Synergistic Effect of Interferon-Gamma and Tumor Necrosis Factor-Alpha on Coxsackievirus and Adenovirus Receptor Expression: An Explanation of Cell Sloughing During Testicular Inflammation in Mice

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ABSTRACT

Coxsackievirus and adenovirus receptor (CAR) is a junction molecule that expresses on Sertoli and germ cells. It mediates Sertoli-germ cell adhesion and facilitates migration of preleptotene/leptotene spermatocytes across the blood–tissue barrier, suggesting that CAR-based cell adhesion and migration are crucial for spermatogenesis. Interferon-gamma (IFNG) and tumor necrosis factor alpha (TNF) are two major cytokines that are elevated during testicular inflammation and cause reduced fertility. We investigated the mechanism by which IFNG and TNF exert their disruptive effects on testicular cell adhesion. We have demonstrated that combined treatment with IFNG and TNF (IFNG+TNF) exerts a synergistic effect by downregulating CAR mRNA and protein levels. Immunofluorescence staining revealed that IFNG+TNF treatment effectively removes CAR from the site of cell–cell contact. Using inhibitor and co-immunoprecipitation, we confirmed that IFNG+TNF mediates CAR protein degradation via ubiquitin-proteasome and NFKB pathways. Blockage of ubiquitin-proteasome pathway significantly inhibits CAR degradation, as indicated by the reappearance of CAR at the site of cell–cell contact. Additionally, IFNG+TNF reduces CAR mRNA via transcriptional regulation. Mutational studies have shown that IFNG+TNF-induced CAR repression is achieved by suppression of the basal transcription. Electrophoretic mobility shift assay and chromatin immunoprecipitation assays further confirmed that IFNG+TNF treatment not only inhibits binding of the basal transcription factors but also promotes binding of NFKB subunits and Sp1 (negative regulators) to the CAR promoter region. Taken together, IFNG+TNF treatment significantly downregulates CAR expression, which provides an explanation of how cell sloughing in the epithelium mediates, by loss of CAR-based cell adhesion, during testicular inflammation.

INTRODUCTION

Coxsackievirus and adenovirus receptor (CAR) was originally identified as the receptor for coxsackievirus group B and adenovirus groups 2 and 5 [1]. Recently, CAR has been recognized as a component of tight junction and adherens junction complexes [2, 3]. In testes, CAR is expressed in Sertoli cells and all types of germ cells [4–6] and mediates homophilic and heterophilic interactions with junctional adhesion molecule-C and junctional adhesion molecule-like protein [4, 7]. It is a major structural component for Sertoli-Sertoli and Sertoli-germ cell adhesion [6]. Also, CAR functions as a regulatory protein at the blood–tissue barrier (BTB). Studies have shown that CAR in Sertoli cells reinforces the BTB function, whereas knockdown of CAR causes disruption of the BTB via promoting the endocytosis of occludin [8]. These findings suggest that CAR plays an important role in facilitating the transit of preleptotene/leptotene spermatocytes across the BTB at stage VIII of the epithelial cycle [6, 8, 9]. Taken collectively, CAR is a component and regulator of BTB function and Sertoli-germ cell adhesion.

BTB and Sertoli-germ cell adhesion are crucial for spermatogenesis. Dysregulation could result in premature release of germ cells or retention of mature spermatids in the seminiferous epithelium and lead to male infertility. Numerous studies have revealed that cytokines and hormones are crucial for junctional restructuring in the seminiferous epithelium. For instance, transforming growth factor-beta and testosterone work cooperatively to control the turnover and recycling of junctional proteins at the site of cell–cell contact, resulting in modulation of junctional integrity [10]. In addition, tumor necrosis factor alpha (TNF) could reversibly disrupt the BTB and impair Sertoli-germ cell adhesion in the seminiferous epithelium of rat testes [11]. It is apparent that cytokines exert their effects in spermatogenesis and male fertility. In the normal testes, TNF is produced by pachytene spermatocytes, round spermatids, Sertoli cells, and interstitial macrophages [12, 13]. Low levels of interferon-gamma (IFNG) is expressed by early spermatids [14]. During testicular inflammation, interstitial macrophages express high levels of TNF and IFNG, and lymphocytes also release the membrane-bound form of TNF [15]. Abnormal elevation of IFNG and TNF has been found to be associated with reduced fertility in inflamed testes [15]. In vivo studies have also shown that chronic administration of IFNG reduces male fertility by causing sperm abnormalities, reduction of sperm count and concentration. Severe cell sloughing following aspermatogenesis and tubular atrophy is observed in those mice [15, 16]. However, the underlying mechanism of how IFNG and TNF exert their effects during testicular inflammation remains enigmatic. Because TNF has been known to regulate cell junction dynamics, we hypothesize that IFNG may work synergistically with TNF to cause pronounced disruptive effects on the BTB and Sertoli-germ cell adhesion in the seminiferous epithelium, which results in reduced fertility.

In this study, we report for the first time that IFNG and TNF work synergistically to downregulate CAR via repression of CAR gene transcription and promotion of turnover of CAR.
TABLE 1. Nucleotide sequence of primers used in RT-PCR, plasmid construction, EMSA, and ChiP.

