

Modulation of Interferon Activity-Associated Soluble Molecules by Appendicitis and Appendectomy Limits Colitis—Identification of Novel Anti-Colitic Targets

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The therapeutic efficacy of interferons (IFNs) in ulcerative colitis is minimal. However, IFN activity-associated molecules have been inadequately investigated. Appendicitis and appendectomy (AA), when done while young, protect against colitis development later. Our novel murine AA model protects against colitis. This therapeutic target-identifying study enumerates IFN activity-associated molecules involved in this protection. Mice with 2 laparotomies were controls (sham-sham/SS). Distal colons were harvested (4 AA-group colons and 4 SS-group colons). Microarray-analysis/reverse transcriptase–polymerase chain reaction-validation was done from RNA from each (3-days/28-days-post-AA). Gene set enrichment analysis (GSEA) software was used to analyze distal colonic gene sets associated with 46 IFN activity-related genes. More AA-upregulated gene sets were associated with *IFIT1*, *IFIT2*, *IFIT3*, *IRF7*, *IFI35*, and *IFI44* (False Discovery Rate-FDR <5% and $P < 0.001$), although only *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44* showed individual gene upregulation ($P < 0.05$). More AA-downregulated gene sets were associated with *IRF1*, *IRF2*, *IRF4*, *IRF8*, *IRF9*, *IRF2BP1*, *IFRD1*, *IFRD2*, and *IFIH1* (FDR <5%/ $P < 0.001$); although only *IRF2BP1* showed individual gene downregulation ($P < 0.05$). There was significant upregulation ($P < 0.05$) of *IFNZ*; and downregulation of *IRF2BP2* and *IFI30*, despite no major associated GSEA differences. *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44*, with profound AA-induced individual/GSEA upregulation, and their immunomodulatory/ antiproliferative activity, are the best molecules to investigate therapeutic potential. *IRF4*, *IRF8*, *IRF2BP1*, *IFRD1*, and *IFRD2*, owing to their profound AA-induced gene set downregulation, and because of their diverse lymphocytic activity, are good targets to competitively inhibit or to treat with exogenous products in knockout animals.

Introduction

IN 1957, ISAACS AND LINDENMANN came across a factor that protected cells from viral infection, and named it interferon (IFN) (Isaacs and Lindenmann 1957). The IFNs (referring to type I IFNs in this article, unless otherwise specified) are proteins that play key roles in antiviral, anti-growth, and immunomodulatory responses (Stark and others 1998). The IFNs were the first cytokines discovered and the first to be used therapeutically (Pestka and others 1987). The main IFN signaling pathways are direct and rapid via tyrosine phosphorylation signaling by Janus kinases at the cell membrane, followed by effector gene expression (Stark and others 1998). IFNs induce MHC class I proteins and prime CD8⁺ T-cell responses (Boehm and others 1997). IFN- γ (not IFN- α or IFN- β) plays a crucial role in macrophage activation (Dalton and others 1993; Huang and others 1993), and induced nitric oxide production (MacMicking and others 1997). IFNs are

crucial to antigen processing and modification of proteasome activity (York and Rock 1996; Boehm and others 1997). IFNs directly or indirectly exert effects on B cells (Stark and others 1998).

A Cochrane systematic review (2008) found that extant literature does not support therapeutic efficacy of IFNs in ulcerative colitis (Seow and others 2008). In 6 clinical studies reviewed (Danese and others 2008), IFN types do not appear promising in ulcerative colitis treatment. Similarly, early promise for anti-IFNG therapy in Crohn's disease (Hommes and others 2006) has not been borne out by subsequent studies (Reinisch and others 2010). Despite these inauspicious clinical results, other IFN activity-associated molecules require examination as therapeutic possibilities.

IFIT1 (ISG56), *IFIT2* (ISG54), and *IFIT3* (ISG60) are characterized by multiple repeats of tetratricopeptide repeat helix-turn-helix motifs, and are conserved from amphibians to mammals (Fensterl and Sen 2011). They are induced by

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IFNs, virus infections, and molecular patterns (lipopolysaccharide, double-stranded DNA, etc); and mediate a variety of protein–protein interactions, and display immunomodulatory and antiproliferative activity (Guo and others 2000b; Fensterl and Sen 2011). The IRF family (IRF1 to IRF9) of DNA-binding transcription factors recognize and bind to consensus gene sequences (IFN-stimulated response elements and virus-responsive elements), assisting in IFN production/feedback-inhibition, cell growth regulation, T-/B-lymphocyte function, and IFN-induced gene expression (Honda and Taniguchi 2006; Zhao and others 2014). IFIH1 (RNA helicase), IFI30 (lysosomal thiol reductase), IFI47 (B cell-GTP-binding protein), IFI35 (leucine zipper protein), and IFI44 (cell proliferation inhibitor) are other important IFN activity-associated molecules (Gilly and Wall 1992; Bange and others 1994; Hallen and others 2007; Moura and others 2013; West and Cresswell 2013).

