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TNF-α inhibits GDNF levels in Sertoli cells, through a NF-κB-dependent, HES1-dependent mechanism

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Abstract

Background: Glial cell line-derived neurotrophic factor (GDNF) is a soluble molecule crucial for the regulation of the spermatogonial stem cells (SSC) of the testis. The effects of GDNF on target cells have been extensively described, but mechanisms underlying GDNF regulation are currently under investigation. In the nervous system, GDNF expression is regulated by pro-inflammatory cytokines including lipopolysaccharide (LPS), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) but the effect of these cytokines on GDNF expression in the testis is unclear.

Objectives: The aim of the present study was to investigate the impact of TNF-α on GDNF expression levels using primary murine Sertoli cells as experimental model.

Material and methods: The expression of TNF-α-regulated genes including *Gdnf* in different culture conditions was determined by real-time PCR. GDNF protein levels were determined by ELISA. The activation of the NF-κb pathway and HES1 levels were assessed by Western Blot analysis and immunofluorescence. HES1 expression was downregulated by RNAi.

Results: In primary Sertoli cells, TNF-α downregulates GDNF levels through a nuclear factor-κB (NF-κB)-dependent mechanism. Mechanistically, TNF-α induces the transcriptional repressor HES1 by a NF-Kb-dependent mechanism, which in turn downregulates GDNF.

Discussion: Under physiological conditions, TNF-α is secreted by germ cells suggesting that this cytokine plays a role in the paracrine control of SSC niche by modulating GDNF levels. HES1, a well-known target of the Notch pathway, is implicated in the regulation of GDNF expression. In Sertoli cells, TNF-α and Notch signaling may converge at molecular level, to regulate the expression of HES1 and HES1- target genes, including GDNF.

Conclusions: Because of the importance of GDNF for spermatogonial stem cell self-renewal and proliferation, this data may give important insights on how cytokine signals in the testis modulate the expression of niche-derived factors.

KEYWORDS
TNF-α, GDNF, Sertoli cells, HES1, NF-κB

1 | INTRODUCTION

GDNF belongs to the transforming growth factor β superfamily molecules and is a founding member of the GDNF family of ligands (GFL), which includes neurturin, artemin, and persephin. GDNF family ligands exert their cellular responses by activating a multicomponent receptor complex consisting of the membrane-anchored GDNF family ligand receptor GFRA1-4 and transmembrane RET receptor tyrosine kinase.[1] GDNF plays an important role in male germ cell development, controlling the balance between self-renewal and differentiation of the spermatogonial stem cells.[2,3] In the testis, GDNF is expressed by several cell types such as Sertoli, peritubular and endothelial cells.[4-6] In Sertoli cells, GDNF levels are regulated by pivotal pathway such those activated by FSH, Notch, and retinoic acid.[7-9] Although the effect of GDNF on spermatogonial stem cells has extensively explored, the mechanisms through which GDNF expression is regulated are currently under investigation.

As a neurotrophic factor, GDNF is a potent survival factor for motoneurons[10] and several evidence argue for a role of pro-inflammatory cytokines and the NF-κB pathway in the induction of GDNF in the nervous system.[11] However, nothing is known about the role of cytokines on the regulation of GDNF expression and production in the testis. In the testis, pro-inflammatory cytokines are produced also in physiological conditions, that is, in the absence of inflammation or immune activation.[12] Among testicular cytokines, the function of tumor necrosis factor alpha (TNF-α) has been largely described. In the testis, TNF-α mRNA is produced by pachytene spermatocytes, round spermatids, and activated macrophages, while the protein has been shown to be released just by round spermatids.[13] TNF-R1 receptor has been found on Sertoli and Leydig cells.[14] TNF-α regulates several testicular functions such as Sertoli cell secretory function and metabolism,[15,16] production of Sertoli-derived immunomodulatory factors,[17-20] steroidogenesis,[21-23] germ cell survival,[24,25] and blood-testis barrier rearrangement.[26] Finally, TNF-α is implicated in the pathogenic response associated to testis injury following toxicant exposure, autoimmune orchitis, and inflammation.[27-29] Sertoli cells play a Janus-faced role in the testicular immune response and express both TNF-α receptors, TNF-R1 (p55) and TNF-R2 (p75).[13,30-32] Previous studies indicate that in primary Sertoli cells, TNF-α activates several signaling pathways, including the transcription factor NF-κB.[17,19] NF-κB family includes different members that can form homo- and heterodimers, comprising typically p50 e p65 (Rel A) subunits, but also other members such as p52, c-Rel, and Rel-B. In the inactivated state, NF-κB is sequestered in the cytoplasm through its association with various isoforms of the inhibitor protein IκB. Activation of the NF-κB signaling cascade results in the phosphorylation and subsequent degradation of IκB, which allows the translocation of NF-κB to the nucleus.[33] It has been previously shown that in TM4 Sertoli cell line, TNF-α and IL-1b induce the expression of *Gdnf* mRNA,[34] but the mechanisms behind were not clarified. In the present study, we tested the hypothesis that in primary Sertoli cells, TNF-α modulates GDNF expression by the activation of NF-κB pathway. Here, we show that TNF-α down-regulates GDNF both at the mRNA and at the protein level in a NF-κB-dependent, HES1-dependent fashion.

