

A long noncoding RNA signature for ulcerative colitis identifies IFNG-AS1 as an enhancer of inflammation

A long noncoding RNA signature in Ulcerative Colitis

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Disclosures: No conflicts of interest, financial or otherwise, are declared by the author(s).

Running title: IFNG-AS1 lncRNA regulates inflammation

Author Contributions: D.M.P., C. Polytaichou, and D.I. conception and design of research; D.M.P., I.K.M.L., J.P.V., and D.Q.S. performed experiments; D.M.P., S.M.-J., and I.K.M.L. analyzed data; D.M.P. interpreted results of experiments; D.M.P. prepared figures; D.M.P. drafted manuscript; D.M.P., J.R.P., D.Q.S., D.I., and C. Pothoulakis edited and revised manuscript; D.I. and C. Pothoulakis approved final version of manuscript.

Funding: Supported by NIH/NIDDK T32 AM 41301 grant (DP), and R01 DK060729 (CP). Support was also provided by NIH/NIDDL P30 DK 41301 Center Grant (DI and CP).

New and Noteworthy: Long noncoding RNA (lncRNA) biology represents an exciting new field with implications in gene regulation. This field has only recently been investigated in the pathogenesis of ulcerative colitis. By utilizing high-throughput technologies on clinical samples, a lncRNA signature was generated and the lncRNA IFNG-AS1 was shown to regulate the important inflammatory cytokine interferon- γ . This work represents one of the first mechanistic studies of lncRNA biology in inflammatory bowel disease.

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Abstract:

High-throughput technologies revealed new categories of genes, including the long noncoding RNAs (lncRNAs), involved in the pathogenesis of human disease; however, the role of lncRNAs in the ulcerative colitis (UC) has not been evaluated. Gene expression profiling was used to develop lncRNA signatures in UC samples. Jurkat T cells were activated by PMA/ionomycin subsequently interferon- γ (IFN γ) and tumor necrosis factor (TNF)- α protein levels were assessed by ELISA. Antisense molecules were designed to block IFN γ -AS1 expression. A unique set of lncRNAs was differentially expressed between UC and control samples. Of these, IFN γ -AS1 was among the highest statistically significant lncRNAs (fold change: 5.27, P value: 7.07E-06). Bioinformatic analysis showed that IFN γ -AS1 was associated with the IBD susceptibility loci SNP rs7134599 and its genomic location is adjacent to the inflammatory cytokine IFN γ . In mouse models of colitis, active colitis samples had increased colonic expression of this lncRNA. Utilizing the Jurkat T cell model, we found IFN γ -AS1 to positively regulate IFN γ expression. Novel lncRNA signatures differentiate UC patients with active disease, patients in remission, and control subjects. A subset of these lncRNAs was found to be associated with the clinically validated IBD susceptibility loci. IFN γ -AS1 was one of these differentially expressed lncRNAs in UC patients and found to regulate the key inflammatory cytokine, IFN γ , in CD4 T cells. Taking these findings together, our study revealed novel lncRNA signatures deregulated in UC and identified IFN γ -AS1 as a novel regulator of IFN γ inflammatory responses, suggesting the potential importance of noncoding RNA mechanisms on regulation of inflammatory bowel disease-related inflammatory responses.

Keywords: inflammatory bowel disease; ulcerative colitis; lncRNA; inflammation; Interferon- γ

Introduction

INFLAMMATORY BOWEL DISEASES (IBDs) are common gastrointestinal disorders that are clinically comprised of ulcerative colitis (UC) and Crohn's disease (CD) (14, 24). These diseases have a devastating toll on a patient's morbidity and quality of life (27). The etiology of IBD, however, still remains elusive. Studies have shown that IBD may result from an inappropriate inflammatory response to intestinal microbes and foreign antigens in genetically susceptible hosts (8, 40). Key mediators of this inflammatory process such as TNF- α , IL-23, IL-12, and interferon- γ (IFNG) have been implicated in the pathogenesis of UC (1, 6, 10, 20, 25, 26). Therapies directed against these cytokines have been relatively successful in treating UC symptoms; however, a subpopulation of patients may fail to respond to these treatments (32, 33, 35). In addition to these cytokines, researchers have also focused on the genetic underpinnings of the disease. Familial clustering and twin studies have shown a role for genetic factors for UC (3, 14). Indeed, meta-analyses of multiple genome-wide association studies have implicated 163 genetic loci in both CD and UC pathogenesis (14). However, the mechanisms of action for many of the genes in close association with these loci have yet to be determined.

The IBD susceptibility loci point to key genomic positions and highlight areas of the genome for further mechanistic studies. In addition to the associated protein-coding transcripts previously reported, noncoding transcripts may be associated with these loci. Recently, investigators have begun to address the noncoding transcripts and their role in disease pathogenesis. One such category of noncoding transcript are the long noncoding RNAs (lncRNAs) (21). The central dogma, wherein "DNA makes RNA makes protein," has long relegated RNA as an intermediary between gene and protein. Recent research has challenged the notion that the noncoding RNAs are innocent bystanders and may indeed be critical to gene regulation (30, 31). Long noncoding RNAs are defined as sequences of greater than 200 nucleotides in length. They can express introns and exons, which can be alternatively spliced and generally lack open reading frames for protein translation (29). lncRNAs appear to have a diverse set of functions in chromatin remodeling, telomere activity, and subcellular structural organization (4). They can mediate epigenetic changes by recruiting chromatin-remodeling complexes to specific loci in the genome. As an example, the lncRNA HOTAIR has been implicated in inducing a repressive chromatin state by recruiting the Polycomb chromatin remodeling complex PRC2 (12). In addition, lncRNAs can regulate transcription by altering RNA polymerase II activity and thereby influence promoter activation (30). Finally, lncRNAs have also been implicated in various steps in the posttranscriptional processing of messenger RNAs, including splicing, editing, transport, translation, and degradation (21).

Based on these above findings, it has been suggested that noncoding RNAs play a role in the pathogenesis of UC (15, 16, 28). In addition, lncRNAs have been shown to participate in multiple

inflammatory processes and contribute to the pathogenesis of autoimmune and immune-related disorders; however, their role in UC has yet to be fully determined (2, 13, 18, 23). To this end, we have profiled the expression of both coding and noncoding transcripts in UC patients. By comparing their expression to those of control patients, we have identified a set of differentially expressed lncRNAs in adult UC patients. To evaluate the relationship of this set of lncRNAs with known IBD genomic alterations, we compared the chromosomal location of the lncRNAs with those of the IBD susceptibility loci. The lncRNA IFNG-AS1 was associated with the IBD susceptibility loci SNP rs7134599 and is in close proximity to the inflammatory cytokine IFNG. It was found to be among the most statistically significant differentially expressed lncRNAs. Given the close proximity to IFNG within the human genome, we investigated its cellular mechanism of action and found a positive regulatory mechanism between IFNG-AS1 and IFNG expression.