The mammalian vermiform appendix contains abundant lymphoid tissue, and it is perpetually exposed to intestinal flora. Inflammation of the appendix, appendicitis, is the most common gastrointestinal emergency requiring surgical intervention (Addiss and others 1990). The highest occurrence of uncomplicated appendicitis is between 10 and 30 years (Marudanayagam and others 2006). The complex interplay between genetic predisposition, gastrointestinal bacteria, and gut immunity in inflammatory bowel disease (or IBD, comprising of Crohn's disease and ulcerative colitis) is yet to be deciphered. It has been shown in over a dozen clinical studies that appendicitis and appendectomy (AA) limit or prevent ulcerative colitis from developing (Koutroubakis and others 2002), this protection is seen in patients undergoing AA prior to the age of 20. Therefore, AA in young patients induces long-lasting distal colonic changes that protect against future colitis. Our AA mouse model is an excellent simulation of that. We used this simulation to investigate the AA-induced "lingering distal colonic immunological environment" that protects against colitis later in life. In mice, the equivalent of the human appendix is the major caecal lymphoid patch. Experimental colitis development was prevented by caecum removal in 3 murine colitis models, namely T cell receptor- α mutants (Mizoguchi and others 1996), dextran sulfate sodium (DSS) model (Kriegelstein and others 2001) and adoptive T cell transfer colitis model (Farkas and others 2005). The first murine model of AA was developed by our group (Watson Ng and others 2007). In our model, appendiceal pathology resembles that of human appendicitis (Watson Ng and others 2007), and AA offers an age-, bacteria-, and antigen-dependent protection against TNBS-colitis (Luo and others 2009). We have also shown that AA curbs T helper 17 cell activities and curtails autophagy in the most distal colon (Cheluvappa 2014).

Studies involving individual gene expression differences between 2 or more experimental groups are commonplace. However, this simplistic approach does not consider the reality of cellular processes contiguously or conjointly effecting changes as groups of genes (gene sets). These changes may be minimal when individual genes are examined, but these are strikingly obvious and statistically pronounced when corresponding gene sets are examined. We have previously demonstrated the utility of this approach in exploring mechanisms of immune protection in the colon (Cheluvappa and others 2011, 2014a, 2014b). This study uses microarray

analysis and gene set enrichment analysis (GSEA)(Subramanian and others 2005) to identify and characterize the role of IFNs and IFN activity-associated molecules in the amelioration of colitis by AA in our animal model.

Materials and Methods

Animal experiments

All animal experiments were approved and monitored by the University of New South Wales Animal Care and Ethics Committee. Specific pathogen free Balb/c mice (Male, 5 weeks) from the Animal Resource Centre (Perth, Western Australia) were kept at the University of New South Wales holding and care facility. Mice were anesthetized with intraperitoneal xylazine (5 mg/kg; Sigma-Aldrich, $\times 1251$) and ketamine (100 mg/kg; Sigma-Aldrich, K1884). Mice were randomized to have either appendicitis or sham operation. Appendicitis was induced by constructing an appendiceal pouch (using a sterile rubber band) from the caecal lymphoid patch. Sham surgery entailed a similar procedure, but without continuous obstruction by band ligation of the caecal patch, and with the placement of a sterile rubber band in the abdominal cavity as a control. Seven days following initial surgery, postappendicitis mice underwent appendectomy (AA, group) while sham mice underwent a second sham surgery (sham and sham, SS, group). All mice were monitored daily. Transmural distal colonic segments were harvested 3, 14, and 28 days after AA or SS. Samples from the 3, 14, and 28-day time points were used for reverse transcriptase–polymerase chain reaction (RT-PCR) analyses. Samples from the 3 and 28-day time points were used for microarray analyses. The details of, and the rationale for each major step has been systematically posited in our previous publication (Cheluvappa and others 2014a).

