 6-week-old C57 WT male mice and REG γ ^{-/-} mice. Then, testes from these mice were collected for western blot analysis, qPCR assays and H&E staining. The western blot analysis results revealed that the expression level of p-p65 was significantly decreased in REG γ ^{-/-} mice (Fig. 2B) compared with WT mice. These results were confirmed by H&E staining (Fig. 2A). The results of qPCR analyses demonstrated that the expression levels of TNF- α , IL-1 β and IL-6 were significantly increased in the C57 WT mice testes compared with the REG γ ^{-/-} mice testes following LPS treatment (Fig. 2C).

REG γ deficiency decreases LPS-induced inflammation in vitro. Primary Leydig cells were extracted from C57 WT and REG γ ^{-/-} mice testes and divided into LPS⁻ or LPS⁺ groups. Then, western blot analysis and qPCR assays were performed. The western blot analysis results revealed that p-p65 expression was significantly decreased in REG γ ^{-/-} mice compared with the WT group (Fig. 3A). The qPCR results indicated that the expression levels of TNF- α were significantly decreased in REG γ ^{-/-} mice compared with the WT group (Fig. 3B).

Western blot analysis confirmed that REG γ was successfully knocked down (Fig. 3C). shN and shR cells were treated

with LPS (5 mg/ml) for 0, 10, 20, 40 and 60 min. The western blot analysis results revealed that the expression level of p-p65 in the shN groups was increased compared with in the shR groups, with increasing incubation time (Fig. 3D).

REG γ deficiency promotes apoptosis. Flow cytometry was used to detect levels of cell apoptosis. Briefly, shN and shR cells were treated with 5 mg/ml LPS for 10 min. The results indicated that the number of apoptotic cells was significantly increased in the shR cells compared with the shN cells (Fig. 3E).

Reciprocal regulation of REG γ and NF- κ B in vivo and in vitro. Inflammation models were established in two groups of REG γ ^{+/+} and REG γ ^{-/-} mice. The testes of these mice were collected, sliced and stained with an anti-REG γ antibody. The immunohistochemical staining results revealed no REG γ expression in REG γ ^{-/-} mice testes, whereas REG γ was highly expressed in REG γ ^{+/+} mouse testis (Fig. 4A). The expression level of p-p65 was markedly decreased in the REG γ ^{-/-} mice compared with the REG γ ^{+/+} mice following LPS treatment (Fig. 4B). LPS treatment increased the expression level of REG γ in Leydig cells of REG γ ^{+/+} mice (Fig. 4C). In addition, LPS treatment upregulated the expression levels of REG γ in TM3 WT cells compared with the control group (Fig. 4D).

REG γ promotes NF- κ B activity by degrading I κ B ϵ . To explore the mechanism behind the association between REG γ and