2 | MATERIALS AND METHODS

2.1 | Sertoli cell culture and treatments

Primary Sertoli cells culture was obtained from 17-day CD1 mice, as previously reported.[8] Briefly, seminiferous tubules were obtained by two consecutive enzymatic digestions and sedimentation at unit gravity. At the end, the fragmented tubules were collected in a polystyrene tube and subjected to centrifugation at 18 g for 2 minutes (min). The pellet was then resuspended in MEM 1X (Minimum Essential Medium) (Gibco, Milan, Italy), containing 20 mM glutamine, Hepes 0.01 M, non-essential 0.1 mM amino acids, and antibiotics. Cells were maintained at 34° C in a controlled atmosphere of 5% CO_2. The residual germ cells were eliminated after 48 hours (h) of culture with a short hypotonic treatment.[35] The purity of Sertoli cell cultures was routinely higher than 95%.[8] Sertoli cells cultures were used after 24 h from hypotonic treatment. Sertoli cells were treated with different concentration of TNF-α (Roche, Milan, Italy), different concentration of BAY11-7082 (Sigma-Aldrich, Milan, Italy), and different concentration of DAPT (Sigma-Aldrich, Milan, Italy).

2.2 | GDNF secretion

To measure GDNF secretion, Sertoli cells were left untreated or treated with 20 ng/ml TNF-α for 24 h. Conditioned medium was collected, centrifuged at 3000 ***g*** for 5 min at 4°C, and the supernatant was stored at −80°C. GDNF levels in the culture medium were measured using the GDNF Emax ImmunoAssay System Kit (Promega, Madison, WI) according to the manufacturer's instructions. The ELISA reaction was carried out in 96-well plates, and the optical densities were recorded by the Microplate reader at 450-nm wavelength. Results are from three experiments and are showed as box plots displaying the five-number summary of the set of data (the minimum, first quartile, median, third quartile, and maximum).

2.3 | RNA extraction and real-time PCR

Total RNA extraction was performed using the TRIzol reagent (Invitrogen) following the manufacturer's instructions and quantified by Nanodrop 1000 (Thermo Fisher Scientific). The cDNA synthesis, from 1 μg of RNA for each sample, was performed using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific) following the manufacturer's instructions. In the control samples, reverse transcriptase was omitted to monitor genomic DNA contamination. The list of genes analyzed and respective primers used are reported

in Table S1. The cDNA was subjected to real-time PCR analysis with FluoCycleTMII SYBR Green Mix (Euroclone) in 7500 Real-Time PCR System (Applied Biosystems, Life Technologies). Blank controls were assayed in each reaction and for each primer pair to detect reagent contamination. Data were analyzed with the comparative $2^{-\Delta\Delta CT}$ method using *Actb* as reference gene.[36] Each real-time PCR assay was repeated at least two times, using duplicate samples in three different experiments. The mean values with standard error of the mean (SEM) or the medians were used for comparison.

2.4 | Western blot

Total mouse primary Sertoli cell lysates were prepared by scraping the cells off the culture plate with Cell Lysis Buffer (New England Biolabs) containing 1 μg/ml of leupeptin and 1 mM PMSF (Sigma-Aldrich). Protein concentration was determined by using the micro BCA method (Pierce). Equal amounts of proteins (25 μg) were subject to SDS polyacrylamide gel electrophoresis and then transferred onto nitrocellulose. The membranes were saturated with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and then incubated overnight with primary antibody at 4°C. The primary antibodies employed are listed in Table S2. After washing 3 times for 15 min with TBST, blots were further incubated with horseradish-conjugated secondary antibody (Biorad) for 1 h at room temperature. Membranes were washed again with TBST and developed using the chemiluminescence system (ECL Advance Western blotting detection kit; GE) by using a Chemidoc Biorad.