Processing of colonic specimens for RNA extraction

Distal colonic segments were cleaned of fecal contents with normal saline and immediately transferred to TRIzol[®] reagent (50–75 mg of tissue in 600 μ L of TRIzol reagent; Invitrogen Australia Pty Ltd., 15596-026), snap-frozen in liquid nitrogen and stored at -80°C until the microarray analysis. Further extraction involved chloroform and isopropanol treatment and centrifugation, followed by washing the resultant pellet with 75% ethanol, air-drying, and final H₂O reconstitution. Concentration and purity of RNA were determined by automated optical density evaluation (OD 260/OD 280 ≥ 1.8 and OD 260/OD 230 ≥ 1.8) using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). The degree of RNA degradation was analyzed by the Agilent electrophoresis bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA) with the RNA integrity number (RIN) values consistently above 7.

Validation and analysis of gene expression with RT-PCR

Reverse transcription to produce cDNA was performed using RT² First Strand Kits (SA Biosciences, Frederick, MD) and RT-PCR was performed utilizing the LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany): with RT² SYBR Green PCR Master

Mix (SA Biosciences). Predesigned primers for genes of interest were obtained from SA Biosciences. β -actin, β -glucuronidase, and 18S rRNA were the reference genes evaluated. Further methodological details have been published previously (Cheluvappa and others 2011).

Affymetrix array process—labeling, hybridization, scanning, and normalization

All experiments are compliant with minimum information about a microarray experiment (MIAME) standards (Brazma and others 2001). Four individual test samples (for 3-day post-SS/AA time point) or 3 individual test samples (for 28-day post-SS/AA time point) were used per group (AA group versus SS group; 1 colonic sample per mouse) with each sample hybridized to an individual slide. RNA (100 ng) from each sample was labeled using the Whole Transcript Sense Target Labeling Assay as described (Affymetrix). Labeled cRNA samples were hybridized to Affymetrix Mouse Gene 1.0 ST Arrays (28,853 well-annotated genes) (Ramaciotti Centre for Gene Function Analysis, University of New South Wales) before being scanned using an Affymetrix GCS3000 7G 4-color Gene Array scanner with autoloader (Affymetrix). The Gene Expression Omnibus accession number for microarray data reported here is GSE23914. Further methodological details are available from our previous publication (Cheluvappa and others 2011).

Microarray preprocessing and filtering

All noncontrol probe sets from the 8 arrays were imported into Partek (Version 6.4, Partek, Inc.), and then normalized using Robust Multi-array Analysis (Irizarry and others 2003). The probability of each probe set being expressed was determined using the detected above background procedure, using Affymetrix Power Tools (v1.10.2), excluding 13 probes from probe set 10338063 that had very low GC, and thus did not have matched controls. Probe sets were excluded if none of the samples were detected above background ($P = 10^{-5}$). To assess the degree of differential expression between AA and SS groups, a 2-way ANOVA on treatment and batch was fitted to each probe set using Partek. To correct for multiple hypothesis testing, we used the qValue/positive False Discovery Rate (FDR) (Storey and Tibshirani 2003).

Gene set enrichment analysis

We utilized GSEA (Subramanian and others 2005) that merges data from gene sets (experimentally derived or from expert curated pathway databases) to detect significant expression differences. We compared gene expression profiles to the c2_all collection of curated gene sets from the molecular signatures database (version 2.5). A preranked file was created, containing the average difference between AA and SS for each probe set, sorted from most upregulated in SS to most downregulated. We used the na28 annotation csv file from www.affymetrix.com to determine the gene symbol for each probe set and collapsed probe sets to unique genes using the default, max_probe option, resulting in 18,600 unique genes. GSEA (version 2.0) (Subramanian and others 2005), was run in preranked mode, using default parameters (gene set sizes between 15 & 500 leaving 1387 gene sets, 1000 permutations, images on the top 50 gene sets). We utilized stringent statistical cutoff [False Dis-

covery Rates (FDR) values <5% and P value <0.001] to delineate IFN-, and IFN-related molecule-associated gene sets, which were consistently altered in the distal colons of all AA mice when compared to control SS mice.

Statistics

Group comparisons were analyzed using the Mann–Whitney U test with GraphPad Prism (Graphpad software, San Diego, CA). Data are expressed as mean \pm standard error of mean and the differences were considered to be significant if P value <0.05.

Results

Quantitative RT-PCR validation of our gene-expression study

Previously published RT-PCR confirmation of our gene expression studies (Cheluvappa and others 2011) involved 14 genes from 4 major groups, namely; innate immunity, immune mediators, cell migration-chemokines, and cell migration-receptors.