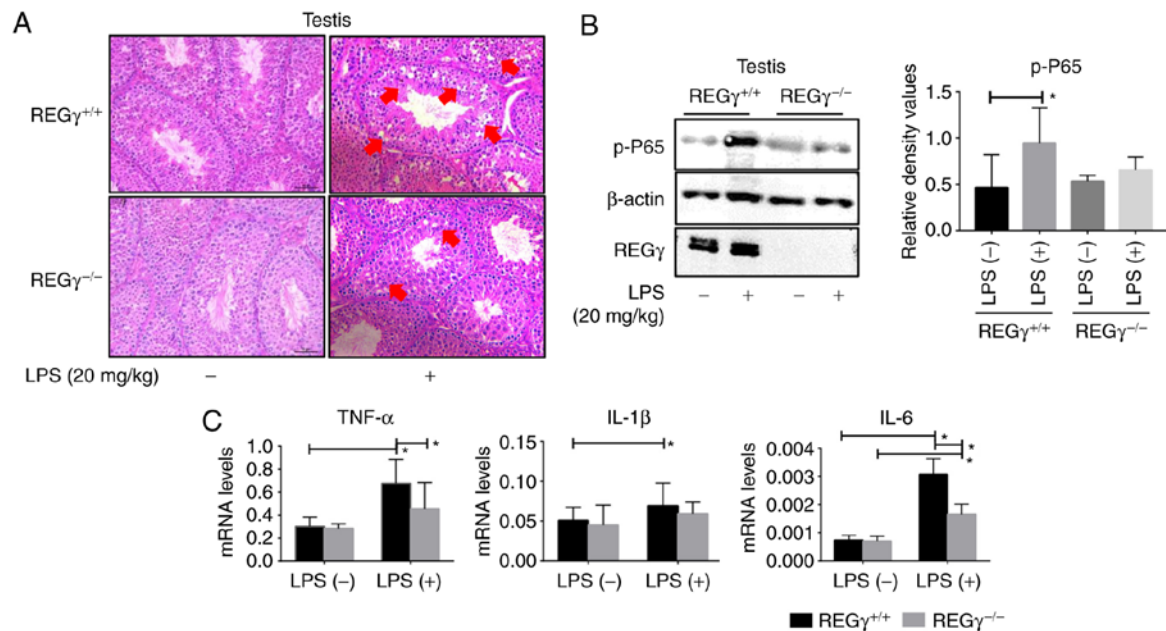


Figure 2. REG γ deficiency decreases LPS-induced inflammation *in vivo*. Testis tissues were collected from REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice treated with double distilled water and LPS (20 mg/kg) for 6 h. (A) The testis tissues collected from each group were subjected to hematoxylin and eosin staining. The damaged testis tissues are highlighted by the red arrows. (B) Proteins were collected from the testis tissues of each group for western blot analysis. (C) The mRNA expression of TNF- α , IL-1 β and IL-6 in the testis tissues of each group was evaluated by quantitative polymerase chain reaction. *P<0.05. Representative data from 3 replicates are presented. REG γ , proteasome activator complex subunit 3; LPS, lipopolysaccharide; IL, interleukin; p, phosphorylated; p65, transcription factor p65; TNF- α , tumor necrosis factor- α ; IL, interleukin.