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<th>Primer</th>
<th>Location (nucleotides)</th>
<th>Orientation</th>
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* Italics indicate mutated nucleotides. † MUP, mutagenic universal primer.

protein through ubiquitin-proteasome and NFKB-dependent pathways. Significant reduction of CAR in germ cells after IFNG+TNF treatment could possibly explain the cause of IFNG+TNF-induced infertility during testicular inflammation. It is apparent that, at the BTB and site of Sertoli-germ cell contact, CAR is the target protein in IFNG+TNF-mediated testicular inflammation, which disrupts Sertoli-germ cell adhesion and thus results in reduced fertility.

MATERIALS AND METHODS

Animals

Male C57 mice were obtained from the Laboratory Animal Unit of The University of Hong Kong. The procedures of animal care and handling were in performed accordance with the protocol approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong.

Cell Culture and Treatment

GC-2spd (ts) cells (mouse germ cell line) were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in humidified atmosphere with 5% CO2 in air. In the experiment with cytokines, IFNG and TNF alone or in combination were added to a final concentration of 5 ng/ml and 10 ng/ml, respectively. For post-translational studies, cells were pretreated with cycloheximide (CHX; 5 μg/ml, for 30 min) followed by inhibitors for 30 min to 5 h before the addition of IFNG and TNF.

Seminiferous Tubule Culture

Seminiferous tubules were isolated from testes of adult C57 mice. Decapsulated testes were incubated in 0.5 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) at 35°C for 25 min, and Leydig cells were removed by sedimentation. Isolated seminiferous tubules were trimmed into pieces approximately 3 mm and cultured in F12/DMEM (Invitrogen) supplemented with growth factors, as previously described [17]. Seminiferous tubules were seeded into 12-well plates and incubated at 35°C in humidified atmosphere with 5% CO2 in air.

Antibodies and Reagents

Rabbit anti-CAR (code sc-15405; Western blot [WB], 1:1000 dilution; immunofluorescence [IF], 1:50 dilution), rabbit anti-NFKB p50 (code sc-718; WB 1:1000 dilution), rabbit anti-NFKB p65 (code sc-372; WB 1:1000 dilution), rabbit anti-SP1 (code sc-59X; WB 1:1000 dilution), rabbit anti-SP3 (code sc-644; WB 1:500 dilution), goat anti-KLF4 (code sc-12538; WB 1:1000 dilution), rabbit anti-ubiquitin (code sc-9133; WB 1:1000 dilution), goat anti-HDAC1 (code sc-6298; WB 1:1000 dilution), and goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP; WB 1:10000 dilution) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (IF 1:100 dilution) was from Zymed (South San Francisco, CA). TNF, nystatin, chlorpromazine (CPZ), wortmannin, helenalin (HLN), and lactacytin were purchased from Calbiochem (San Diego, CA). Cycloheximide (CHX) and 5-(N-ethyl-N-isopropyl)amidole (EIPA) were from Sigma-Aldrich. IFNG was obtained from R&D Systems (Minneapolis, MN).

Preparation of CAR Promoter-Luciferase Constructs and Site-Directed Mutagenesis

The 5'-flanking region of the CAR gene was generated by PCR amplification using gene-specific primers (Table 1) and mouse genomic DNA. Progressive 5'-deleted fragments generated by PCR were cloned into the pGL-3 Basic vector (Promega Corp., Madison, WI). Mutant plasmids were generated by 3-step PCR mutagenesis using mutagenic primers (Table 1) as described in previous studies [18, 19]. All plasmids were prepared by Plasmid Midi Kits (Qiagen, Chatsworth, CA) and confirmed by sequencing analysis.

RT-PCR and Quantitative Real-Time PCR

Total RNA was isolated from GC-2spd (ts) cells using TRIzol reagent (Invitrogen), and complementary DNA was synthesized with Moloney murine leukemia virus reverse transcriptase kit (Invitrogen). RT products were used as templates for subsequent PCR, with a pair of CAR and GAPDH primers, respectively (Table 1). Coamplifications of CAR and GAPDH were carried out in their linear phases. Authenticity of the PCR product was confirmed by sequencing analysis. The gel images were captured and analyzed using Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA). For real-time PCR, CAR mRNA levels were analyzed by 7300 real time PCR system (Applied Biosystems, Foster City, CA) with Power SYBR Green Master Mix (Applied Biosystems) according to the manufacturer’s instructions (n = 3, each in triplicate) [19]. GAPDH was used as an internal control for normalization. The specificity of the fluorescence signal was confirmed by both melting curve analysis and agarose gel electrophoresis. The expression level of the target gene was determined by using the 2−ΔΔCt method.