2.5 | Immunofluorescence

Sertoli cells were isolated as described above and cultured on coverslips for 48 h before hypotonic treatment. After 24 h, cells were fixed with 4% PFA for 10 min at room temperature. To reduce unspecific background signal, Sertoli cells were incubated with 1 M glycine (Sigma-Aldrich) and subsequently with 3% BSA (Sigma-Aldrich), 5% Normal Donkey Serum (Jackson Laboratories Immuno Research Europe Ltd), and 0.1%Triton in PBS. Subsequently, Sertoli cells were incubated one hour at room temperature with appropriate primary antibodies. The primary antibodies employed are listed in Table S2. After the washes, Sertoli cells were incubated with species-specific secondary antibodies, for one hour at room temperature. All secondary ALEXA488-, Cy3-conjugated antibodies were from Jackson Immuno Research Europe Ltd (Newmarket, UK). At the end, the nuclei were counterstained with TO-PRO-3 Iodide (642/661) (T3605 Thermo Fisher Scientific). Images were acquired using a Leica TCS SP2 confocal microscope with 40× oil immersion objective. For quantification of p65 and HES1 nuclear expression levels, Z-stacks were acquired e at 1 μm increments between z-slices and the mean nuclear fluorescence intensity was quantified using LAS AF Software.[37] In the quantification procedure, the region of interest (ROI) was drawn around the nuclear profile revealed by TO-PRO-3 staining.

2.6 | Short interference RNA (siRNA)

To interfere with HES1 expression, primary Sertoli cells were transfected with scrambled siRNA (MISSION siRNA Universal Negative control, Sigma) or with siRNA specific for HES1 (5'-UCGUUUUUAGUGUCCGUCAGAAGAG-3' from Sigma) as described[9]. After the hypotonic treatment, Sertoli cells were transfected for 24 h with Lipofectamine 2000 (Invitrogen) and 80 picomoles of the anti HES1 siRNA or not specific siRNA in Opti-MEM medium (Invitrogen). At the end, the medium was removed, and cultures were treated in the presence or absence of 20 ng/ml TNF-α for additional 2 h. *Gdnf* expression level was evaluated by real-time PCR, and cDNA levels were standardized by normalizing to a β-actin control. The expression levels are presented as fold induction over the basal condition of the not specific siRNA sample. The results are from three independent experiments and are showed as box plots displaying the five-number summary of the set of data (the minimum, first quartile, median, third quartile, and maximum).

2.7 | Statistical analysis

Statistical analysis was performed using the Sigma Plot 11.0 program. Quantitative data are shown as the mean ± (SEM), mean ± standard deviation of the mean (SD), or Tukey boxplots. To define the significance of the differences between two groups, data were analyzed using a t test. To compare many groups, data were analyzed using a one-way analysis of variance (ANOVA) followed by an appropriate post hoc method, detailed in the figure legends. The significance level was fixed at $\alpha = 0.05$.

3 | RESULTS

3.1 | TNF-α downregulates GDNF mRNA and protein in mouse Sertoli cells

It has been demonstrated that TNF-α upregulates *Gdnf* in glioblastoma cells, astrocytes, and TM4 murine Sertoli cell line.[34,38,39] We asked whether TNF-α has a similar effect on GDNF expression at the mRNA and at the protein level in murine primary Sertoli cells. To this aim, Sertoli cells were treated with different TNF-α concentrations for 2 h prior to RNA extraction. Data in Figure 1A show that TNF-α significantly downregulates *Gdnf* expression in a dose-dependent fashion. As a positive control for TNF-α, we assayed the levels of *Fas* which has previously been shown to increase following TNF-α stimulation[18] (Figure S1A). Subsequently, we performed a time-course experiment by stimulating Sertoli cells with TNF-α at the concentration of 20 ng/ml. Data show that a significant reduction of *Gdnf* mRNA is detectable already after 1 h stimulation and is maintained at all time points up to 24 h (Figure 1B). In contrast on the same samples, *Fas* is strongly upregulated (Figure S1B). To verify that the downregulation of *Gdnf* also

occurs at the protein level, Sertoli cells were treated for 24 h with 20 ng/ml TNF-α or with control medium and GDNF in the conditioned media was assayed by ELISA. A significant reduction of GDNF protein (~30%) triggered by TNF-α is observed (Figure 1C).