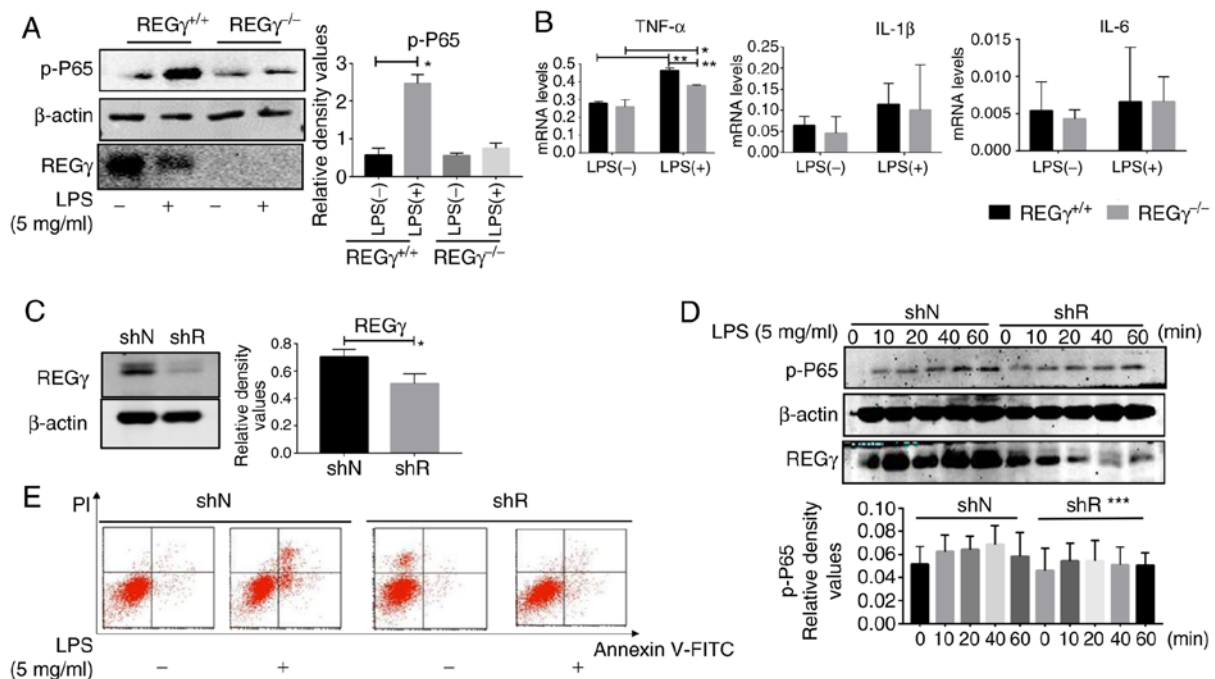


Figure 3. REG γ deficiency decreases LPS-induced inflammation *in vitro*. (A) Testis tissues were collected from REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice treated with double distilled water and LPS (20 mg/kg) for 6 h. Leydig cells were extracted from the testis tissues of each group, and proteins were then collected from Leydig cells for western blot analysis. (B) mRNA expression of TNF- α , IL-1 β and IL-6 in the Leydig cells of each group was evaluated by quantitative polymerase chain reaction. (C) shR TM3 cells and shN TM3 cells were subjected to western blot analysis to evaluate the expression of REG γ . (D) shN and shR cells were treated with 5 mg/ml LPS for different times (0, 10, 20, 40 and 60 min) prior to being subjected to western blot analysis. (E) shN and shR cells treated with 5 mg/ml LPS for 10 min were collected for flow cytometry analysis to evaluate apoptosis. *P<0.05, **P<0.01 and ***P<0.001. Representative data from 3 replicates are presented. REG γ , proteasome activator complex subunit 3; LPS, lipopolysaccharide; p, phosphorylated; p65, transcription factor p65; TNF- α , tumor necrosis factor- α ; IL, interleukin.; shR, REG γ -knockdown; shN, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide.

NF- κ B in Leydig cells, several upstream signaling pathways were identified from previous studies (5-7,12). Of these, the

present study focused on the I κ B family proteins. Western blot analysis of I κ B α , I κ B β and I κ B ϵ were performed in shN and