Materials and Methods

Human tissue collection and RNA extraction

Colonic tissues were obtained from two sources. For the microarray analysis, RNA extracted from colonic surgical samples were obtained from Origene. A total of 50 anonymized samples, 19 for the initial profiling experiment and 31 for the validation cohort, were included in this study. For the profiling experiment, 7 control patients, 8 UC-active (UCA), and 4 UC-inactive (UCI) were collected. Diagnoses were confirmed by an independent set of pathologists associated with Origene. The samples were obtained through institutional review board (IRB) protocols and with documented patient consent, all from accredited U.S.-based medical institutions (www.origene.com). A second set of colonic samples was obtained from colonic surgeries performed at Cedars-Sinai Medical Center, Los Angeles, CA. These samples were collected under an IRB-approved protocol. Patients that participated in the IRB tissue recruitment were undergoing colonic resections as part of their regular medical care. A total of 31 anonymized patient samples were included in this part of the study, 16 control patients and 15 UC patients. A segment of the colon resection was flash frozen in liquid nitrogen and stored at -80°C. The diagnoses were confirmed on histopathology. Available patient information is summarized in Table 1.

RNA extraction

Total RNA was extracted from frozen colonic tissues and cells in culture using TRIzol (Qiagen, Valencia, CA; cat. no. 79306) and miRNeasy Mini Kit (Qiagen; cat. no. 217004) following the product instructions and utilizing DNase I digestion (cat. no. 79254). RNA was stored at -80°C and measured with a Nano-Drop 1000 Spectrophotometer (Thermo Scientific). The integrity of the RNA

submitted for microarray analysis was assessed with either the Agilent Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies) or gel analysis.

Microarray analysis for coding and non-coding transcripts

For microarray analysis, the RNA samples were submitted to Arraystar for coding and noncoding RNA expression profiling. An Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar). The labeled cRNAs were hybridized onto the Human LncRNA Array v3.0 (8 X 60 K, Arraystar). After slides were washed, the arrays were scanned by the Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that at least 19 samples have flags in Present or Marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs were identified using Student's t-test between two groups with a fold change cut-off >2 and $P < 0.05$. Pathway analysis and GO analysis were applied to determine the roles of these differentially expressed mRNAs played in these biological pathways or GO terms. Finally, Hierarchical Clustering was performed to show the distinguishable lncRNAs and mRNAs expression patterns among samples.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (9) and are accessible through GEO Series accession number GSE77013.

Quantitative-Real-time PCR for lncRNA and mRNA

To analyze human gene expression, 500 ng of RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad; cat. no. 1708891). Real-time PCR was performed in triplicate using the SYBR Green mastermix (Bio-Rad; cat. no. 203450). qPCR primers were designed for the reference genes ACTIN and GAPDH, as well as IFNG-AS1, and IFNG. The SYBR-Green primers were ordered from Integrated DNA Technologies. The sequences are *ACTIN-F*: 5'-cccagcacaatgaagatcaa-3'; *ACTIN-R*: 5'-acatctgctggaaggtggac-3'; *GAPDH-F*: 5'-

atgttcgtcatgggtgtgaa-3'; *GAPDH-R*: 5'-ggtgctaagcagttggtgt-3'; *IFNG-AS1-F*: 5'-tgcttagtgctctctgatg-3'; *IFNG-AS1-R*: 5'-ggcacaacccatggaagac-3'; *IFNG-F*: 5'-gagtgtggagaccatcaagga-3'; *IFNG-R*: 5'-gtattgcttgcgttgaca-3'. The qPCRs were performed in a CFX384 Real Time PCR detection system (Bio-Rad). For gene expression of mouse genes 18S and Tmevpg1, TaqMan master mix (Applied Biosystems; cat. no. 4352042) was used and primers were ordered from Applied Biosystems (18S:Mm03928990_g1; Tmevpg1: Mm01161206_m1).

Cell culture

Jurkat CD4 T cells were generously provided by Dr. Otto Yang's laboratory at UCLA. The cells were grown in RPMI+10%FBS+1% penicillin+1% streptomycin. Experiments were performed in 24-well plates with 1 million cells plated in 1 ml of media. Activation of T cells was achieved by 6 h incubation at 37°C with phorbol-12-myristate-13-acetate (PMA) at a final concentration of 50 ng/ml and ionomycin at 1 µM concentration (PMA: Sigma-Aldrich; cat. no. P8139. Ionomycin: Sigma-Aldrich; cat. no. I3909).

Peripheral mononuclear blood cells were obtained from blood draws at Cedars-Sinai Medical Center, Los Angeles, CA. The samples were obtained from control patients under the IRB no. 23705. CD4 cells were isolated using the EasySep Human CD4+ T cell enrichment kit (Stemcell; cat. no. 19052).

Knockdown and overexpression of lncRNA IFNG-AS1

Reductions in *IFNG-AS1* lncRNA expression were achieved using two custom-made siRNA constructs: siIFNG-AS1#1 sense, caaucuaaggauacagaatt; siIFNG-AS1#2 sense, caaugcauuuuuuuucutt (Ambion). siRNA control was transfected for comparison (Ambion; cat. no. AM4613). Transfections were performed using Lipofectamine RNAiMax (Invitrogen; cat. no. 13778-075) following the product instructions. Briefly, 5 µl of a 20 µM concentration of siRNA was mixed with 7.5 µl of Lipofectamine RNAiMax in 200 µl of Optimem media (Thermo Scientific; cat. no. 31985-062). The sample was incubated for 15 min and then added to 1 million Jurkat cells for 24 h. Overexpression of the lncRNA was achieved by transfecting 4 µg of pCMV6-AC-IRES expression vector with *IFNG-AS1* (Blue Heron). Control cells were transfected with the expression vector alone without insert.

Protein analysis

IFNG protein production was quantified using the Quantikine ELISA (R&D Systems; cat. no. DIF50). TNF- α protein production was assayed using the Milliplex Human High Sensitivity T cell Magnetic Bed Panel (Millipore; cat. no. HSTCMAG28SPMX13). Jurkat cells were grown in 24-well plates in 1 ml of volume. The cells were activated with PMA/ionomycin for 6 h and medium was collected 24 h after activation. The medium was centrifuged to remove excess cells. Manufacturer's instructions were followed for quantification of protein using both kits. The quantity of protein expression was normalized to cell numbers at the time of collection.

Mouse models of colitis

Colitis was induced in mice via trinitrobenzenesulfonic acid (TNBS) treatment with modifications of previously described protocols (17). All animal studies were approved by the IACUC. Total RNA from the colonic tissues of the TNBS model was purified by use of the miReasy Mini Kit (Qiagen). Tissue sections were scored for histopathology analyses in a double-blinded manner, as previously reported (17). Animals were maintained at University of California-Los Angeles animal research facility and received standard pelleted chow and water ad libitum. The 6- to 8-wk-old male C57BL/6J mice ($n = 8$ per group) received a 50 μ l intracolonic injection of 40 mg/kg TNBS (Fluka) in 30% ethanol, using a 1-ml syringe (Becton Dickinson) fitted with a polyethylene cannula (Intramedic PE-20 tubing). The control groups were injected with 50 μ l of 30% ethanol alone into the colon. The mice were held head down for 1 min after the enema administration to ensure the containment of the TNBS solution into the colon. The colons were fixed in 10% buffered formalin for histological analysis. Paraffin sections were stained with hematoxylin/eosin.

IL10 $^{-/-}$ C57BL/6 male ($n = 8$) and female ($n = 8$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were kept under specific pathogen-free conditions and fed a standard diet and water ad libitum, under the approval by the institutional animal care and use committee (IACUC) at the Department of Veteran Affairs. Mice were weighed weekly and monitored for appearance of diarrhea and blood in the stools. Mice that developed severe rectal prolapse that was evident at all times were euthanized. Control mice were of the same background and euthanized at the same age. After euthanasia, the entire colon was excised and a portion of the proximal and distal colon was fixed in 10% buffered formalin for histological analysis. Paraffin sections were stained with hematoxylin/eosin.