Transfection and Luciferase Reporter Assays

GC-2spd (ts) cells (0.5×10^5 cells/well) were seeded into a 12-well plate 1 day before transfection. Luciferase constructs (0.5 μg) and pEGFP vector (0.1 μg) were cotransfected using Genejuice transfection reagent (Novagen, Madison, WI). Cytokines were added 18 h before harvest. For inhibitor studies, cells were pretreated with HLN for 1 h before the addition of cytokines. Luciferase activity was detected at 48 h post-transfection using a Perkin-Elmer
FIG. 1. Synergistic effects of IFNG and TNF are shown on CAR expression and localization in GC-2spd (ts) cells. Cells treated with vehicle (V) or IFNG (I, 5 ng/ml) TNF (T, 10 ng/ml) for 18 h were subjected to RT-PCR analysis (A), real-time PCR (B), and immunoblotting (C). For RT-PCR and real-time PCR, CAR mRNA levels were normalized with GAPDH. The authenticity of the PCR product was confirmed by sequencing analysis. The synergistic effect of IFNG and TNF was studied by dose-dependent analysis (D). Analysis of CAR mRNA (E–F) and protein (G) levels in cells treated with vehicle and I+T at specified time-points. Immunofluorescence staining was performed in vehicle- and I+T-treated cells (H). Cells were incubated with rabbit anti-CAR antibody, followed by goat anti-
2030 multilabel reader (Perkin-Elmer, Waltham, MA). pEGFP activity was determined by using a CytoFluor multiwell plate reader (PerSeptive Biosystems, Framingham, MA) and used to normalize transfection efficiency.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared as described previously [20]. Oligonucleotides containing the putative Elk-1 or Sp1a motif were synthesized and annealed to form double-stranded DNA. Probes were end-labeled with $\gamma^{32}$P-ATP (Perkin-Elmer) by using T4 kinase (Invitrogen) and separated from unincorporated nucleotides via Microspin G25 columns (GE Healthcare, Buckinghamshire, U.K.). For competition assay, electrophoretic mobility shift assay (EMSA) was performed in 20-$\mu$l reaction mixtures containing 20 mM HEPES (pH 7.6), 50 mM NaCl, 1.5 mM MgCl$_2$, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 $\mu$g of poly(dI:dC), radiolabeled probe (100,000 cpn), and nuclear extract (1–15 $\mu$g). Unlabeled oligonucleotides (100–500$\times$) were added to the reaction as competitors. For supershift assay, EMSA was conducted in 35-$\mu$l reaction mixtures and further incubated with specified antibodies (6 $\mu$g) or serum at room temperature for 30 min before gel electrophoresis. The reaction products were separated on a 5% polyacrylamide gel. Gels were dried and then exposed to x-ray film at −80°C overnight.

**Chromatin Immunoprecipitation (ChIP) Assay**

Chromatin immunoprecipitation (ChIP) assay was performed using EZ-ChIP kit (Millipore Corp., Billerica, MA). GC-2spd (ts) cells were seeded onto a 12-well plate for 4 days. Cells were treated with IFNG and TNF for 18 h before harvest. Lysates were sonicated (Sonifier 450; Branson, Danbury, CT) [21] and precleared with protein G agarose. Pre-cleared lysates were incubated with antibodies (4 $\mu$g) or corresponding serum at 4°C overnight. Protein G agarose with protein/DNA complexes were washed and reverse to free DNA at 65°C for 5 h. DNA was purified using spin columns and analyzed by PCR, using specific primers (Table 1).

**Western Blotting**

Protein extracts from GC-2spd (ts) cells were prepared in IP lysis buffer (50 mM Tris, 0.15 M NaCl, 2 mM EDTA, 2 mM PMSF, 1% NP-40, and 10% glycerol [v/v], pH 7.4 at 22°C). Protein concentration was determined. Equal amount of protein lysate were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat milk powder [v/v] in PBS/Tris/0.1% Tween-20 for 1 h, followed by incubation with primary antibody at 4°C overnight. Membranes were then incubated with corresponding secondary antibody (Santa Cruz Biotechnology) for 1 h. Enhanced chemiluminescence Western blotting detection reagents (GE Healthcare) were used for protein detection.

**Immunoprecipitation**

Approximately 100 $\mu$g of protein extracts from GC-2spd (ts) cells was diluted in IP lysis buffer and incubated with antibody against CAR (0.3 $\mu$g) at 4°C overnight. Protein A/G PLUS-agarose (20 $\mu$L) was added and incubated at 4°C for 4 h. Immunocomplexes were washed with IP lysis buffer. Proteins were extracted in SDS sample buffer and used for immunoblotting.

**Immunofluorescence Microscopy**

GC-2spd (ts) cells were cultured on Matrigel (BD Biosciences, Bedford, MA)-coated glass coverslips for 4 days, cytokines were added 18 h before harvest. For inhibitor studies, cells were pretreated with CHX (5 $\mu$g/ml) for 18 h, followed by lactacystin (10 $\mu$M) for 5 h before addition of cytokines (12 h). Cells were fixed in 4% paraformaldehyde (w/v) in PBS/Tris/0.1% Tween-20 for 1 h, followed by incubation with primary antibody at 4°C overnight. Cells were then incubated with FITC-goat anti-rabbit IgG (1:100 dilution) (Zymed) for 1 h. Cells were washed with PBS and mounted in Vectashield Hardset with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images were obtained using confocal laser scanning microscopy (model LSM 710 NLO; Carl Zeiss, Jena, Germany).

**Data Analysis**

For transfection assays, data were shown as means ± SD of duplicate assays from 3 independent experiments. For EMSA and ChIP assays, experiments were repeated 3 times, and consistent results were obtained. For time-course experiments, statistical analyses were performed using one-way ANOVA with Tukey multiple comparison tests. For all other studies, Student $t$-tests were performed using Prism software (GraphPad Software, Inc., San Diego, CA).