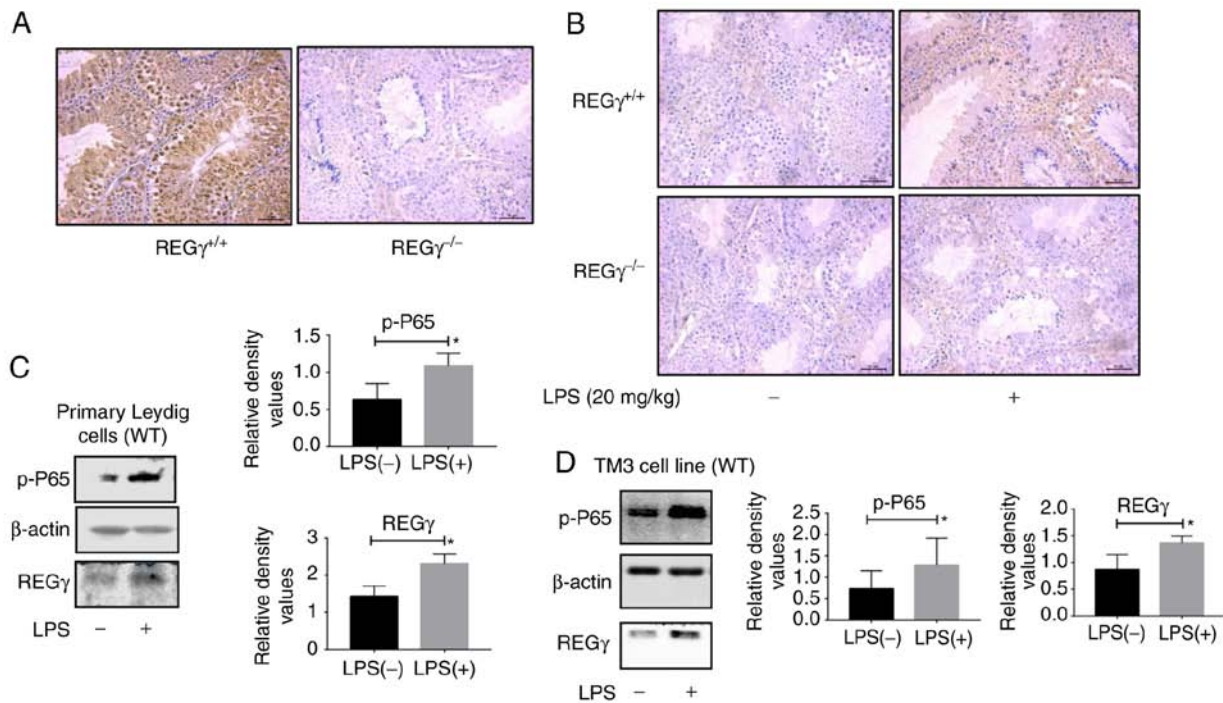


Figure 4. Reciprocal regulation of REG γ and nuclear factor- κ B *in vivo* and *in vitro*. (A) Testis tissues collected from REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice were analyzed by immunohistochemical staining. (B) Testis tissues were collected from REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice treated with dd water and LPS (20 mg/kg) for 6 h and then evaluated by immunohistochemical staining. (C) Proteins were collected from Leydig cells of REG $\gamma^{+/+}$ mice treated with dd water and LPS (20 mg/kg) for 6 h for western blotting. (D) Proteins were collected from wild-type TM3 cells treated with or without LPS (5 mg/ml) for 10 min for western blot analysis. *P<0.05 by Student's t-test. Representative data from 3 replicates are shown. REG γ , proteasome activator complex subunit 3; LPS, lipopolysaccharide; IL, interleukin; dd, double distilled.

shR cells. The results demonstrated significant differences in I κ B ϵ expression levels between shN and shR cells (Fig. 5A). The results of the immunohistochemical staining also revealed that I κ B ϵ expression levels were increased in the testicular tissues of REG $\gamma^{-/-}$ mice compared with REG $\gamma^{+/+}$ mice (Fig. 5B). Based on these results, cycloheximide degradation analyses were conducted. The results revealed that I κ B ϵ degradation was increased in shN cells compared with in shR cells treated for the same time interval. These results demonstrated that the degradation of I κ B ϵ increased with increased expression of REG γ (Fig. 5C).

REG γ /I κ B ϵ double knockdown (dKD) restores inflammation levels. shRNA was used to knock down the REG γ and I κ B ϵ genes in the TM3 cell line. shN, shR (I κ B ϵ knockdown) and dKD (REG γ and I κ B ϵ knockdown) cells were treated with 5 mg/ml LPS for 10 min. The western blot analysis results demonstrated that the expression levels of REG γ and I κ B ϵ were significantly decreased in the dKD cells compared with the shN cells (Fig. 5D). In addition, p-p65 was highly expressed in dKD cells, which was similar to the expression levels observed in WT cells (Fig. 5D).

Discussion

The results of the present study demonstrated that the protease activator REG γ was involved in the development of Leydig cell inflammation in a mouse model of LPS-induced inflammation. These results were additionally validated in primary Leydig cells and the TM3 cell line. Deletion of REG γ increased

the accumulation of I κ B ϵ and inhibited the activation of the NF- κ B signaling pathway, thereby inhibiting inflammation. Furthermore, dKD of REG γ and I κ B ϵ produced a successful cell model of inflammation.

