Published in final edited form as:

Inflamm Bowel Dis. 2012 December ; 18(12): 2342–2356. doi:10.1002/ibd.22957.

Immune Markers and Differential Signaling Networks in Ulcerative Colitis and Crohn's Disease

George P. Christophi, MD, PhD^{1,4,*}, Rong Rong, MD, PhD², Philip G. Holtzapple, MD³, Paul T. Massa, PhD¹, and Steve K. Landas, MD²

¹Department of Microbiology & Immunology, SUNY Upstate Medical University, Syracuse NY

²Department of Pathology, SUNY Upstate Medical University, Syracuse NY

³Department of Gastroenterology SUNY Upstate Medical University, Syracuse NY

⁴Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

Abstract

Background & Aims—Cytokine signaling pathways play a central role in the pathogenesis of inflammatory bowel disease (IBD). Ulcerative colitis (UC) and Crohn's disease (CD) have unique as well as overlapping phenotypes, susceptibility genes, and gene expression profiles. This study aimed to delineate patterns within cytokine signaling pathways in colonic mucosa of UC and CD patients, explore molecular diagnostic markers, and identify novel immune-mediators in IBD pathogenesis.

Methods—We quantified 70 selected immune genes that are important in IBD signaling from formalin-fixed, paraffin-embedded (FFPE) colon biopsy samples from normal control subjects and UC and CD patients having either severe colitis or quiescent disease (n=98 subjects). We utilized and validated a new modified real-time RT-PCR technique for gene quantification.

Results—Expression levels of signaling molecules including IL-6/10/12/13/17/23/33, STAT1/3/6, T-bet, GATA3, FOXP3, SOCS1/3, and downstream inflammatory mediators such as chemokines CCL-2/11/17/20, oxidative stress inducers, proteases, and mucosal genes were differentially regulated between UC and CD and between active and quiescent disease. We also document the possible role of novel genes in IBD, including SHP-1, IRF-1, TARC, Eotaxin, NOX2, Arginase I, and ADAM 8.

Conclusions—This comprehensive approach to quantifying gene expression provides insights into the pathogenesis of IBD by elucidating distinct immune signaling networks in CD and UC. Furthermore, this is the first study demonstrating that gene expression profiling in FFPE colon biopsies might be a practical and effective tool in the diagnosis and prognosis of IBD and may help identify molecular markers that can predict and monitor response to individualized therapeutic treatments.

Keywords

inflammatory bowel disease; gene expression; cytokines; JAK/STAT signaling; pathology of colon biopsies

*Corresponding Author: Mailing Address: Washington University School of Medicine Department of Internal Medicine, 660 S. Euclid Ave., Box 8121, St. Louis, MO 63110, gchristo@dom.wustl.edu, Tel: 314-956-9640 .

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic immune-mediated disease of the gastrointestinal tract that affects more than 1.4 million Americans (1). The two broad phenotypic variants of IBD are ulcerative colitis (UC) and Crohn's disease (CD). UC is characterized by diffuse, continuous, superficial and ulcerating inflammation confined to the colon. CD is characterized by patchy mucosal and submucosal inflammation, presence of granulomas in 20% of patients, and formation of fissures, fistulae, and strictures that occur throughout the gastrointestinal tract.

The current working model of IBD pathogenesis posits a dysregulated immune response to gut microbiota, leading to exaggerated self-injurious inflammatory responses (2). Genome wide association studies (GWAS) have identified IBD susceptibility genes, the majority of which are immunoregulatory (3, 4). Although some risk alleles confer increased susceptibility to both UC and CD, other genes are uniquely associated with either UC or CD, suggesting unique pathways of pathogenesis (5, 6). GWAS have uncovered important novel genes involved in cytokine signaling, underscoring the importance of delineating those signaling pathways in IBD.

Inflammatory cytokine pathways play a central role in the pathogenesis of IBD. Cytokine levels are elevated in both IBD mouse models as well as peripheral and mucosal samples of IBD patients (2). Furthermore, blocking cytokine signaling has therapeutic potential in IBD (7). Cytokines activate several pathways including phosphorylation of tyrosine residues of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins, as well as phosphorylation of the inactive I κ B-bound NF- κ B to undergo proteosomal degradation of I κ B and nuclear translocation of NF- κ B (8, 9). Other transcription factors such as interferon regulatory factors (IRFs), T-bet, GATA3, and Foxp3 are transcriptionally regulated and induced by cytokine signaling (10, 11). Specific transcription factor translocation into the nucleus and interaction with conserved regulatory DNA sequences results in the induction of target genes, including more cytokines, receptors, signaling regulatory genes, chemokines, proteases, oxidative stress inducers and other inflammatory molecules.