3.2 | NF-κB is involved in TNF-α-induced *Gdnf* downregulation

In mouse Sertoli cells, TNF-α activates the transcription factor NF-κB[19]. In order to test the involvement of NF-κB in *Gdnf* downregulation following TNF-α stimulation, we sought to inhibit NF-κB activity by using the pharmacological inhibitor BAY11-7082 37. BAY11-7082 inhibits IκBα phosphorylation, blocking its proteasomal degradation and allowing NF-κB to be sequestered in the cytoplasm in an inactivated state. We first performed a dose-response experiment to choose the lowest efficacious concentration of BAY11-7082. To this aim, Sertoli cells were pretreated for 1 hour with different BAY11-7082 concentrations and then stimulated with 20 ng/ml TNF-α for 5 and 20 min, which are respectively the time points of high IκBα phosphorylation and of total IκBα degradation.[20] Results shown in Figure 2A show that 10 μM was sufficient to fully inhibit both IκBα phosphorylation and its subsequent degradation. We then pretreated Sertoli cells for 1 hour with 10 μM BAY11-7082 and then stimulated with 20 ng/ml TNF-α for 2 h prior to RNA extraction. Data in Figure 2B show that BAY11-7082 pre-treatment significantly blocked *Gdnf* downregulation, thus suggesting that NF-κB is involved in *Gdnf* downregulation. As control, *Fas* expression induced by TNF-α is also inhibited by BAY11-7082 (Figure S2), in agreement with previous data demonstrating that *Fas* induction following TNF-α stimulation is NF-κB-dependent.[19]

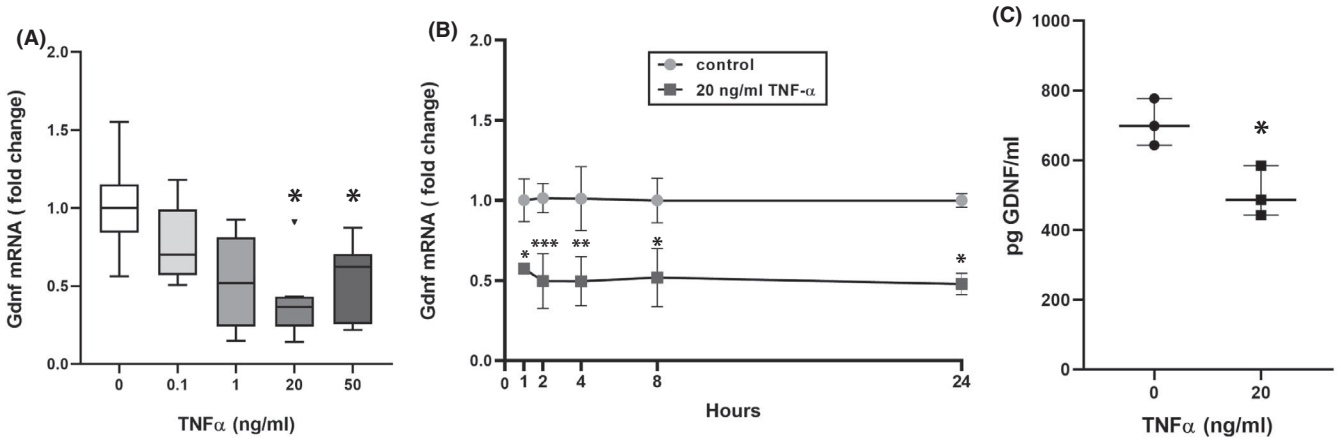

FIGURE 1 TNF-α inhibits *Gdnf* levels in dose- and time-dependent fashion. (A, B) Sertoli cells were cultured for 24 h without or with increasing concentrations of TNF-α (A), or for different time lengths in the presence or absence of 20 ng/ml TNF-α (B) *Gdnf* mRNA levels were measured by real-time PCR, normalized to *Actb* levels, and expressed as arbitrary units. (C) The boxplots show the effect of 24 h TNF-α treatment on secreted GDNF protein in Sertoli cells conditioned medium. In A and C, data are shown using Tukey's boxplots ($n = 3$). In B, data are presented as the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* control condition

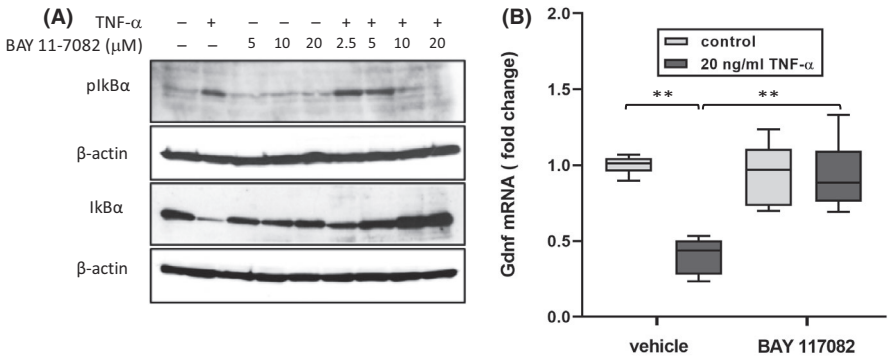

FIGURE 2 NF-κB is implicated in TNF-α-mediated *Gdnf* downregulation. (A) Sertoli cells were pretreated with increasing BAY 11-7082 concentration one hour before stimulation with 20 ng/ml TNF-α for 5 and 20 min. Whole extracts (25 μg) were subjected to Western blot analysis using anti-phospho-specific IκBα Ab (5 min treatment) and anti-IκBα polyclonal Ab (20 min treatment). The blot was then reprobed with anti-β-actin Ab as control of equal amount of protein loaded. This experiment is representative of three independent experiments. (B) Real-time PCR analysis of *Gdnf* in Sertoli cells treated for 2 h with TNF-α 20 ng/ml in the presence or absence of 10 μM BAY11-7082. Data are shown using Tukey's boxplots ($n = 3$). $^{**}p < 0.01$