With environmental degradation and unhealthy habits, male fertility continues to exhibit a downward trend (14). Numerous factors contribute to male infertility (15-17), including congenital genital abnormalities, genetic and endocrine diseases, reproductive system infection and inflammation, and physical and chemical factors. Inflammation of the male reproductive system is an important pathogenic factor of male infertility (16).

LPS is the primary constituent of the cell wall of gram-negative bacteria and a key cause of infection (18). LPS has been widely used to generate animal models of inflammation to study the effect of inflammation *in vivo*. Injection of LPS produces an inflammatory response and inhibits testicular steroid production and subsequent spermatogenesis, and may also damage the blood-testis barrier, thereby decreasing fertility (19). In a previous study, C57 mice were intraperitoneally injected with LPS to induce an inflammatory response, in order to observe the histopathological changes in the cells (19). In the present study, the serum testosterone levels decreased following LPS treatment, which indicated that inflammation damaged the normal functions of the Leydig cells.

Previous studies have demonstrated the associations between REG γ regulation and various diseases, which have confirmed the important roles of REG γ in the regulation of biological processes (20-23). The majority of these studies on REG γ have focused on cancer, neurological diseases and heart

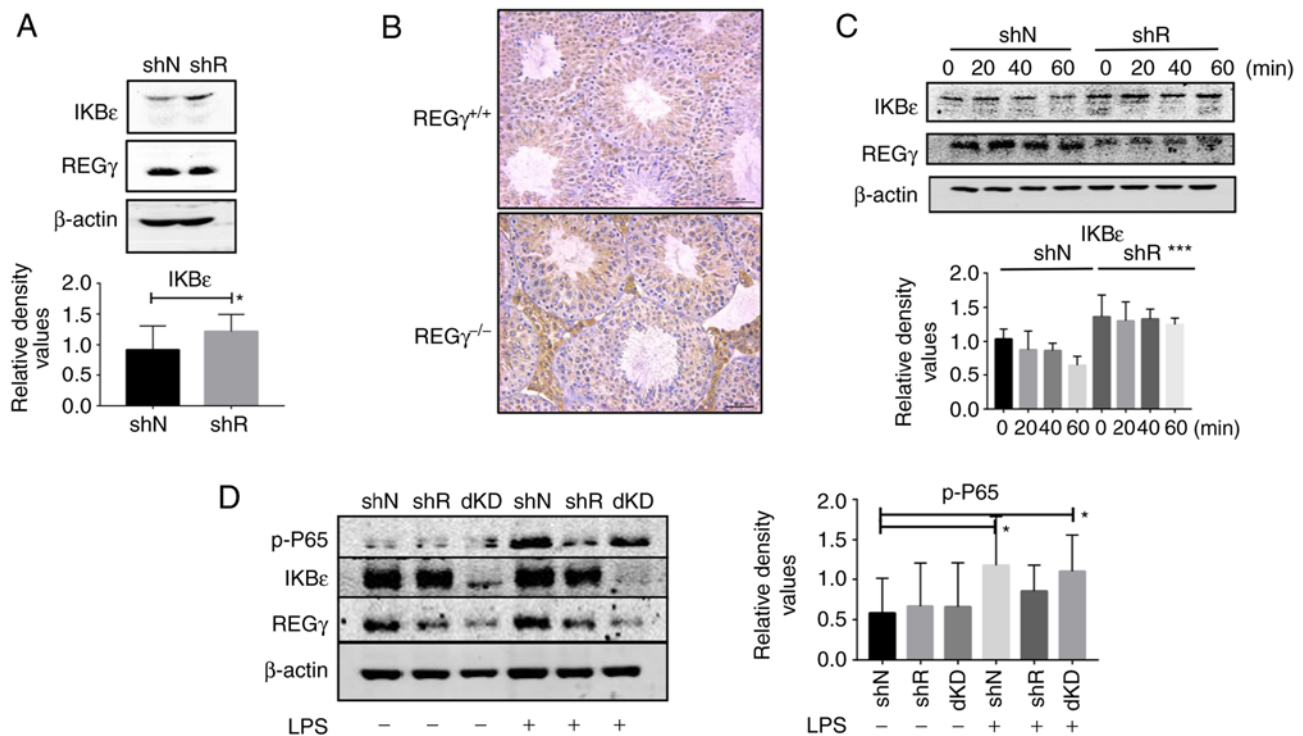


Figure 5. REGγ/IκBε dKD restores inflammation levels. (A) Proteins were collected from shN and shR cells for western blotting. (B) Testis tissues collected from REGγ^{+/+} and REGγ^{-/-} mice were analyzed by immunohistochemical staining. (C) Proteins were collected from shN and shR cells treated with cycloheximide for different times (0, 20, 40 and 60 min) for western blot analysis. (D) Proteins were collected from shN, shR and REGγ/IκBε dKD cells with or without lipopolysaccharide (5 mg/ml) treatment for western blotting. *P<0.05, ***P<0.001 by Student's t-test and one-way ANOVA followed by post hoc test for multiple comparisons (Fisher's Least Significant Difference test). Representative data from 3 replicates are shown. REGγ, proteasome activator complex subunit 3; IκBε, nuclear factor light-chain-enhancer of activated B cells inhibitor ε; dKD, double knockdown; shR, REGγ-knocked down; shN, negative control.

disease (20-23), while few have investigated the association between REGγ and inflammation. A previous study revealed that REGγ was involved in the development of inflammatory bowel disease in a mouse model of enteritis (12). Therefore, the present study focused on the role of REGγ in testicular inflammation.