Experimental statistical analysis

The Student's *t*-test for two-group comparisons was performed to determine any statistically significant differences between the experimental groups. $P < 0.05$ was considered statistically significant. All results are expressed as means \pm standard error on the mean.

Results

lncRNA signatures in UC colonic tissues.

Clinical samples from a total of 19 patients consisting of colon resections from 8 UCA, 4 UCI, and 7 control patients (Table 1), were used to perform lncRNA expression profiling analysis. The lncRNA expression profiling was performed using the Arraystar lncRNA microarray V3.0, which consists of ~30,600 lncRNAs. This analysis (>2 -fold and $P < 0.05$) revealed that there were 1,931 differentially expressed lncRNAs between UCA and control, while 1,361 lncRNAs were differentially expressed between UCA and UCI colonic tissues (Figs. 1A and 2). Furthermore, 287 lncRNAs were found to be differentially expressed in UCI relative to control tissues (Fig. 2).

A focus was placed on the comparison between UCA vs. control samples due to the robust statistical differences seen between these groups as well as access to a separate cohort of UC and control samples for validation studies. As a representative sample of these lncRNAs, Fig. 1, B and C demonstrates the top 15 most statistically significant upregulated and 15 downregulated lncRNAs differentially expressed between the UCA and control samples. As shown, the expression of these 30 lncRNAs enables the clustering of the UCA samples away from the control samples, indicating that these IBD samples express a unique lncRNA expression signature that is distinct from control samples. Figure 1C identifies the 30 differentially expressed lncRNAs and shows the range of expression changes from over 20-fold upregulated to over 20-fold downregulated between the UCA and control patients. These results demonstrate that lncRNAs are significantly differentially expressed between UCA samples and controls and that the identity of the majority of these differentially expressed lncRNAs is unique between these comparisons. Taken together, these analyses show novel lncRNA signatures that were deregulated between UCA, UCI, and control colonic tissues.

Differentially expressed lncRNAs are associated with known IBD loci.

The list of lncRNAs differentially expressed in UCA samples compared with control samples spans the human genome. Previous research has identified 163 distinct single nucleotide polymorphism (SNP) loci as IBD susceptibility loci (14). A comparison of the chromosomal locations was performed between the known IBD susceptibility loci and a list of lncRNAs differentially expressed in IBD samples. A list of lncRNAs was generated to compare the common differentially expressed lncRNAs between the UCA samples and the controls as well as the UCA and the inactive UC

samples. This comparison was performed to assess for lncRNAs that were associated with active inflammation in UC samples. The analysis revealed that, of the 767 lncRNAs evaluated, only 31 lncRNAs were found to be within 250 kilobases from the IBD loci (Fig. 3). The 250-kb distance was selected based on the analysis performed by Jostins et al. (14) where a locus was defined as at 500-kb region with the SNP at its center. This comparison demonstrates that a portion of the lncRNAs identified as differentially expressed transcripts between IBD samples and control samples are closely associated with clinically validated IBD susceptibility loci.

Validating lncRNA expression in a second cohort of UC patient samples.

The lncRNA expression differences seen on the microarray analysis showed robust expression changes between the comparison groups. To validate these findings, a separate second cohort of UC samples was assayed for lncRNA expression. The samples consisted of 15 UCA samples and 16 control colonic biopsy samples (Table 2). Total RNA from these samples was extracted and the level of expression for a select number of lncRNAs was performed using quantitative real-time polymerase chain reaction (qRT-PCR). We chose to validate the lncRNAs that were associated with the IBD susceptibility loci and were among the most statistically significant differentially expressed lncRNAs (Fig. 3). The lncRNAs *RP11-81H14.2* and *IFNG-AS1* were selected for validation (Fig. 4, A and B). *RP11-81H14.2* expression analysis did not reveal a statistically significant difference between UC and control samples (Fig. 4D). *IFNG-AS1*, however, was found to be statistically different between the two sample cohorts and was chosen as a lncRNA of interest for further analysis (Fig. 4C).

lncRNA-associated gene pathway analysis.

To understand how changes in lncRNA expression may affect phenotypic changes in UC, an analysis of the changes in coding transcript expression was performed. It was hypothesized that changes in long noncoding RNA expression may affect coding transcript expression and as a result alter distinct gene pathways. To address this, the coding transcripts of each of the UC samples and control samples were assayed using the microarray platform from Arraystar. Nineteen patient samples were then divided into two groups: those expressing relatively high quantities of *IFNG-AS1* and those expressing relatively low amounts *IFNG-AS1* as determined by their expression level on the microarray analysis (Fig. 5). In dividing the clinical samples into these two groups, the coding transcripts could then be assessed for differences in expression between the two groups. A threshold of a fold change of 1.5 upregulated and 2 downregulated was used to generate the coding transcript list. A total of 426 coding transcripts were differentially expressed using these cutoffs. By averaging the expression of the differentially expressed coding transcripts, a gene signature

associated with relative *IFNG-AS1* expression could be generated. Taking this list of differentially expressed coding transcripts, a bioinformatics gene pathway analysis was performed using Ingenuity Pathway Analysis software. Based on this analysis, several upstream regulators were shown to be activated in the *IFNG-AS1* high samples including IFNG, IL1, IL6, and TNF- α (Fig. 5). Of particular interest, was the enhancement of IFNG signaling in the group that expresses higher levels of *IFNG-AS1* as this had the lowest associated *P* value (Fig. 5).

IFNG-AS1 expression in CD4 T cells.

The lncRNA *IFNGAS1* has previously been implicated in immune cell biology in both mouse and human studies (11, 39). In an attempt to identify a target cell for our selected lncRNA, we first examined peripheral CD4 T cells isolated from control patients. The expression of *IFNG-AS1* was compared in the total peripheral mononuclear cells with the selected CD4 T subpopulation. Figure 6A shows qRT-PCR data showing that CD4 T subpopulations have enriched expression of *IFNG-AS1* consistent with recently published data that *IFNG-AS1* is expressed in the Th1 immune cells (39).

Given these results, we chose to study the function of *IFNG-AS1* using the Jurkat immune T cell model. Jurkat CD4 T cells can be activated via PMA and ionomycin stimulation and can be induced to secrete a variety of immune mediators such as *IFNG* and *TNF- α* (Fig. 6, B and C). Analyzing the RNA expression, PMA/ionomycin was found to induce *IFNG-AS1* as well as *IFNG* and *TNF- α* (Fig. 6D). Figure 7 highlights the time course of *IFNG-AS1* and *IFNG* expression with *IFNG-AS1* reaching maximal induction 24 h after stimulation with PMA/ionomycin.

IFNG-AS1 regulates IFNG expression at the RNA and protein level.

Previous studies in mouse immune cells have suggested that *lfn-g-as1* can directly regulate the expression of its genomic neighbor, *lfn-g* (11, 36). These studies reveal a pattern of histone methyltransferase recruitment to the *lfn-g* promoter resulting in positive regulation of *lfn-g* expression. We sought to show that the human ortholog, *IFNG-AS1*, could regulate *IFNG* production in a similar positive relationship. Using two siRNA constructs, we transiently transfected Jurkat T cells and assessed the expression of *IFNG-AS1* and *IFNG* in the context of T cell activation via PMA/ionomycin. The induction of *IFNG-AS1* was abrogated in both siRNAs by ~30-40% and resulted in reduced expression of IFNG RNA (Fig. 8, A and B).