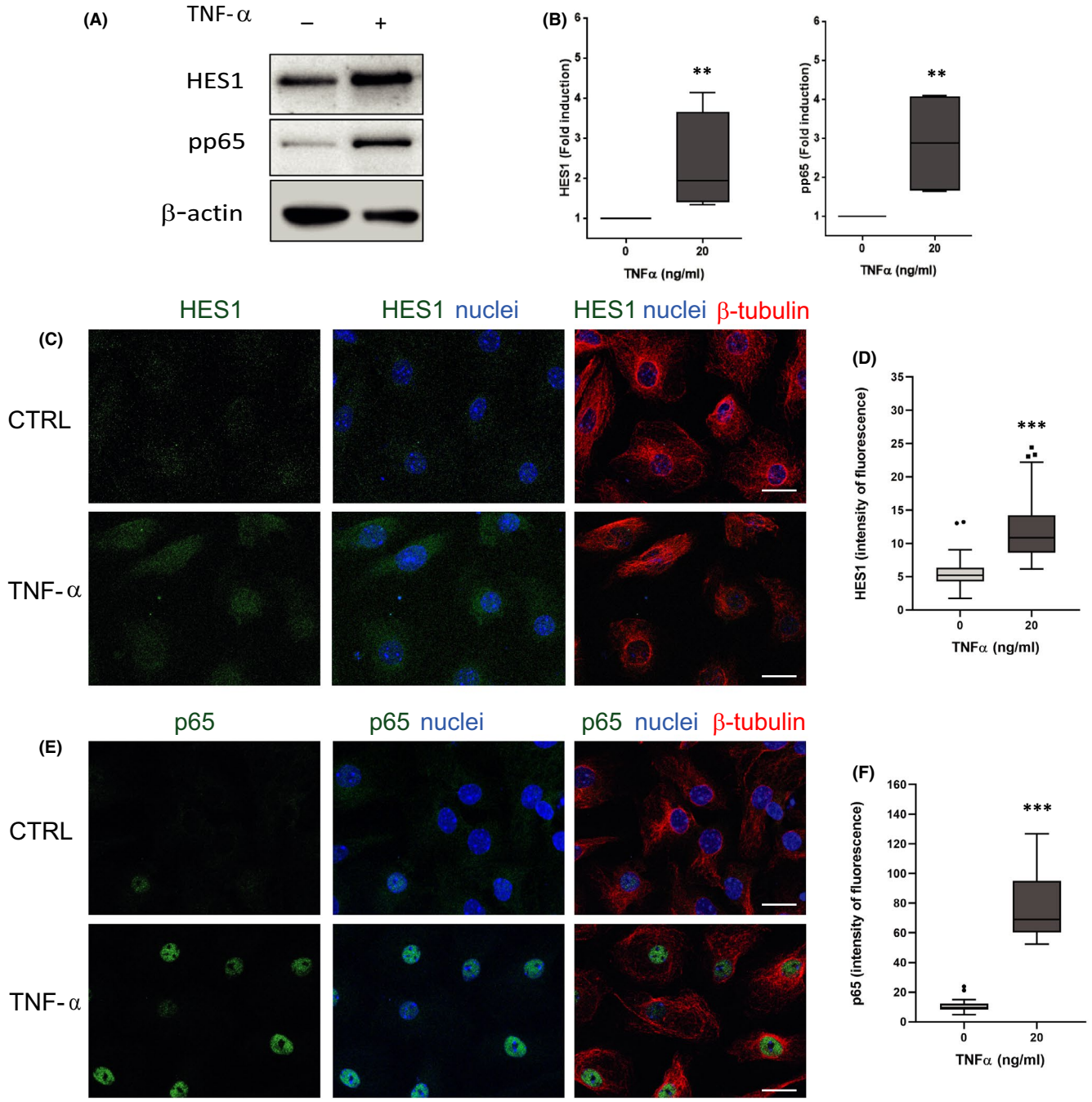

FIGURE 3 TNF-α induces the nuclear repressor HES1. (A, B) Sertoli cells were treated with 20 ng/ml TNF-α for 2 h. Whole cell extracts (25 μg) were subjected to Western blot analysis using anti- HES1 Ab. The blot was reincubated first with a phospho-specific p65 (pp65) Ab and then with anti-β-actin Ab as control of equal amount of protein loaded. This Western blot is representative of three independent experiments (A). (B) Average data obtained by densitometric analysis of the HES1 (left) and the p65 (right) immunoreactive bands from three different experiments. (C, F) Sertoli cells were treated or untreated with 20 ng/ml TNF-α for 2 h, fixed and subjected to immunodetection of HES1 (green), and β-tubulin (red) (C) or with p65 (green) and β-tubulin (red) (E). Nuclei were counterstained with TO-PRO-3 (blue) (C, E). Micrographs in central and right columns are merge of the indicated stainings. Scale bar, 20 μm. The Tukey boxplots show the nuclear fluorescent intensity of HES1 (D) or p65 (F) in treated or untreated Sertoli cells. Data are from $n = 2$ experiments for a total of 40 cells analyzed in each condition. $^{**}p < 0.05$, $^{***}p < 0.001$ *vs* control condition

3.3 | TNF-α induces the nuclear repressor HES1

As our previous data indicated that TNF-α-mediated activation of NF-κB pathway in Sertoli cells downregulated *Gdnf*, we sought to test whether NF-κB activation may indirectly influence GDNF by affecting the levels of known transcriptional repressors. It has been recently shown that activation the Notch pathway in Sertoli cells induces downregulation of GDNF through the nuclear repressors

HES1 and HEY1.[7] We therefore analyzed the effect of TNF-α treatment on HES1. To this aim, Sertoli cells were treated with 20 ng/ml TNF-α for 2 h prior to protein isolation. Western blot analysis shown that TNF-α induces HES1 and phosphorylation of p65 (Figure 3A,B). To further verify the induction of HES1 by TNF-α treatment, we performed immunofluorescence analysis on Sertoli cells. Cells were treated or left untreated with TNF-α and then stained for HES1, p65, and β-tubulin to label Sertoli cytoplasmatic microtubules. As expected, TNF-α treatment induced a nuclear translocation of p65 (Figure 3E) and, in line with Western blot analysis, a significant increase of HES1 levels, both in the cytoplasm and in the nuclei of Sertoli cells (Figure 3C). Quantification of immunofluorescence signals showed a significant increase of nuclear p65 and HES1 in TNF-treated cells compared with untreated cells (Figure 3D,F). Taken together, this indicated that in Sertoli cells, TNF-α induces expression level of nuclear repressor HES1.