The NF-κβ signaling pathway has been recognised as a key signaling pathway in the development of inflammatory-associated diseases (24,25). As IκBε is activated upstream of NF-κβ, previous studies explored the association between REGγ and inflammation (26-28). The results of the present study demonstrated the role of the protein kinase-activating factor REGγ in testicular inflammation in animal and cell models.

Testicular tissue damage and NF-κβ activation induced a high expression level of REGγ in Leydig cells, and REGγ upregulated the activity of the NF-κβ signaling pathway by degrading IκBε, which led to the secretion of proinflammatory cytokines and the persistence of testicular Leydig cell and testis injury, ultimately promoting the development of testicular Leydig cell inflammation. Therefore, the absence of REGγ may promote the accumulation of IκBε, thereby inhibiting the activity of the NF-κβ signaling pathway in testicular Leydig cells in mice.

To the best of our knowledge, the present study demonstrated the physiological functions of REGγ and IκBε in testicular inflammation for the first time. REGγ is a specific regulatory factor of IκBε in testicular Leydig cells, which extends awareness of the IκBε regulatory pathway. The testicular tissue of REGγ^{-/-} mice exhibited decreased levels of

inflammatory molecules. However, the roles of other testicular tissue cells in this REGγ testicular inflammation model were not investigated. Therefore, the effects of other types of cells in this process cannot be ruled out. The knockdown of IκBε was performed in the present study, but the results were not satisfactory as no significant differences were identified. This is a limitation of the study, and will be a focus in future studies.

Various anti-inflammatory drugs, including glucocorticoids, methotrexate and anti-TNF-α antibodies, completely or partially inhibited the NF-κβ signaling pathway (29). However, these anti-inflammatory drugs do not specifically inhibit NF-κβ, and the response to these drugs differs among individuals, which highlights the ongoing challenges in proteasome studies. Notably, the results of the present study indicated that REGγ regulates NF-κβ activity by specifically degrading IκBε, thereby providing novel molecular targets of the atypical proteasomal pathway. It may be possible to prevent and treat inflammation by attenuating, as opposed to completely inhibiting the NF-κβ signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL and YX designed the experiments. TX, HC, SS, GH and BH performed the experiments and TH analyzed the data. TX wrote the manuscript.

Ethics approval and consent to participate

All animal procedures were reviewed and approved by the Animal Care Committee of East China Normal University, which followed the Guide for the Care and Use of Laboratory Animals by the National Research Council.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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