Distal colonic expression of individual genes associated with IFN activity

Distal colonic gene expression of 46 genes associated with IFN activity (Table 1) was done 3 and 28 days after AA ($*P < 0.05$). At 3 days post-AA, *IRF7* and *IFI35* were significantly upregulated ($*P < 0.05$); and *IFNK* and *IFRD2* were significantly downregulated ($*P < 0.05$). At 28 days post-AA, *IFNZ*, *IFIT1*, *IFIT2*, *IFIT3*, *IRF9*, *IFIH1*, and *IFI44* were significantly upregulated ($*P < 0.05$); and *IRF2BP1*, *IRF2BP2*, and *IFI30* were significantly downregulated ($*P < 0.05$). The only upregulated IFN gene at either time point is *IFNZ*.

Distal colonic 28 days post-AA expression of gene sets associated with IFN activity—genes with minimal differences in gene set expression

GSEA analysis of distal colonic gene sets (28 days after AA) associated with 46 genes involved in IFN activity was performed. Genes with minimal differences in gene set regulation are *IFNA1*, *IFNA2*, *IFNA3*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA7*, *IFNA8*, *IFNA9*, *IFNA10*, *IFNA11*, *IFNA12*, *IFNA13*, *IFNA14*, *IFNA15*, *IFNA16*, *IFNA17*, *IFNB1*, *IFND*, *IFNE*, *IFNK*, *IFNT*, *IFNW*, *IFNG*, *IRF3*, *IRF5*, *IRF6*, and *IFI47* (Table 2). However, there was statistically significant ($P < 0.05$) individual gene upregulation of *IFNZ*; and downregulation of *IRF2BP2* and *IFI30* (Tables 1 and 2).

Distal colonic 28 days post-AA expression of gene sets associated with IFN activity—genes with more gene sets upregulated

GSEA analysis of distal colonic gene sets (28 days after AA) associated with 46 genes related to IFN activity was done. More AA-upregulated gene sets were associated with the genes *IFIT1*, *IFIT2*, *IFIT3*, *IRF7*, *IFI35*, and *IFI44* (FDR <5% and $P < 0.001$) (Table 3), although only the genes *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44* showed statistically significant AA-induced individual gene upregulation ($P < 0.05$) (Tables 1 and 3).

TABLE 1. EXPRESSION OF IFNs AND IFN-INDUCED SOLUBLE FACTOR GENES IN THE DISTAL COLON, 3 AND 28 DAYS AFTER AA

No.	Gene	3-Day post-AA		28-Day post-AA	
		Fold-change	P value	Fold-change	P value
1	<i>IFNA1</i>	0.92	0.230	1.15	0.296
2	<i>IFNA2</i>	—	—	1.15	0.209
3	<i>IFNA4</i>	—	—	0.96	0.597
4	<i>IFNA5</i>	0.90	0.155	1.04	0.615
5	<i>IFNA6</i>	—	—	1.11	0.215
6	<i>IFNA7</i>	0.86	0.116	1.03	0.687
7, 8	<i>IFNA8/IFNA10</i>	—	—	—	—
9	<i>IFNA13</i>	—	—	1.12	0.173
10	<i>IFNA14</i>	—	—	1.13	0.232
11, 12, 13	<i>IFNA16, IFNA17, IFNA3</i>	—	—	—	—
14	<i>IFNA9</i>	0.96	0.604	0.93	0.321
15	<i>IFNA11</i>	—	—	1.06	0.465
16	<i>IFNA12</i>	—	—	0.85	0.079
17	<i>IFNA15</i>	—	—	—	—
18	<i>IFNB1</i>	0.92	0.295	0.89	0.218
19	<i>IFNE</i>	1.04	0.312	1.10	0.280
20	<i>IFNK</i>	0.91	0.035*	0.96	0.512
21, 22, 23	<i>IFNW, IFND, IFNT</i>	—	—	—	—
24	<i>IFNZ</i>	0.83	0.135	1.36 [#]	0.016*
25	<i>IFNG</i>	—	—	1.02	0.758
26	<i>IFIT1</i>	2.60 [#]	0.062	3.55 [#]	0.008*
27	<i>IFIT2</i>	2.26 [#]	0.065	1.65 [#]	0.007*
28	<i>IFIT3</i>	2.13 [#]	0.151	2.32 [#]	0.012*
29	<i>IRF1</i>	1.18	0.086	0.90	0.140
30	<i>IRF2</i>	0.91	0.162	1.11	0.230
31	<i>IRF3</i>	1.04	0.632	0.88	0.085
32	<i>IRF4</i>	—	—	1.15	0.204
33	<i>IRF5</i>	1.04	0.733	0.93	0.388
34	<i>IRF6</i>	1.05	0.679	0.98	0.801
35	<i>IRF7</i>	1.72 [#]	0.024*	1.42 [#]	0.081
36	<i>IRF8</i>	1.04	0.490	0.91	0.173
37	<i>IRF9</i>	1.35 [#]	0.079	1.47 [#]	0.037*
38	<i>IRF2BP1</i>	0.91	0.197	0.83	0.026*
39	<i>IRF2BP2</i>	1.17	0.134	0.84	0.048*
40	<i>IFRD1</i>	1.21	0.154	0.86	0.084
41	<i>IFRD2</i>	0.89	0.043*	0.92	0.388
42	<i>IFIH1</i>	1.19	0.062	1.30 [#]	0.035*
43	<i>IFI30</i>	1.01	0.956	0.87	0.044*
44	<i>IFI47</i>	2.51 [#]	0.062	1.26	0.058
45	<i>IFI35</i>	1.43 [#]	0.005*	1.03	0.708
46	<i>IFI44</i>	2.2 [#]	0.108	2.65 [#]	0.000*