Early work from patients and IBD mouse models suggested that CD might be predominately mediated by a Th1 immune response through activation of IL-12/STAT4 and IFN- γ /STAT1 signaling pathways whereas UC might be mediated by IL-4/IL-13/STAT6 pathway (12, 13). Although there may be a partial Th1/Th2 polarization of the immune response in CD versus UC, recent studies suggest that other cytokines like TNF- α , IL-1 β , and IL-17A which primarily activate NF- κ B, IL-6 and IL-10 which primarily activate STAT3 have overlapping roles in UC and CD, blurring the Th1/Th2 paradigm (14-17).

This study aims to further expand our knowledge of the signaling pathways involved in IBD. We quantified the expression of 70 immune-related genes implicated in IBD signaling network in 98 colonic biopsy samples having either active or quiescent disease. Many genes previously identified by GWAS or in IBD mouse models or genes shown to be important in signaling pathways are shown to be differentially regulated in UC vs. CD or in active vs. quiescent disease. Importantly, we identified novel genes (SHP-1, IRF-1, TARC, Eotaxin, NOX2, Arginase I, ADAM 8) that are differentially expressed in IBD and represent new potential therapeutic targets. Furthermore, for the first time we demonstrate the feasibility of using formalin-fixed, paraffin-embedded (FFPE) colon biopsy samples to quantify gene expression, which can serve as a novel, practical, and reliable diagnostic and investigative tools in IBD. Our data provide insights into the pathogenesis of IBD through identifying novel immune-mediators and further delineating distinct signaling networks in CD and UC.

METHODS

Colon Biopsy Sample Selection

The study was approved by the Upstate Medical University Institutional Review Board. Archived formalin-fixed paraffin-embedded (FFPE) colon biopsy pathology samples were used for the study. Inclusion criteria were a clinical diagnosis of UC or CD by a gastroenterologist based on standard clinical, laboratory, endoscopic, radiologic, and pathologic criteria. We excluded patients who were receiving biological treatment including anti-TNF- α antibodies within the past year, because the TNF α /NF- κ B pathway is selectively affected in those individuals. We also excluded patients with other immune-mediated diseases such as multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune hepatitis, or active infection including tuberculosis, human immunodeficiency virus infection, cytomegalovirus infection or documented bacterial or protozoan infection. Given the retrospective nature of the study, clinical disease severity was not assessed.

In addition to the clinical diagnosis, the pathologic inclusion criteria for sample selection were applied as previously described (18, 19). Two pathologists independently reviewed slides. The following criteria were used to select samples for each category: 1) normal subjects (NS): normal appearing colon. 2) active UC (aUC): Diffuse continuous colonic disease and no evidence of ileal involvement. Histopathology showed irregularly arranged, branched and shortened crypts: homogeneous, dense, lymphoplasmacytic infiltrate causing expansion of the lamina propria and basal plasmacytosis, crypt abscess and absence of epithelioid granulomas. 3) Quiescent UC (qUC): colonic mucosa from patients having documented UC but no current active inflammation (no evidence of crypt distortion or leukocyte infiltrate). 4) active CD (aCD): segmental disease with no rectal involvement and presence of irregularly arranged, branched and shortened crypts, aphthous or fissuring ulcers and focal, patchy lymphoplasmacytic or neutrophilic infiltrate and in some cases non-mucinous, non-necrotizing, paracryptal granuloma. 5) Quiescent CD (qCD): Colonic mucosa from patients having documented CD without current active inflammation (with no crypt distortion or leukocyte infiltrate). Patients from each category were matched for gender and age. The disease duration in each patient group was calculated by subtracting the age at disease onset from the age at which the sample was taken (Figure 1, Table 1).