3.4 | HES1 is involved in the TNF-α-mediated, NF-κB-dependent *Gdnf* downregulation

Our previous data indicated that inhibition of NF-κB pathway was able to block TNF-α-mediated downregulation of *Gdnf*. We next asked whether blunting the TNF-α-mediated activation of NF-κB pathway in Sertoli cells could also impact on the HES1 levels. To this end, Sertoli cells were treated without or with 20 ng/ml TNF-α in the presence or absence of BAY11-7082 for 2 h prior to RNA and protein isolation. Results in Figure 4A show that while TNF-α induced a small but significant upregulation of *Hes1* mRNA, in Sertoli cells treated with BAY11-7082 both basal and TNF-α-induced *Hes1* levels were reduced. This was also observed at protein level as shown in Figure 4B. The induction of HES1 by TNF-α was blunted in the presence of BAY11-7082. The levels of phospho-p65 were monitored to verify the effective inhibition of NF-κB pathway. Taken together, this indicates that in Sertoli cells, TNF-α induces HES1 in a NF-κB-dependent fashion.

Finally, we aimed to evaluate a direct involvement of HES1 on the TNF-α-dependent negative regulation of *Gdnf* in Sertoli cells. We reasoned that if HES1 is involved in the negative regulation, reducing HES1 levels could inhibit TNF-α-mediated effect on the levels of *Gdnf* transcript. To this end, HES1 levels were manipulated by short interference RNA. Sertoli cells were transfected for 24 h either with Hes1-specific siRNA or with scrambled siRNA as a control and then treated or left untreated with 20 ng/ml TNF-α for 2 h. Data in Figure S3 show that HES1 levels were reduced by RNAi of about 70%. Then, *Gdnf* was evaluated by real-time PCR in transfected cells untreated or treated with TNF-α (Figure 4C). As expected, in Sertoli cells transfected with a scramble RNA, TNF-α significantly downregulated *Gdnf*. Importantly, HES1 knockdown significantly inhibited, even though not completely, TNF-α-dependent *Gdnf* downregulation. These data indicated that HES1 is involved, at least in part, in the negative regulation of *Gdnf* levels by TNF-α.