Distal colonic gene expression of 46 IFN activity-associated genes was done 3 and 28 days after AA. At 3 days post-AA; *IRF7* and *IFI35* were significantly upregulated (*); and *IFNK* and *IFRD2* were significantly downregulated (*). At 28 days post-AA; *IFNZ*, *IFIT1*, *IFIT2*, *IFIT3*, *IRF9*, *IFIH1*, and *IFI44* were significantly upregulated (*); and *IRF2BP1*, *IRF2BP2*, and *IFI30* were significantly downregulated (*). **P* value < 0.05. [#]Fold-increase ≥ 1.3 . Please peruse Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/jir) for a detailed version of this table with a columns for gene abbreviation expansion, gene-product functions and corresponding references. **P* value < 0.05. [#]Fold-increase ≥ 1.3 .

AA, Appendicitis and appendectomy group; IFN, interferon.

Distal colonic 28 days post-AA expression of gene sets associated with IFN activity—genes with more gene sets downregulated

GSEA analysis of distal colonic gene sets (28 days after AA) associated with 46 genes related to IFN activity was done. More AA-downregulated gene sets were associated with the genes *IRF1*, *IRF2*, *IRF4*, *IRF8*, *IRF9*, *IRF2BP1*, *IFRD1*, *IFRD2*, and *IFIH1* (FDR < 5% and *P* < 0.001) (Table 4); although only the gene *IRF2BP1* showed statistically significant AA-induced individual gene downregulation

(*P* < 0.05) (Tables 1 and 4), and the genes *IRF9* and *IFIH1* paradoxically showed significant AA-induced individual gene upregulation (*P* < 0.05) (Table 1 and Table 4).

Discussion

The broad spectrum of properties (antiviral, differentiation-priming, proapoptotic, and MHC I-inducing) and activities (antiproliferative, antiviral, antiangiogenic, and cytotoxic) displayed by IFNs endow each of them with therapeutic potential for a vast array of prevalent diseases (Pestka and

TABLE 2. IFNs AND IFN-INDUCED SOLUBLE FACTOR GENES WITH MINIMAL DIFFERENCES IN UPREGULATED VERSUS DOWNREGULATED GENE SETS IN THE DISTAL COLON 28 DAYS AFTER AA

No.	Gene	Individual gene change (Table 1)	Upregulated gene sets in AA	Downregulated gene sets in AA
1	<i>IFNA7</i>	—	0	1
2	<i>IFNB1</i>	—	1	1
3	<i>IFNE</i>	—	0	1
4	<i>IFNG</i>	—	2	1
5	<i>IRF3</i>	—	1	3
6	<i>IRF5</i>	—	0	1
7	<i>IRF6</i>	—	0	3
8	<i>IRF2BP2</i>	↓	0	3
9	<i>IFI30</i>	↓	6	6