RNA isolation and cDNA conversion

RNA was extracted from FFPE tissue using the High Pure FFPE RNA Micro Kit and based on the manufacture's recommendations (Roche cat # 04823125001). Briefly, five shavings ten microns thick from a paraffin block of colon biopsies were deparaffinized by washing the tissue with xylenes subsequently, ethanol (20). Tissue was then digested in SDS and protease K and allowed to adhere to the RNA-binding column. The columns were incubated in DNase and washed with ethanol before RNA was eluted in 50 μ l of water. The quantity of the purified RNA was assessed using the RNA Pico Lab Chip Kit with the Agilent Technologies Bioanalyzer and agarose gel electrophoresis. In addition, RNA was quantified spectrophotometrically and only samples with sufficient quantity (500ng total yield) and appropriate optical density (OD 260/280 ratio = 1.7-2.1) were used for subsequent analysis (21). Samples that did not meet the RNA quality and quantity requirements were excluded from the study. For cDNA synthesis, 0.5 μ g of total RNA from each sample was used. Briefly, 25 μ l volume of the RNA and random hexameric primers (Invitrogen, Carlsbad, CA) were incubated at 72 degrees for 10 minutes. Reverse transcription was performed using the Superscript II RT enzyme (Invitrogen, Carlsbad, CA) and followed the specifications of the manufacturer and were incubated for 30 min at 50 degrees followed by 70 degrees for 15 minutes. cDNA was diluted with sterile water into a volume of 150 μ l (22).

Real time quantitative PCR

Primers were designed to amplify amplicons of 50-150 bp from specific NCBI reference sequences. Primers were designed to span exons and were blasted through NCBI GenBank to ensure lack of homology to other known human cDNA sequences. Quantitative real time PCR was performed with the SYBR Green kit (Abgene, Epsom, UK) and using 2 µl of the cDNA mixture and 10 nM gene-specific DNA primers in a 10 µl reaction. The Roche LightCycler® 480 Real-Time PCR System with 384-well block was used for amplification and the PCR parameters were 15 minutes for 95 degrees, and 45 cycles of 95 degrees for 15 seconds and 60 degrees for 1 minute. Serial dilutions of cDNA containing a known copy number of each gene were used in each quantitative PCR run to generate a standard curve relating copy number with threshold amplification cycle (23). Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve. Amplification of each gene specific fragment was confirmed both by examination of melting peaks and by agarose gel electrophoresis. To further control for equal amount of total RNA loading, parallel examination of the housekeeping genes glyceraldehyde phosphate 3-dehydrogenase (GAPDH), the ribosomal RNA 18S, and the β -actin gene were quantified in each sample (22).

Statistical Analysis

Histograms contain statistical means with the standard error values. The SigmaSTAT software using the unpaired Student's t-test generated the p-values and a p-value of less than 0.05 was chosen to indicate statistical significance between two sample means. The data are presented in histograms showing the average value with error bars representing standard error. In histograms, * designates a p value of 0.05-0.005 between the group and normal subjects, ** if $p < 0.005$ between group and normal subjects, and δ designates a $p < 0.05$ between aUC and aCD.

RESULTS

Validation of methodology

New techniques including more efficient RNA isolation and enzymes, cDNA conversion using random hexamers, and gene-specific primers generating short amplicons (50-200bp) have allowed successful quantification of gene expression using formalin-fixed paraffin embedded tissue. This approach has demonstrated clinical utility, i.e. in assessing recurrence risk in stage II colon cancer (24). Our study utilized several additional measures to validate the technique, including primers designed to span introns, primers that are non-homologous with other human cDNA sequences, and inclusion of negative and positive controls (cDNA vectors with verified DNA sequence of each gene) for each reaction (25-27). Furthermore, the generated PCR product was confirmed with examination of melting peaks and by agarose gel electrophoresis (data not shown). Quantification of abundant internal control genes like GAPDH, β -actin, and S18 rRNA showed similar expression levels among the different samples, suggesting valid RNA quantification and equal input for RT-PCR (Figure 2L, Table II).

Indeed, several studies have shown significant correlation of quantitative PCR gene expression between formalin-fixed and fresh-frozen tissue from the same samples with correlation coefficients of 0.93-0.98 ($P < 0.01$) (25-27). In order to document whether there was any substantial difference in gene expression between fresh tissue and formalin-fixed tissue we quantified the expression of the housekeeping control genes GAPDH, beta-actin, and S18 ribosomal RNA between RNA extracted from ten control fresh frozen tissue and ten control FFPE tissue. There was a significant correlation between the fresh frozen tissue and FFPE tissue in the expression of GAPDH, beta-actin, and S18 rRNA ($P < 0.005$). This

suggests that formalin fixation did not significantly affect the quantification of gene expression when this modified RNA isolation and cDNA amplification technique was utilized.