Protein expression was then analyzed with an IFNG-specific ELISA kit. IFNG protein levels were similarly abrogated when *IFNG-AS1* expression was reduced (Fig. 8, C and D).

In addition to the knockdown studies, we generated an IFNG-AS1 overexpression construct and transiently transfected it to Jurkat T cells. Figure 9A demonstrates the level of overexpression of IFNG-AS1 achieved with this construct. When transfected into cells and induced with PMA/ionomycin, there was an increase in IFNG RNA levels as early as 4 h of PMA/ionomycin induction (Fig. 9B). Under these conditions, protein expression of IFNG was increased by an average of 45% (Fig. 9C). These data suggest that IFNG-AS1 has a direct relationship with the expression of IFNG within immune cells.

*Mouse models of colitis have increased *lfng-as1* levels.*

We then assessed whether colitis models would show a similar increase in the expression of the mouse ortholog *lfng-as1*. We utilized two mouse models of colitis, the acute TNBS colitis model at 48 h post-TNBS administration as well as the IL10 knockout mouse model of spontaneous colitis at age 12 wk. Colonic tissues were obtained from each mouse model and compared with controls. Expression of the lncRNA was then assessed by qRT-PCR. In both models, the mouse ortholog *lfng-as1* was found to be significantly elevated in the colitis setting compared with control (Fig. 10, A and B). These results are consistent with our human clinical data showing increased *IFNG-AS1* expression in colitis tissues.

Discussion

The diagnosis and treatment of IBD faces many challenges that can only be addressed by gaining a deeper understanding of the molecular mechanisms involved in the pathogenesis of the disease. Noncoding RNA biology has emerged as a new and potent form of gene regulation and has only recently begun to be evaluated in IBD (7, 42). In the past year, Mirza et al. (22) performed the first transcriptomic profiling of lncRNAs in IBD patients and revealed hundreds of differentially expressed lncRNAs between UC, CD, and control patients. Additionally, Wu et al. (41) also reported a profiling analysis of lncRNAs differentially expressed in UC patients vs. controls. They evaluated over 60 patients with and without IBD and identified 455 lncRNAs that were differentially expressed. This group focused on one of the differentially expressed lncRNAs, BC012900, and showed that it can regulate epithelial cell apoptosis. Our IBD studies investigated differentially expressed lncRNAs among UC patients and in addition uncovered new potential mechanisms of action. Through a noncoding RNA expression analysis, we identified a set of lncRNAs that are differentially expressed between UC samples and controls. By focusing on those lncRNAs differentially expressed in UC patients, we then filtered our list to identify lncRNAs that were associated with the clinically validated IBD susceptibility loci. We were able to validate the

differential expression of one of these lncRNAs, *IFNG-AS1*, with the use of a separate clinical cohort of colonic samples. Of note, Mirza et al. also found this lncRNA to be differentially expressed in their IBD patient samples, thereby providing further validation (22). Given its discovery on two independent analyses, *IFNG-AS1* presents itself as an ideal candidate for further study. As one of the first mechanistic studies for lncRNAs in the IBD field, this work focused on *IFNG-AS1*. Future studies will be needed to investigate additional lncRNAs involved in IBD pathophysiology.

IFNG-AS1 is a noncoding transcript located on chromosome 12 and adjacent to the *IFNG* gene. Prior studies have suggested that *IFNG-AS1* can positively regulate *IFNG* expression; however, much of the data implies function from the mouse ortholog *ifng-as1/NeST/tmevpg1*. These previous investigations have shown that *ifng-as1* is expressed in immune cells such as CD8 and CD4 T cells (5, 11, 37). The prior studies highlighted a mechanism wherein *ifng-as1* recruits a subunit of MLL/SET1 H3K4 methyltransferase complex to the *ifng* promoter and lays down an activating histone methylation signal that promotes expression of *ifng* in mouse immune cells (11). This mechanism, however, has yet to be validated in the human context. Indeed, the human studies of *IFNG-AS1* by Collier et al. (5) in the context of general immune cell biology and Wang et al. (39) in the context of Sjogren syndrome have shown expression of this noncoding transcript within CD4+ T cells. Our own studies show that a site of expression of *IFNG-AS1* is within these CD4+ cells. Using knockdown and overexpression constructs in human T cells, we directly show that *IFNG-AS1* expression can positively regulate IFNG production. We suspect the relatively low abundance of the IFNG-AS1 transcript results in modest knockdown efficiencies seen in our model system. However, our data suggest that within immune cells there is a mechanistic link between the overexpression of this noncoding RNA in colitis samples and the production of a key inflammatory cytokine. In addition, lncRNAs have also been implicated in the stability of mRNA transcripts, thereby providing additional modes by which IFNG-AS1 could potentially regulate its targets (38).

Through this work, we have identified a novel set of lncRNAs implicated in UC pathophysiology. Additional studies that are focused not only on *IFNG-AS1* but also on the other noted lncRNAs presented in this work will be essential in expanding our knowledge of UC pathogenesis. lncRNAs have served as novel targets in preclinical studies from other fields of medicine such as cancer biology and may serve as important therapeutic targets in UC (19). Additionally, lncRNAs could also serve as important biomarkers with a range of uses (34). lncRNA biology is a nascent field in IBD and much work is needed to expand its potential applications. Future studies evaluating the effects of the IBD susceptibility SNPs on expression of lncRNAs will help to link the genotypic changes seen in patients with specific SNPs with expression changes in SNP-associated genes. Additionally, mouse models of colitis will need to be employed to better define the mechanisms for lncRNA biology. To this end, this study has identified that the murine ortholog *ifng-as1* is also

overexpressed in the context of mouse models of colitis. This opens up additional avenues of research to carefully dissect other mechanisms of action for this noncoding RNA. The generation of *ifng-as1* knockout and transgenic animals provides additional tools to investigate its function (11). All told, the lncRNA biology serves as an additional layer of gene regulation adding to the complexity of IBD pathophysiology but also providing new tissue-specific targets.

Acknowledgements

We thank I. Karagiannides, K. Bakirtzi, M. Hatzia Apostolou, C. Vorvis, and M. Koutsioumpa for their helpful discussions and technical suggestions. We would also like to acknowledge E. Faure-Kumar in the Center for Systems for Biomedicine for expert technical assistance.

Figures

Figure 1.