GSEA analysis of distal colonic gene sets (28 days after AA) associated with 46 genes involved in IFN activity was done. Genes showing no gene set differences were *IFNA1*, *IFNA2*, *IFNA3*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA8*, *IFNA9*, *IFNA10*, *IFNA11*, *IFNA12*, *IFNA13*, *IFNA14*, *IFNA15*, *IFNA16*, *IFNA17*, *IFND*, *IFNK*, *IFNT*, *IFNW*, *IFNZ*, and *IFI47*. Genes with minimal differences in gene set regulation are listed in this table (FDR < 5% and *P* value < 0.001). The arrows refer to the genes that individually showed statistically significant upregulation (↑) or downregulation (↓) according to Table 1 (*P* value < 0.05). Please peruse Supplementary Table S2 for a detailed version of this table - Tables 2–4 listed here are integrated into 1 detailed table and 1 concise table therein. The Supplementary Table S2 enumerates all gene sets enriched, the number of genes enriched in each gene set, and the FDR value of each gene set enriched (each enriched gene set had *P* value < 0.001). GSEA, gene set enrichment analysis; FDR, False Discovery Rate.

others 2004). The challenge is to convert this potential into therapy with minimal adverse effects, via molecular modifications and targeted delivery techniques. However, IBD clinical trials with IFNs have shown no statistical benefit in disease amelioration (Danese and others 2008; Seow and others 2008;

TABLE 3. IFN-INDUCED SOLUBLE FACTOR GENES WITH MORE UPREGULATED GENE SETS IN THE DISTAL COLON 28 DAYS AFTER AA

No.	Gene	Individual gene change (Table 1)	Upregulated gene sets in AA	Downregulated gene sets in AA
1	<i>IFIT1</i>	↑	14	3
2	<i>IFIT2</i>	↑	10	1
3	<i>IFIT3</i>	↑	14	0
4	<i>IRF7</i>	—	11	4
5	<i>IFI35</i>	—	11	2
6	<i>IFI44</i>	↑	14	0

GSEA analysis of distal colonic gene sets (28 days after AA) associated with 46 genes involved in IFN activity was done. Genes with more upregulated gene sets are listed in this table (FDR < 5% and *P* value < 0.001). The arrows refer to the genes that individually showed statistically significant upregulation (↑) or downregulation (↓) according to Table 1 (*P* value < 0.05). Please peruse Supplementary Table S2 for a detailed version of this table - Tables 2, 3, and 4 listed here are integrated into 1 detailed table and 1 concise table therein. The Supplementary Table S2 enumerates all gene sets enriched, the number of genes enriched in each gene set, and the FDR value of each gene set enriched (each enriched gene set had a *P* value of less than 0.001). AA, Appendicitis and appendectomy group; GSEA, gene set enrichment analysis; FDR, False Discovery Rate.

TABLE 4. IFN-INDUCED SOLUBLE FACTOR GENES WITH MORE DOWNREGULATED GENE SETS IN THE DISTAL COLON 28 DAYS AFTER AA

No.	Gene	Individual gene change (Table 1)	Upregulated gene sets in AA	Downregulated gene sets in AA
1	<i>IRF1</i>	—	4	10
2	<i>IRF2</i>	—	0	5
3	<i>IRF4</i>	—	0	7
4	<i>IRF8</i>	—	1	9
5	<i>IRF9</i>	↑	4	9
6	<i>IRF2BP1</i>	↓	0	10
7	<i>IFRD1</i>	—	1	9
8	<i>IFRD2</i>	—	0	14
9	<i>IFIH1</i>	↑	0	8

GSEA analysis of distal colonic gene sets (28 days after AA) associated with 46 genes involved in IFN activity was done. Genes with more downregulated gene sets are listed in this table (FDR < 5% and *P* value < 0.001). The arrows refer to the genes that individually showed statistically significant upregulation (↑) or downregulation (↓) according to Table 1 (*P* value < 0.05). Please peruse Supplementary Table S2 for a detailed version of this table - Tables 2, 3, and 4 listed here are integrated into 1 detailed table and 1 concise table therein. The Supplementary Table S2 enumerates all gene sets enriched, the number of genes enriched in each gene set, and the FDR value of each gene set enriched (each enriched gene set had a *P* value < 0.001). AA, Appendicitis and appendectomy group; GSEA, gene set enrichment analysis; FDR, False Discovery Rate.

Reinisch and others 2010). Crucially, IFN-induced factors, or downstream factors with immunoregulatory or immunosuppressive function have not been enumerated and assessed for further investigation of therapeutic potential in IBD. This study addresses this deficiency.