4 | DISCUSSION

This study demonstrates that, in immature primary Sertoli cells, TNF-α downregulates GDNF levels through activation of the NF-κB pathway. It has been previously shown that in neuronal cells, such as glioblastoma cells and astrocytes, TNF-α induces GDNF expression.[38-41] However, in neuroblastoma cells, TNF-α treatment inhibits GDNF release.[41] The present data further suggest that the response to TNF-α treatment in terms of GDNF modulation is cell type-dependent.[11] Our finding contrasts with a previous study

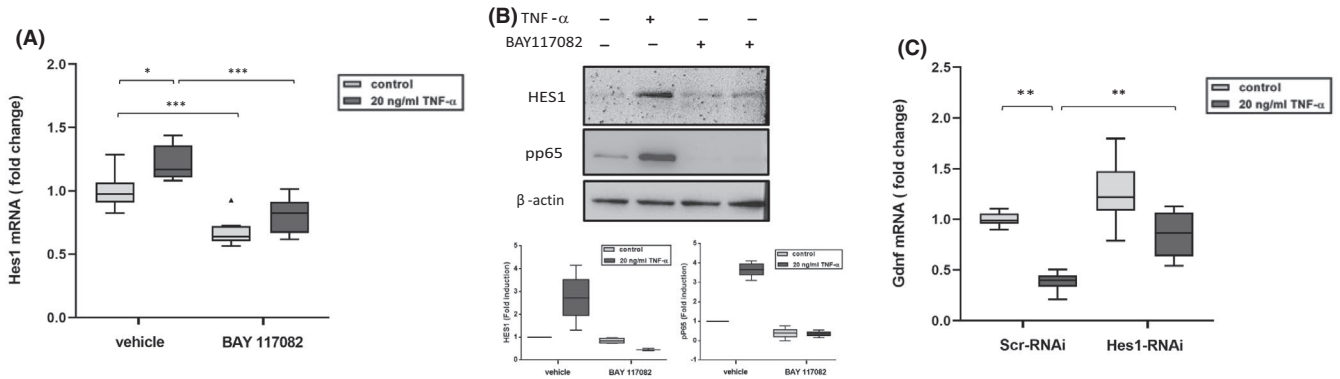

FIGURE 4 TNF-α induces HES1 in a NF-κB-dependent fashion. (A, B) Sertoli cells were pretreated with 10 μm BAY 11-7082 concentration one hour before stimulation with 20 ng/ml TNF-α for 2 h. (A) *Hes1* mRNA levels were measured by real-time PCR, normalized to *Actb* levels, and expressed as fold changes. (B) Whole extracts (25 μg) were subjected to Western blot analysis using anti-HES1 and anti-pp65 antibodies. The blot was then reprobed with anti-β-actin Ab as control of equal amount of protein loaded. This Western blot is representative of three independent experiments, while box plots below show the average data obtained by densitometric analysis of the HES1 (left) and the p65 (right) immunoreactive bands from three different experiments. (C) Sertoli cells were transfected with scramble RNAi (scrb-RNAi) or with a specific anti-HES1 RNAi (HES1-RNAi) for 24 h and then treated with 20 ng/ml TNF-α or left untreated for 2 h. *Gdnf* levels were quantified by real-Time PCR, normalized to *Actb* levels. Data are shown using Tukey's boxplots from three independent experiments and expressed in fold change compared with the untreated control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

showing TNF-α-dependent induction of *Gdnf* mRNA, in TM4 murine Sertoli cell line.[34] Differences on cell type employed could account for these different results. Even though TM4 cell line retains major characteristics of primary Sertoli cells, they do not completely replicate primary Sertoli cells.[42]

The analysis of early biochemical events, triggered by TNF-α in murine Sertoli cells, has been elucidated. The binding of TNF-α to TNF-R1 triggers a series of intracellular events that result in the activation of different pathways, including the mitogen-activated protein kinase (MAPK) pathways p42/p44 MAPK, JNK/SAPK, and p38, and NF-κB.[17,20] In the present study, pre-treatment of Sertoli cells with BAY 11-7082, a specific NF-κB inhibitor, was able to completely inhibit the TNF-α-dependent GDNF downregulation, suggesting the NF-κB pathway as the only player responsible for the observed regulation.