Cytokines

This study takes a comprehensive approach to quantify selected cytokines, genes mediating signaling, or downstream immunomodulatory or effector molecules shown to be previously important in IBD mouse models, human IBD phenotypes, or novel genes implicated in these signaling networks (Figure 6). Cytokine elevation both in the periphery and within inflamed colon tissue is a predominant feature in IBD (2, 14, 17, 28). Using quantitative real-time RT-PCR we quantified the mRNA expression of several cytokine genes from FFPE colon biopsy tissue of normal subjects (NS), active UC (aUC) and active CD (aCD) and quiescent UC (qUC) and CD (qCD) patients (Figure 1). Biopsy samples labeled having quiescent disease were taken from non-inflamed, normal-appearing colon of IBD patients rather than tissue that had signs of minimal or residual inflammation. Biopsy tissue without inflammatory infiltrate from IBD patients would potentially provide more useful information by correlating with GWAS and underlying genetic or epigenetic changes in immune genes that are not masked by an overt inflammatory response.

The NF- κ B-induced cytokines TNF- α , IL-8, IL-17A, and IL-6 are elevated in active IBD and have important roles in IBD pathogenesis (2). TNF- α was similarly elevated close to 10-fold in aCD and aUC, IL-8 was elevated close to 80-fold in aUC and aCD, IL-17A was 6000-fold and 2000-fold higher in aUC and aCD respectively, and IL-6 was 50-fold and 140-fold higher in aUC and aCD, respectively (Figure 2, Table II). Also slightly higher, but not significant levels, of these cytokines were observed in qUC and qCD compared to NS. The p40 subunit that is shared by IL-12 and IL-23 cytokines which have an important role in polarizing the immune response into Th1 and Th17, respectively (2), was significantly higher in both aUC and aCD compared to normal subjects and p40 was four-fold higher in aCD versus aUC (Figure 2E). IFN- γ , the Th1 hallmark cytokine that is IL-12/STAT4 inducible was slightly elevated in all IBD groups, but was significantly elevated only in aCD compared to NS.

In contrast, IL-13, an important Th2 promoting cytokine that primarily activates STAT6 (29), was significantly elevated in both aUC and qUC compared to NS. Similarly, IL-33, which is highly expressed in colonic epithelial cells and can induce a polarized Th2 response and activate macrophages and mast cells (30, 31), showed a significant 10-fold increase in aUC, and a significant 4- and 3-fold increase in qUC and aCD respectively (Figure 2H). Lastly, both the anti-inflammatory cytokines IL-10 and TGF- β 1 were elevated 4-5 fold in aCD, but only significantly elevated in aUC compared to NS (32, 33). Importantly, we examined the gene expression of innate immunity and cytokine receptors including TLR4, CARD 9, IFN- γ R1, IL-4R α , IL-12R β , and IL-17Ar and found no significant changes among UC, CD, and NS (data not shown).

Transcription Factors

We examined the mRNA expression of the STAT transcription factors, STAT1, STAT3, STAT4, and STAT6. We found no significant differences with the exception of STAT3, which was significantly decreased in aCD compared to normal subjects (Figure 3A, D, F). Next, we examined the expression of the transcription factors T-bet and IRF-1 that are central in promoting a Th1 response and are transcriptionally regulated by STAT4/STAT1/NF- κ B (10). Both T-bet and IRF-1 were significantly elevated in aCD compared to NS and aUC (Figure 3B & C). In contrast, GATA3, which is associated with Th2 responses (11) was significantly elevated 2-fold in UC and significantly reduced 10-fold in aCD compared

to NS and aUC (Figure 3E). Lastly we found that Foxp3, a transcription factor regulated by IL-2/STAT5 and TGF- β 1/SMADs and considered essential for T-regulatory cell differentiation (34, 35) was slightly elevated in aUC but is significantly 3-fold deficient in qUC (Figure 3G).

Signaling Molecules

Next, we examined the expression of genes that mediate cytokine signal transduction. Janus family tyrosine (JAK) kinases are activated following cytokine receptor binding and in turn phosphorylated STATs to mediate gene induction (Figure 6). JAK1, JAK2, and JAK3 mRNA expression was not different among UC, CD, and NS (Figure 3H, Data not shown). We also quantified the expression of suppressors of cytokine signaling (SOCS) that inhibit signaling by direct binding or by preventing access to the signaling complex and through targeting signal transducers for proteasomal destruction (36). SOCS1, which mainly inhibits STAT1 signaling and has been involved in IBD pathogenesis (37), showed a non-significant 4-fold increase in aUC and had a significant 8-fold increase in aCD versus NS (Figure 3I). SOCS3, which primarily inhibits STAT3 signaling, was significantly 14-fold higher in aUC and 6-fold higher in aCD compared to NS (Figure 3J).