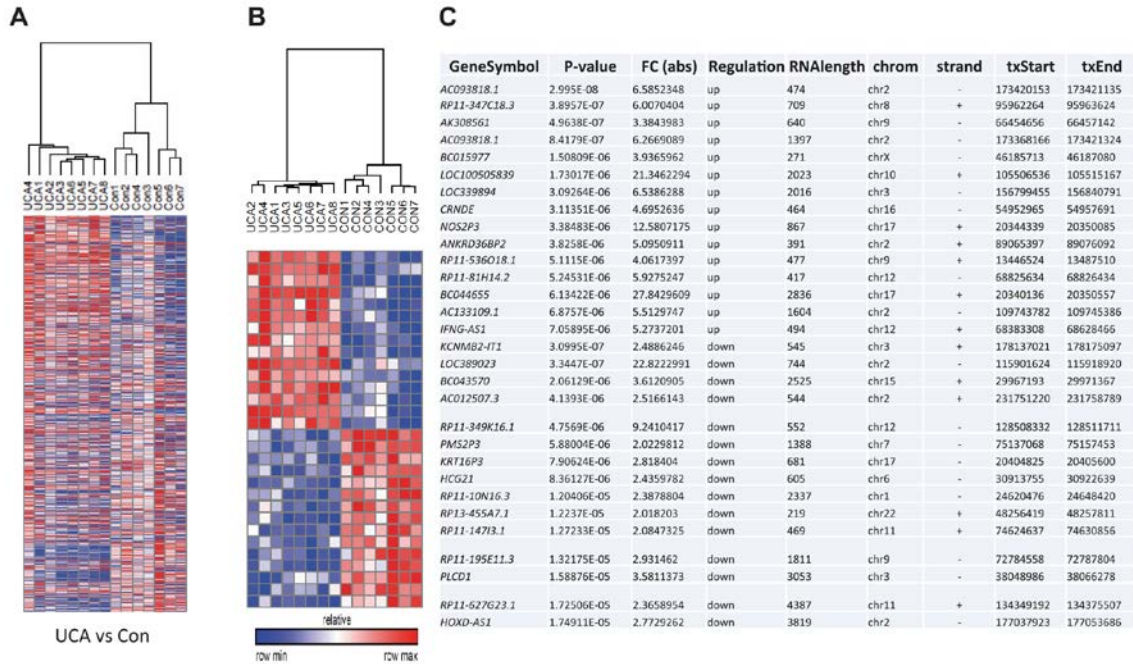


Fig. 1. A: heat map representation of expression changes between UCA and control (Con) groups with a fold change greater than twofold and P value less than 0.05. B: heat map representation of the top 15 upregulated and 15 downregulated lncRNAs in the UCA vs. Con comparison. C: table shows the identity of the lncRNAs along with their associated P value, fold change, direction of regulation, RNA length, chromosomal location (chrom), strand location (strand), and transcriptional start (txStart) and end (txEnd) positions. Row min and max refer to the minimum and maximum expression levels.

Figure 2.

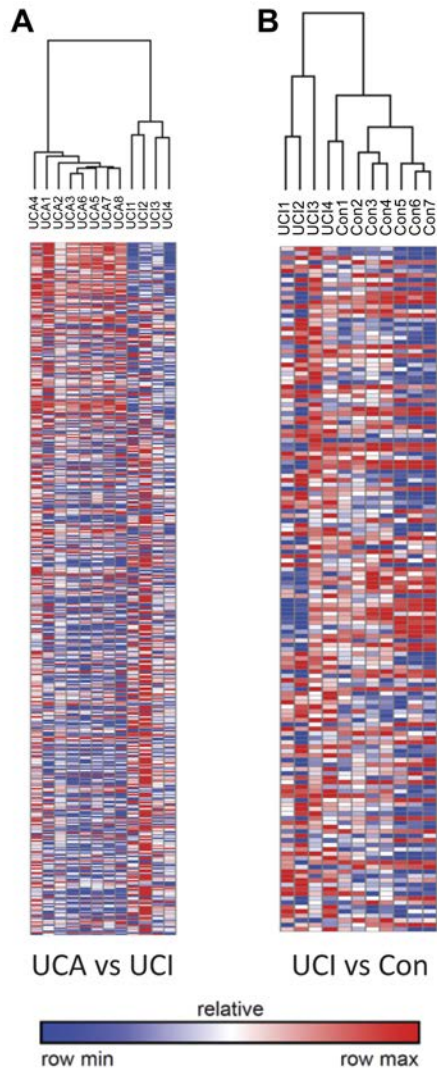


Fig. 2. Heat map representations of expression changes in lncRNA transcripts between UCA vs. UCI, UCI vs. Con. The number of differentially expression lncRNAs with a fold change cutoff of 2 and a *P* value cutoff of 0.05.

Figure 3.

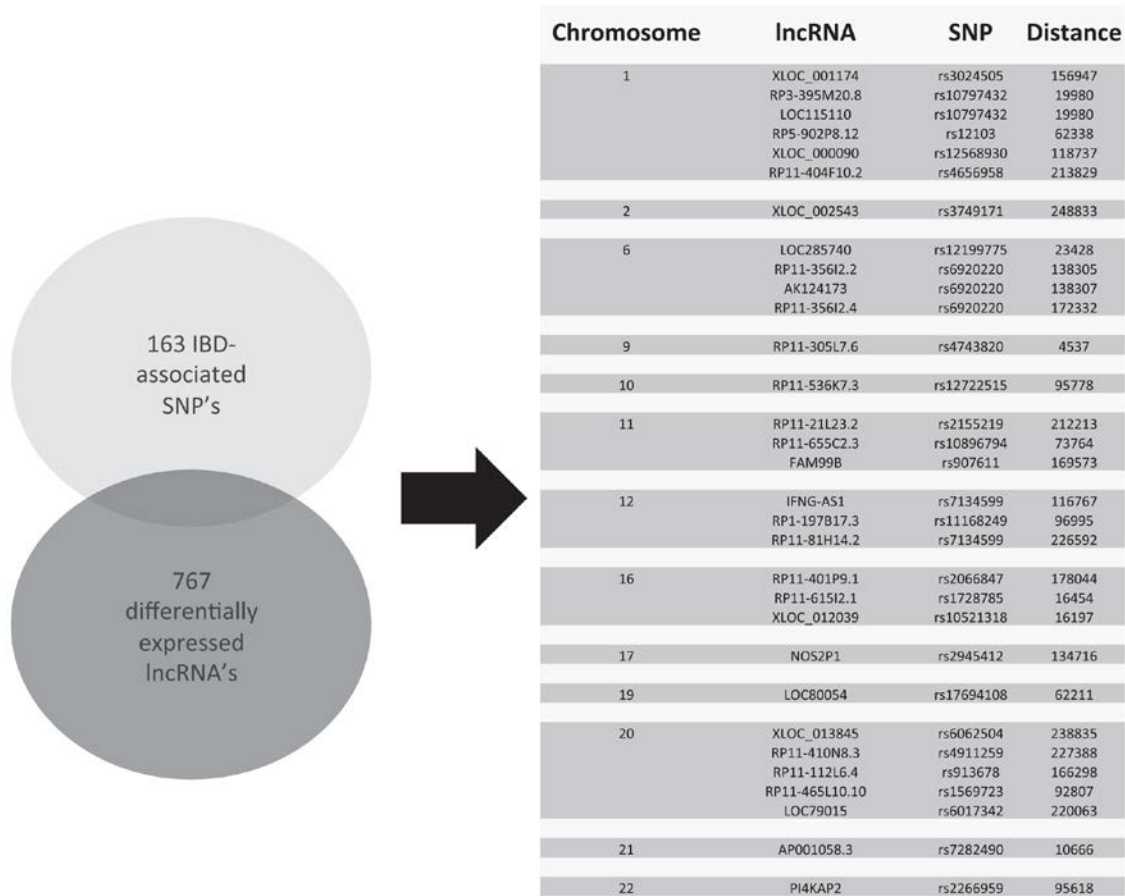


Fig. 3. Graphical representation of the overlap between the 163 IBD associated SNPs and the lncRNAs commonly differentially expressed between UCA vs. Con and UCA vs. UCI. The chromosomal location, lncRNA identity, associated SNP, and genomic distance from the SNPs are presented.

Figure 4.