**RESULTS**

**IFNG and TNF Synergistically Downregulate CAR mRNA and Protein Levels in a Time-Dependent Manner**

In normal adult rodent testis, TNF is expressed at a concentration of 0.5 ± 0.15 $\mu$g/testis, but IFNG is barely detected [11, 22]. During testis inflammation, elevated levels of IFNG and TNF were observed with reduced fertility. As previous studies in other epithelial cells have reported that IFNG and TNF work synergistically to disrupt the barrier function. We hypothesized that coexistence of IFNG and TNF during testis inflammation caused reduced male fertility possibly via disruption of the cell–cell interaction/adhesion in testicular cells. Because primary germ cells failed to retain their viability beyond ∼20 h when cultured in vitro in serum-free chemically defined medium and to display low transfection efficiency [23], a germ cell line GC-2spd (ts) cell was used to test this hypothesis. Some of the findings were further validated in seminiferous tubule culture. GC-2spd (ts) cells were treated with IFNG (5 ng/ml) and TNF (10 ng/ml) for 18 h separately or in combination, followed by RT-PCR (Fig. 1A) and real-time PCR (Fig. 1B). Results showed that IFNG alone had no effect on CAR mRNA level, whereas TNF reduced CAR mRNA by 40%. Moreover, combined treatment of IFNG and TNF led to a more significant reduction of CAR mRNA level (60% reduction), suggesting that IFNG exerts synergistic effect with TNF on CAR mRNA reduction (Fig. 1, A and B). Immunoblots also showed that IFNG alone caused no significant change in CAR protein level, whereas TNF slightly reduced CAR expression. When cells were cotreated with IFNG and TNF for 18 h, CAR protein level was significantly decreased by ∼50% (Fig. 1C). Dose-dependent analyses showed that the synergistic effect of IFNG and TNF together was consistently observed when the concentration of IFNG reached 5 ng/ml or above (Fig. 1D). Subsequent time-course studies have shown that the synergistic effect of IFNG with TNF caused reduction of the CAR mRNA and protein levels in a time-dependent manner (Fig. 1, E–G). Taken together, these results suggest that IFNG and TNF work synergistically to reduce CAR mRNA and protein levels in GC-2spd (ts) cells. Because both IFNG and TNF are coelevated during testicular inflammation, it is worth examining their synergistic effects on CAR expression, despite the fact that TNF alone could cause a significant reduction of CAR mRNA and protein levels.

**CAR Disappears at the Site of Cell–Cell Contact Upon Combined IFNG+TNF Treatment**

Immunofluorescence staining was performed to examine the subcellular localization of CAR in vehicle-treated and IFNG+TNF-treated GC-2spd (ts) cells. As shown in Figure 1H (upper panels), CAR protein was localized at the cell.
FIG. 2. Post-translational regulation of CAR protein by IFNG+TNF (I+T) treatment in GC-2spd (ts) cells. CAR protein stability was analyzed by CHX assay (A). Cells were pretreated with CHX (5 μg/ml, 30 min) before vehicle (Veh) or I+T treatment. Western blotting was performed to determine CAR protein level. To determine the potential post-translational pathway, cells were pretreated with CHX, followed by caveolin inhibitor nystatin for 30 min (B), clathrin inhibitor CPZ for 1 h (C), macropinocytosis inhibitor EIPA for 30 min (D), wortmannin for 30 min (E), proteasome inhibitor lactacystin for 5 h (F), NFKB inhibitor HLN for 1 h (H) prior to I+T treatment. CAR protein level was then analyzed by Western Blotting. Immunoprecipitation was performed...
**IFNG+TNF reduces CAR Protein Level via Post-Translational Regulation**

Studies have demonstrated that post-translational regulation plays a crucial role in regulating rapid turnover of junction proteins at the BTB to facilitate germ cell movement [19, 24, 25]. To examine whether post-translational regulation was involved in IFNG+TNF-induced CAR protein reduction, CHX was used to block protein synthesis to examine the turnover of CAR upon IFNG+TNF treatment. Cells were pretreated with CHX prior to IFNG+TNF treatment. It was found that IFNG+TNF treatment was capable of reducing CAR protein level by ~40% compared to vehicle (Fig. 2A), suggesting that IFNG+TNF acts post-translationally on CAR protein expression.

**IFNG+TNF Promotes CAR Protein Turnover via Ubiquitin-Proteasome and NFKB Pathways**

Endocytosis and ubiquitination are the common pathways to remove membrane proteins from cell–cell interface to allow protein turnover. To examine IFNG+TNF-induced CAR protein turnover, inhibitors were used to screen the potential degradation pathways. Nystatin (an inhibitor of caveolin-dependent endocytosis) and CPZ (an inhibitor of clathrin-dependent endocytosis) could not abolish the effect of IFNG+TNF on CAR protein degradation (Fig. 2, B and C), indicating that neither caveolin- nor clathrin-dependent endocytosis is involved in IFNG+TNF-induced CAR degradation. Studies in other epithelia have revealed that IFNG could initiate macropinocytosis of tight junction proteins [26, 27]. To test this possibility, two inhibitors of macropinocytosis, namely EIPA and wortmannin, were used. However, both of them could not cause any rebound in CAR protein level in the presence of IFNG+TNF (Fig. 2, D and E), suggesting that IFNG+TNF-induced CAR protein turnover is not mediated via macropinocytosis.