Using a murine appendicitis-appendectomy model previously reported by us (Watson Ng and others 2007), we showed that AA ameliorated TNBS colitis (Luo and others 2009), using microarray analyses, GSEA, and RT-PCR validation to delineate genetic pathways entailed in this protection or prevention (Cheluvappa and others 2011). The novelty of our study is that the most distal regions of the colon sustain major persistent changes (protective against colitis), by manipulation at the caecum, the most proximal region of the colon.

Twenty-eight days after AA, GSEA analysis of distal colonic gene sets associated with 46 genes involved in IFN activity was done (Table 2). There was significant gene upregulation of *IFNZ*; and downregulation of *IRF2BP2* and *IFI30* although no major differences in gene set numbers were noted (Table 1, Table 2). More AA-upregulated gene sets were associated with the genes *IFIT1*, *IFIT2*, *IFIT3*, *IRF7*, *IFI35*, and *IFI44* (Table 3). More AA-downregulated gene sets were associated with the genes *IRF1*, *IRF2*, *IRF4*, *IRF8*, *IRF9*, *IRF2BP1*, *IFRD1*, *IFRD2*, and *IFIH1* although the genes *IRF9* and *IFIH1* paradoxically showed significant AA-induced individual gene upregulation (Tables 1 and 4). This paradoxical phenomenon may be an indication of compensatory changes (positive regulatory changes), or the initial changes which, in turn, led to the downregulation of the many gene sets downregulated by AA, as observed earlier (Cheluvappa and others 2014a).

These data are commensurate with a recent study demonstrating imiquimod-induced colonic upregulation of IFN activity-associated genes or their gene sets (*IFIT1*, *IFIT2*,

IFIT3, *IRF7*, *IFI44*, and *IFIH1*) that protected against DSS colitis (Sainathan and others 2012). However, a thorough literature search (te Velde and others 2007; Hansen and others 2009; Frantz and others 2012) shows that these genes have not been investigated or shown to be differentially regulated in murine colitic models *per se*, including a comparative (but selective) gene expression study of different murine colitic models (te Velde and others 2007).

The only upregulated IFN gene at either time point is *IFNZ*. The murine IFN *IFNZ* (Limitin) displays immunomodulatory and antiviral effects, initiated by its IFN- α/β receptor binding (Oritani and Kanakura 2005). However, its suppression of B-cell lymphopoiesis is far milder than that of IFN- α (Oritani and Kanakura 2005).

IFIT1 significantly suppresses global protein synthesis in HT1080 cells (Guo and others 2000a). IFIT1 disrupts interactions between key components in the virus-triggered IFN signaling pathways, suggesting that it is a negative feedback regulator of IFNs (Li and others 2009). In transfected macrophages, IFIT2 overexpression inhibits LPS-induced expression of TNF- α , IL-6, and MIP-2, at the post-transcriptional levels (Berchtold and others 2008). In monocytic U937 cells, IFIT3 expression leads to cell cycle negative regulator (P27 and P21) upregulation and inhibition of cellular proliferation (Xiao and others 2006). The antiproliferative effect of IFITs on immune cells, in addition to their suppression of pro-inflammatory cytokines may have contributed to the limitation of colitis by AA. The IRF family of DNA-binding transcription factors bind to consensus gene sequences, assisting in IFN production/feedback-inhibition, IFN-induced gene expression, and lymphocyte function (Honda and Taniguchi 2006; Zhao and others 2014). IRF1, IRF3, IRF5, and IRF7 are positive IFN regulators (Honda and Taniguchi 2006; Zhao and others 2014). IRF1 is required for IFNs to induce MHC class I proteins and prime CD8⁺ T-cell responses (Reis and others 1992). IRF-1 regulates IL-15 gene expression and controls the development of NK1 + T cells, NK cells, and CD8- α/α^+ intestinal intraepithelial lymphocytes (Ohteki and others 1998). IRF2 attenuates IFN responses, and antagonizes IRF1 and IRF9 (Honda and Taniguchi 2006). IRF2 also promotes Th1 responses and NK-cell maturation (Lohoff and Mak 2005). IRF2BP1 and IRF2BP2 are IRF2-dependent transcriptional co-repressors (Childs and Goodbourn 2003). IRF4 promotes Th2 responses, promotes or inhibits apoptosis, and generates antibody and CD8 α -dendritic cells (Lohoff and Mak 2005). Suppression of these pro-inflammatory cell-mediated and cytokine-mediated activities via downregulation of *IRF1*-, *IRF2*-, *IRF4*-, and *IRF2BP1*-associated gene sets may have contributed to the limitation of colitis by AA. IRF7 dimerizes with IRF3 to form a holocomplex, which bind IFN promoters to facilitate IFN expression (Honda and Taniguchi 2006). IRF7 is a key mediator of the IFN positive feedback amplification loop (Honda and Taniguchi 2006). IRF8 promotes Th1 responses and macrophage and dendritic cell differentiation (Lohoff and Mak 2005). IRF9 contributes toward a heterotrimeric complex, which induces IRF7 via Jak-STAT signaling, and autocrine IFN-receptor activation (Honda and Taniguchi 2006). Suppression of these pro-inflammatory cell-mediated and cytokine-mediated activities via downregulation of *IRF8*- and *IRF9*-associated gene sets may have contributed to the limitation of colitis by AA. *IFIH1* is a RNA helicase involved in translation