Furthermore, we quantified the expression of protein tyrosine phosphatases that inhibit signaling through dephosphorylation of cytokine receptors or transcription factors (38). The protein tyrosine phosphatase non-receptor type 1 (PTPN1) did not show any significant expression difference in colon tissue from IBD patients and normal subjects (data not shown). PTPN2, which has been genetically linked to UC and CD and regulates STAT1, ERK, and p38 signaling (39, 40) was non-significantly elevated in aUC and aCD (Figure 3K). Importantly, the phosphatase SHP-1 (PTPN6) that negatively regulates STAT1/3/6 and NF- κ B signaling (41) was significantly reduced in aUC, qUC, and aCD compared to NS (Figure 3L). Lastly, the phosphatase SHP-2 (PTPN11), which modulates STAT1/3/5 signaling (38) and PTPN22 that modulates T-cell receptor signaling, did not show any gene expression changes in UC or CD compared to NS (data not shown).

Chemokines and Adhesion molecules

Chemokines recruit immune cells to inflammatory sites and play a major role in the pathogenesis of IBD (42, 43). Chemokines have distinct chemoattractant properties dictated by their cell-specific expression, differential cytokine regulation, and chemokine receptor cell-specific expression, abundance, and specificity. The monocyte chemoattractant protein MCP-1 (CCL2), which is NF- κ B-inducible, mediates monocytes chemoattraction and bone marrow egress, and had been genetically associated with CD (44, 45) showed a 20- and 30-fold increase in aUC and aCD respectively compared to NS (Figure 4A, Table II). In turn, the MCP-1 chemokine receptor CCR2, which is a marker for activated monocytes and macrophages, showed a significantly 20- and 40-fold higher expression in aUC and aCD respectively compared to NS (Figure 4B). In addition, the chemokine CCL20, which predominantly chemoattracts dendritic cells and lymphocytes (46, 47), showed a significant 15- and 10-fold increase in aUC and aCD, respectively (Figure 4C). Importantly, the CCL20 chemokine receptor CCR6, which is also genetically associated with IBD, showed a significant 4-fold increase in aUC and a significant 7-fold decrease in qCD (Figure 4D).

Next we examined the expression of the interferon gamma-induced protein IP-10/CXCL10, which showed a significant 15-fold and 40-fold increase in aUC and qCD, respectively, compared to NS. In contrast, the thymus and activation-regulated chemokine TARC/CCL17 that is STAT6 inducible and attracts Th2 cells showed a significant 20-fold increase in colon tissue from aUC. Similarly, eotaxin-1/CCL11 showed a significant 15-fold increase in aUC and a significant 50-fold increase in qUC. In addition, we quantified the expression of the

chemokine receptors CCR4 that binds MCP-1, CCL5, and CCL22, CCR8 that binds TARC and MIP-1 β , and CXCR3 that binds IP-10 and found no gene expression difference among UC, CD, and NS (data not shown).

Lastly, we quantified the expression adhesion molecules that are expressed in endothelial cells and bind to integrins on immune cells to facilitate trafficking into areas of inflammation (48). Intercellular adhesion molecule-1 (ICAM-1) that binds $\alpha_2\beta_2$ integrin (LFA-1) was slightly increased in aUC and qCD, but showed a significant 9-fold increase in aCD (Figure 4H). Furthermore, vascular cell adhesion molecule-1 (VCAM-1) that binds $\alpha_4\beta_1$ integrin (VLA-4) was slightly increased in aCD, but showed a significant 10- and 5-fold increase in aUC and qUC, respectively (48, 49).

Oxidative Stress inducers

Next we quantified the expression of downstream inflammatory genes that directly cause tissue damage by mediating the production of reactive oxygen species and toxic molecules that directly contribute to tissue injury (50). Gene expression for cyclooxygenase 2 (COX2), which may play an important role in IBD and mediates prostaglandin production through fatty acid oxidation and radical reactions (51, 52), was significantly increased 100-fold in both aUC and aCD compared to normal subjects and did not show any changes in qUC and qCD (Figure 5A). The NADPH oxidase subunit gp91phox (Nox2), which is highly expressed in phagocytes and produces reactive oxygen species (53), was elevated 4-fold in aUC and 6-fold in aCD. Additionally the inducible nitric oxide synthase (iNOS) that also contributes to apoptosis and injury through reactive oxygen species generation (54) was significantly upregulated 5-fold in aUC, 10-fold in aCD, and had a non-significant increase in qCD compared to NS (Figure 5C). Lastly, the enzyme arginase-I that is STAT6-inducible and can mediate inflammation and oxidative stress through polyamine production (55) was significantly 4-fold elevated in aUC and significantly 3-fold reduced in aCD compared to NS.