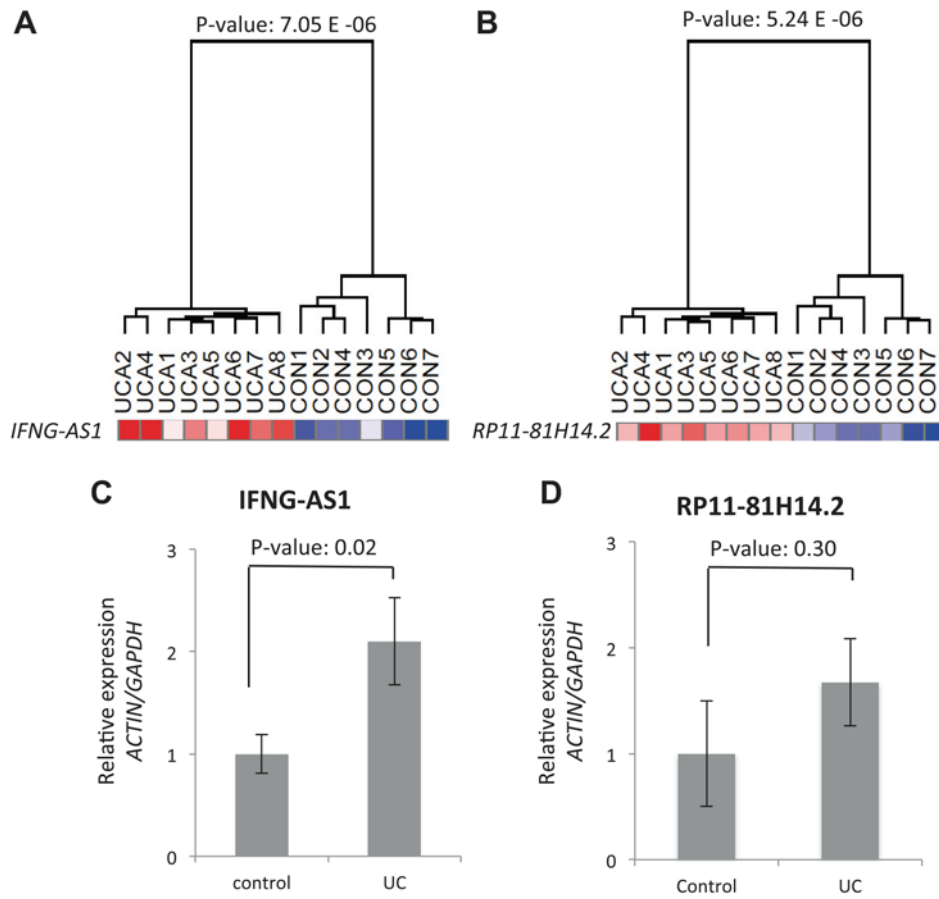
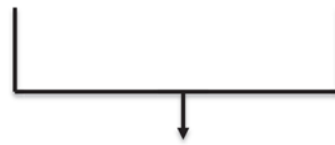
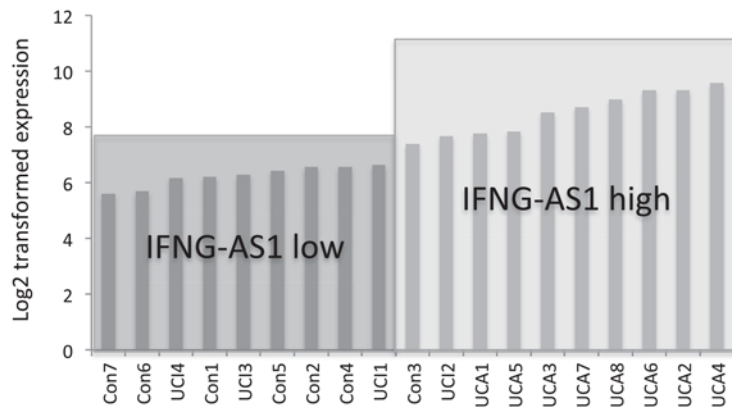


Fig. 4. A and B: microarray expression data for the lncRNAs IFNG-AS1 and RP11-81H14.2 in the analysis comparing UCA vs. Con. C and D: qPCR expression for IFNG-AS1 and RP11-81H14.2 in the validation cohort (control $N = 16$, UC $N = 15$). ACTIN and GAPDH were used as control housekeeping genes. Relative expression was to the average expression in the control samples.

Figure 5.



| Upstream Regulator | Molecule Type | Predicted Activation State | p-value |
|--------------------|---------------|----------------------------|----------|
| <i>TNF</i> | cytokine | Activated | 9.53E-14 |
| <i>IL1B</i> | cytokine | Activated | 1.59E-11 |
| <i>IFNG</i> | cytokine | Activated | 3.03E-17 |
| <i>IL1</i> | group | Activated | 1.96E-15 |
| <i>IL6</i> | cytokine | Activated | 1.55E-14 |

Fig. 5. Expression of IFNG-AS1 based on microarray expression across all UC and control samples. Two groups were created showing relative high and low levels of IFNG-AS1. Bioinformatic analysis was performed on the associated coding transcript expression changes between the two groups. A list of upstream regulators found to be activated in the IFNG-AS1 high group are listed along with the predicted activation state and the associated *P* value.

Figure 6.