initiation, nuclear/mitochondrial splicing, and ribosome assembly (Moura and others 2013). It may be involved in gametogenesis, embryogenesis, mitosis, and pathogenesis of autoimmune diseases. *IFI30* is a lysosomal thiol reductase enzyme expressed from antigen-presenting cells, responsible for antigen processing by reducing disulfide bonds of endocytosed proteins (West and Cresswell 2013). *IFI47* is a GTP-binding protein produced in B cells (Gilly and Wall 1992). *IFI35* is a leucine zipper protein expressed in fibroblasts, macrophages, and epithelial cells (Bange and others 1994). *IFI44* binds intracellular GTP, and impedes cell proliferation (Hallen and others 2007). The inhibition of immune cell proliferation induced by *IFI44* may have contributed to the limitation of colitis by AA.

These antiproliferative/immunomodulatory properties of *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44*; and the diverse lymphocytic/Th1-related activity of *IRF4*, *IRF8*, *IRF2BP1*, *IFRD1*, and *IFRD2* (Table 1) may have substantially contributed to the protection offered by AA against colitis in our model (Watson Ng and others 2007; Luo and others 2009; Cheluvappa and others 2011). The antiproliferative, immunomodulatory properties of *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44* are attributes compatible with expected (and direct) therapeutic value. The diverse lymphocytic/Th1-related activity of *IRF4*, *IRF8*, *IRF2BP1*, *IFRD1*, and *IFRD2* (Table 1) are good targets to competitively inhibit in our colitis model, or to treat with exogenous product in knockout animals.

Elucidating IFN activity-related pathways and molecules involved in the persistent protective effect of AA on colitis (Watson Ng and others 2007; Cheluvappa and others 2011, 2014a, 2014b) will enhance the development of approaches and techniques to manipulate different immunomodulatory genes, enzymes, and proteins related to IFN activity. This will enhance the therapeutic options in IBD. Investigating strategies, involving monoclonal antibodies, combinatorial peptides, and small molecules (identified by high-throughput screening) to manipulate and modulate different aspects of IFN-related molecule activity, would augment the development of new therapeutic options to manage IBD.

Suggested genes/molecules to initially expand research on include:

(1) Knockout animal models or exogenous addition/endogenous hyperexpression - *IFIT1*, *IFIT2*, *IFIT3*, *IRF7*, *IFI35*, *IFI44*, *IFNZ*, [*IRF9* and *IFIH1*]; and their corresponding products

- The genes *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44*, are the best targets owing to the magnitude of their upregulation, both by individual gene expression and by GSEA.
- Additionally, *IFIT1*, *IFIT2*, *IFIT3*, and *IFI44* are immunomodulatory and antiproliferative. These properties are compatible with that of expected therapeutic value.
- The mild suppression of B cell lymphopoiesis, and the mild immunomodulatory effects of *IFNZ* (Limitin) can be further investigated (Oritani and Kanakura 2005).

(2) Competitive inhibition or knockout animal models with exogenous molecule compensation-*IRF1*, *IRF2*, *IRF4*, *IRF8*, *IRF9*, *IRF2BP1*, *IFRD1*, *IFRD2*, *IFIH1*, [*IRF2BP2* and *IFI30*]; and their corresponding products

- The genes *IRF4*, *IRF8*, *IRF2BP1*, *IFRD1*, and *IFRD2* are the best targets owing to the magnitude of their downregulation by GSEA.

Addenda

The raw microarray data are available in Gene Expression Omnibus. The accession number for microarray data reported here is GSE23914, and the relevant link is www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23914

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Author Disclosure Statement

No competing financial interests exist.

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