TNF-α-dependent activation of NF-κB relies on phosphorylation-dependent ubiquitination and degradation of inhibitor of κB (IκB) proteins, which normally retain NF-κB within the cytoplasm of unstimulated cells.[43] In Sertoli cells, the IκBα phosphorylation is maximal after few minutes, followed by its complete degradation within 20 min of TNF-α stimulation.[20] IκB degradation in the proteasome results in rapid and transient nuclear translocation of NF-κB members followed by activation of NF-κB target genes. The analyses of evolutionary conserved binding sites show the presence of several putative NF-κB DNA binding sites on murine *Gdnf* promoter.[8] Given that NF-κB is primarily considered a transcriptional activator, but in some cases can act as a transcriptional repressor, we hypothesized that one or more NF-κB binding sites could be a direct target for activated NF-κB.[44] When we tested this hypothesis by site-directed mutagenesis and promoter-gene-reporter assays, we found that the NF-κB DNA binding sites are not involved in the modulation of *Gdnf* levels by TNF-α (data not shown). This prompted us to hypothesize that NF-κB may induce a transcriptional repressor that in turn inhibits *Gdnf* transcription. A similar mechanism has been described for TNF-α-mediated inhibition of AMH.[45] Following this line of reasoning, we focalized our attention on HES1, a transcriptional repressor recently implicated in the regulation of GDNF expression.[7] HES1 belongs to the HES/HEY family and is a well-known target of the Notch pathway. In the adult testis, HES1 is expressed in Sertoli cells in a stage-dependent fashion, and its expression is regulated by the ciclica activation of Notch signaling.[46] Importantly, HES1 and HEY1 mediated the Notch-dependent GDNF downregulation by binding to the *Gdnf* promoter.[7] Even if HES1 is a well-characterized target of the Notch pathway, several other pathways signal to control its expression.[47-49] In the present study, we show for the first time that in Sertoli cells, TNF-α induces HES1 at both mRNA and protein levels, as shown by Western blot and immunofluorescence analysis. Importantly, pre-treatment of Sertoli cells with BAY11-7082 was able to block the TNF-α-mediated induction of HES1. Our results are in line with a previous study showing that TNF-α induces *Hes1* mRNA synthesis through NF-κB.[50]

To directly test the involvement of HES1 on *Gdnf* regulation in Sertoli cells, we abrogated its expression by transient transfection with RNAi. HES1 knockdown significantly inhibited the TNF-α-dependent reduction of *Gdnf* indicating its involvement as transcriptional repressor of *Gdnf* in line with previous work.[7] Importantly, in adult testis, the activation of Notch signaling and its downstream target genes HES1 and HEY1 have been inversely correlated with GDNF expression level.[7] Our data suggest an alternative pathway in Sertoli cells, activated by TNF-α and converging with Notch signaling at molecular level to regulate the expression of HES1.

Tadokoro and colleagues have previously shown that GDNF expression is induced by FSH in vivo.[4] In our previous study, we identified the cAMP pathway as the one involved in this activation in primary Sertoli cells[8] and here we show that TNF-α opposes GDNF production. These data lend further support to the reciprocal relation between FSH/cAMP and inflammatory cytokine signaling pathways in the control of Sertoli cell function.[51,52] The expression of GDNF varies significantly within the different stages of the cycle of the seminiferous epithelium, and its cyclical expression is required for spermatogonial stem cells homeostatsis.[53-57] As TNF-α is released by round spermatids,[13] our data suggest that this cytokine could act in a paracrine fashion to indirectly control the spermatogonial stem cell compartment by modulating GDNF levels during the first half of the seminiferous cycle. Our hypothesis is also supported by the in vivo evidence, provided by Tadokoro and collegues, that showed how GDNF expression levels negatively correlate with the accumulation of the most advanced germ cells in the testis.[4] Furthermore, Garcia et al. showed that *Hes1* levels increase during stages V-VIII of the cycle where round spermatid can be found.[7] However, future studies are needed to clarify whether TNF-α is able to regulate GDNF also in vivo and to identify at which developmental stage the round spermatids start to release TNF-α. Besides TNF-α, multiple hormones and growth factors have been shown to regulate GDNF expression during the different steps of the spermatogenic process.[4,7-9] In vivo, these factors could counteract or synergize with TNFα leading to a different effect on the GDNF levels. Such a complexity cannot be reproduced using an in vitro system as the one used in this study. Despite this limitation, here we described that TNF-α mediated regulation of GDNF in Sertoli cells and provided a possible mechanism involving a NF-κB-dependent HES1 induction by TNF-α. Because of the importance of GDNF for SSC self-renewal and proliferation, the results obtained may give important insights on how cytokine signals modulate the expression of niche-derived factors.

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CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

SDP, DS, CC, RS, and SF involved in data acquisition. SDP, DS, CC, RS, and EV analyzed the data. SDP, DS, AF, and EV interpreted the

data. SDP and EV involved in writing. AF and EV involved in funding acquisition.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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