To assess the involvement of proteasome in CAR degradation, lactacystin (a proteasome inhibitor) was used, and a significant rebound of CAR protein level was observed (Fig. 2F). To further examine whether IFNG+TNF-induced CAR degradation was ubiquitin-dependent protein degradation, cells were pretreated with lactacystin followed by IFNG+TNF treatment, and protein lysates were used to co-immunoprecipitate with anti-CAR antibody. Ubiquitin-conjugated CAR protein level was immunoprecipitated and detected using anti-ubiquitin antibody. An accumulation of ubiquitin-conjugated CAR protein was observed in the presence of IFNG+TNF (Fig. 2, B and C), suggesting that IFNG+TNF-induced CAR protein turnover is mediated via ubiquitination.

In addition to inhibiting proteasomes, lactacystin also inhibited the NFKB pathway. It will be of interest to determine whether NFKB pathway is involved in IFNG+TNF-induced CAR degradation. When cells were pretreated with HLN (a specific inhibitor of the NFKB p65 subunit), IFNG+TNF-induced CAR degradation was blocked (Fig. 2H).

![FIG. 3. Effect of lactacystin on CAR protein level upon IFNG+TNF (I+T) treatment. Cells were pretreated with CHX, followed by DMSO (A–D) or proteasome inhibitor lactacystin (E–H) for 5 h prior to vehicle (A, B, E, F) or I+T stimulation (C, D, G, H) for 12 h. Immunofluorescence staining was performed afterward. Cells were incubated with rabbit anti-CAR antibody, followed by goat anti-rabbit IgG conjugated with FITC. CAR appeared as green fluorescent. Arrows indicate the re-localization of CAR.](Image)
INFLAMMATORY CYTOKINES DOWNREGULATE CAR EXPRESSION

FIG. 4. Effect of IFNG+TNF (I+T) on CAR expression in seminiferous tubule culture. Isolated seminiferous tubules were treated with vehicle (V), IFNG (I, 5 ng/ml) or/and TNF (T, 10 ng/ml) for 18 h were subjected for immunoblotting analysis (A). For post-translational study, seminiferous tubules were pre-treated with CHX (5 μg/ml, 30 min), followed by proteasome inhibitor lactacystin for 5 h prior to I+T treatment (B). Results are expressed as the mean ± SD of three independent experiments. CHX, cycloheximide; ns, not significant versus vehicle control; **P < 0.001 versus vehicle control.

INFLAMMATORY CYTOKINES DOWNREGULATE CAR EXPRESSION

IFNG+TNF Causes CAR Protein Reduction in Cultured Seminiferous Tubules

The characterization of IFNG+TNF-mediated CAR down-regulation was performed in cell line GC-2spd (ts) due to the limitation of experimental manipulation in primary cell culture. We therefore performed some key experiments in cultured seminiferous tubules to validate our findings of CAR protein reduction in GC-2spd (ts) cells. In seminiferous tubule culture, similar reduction of CAR protein was observed upon IFNG+TNF treatment (Fig. 4A). In addition, inhibitor analyses have revealed that CAR protein level in IFNG+TNF-treated group remained as high as the corresponding control in the presence of lactacystin (Fig. 4B). Taken together, data obtained from cell line and seminiferous tubule culture unequivocally suggest that IFNG+TNF downregulate CAR expression via proteasome-mediated protein degradation.

IFNG+TNF Reduces CAR Level via Repression of CAR Gene Transcription, and Elk-1 and Sp1a Motifs Are Involved in IFNG+TNF-Mediated Repression

As shown in Figure 1, A and B, IFNG+TNF treatment not only reduced CAR protein level but also significantly downregulated CAR mRNA level. To investigate whether IFNG+TNF reduces CAR mRNA level through transcriptional regulation, a series of luciferase constructs with progressive deletion of the 5’ flanking region of the CAR gene were generated using the primers listed in Table 1. Transient transfection using various deletion luciferase constructs revealed that IFNG+TNF causes no significant change on CAR promoter activity when cells were transfected with the p (-110/-1)Luc construct (luciferase construct containing CAR promoter region from nucleotide -110 to -1), but IFNG+TNF reduces the promoter activity significantly when cells transfected with the p (-160/-1)Luc construct (Fig. 5A). Similar reductions were observed in cells transfected with two other deletion constructs (p (-237/-1) and p (-866/-1)Luc) in the presence of IFNG+TNF (Fig. 5A). These results suggest that the 5’ flanking region between nucleotides -110 and -160 is responsive to IFNG+TNF treatment and contains cis-acting motifs crucial for IFNG+TNF-mediated CAR gene repression.