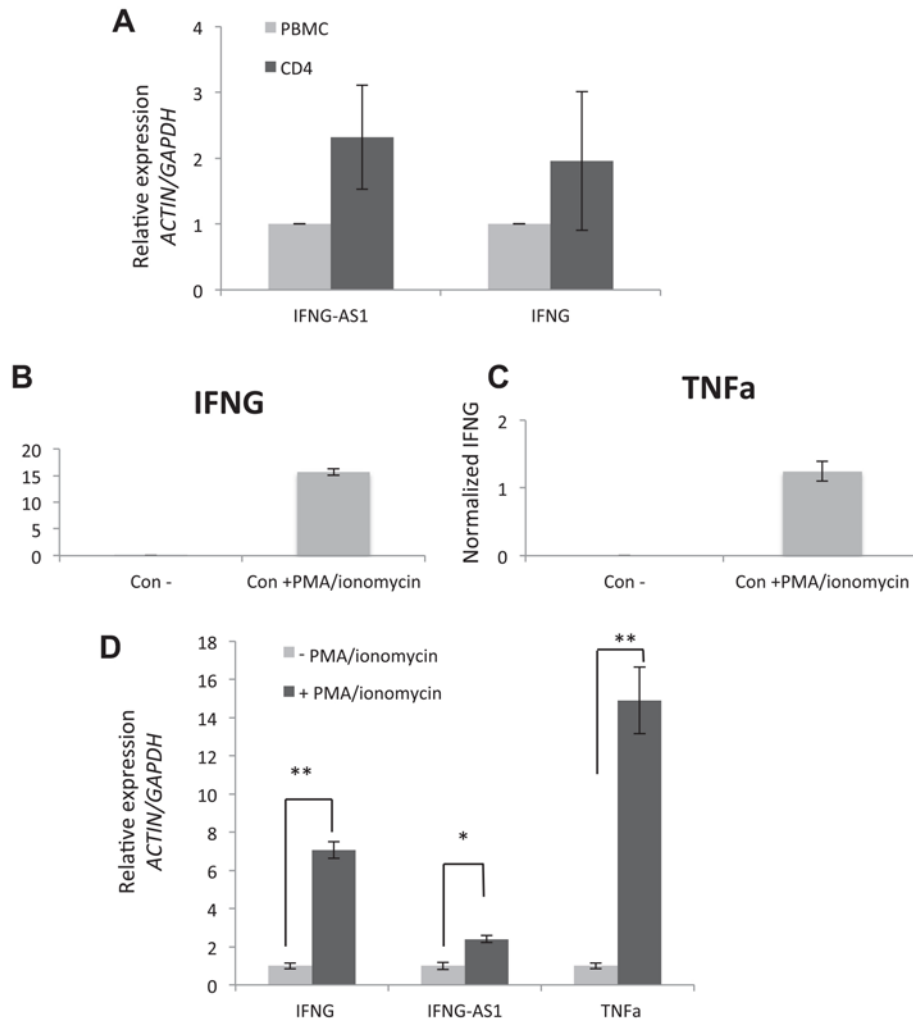


Fig. 6. A: qPCR expression of IFNG-AS1 and IFNG among CD4 cells and total PBMC ($N = 4$). B and C: Jurkat T cells activated with PMA/ionomycin for 6 h and cultured media collected after 24 h show inductions of IFNG and TNF- α protein, respectively. D: Jurkat T cells activated with PMA/ionomycin for 6 h; qPCR analysis of IFNG, IFNG-AS1, and TNF- α . * P value < 0.05 , ** P value < 0.01 ; $n = 3$.

Figure 7.

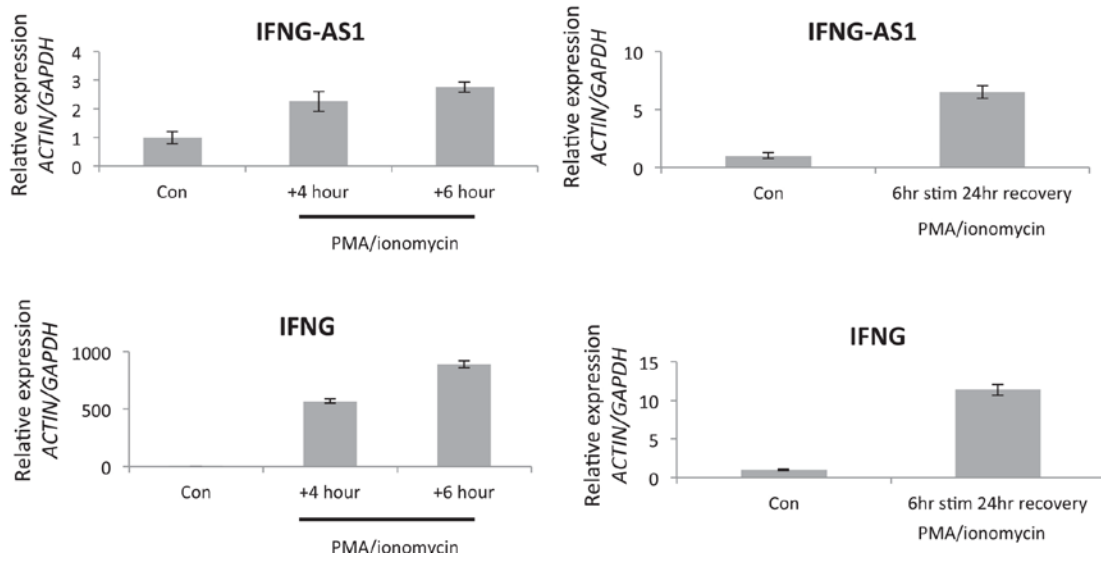


Fig. 7. qPCR expression for a time course evaluating the expression IFNG-AS1 and IFNG in Jurkat T cells.

Figure 8.

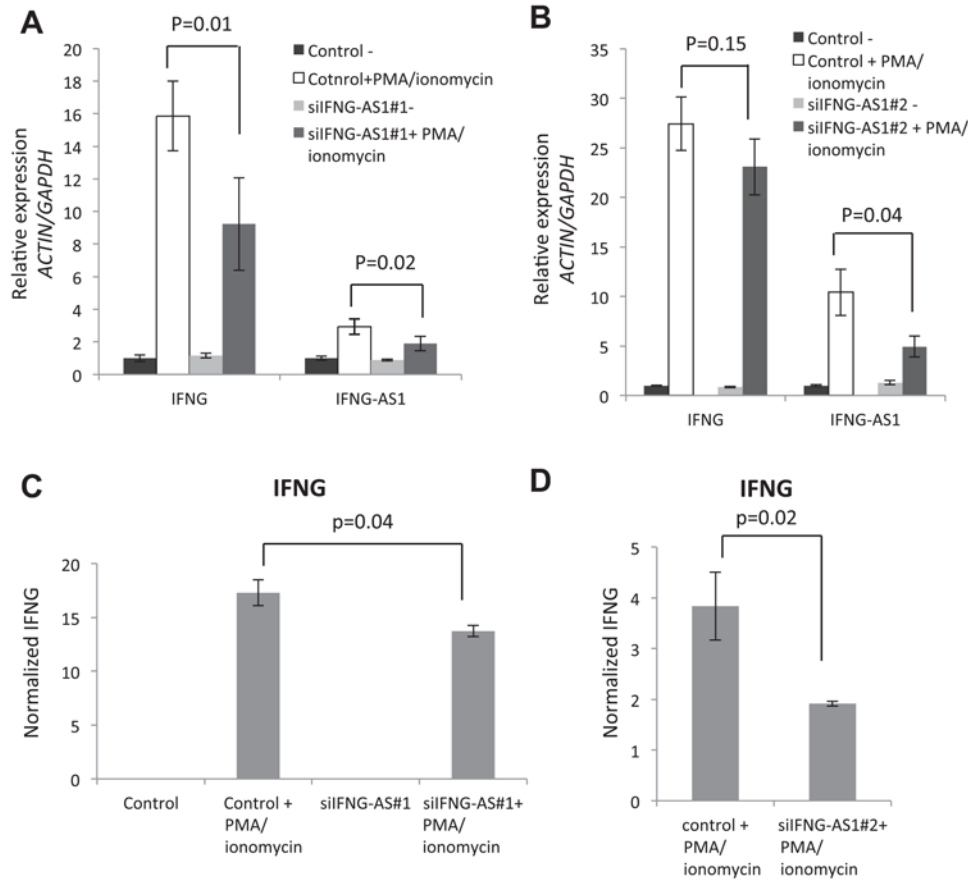


Fig. 8. *A* and *B*: qPCR analysis of Jurkat T cells treated with PMA/ionomycin for 6 h followed by a 24-h recovery period. Cells were treated with 2 independent antisense siRNAs against IFNG-AS1 ($n = 3$). *C* and *D*: protein analysis for IFNG was assessed by measuring the culture medium ($n = 3$). The siRNAs refer to small interfering RNAs with siFNG-AS1#1 and siFNG-AS1#2 referring to two independent small interfering RNAs directed against IFNG-AS1.

Figure 9.