Three putative motifs within the sequence between nucleotides -110 and -160, namely Elk-1, Sp1a, and Sp1b, were identified by MatInspector (Genomatix Software Inc., Ann Arbor, MI) and TFSearch (Parallel Application TRC Laboratory, Japan) (Fig. 5B, upper panel). Site-directed mutants were constructed and transfected into GC-2spd (ts) cells followed by IFNG+TNF treatment. Single mutation of the Sp1b motif (p (-160/-1)Sp1b*) caused a reduction in promoter activity that was as significant as that of the wild-type construct (p (-160/-1)Luc) in the presence of IFNG+TNF (Fig. 5A). Both constructs containing Elk-1 and Sp1a (p (-160/-1)Elk-1*), or Sp1a (p (-160/-1)Sp1a*) caused partial reduction of CAR promoter activity under normal conditions (vehicle control) (Fig. 5B, lower panel), suggesting that Elk-1 and Sp1a are involved in basal transcription.

IFNG+TNF treatment, however, could only cause a minimal reduction or an increase in promoter activity in cells transfected with the construct (p (-160/-1)Sp1a* or p (-160/-1)Elk-1*) compared with the vehicle control (Fig. 5B, lower panel), suggesting that IFNG+TNF-mediated CAR repression is partially abolished if Elk-1 or Sp1a motif is absent. The construct having double mutations (Elk-1 and Sp1a, [p (-160/-1)Sp1a*&Elk-1*]) showed no difference in promoter activity between vehicle and IFNG+TNF treatment, seemingly suggesting that Elk-1 and Sp1a motifs are involved in CAR gene repression in response to IFNG+TNF treatment and that these two motifs may functionally cooperate with each other. Due to the fact that Elk-1 and Sp1a are involved in basal transcription, it is highly possible that IFNG+TNF treatment affects the expression or binding affinity of transcription factors that mediate the basal transcription of CAR gene.
FIG. 5. Effect of IFNG+TNF (I+T) on CAR gene transcription. Progressive 5′-deletion analysis of mouse CAR promoter was performed between nt −866 and −1 (A). A series of 5′-deletion constructs and pEGFP vector were co-transfected into GC-2spd (ts) cells followed by I+T treatment. Three putative cis-acting elements including Elk-1, Sp1a and Sp1b motifs are located within the region between nt −160 and −110 (B, upper panel). Site-directed mutagenic constructs containing single or double mutations and pEGFP vector were co-transfected into GC-2spd (ts) cells followed by I+T treatment (B, lower panel). pGL-3 vector, p(−160)/Luc and pEGFP vector were co-transfected with various overexpression vectors encoding different transcription factors for 48 h.
IFNG+TNF Treatment Alters Expression of NFKB and Sp/KLF Proteins to Mediate CAR Gene Repression

Accumulated evidence shows that TNF regulates gene transcription via the NFKB pathway. To test whether the NFKB pathway is involved in IFNG+TNF-mediated CAR gene repression, overexpression analysis of NFKB p50 (NFKB1) and p65 was performed. Transfection of p50 or p65 expression construct with wild-type p (~160/-1)Luc construct caused reduction in CAR promoter activity compared with that of pGL-3 vector (Fig. 5C), which mimics the effect of IFNG+TNF treatment on CAR gene transcription. This suggests that p50 and p65 are the potential negative regulators of IFNG+TNF-mediated CAR gene repression. We next examined whether two NFKB subunits translocate to the nucleus to regulate CAR gene transcription upon IFNG+TNF stimulation. As shown in Fig. 5D, a significant increase in nuclear p65 level, but not p50 subunit, was observed upon IFNG+TNF stimulation, suggesting that p65 subunit is the major negative regulator of IFNG+TNF-mediated CAR gene repression. To test this, an inhibitor study was performed. Results showed that IFNG+TNF significantly reduced CAR promoter activity, whereas HLN (a specific inhibitor of p65 subunit) successfully blocked the effect of IFNG+TNF in a dose-dependent manner (Fig. 5E), suggesting that p65 subunit plays an indispensable role in regulating CAR gene repression upon IFNG+TNF treatment.

In addition, mutation studies (Fig. 5B) revealed that Sp1a motif was involved in IFNG+TNF-mediated CAR gene repression; overexpression analysis was conducted to find out if Sp/KLF family proteins were involved. As shown in Fig. 5F, cotransfection of Sp1 expression construct with p (~160/-1)Luc significantly decreased CAR promoter activity, whereas overexpression of Sp3 and KLF4 remarkably increased CAR promoter activity. Apparently, members of Sp/KLF family exert opposite effects on CAR gene transcription. We therefore checked if IFNG+TNF alters the levels of these transcription factors in the nucleus so as to regulate CAR gene transcription. In fact, IFNG+TNF treatment reduced the level of nuclear Sp3 (78- to 80-kDa isofrom), whereas Sp1, Sp3 (110-kDa isofrom), and KLF4 levels in the nucleus remained unchanged (Fig. 5G). Taken collectively, significant increase in p65 subunit (a negative regulator) and reduction in Sp3 (78- to 80-kDa isofrom, a positive regulator) were observed upon IFNG+TNF treatment, and the change of components of transcription factors may contribute to CAR gene repression.

IFNG+TNF Causes CAR Gene Repression by Recruiting p50/p65 and Sp1 (Negative Regulators) to Elk-1 and Sp1 Motifs and Inhibiting Binding of Basal Transcription Complex Bound to Sp1a Motif

It is apparent that IFNG+TNF could alter the protein levels of p65 and Sp3 in the nucleus; it remains unknown whether these transcription factors are recruited and bound onto the Elk-1 and/or Sp1a motif to alter CAR gene transcription. EMSA and ChIP assay were used to determine the interaction between these transcription factors and cis-acting motifs in both vehicle- and IFNG+TNF-treated sample. EMSAs showed that DNA-protein complexes (complexes A–C) were formed in a dose-dependent manner when radiolabeled probes containing either the Elk-1 or Sp1a motif interacted with nuclear extracts. Formation of the complexes was inhibited dose-dependently by the addition of cold competitors (Fig. 6A and D). As shown in Figures 6B, antibody super-shift assays indicated that IFNG+TNF treatment promoted p50 and p65 binding onto Elk-1 motif as antibodies against p50 and p65 significantly diminished the complex formation in the presence of IFNG+TNF compared with vehicle (Fig. 6B, lanes 4 and 6 vs. lanes 3 and 5), indicating that p50 and p65 are recruited to Elk-1 motif upon IFNG+TNF stimulation but not under normal conditions. For the Sp/KLF family, Sp3 and KLF4, but not Sp1, were the components of complex A in the normal conditions (vehicle control) as incubation of nuclear extracts with antibodies against Sp3 and KLF4 abolished the DNA-protein interactions (Fig. 6C, lanes 5 and 7 vs. lane 1), suggesting that Sp3 and KLF4 are the transcription factors involved in basal gene transcription. In the presence of IFNG+TNF, Sp3 and KLF4 remain bound to Elk-1 motif (Fig. 6C, lanes 6 and 8 vs. lane 2). Taken together, IFNG+TNF alters the components of DNA-protein complex by promoting the binding of two negative regulators, p50 and p65, onto the Elk-1 motif.

Like the Elk-1 motif, Sp1a motif is critical for the basal transcription of the CAR gene. When IFNG+TNF-treated nuclear extracts were used, we observed that the formation of complexes B and C was significantly decreased (Fig. 6E), suggesting that IFNG+TNF may affect the binding of transcription factors that regulates the basal transcription. As shown in Fig. 6F, antibody supershift assays revealed that p50, p65, Sp3, and KLF4 were the components of complexes B and C when vehicle-treated nuclear extracts were used. In addition to these transcription factors, Sp1 was found to bind effectively to Sp1a motif upon IFNG+TNF treatment (Fig. 6G, lanes 5 vs. Fig. 6F, lane 5). These results suggest that IFNG+TNF induces the binding of a negative regulator, Sp1, to the basal transcription protein complex and alters the gene transcription.

ChIP assays have also revealed that IFNG+TNF promoted the binding of p50, p65, and Sp1 onto CAR promoter, whereas the interaction between the positive regulators Sp3 and KLF4 remained unchanged or were slightly disrupted (Fig. 6H). Taken together, IFNG+TNF-mediated CAR gene repression is mediated via promoting the binding of p50/p65 and Sp1 onto Elk-1 and Sp1a motif, respectively.

DISCUSSION

During spermatogenesis, preleptotene/leptotene spermatocytes must traverse the BTB from the basal to the adluminal compartment for further development at stage VIII of epithelial cycle. At the same stage, mature spermatooza are timely released to the tubule lumen at spermiation. These two important events require extensive junction restructuring at the BTB and apical ectoplasmic specializations, respectively. As a structural block, CAR not only mediates Sertoli-Sertoli cell interaction homotypically [6] but is also involved in Sertoli-germ cell interaction in homotypical and heterotypical manner [4, 6]. In addition, CAR facilitates the transit of preleptotene/leptotene spermatocytes across the BTB at stage

(C, F). Western blotting analysis of the levels of NFKB subunits and Sp/KLF transcription factors in nuclear extract prepared from cells treated with vehicle or I+T-/D. G). Cells were transfected with pGL-3 or p (~160/-1)Luc construct, followed by HLN pretreatment prior to I+T treatment (E). pEGFP activity was used to normalize transection efficiency. Results are expressed as the mean ± SD of three independent experiments. ns, not significant versus vehicle control or pGL-3; *P < 0.01 vehicle control or pGL-3; **P < 0.001 versus vehicle control pGL-3.
FIG. 6. EMSA of Elk-1 and Sp1a motifs and ChIP assay. Dose-dependent and competition assay of Elk-1 motif (A) and Sp1a motif (D) were performed using nuclear extract from vehicle-treated cells. Double stranded oligonucleotides containing the respective motif were radiolabeled with $\gamma$-32P ATP and incubated with nuclear extract (1–15 μg) alone or in the presence of cold competitors (100- to 500-fold excess). Labeled probes were incubated with nuclear extract of vehicle- or 1+T-treated cells in the presence/absence of specified antibodies or serum (B, C, E, F, G). A schematic drawing illustrating the relative location of Elk-1 and Sp1a cis-acting motifs in the CAR promoter and the primers for chromatin immunoprecipitation assay (H, upper panel).
VIII of the epithelial cycle [6, 8, 9]. Apparently, CAR expressed in germ cells is crucial for cell adhesion and migration. TNF is a well-known cytokine that reversibly regulates Sertoli-Sertoli and Sertoli-germ cell junction dynamics in vivo [11] to facilitate the passage of germ cells along the epithelium for development. However, elevated levels of TNF and IFNG in the testis have been reported during testicular inflammation, which is highly associated with male infertility [15, 16]. In this study, we have unraveled the molecular mechanisms of how these inflammatory cytokines work synergistically to terminate CAR gene transcription and promote protein turnover. Apparently, downregulation of CAR is an adverse effect that elevated IFNG+TNF exerts on germ cell adhesion during testicular inflammation and results in reduced fertility.