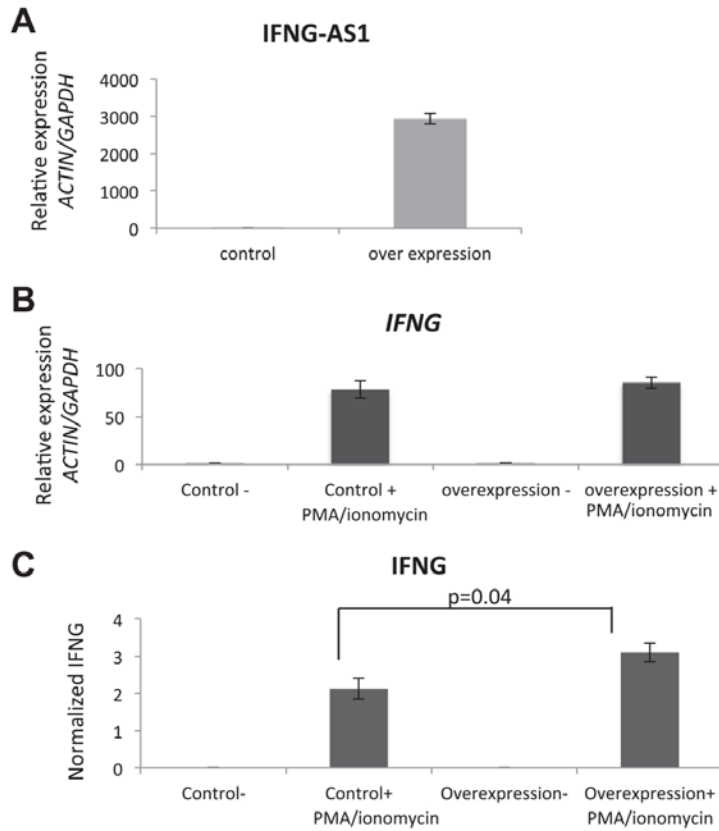


Fig. 9. A: qPCR expression of Jurkat T cells transiently overexpressing IFNG-AS1. B: qPCR analysis of Jurkat T cells with and without transient transfection of an IFNG-AS1-overexpressing construct. Cells were activated with PMA/ionomycin for 4 h ($n = 3$). C: IFNG protein was measured from cultured media ($n = 3$).

Figure 10.

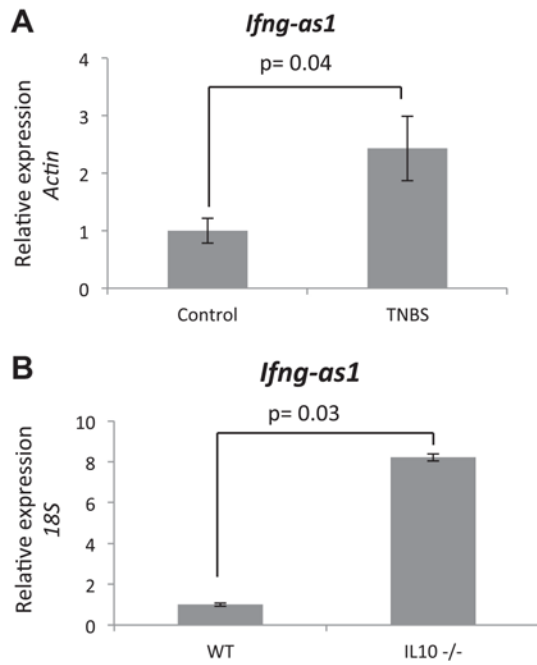


Fig. 10. *A*: *Ifng-as1* qPCR expression analysis. Whole colon samples from TNBS-treated animals vs. controls ($N = 5$) were assayed for *Ifng-as1* expression with *actin* as the control gene. *B*: *Ifng-as1* qPCR analysis from colon tissue derived from IL10 knockout animals compared with controls ($N = 4$).

Tables

Table 1. Patient information

| Sample | Age | Gender | Location |
|--------|-----|--------|----------------|
| Con1 | 31 | Male | Colon |
| Con2 | 45 | Female | colon |
| Con3 | 26 | Male | Colon: sigmoid |
| Con4 | 46 | Male | Colon |
| Con5 | 50 | Female | Colon |
| Con6 | 60 | Female | Colon |
| Con7 | 56 | Male | Colon |
| UCA1 | 30 | Female | Colon |
| UCA2 | 56 | Female | Colon: left |
| UCA3 | 26 | Female | Colon |
| UCA4 | 46 | Female | Colon |
| UCA5 | 29 | Male | Colon |
| UCA6 | 24 | Male | Colon |
| UCA7 | 45 | Female | Colon |
| UCA8 | 26 | Female | Colon |
| UCI1 | 31 | Male | Colon |
| UCI2 | 71 | Female | Colon |
| UCI3 | 51 | Male | Colon |
| UCI4 | 33 | Female | Colon |

Table 2. Ulcerative colitis and control samples

| Control | Disease | Age | Gender | Pathology |
|---------|----------------|-----|--------|-----------------------|
| 1 | Diverticulosis | n/a | n/a | n/a |
| 2 | rectal cancer | 50 | F | benign colonic tissue |

| | | | | |
|-----------|-------------------------|-----|-----|--|
| 3 | rectal villous adenoma | 68 | F | non-dysplastic mucosa |
| 4 | metastatic colon cancer | 53 | M | colonic tissue with no histopathologic abnormality |
| 5 | colon cancer, cecum | 76 | F | invasive adenocarcinoma of cecum with adjacent normal colon |
| 6 | colon cancer, rectum | 70 | M | colonic adenocarcinoma with adjacent normal colon |
| 7 | colon-normal | n/a | n/a | n/a |
| 8 | colon-normal | n/a | n/a | n/a |
| 9 | colon-normal | n/a | n/a | n/a |
| 10 | colon-normal | n/a | n/a | n/a |
| 11 | colon-normal | n/a | n/a | n/a |
| 12 | colon-normal | 42 | F | foci of endometriosis, large intestine colon without significant change |
| 13 | colon-normal | n/a | n/a | n/a |
| 14 | colon cancer | n/a | n/a | n/a |
| 15 | perforation | n/a | n/a | n/a |
| 16 | vaginal prolapse | n/a | n/a | n/a |
| | | | | |
| UC | | | | |
| 1 | ulcerative colitis | 61 | F | moderate to severely active ulcerative colitis, neg for dysplasia |
| 2 | ulcerative colitis | 49 | M | chronic pancolitis with severe activity, extensive ulcerations |
| 3 | ulcerative colitis | 52 | F | moderate to severe diffuse active pancolitis neg for dysplasia |
| 4 | ulcerative colitis | 35 | M | moderate to severe activity, chronic pancolitis, neg dysplasia |
| 5 | ulcerative colitis | 68 | F | severely active ulcerative colitis |
| 6 | ulcerative colitis | 31 | M | chronic active colitis with foci of ulceration |
| 7 | ulcerative colitis | 43 | M | n/a |
| 8 | ulcerative colitis | 57 | M | chronic colitis with moderate to severe activity and extensive ulcerations |

| | | | | |
|----|--------------------|-----|-----|---|
| 9 | ulcerative colitis | 39 | F | moderately active ulcerative colitis, neg dysplasia |
| 10 | ulcerative colitis | 30 | F | colon with moderate chronic colitis, no dysplasia |
| 11 | ulcerative colitis | 36 | M | chronic pancolitis with moderate to severe activity, ulceration and inflammatory polyps |
| 12 | ulcerative colitis | 49 | F | n/a |
| 13 | ulcerative colitis | 64 | M | severe inflammatory bowel disease type indeterminate |
| 14 | ulcerative colitis | 34 | F | Rectosigmoid, Involved |
| 15 | ulcerative colitis | n/a | n/a | n/a |

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