Previous studies have shown that TNF reduces CAR expression reversibly in cultured Sertoli cells [6]. Our current study also demonstrated that TNF alone causes reduction of CAR mRNA and protein levels in germ cells, suggest that TNF secreted by Sertoli and germ cells exerts negative regulatory effects on CAR expression in these two cell types via autocrine or paracrine regulations, thus regulating cell junction restructuring during spermatogenesis. More importantly, our current studies have identified that IFNG itself, a cytokine that is only expressed in the testis during inflammation [22], has no effect on CAR expression. However, it could work with TNF, which mimics the condition of testis inflammation, causing a more prominent reduction of CAR level. It is possible that IFNG might induce an upregulation of TNF receptor or enhance the susceptibility of TNF [28], thus resulting in the synergistic effect on CAR reduction.

In our studies, IFNG+TNF treatment caused a significant reduction of CAR protein level in the presence of CHX, suggesting that these two cytokines work in concert to downregulate CAR expression, in part by post-translational regulation. Effective removal of pre-existing CAR at the germ cell surface by IFNG+TNF provides an explanation for severe germ cell sloughing from the seminiferous epithelium during testicular inflammation as Sertoli-germ cell interaction is disrupted. Previous studies in other epithelia have shown IFNG and TNF separately could trigger the turnover of various junction proteins such as JAM-A and occludin via different endocytic pathways [25, 26]. To our surprise, neither endocytosis (clathrin- or caveolin-dependent) nor macropinoscyosis are involved in IFNG+TNF-induced CAR protein degradation in germ cells. Using proteasome inhibitor coupled with Western blotting or immunofluorescence staining, we found that IFNG+TNF promotes the degradation of CAR protein appeared on germ cell via ubiquitin-proteasome pathway. Co-immunoprecipitation studies further confirmed that an accumulation of ubiquitin-conjugated CAR protein was observed in the presence of proteasome inhibitor upon IFNG+TNF stimulation. Taken together, it unequivocally demonstrates the involvement of ubiquitin and proteasome in IFNG+TNF-induced CAR protein degradation. The ubiquitin-proteasome degradation pathway triggered by IFNG+TNF, but not by individual cytokines, provides new information on how inflammatory cytokines expressed in inflamed testis work together to exert a regulatory mechanism that is different from normal testis in regulating cell junction restructuring.

In addition to post-translational regulation, IFNG+TNF could downregulate CAR level via transcriptional regulation. Two motifs including Elk-1 and Sp1a are found to be involved in the basal transcription of CAR and responsive to IFNG+TNF treatment. To mediate the basal transcription of CAR gene, protein complexes containing p50, p65, Sp3, and KLF4 are bound to Elk-1 and Sp1a motifs under normal conditions. Upon IFNG+TNF stimulation, we observed a change in the ratio of transcription factor components that bind to Elk-1 and Sp1a motifs. For the Elk-1 motif, both EMSAs and ChIP assays showed that IFNG+TNF promotes NFκB p50 and p65 subunits bound to the Elk-1 motif compared to vehicle control group. In the nuclear extract analysis, we also observed that a significant increase in p65 level in nucleus upon IFNG+TNF treatment (Fig. 5D). IFNG+TNF may promote the translocation of p65 to nucleus and p65 binding to the CAR promoter. It is possible that p65 subunit is the major negative regulator of IFNG+TNF-induced CAR repression, as overexpression of p65 could cause a more significant reduction in CAR promoter activity when compared to that of p50 (Fig. 5C) and the inhibitor studies also showed that blockage of p65 function abolished IFNG+TNF-induced CAR gene repression (Fig. 5E).

For Sp1a motif, IFNG+TNF treatment partially inhibited the formation of complexes B and C, suggesting that transcription factors involved in the basal gene transcription could not bind effectively to drive gene transcription upon IFNG+TNF treatment. Also, it is apparent that another negative regulator, Sp1, bound more effectively on Sp1a motif upon IFNG+TNF+TNF treatment when compared to vehicle control. According to the overexpression study, like p50 and p65, Sp1 itself could cause a reduction on CAR promoter activity. In short, IFNG+TNF represses CAR transcription by inhibiting the binding of basal transcription complex to Sp1a motif, inducing an increase in nuclear p65 level, and promoting the binding of negative regulators (p50, p65, and Sp1) onto the CAR promoter.

In summary, we unraveled two mechanisms by which IFNG+TNF exerts synergistic effect on CAR expression that is important for Sertoli-germ cell interaction in the testis. IFNG+TNF promotes CAR protein degradation via ubiquitin-proteasome pathway and represses CAR gene transcription through regulating the binding of NFκB subunits and Sp1/KLF4 proteins to the CAR promoter. This study has provided new information on how inflammatory cytokines work in concert to disrupt cell–cell interaction in the testis during testis inflammation.

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