Proteases

We also quantified the expression of proteolytic enzymes, such as caspase 1 that contributes to apoptosis, cleaves IL-1 β and IL-18 into their active secreted forms, and has been implicated in IBD pathogenesis (56). Caspase 1 was elevated 3-fold in aUC and significantly elevated 4-fold in aCD compared to NS (Figure 5E). Furthermore, the metalloprotease MMP3 that degrades fibronectin, laminin, and collagens and directly correlates with IBD activity (57) was significantly 175-fold and 75-fold higher in aUC and aCD respectively, compared to NS. Lastly, the membrane anchored proteinase with a disintegrin and a metalloproteinase domain-8 (ADAM8) that is involved in extracellular matrix proteolysis, cell adhesion, and shedding of signaling molecules (58) was significantly 3-fold elevated in aUC (Figure 5G).

Mucosal defense and proliferation

In addition, several genes that are important in colonic mucosa homeostasis were characterized (Figure 5 H-L). Mucins are glycosylated proteins expressed on the apical surface of epithelial cells and have important functions in mucosal antimicrobial defense, cell signaling, and carcinogenesis (59). Mucin 1 (MUC1) expression was slightly higher in aUC and aCD and only significantly 4-fold higher in qCD, which may represent an adaptation to mucosal injury. The mRNA expression of the NF- κ B-inducible MUC5ac was significantly 1200-fold and 15-fold higher in aUC and aCD, respectively. In addition, the intestinal trefoil factor TFF3, which is secreted by goblet cells and may be involved in cell migration, mucosa; healing, and tumor suppression (60, 61), showed a significant 2-fold reduction in aUC compared to NS. Defensin β -2, an antimicrobial peptide and part of

mucosa innate immune response (62), was significantly increased around 8-fold in aUC and aCD versus NS (Figure 5K). Importantly, in normal appearing colon of qCD, defensin β -2 was significantly 10-fold elevated. Lastly the NF- κ B-inducible anti-apoptotic molecule Bcl-2 was significantly 5-fold elevated in aUC and had a non-significant increase in aCD.

CONCLUSION

Through a focused approach this study quantified the expression of several cytokines, respective signaling molecules, and their downstream inflammatory gene expression. The importance of these signaling pathways is underscored by recent finding of GWASs studies demonstrating that numerous genes associated with IBD are major cytokine signaling regulators (3, 4) and from promising new IBD treatments that target these pathways (7) (Figure 6). Utilizing improved RNA isolation techniques, cDNA conversion enzymes and random primers, as well as modified quantitative real time PCR, we quantified the expression of more than 60 genes from 98 FFPE colon biopsy samples of IBD patients. Several of the quantified genes are differentially regulated in inflamed colonic mucosa tissue from UC versus CD patients and some genes were shown to be modulated in histologically normal-appearing colon of IBD patients (Table 2). Notably, many immunomodulatory genes including the IBD susceptibility genes TLR4, CARD 9, STAT6, JAK2, and PTPN22 were expressed at equal levels among samples from normal subjects and IBD patients, which suggests the presence of a different molecular mechanism in implicating these genes in IBD pathogenesis such as mRNA splicing, changes in protein expression, localization, and post-translational modification rather than mRNA transcription. Importantly, we also demonstrate significant differences in novel genes like SHP-1, IRF-1, TARC, Eotaxin, NOX2, Arginase I, and ADAM 8 that may represent new potential investigative and therapeutic targets for IBD.

In the active inflamed colon mucosa of UC and CD patients, there is a cytokine storm, and particularly the cytokines TNF- α , IL-17A, and IL-6 were substantially elevated, indicating that an exaggerated/prolonged NF- κ B stimulation is an overlapping characteristic of the immune response in IBD (Table II). The Th2 skewing cytokines IL-13 and IL-33, the Th2 transcription factor GATA3, and the STAT6-inducible genes arginase-1 and the chemokines TARC and eotaxin-1, were substantially increased in the inflamed colon of UC patients compared to CD patients. In contrast, the cytokines IL-12/23 p40 subunit and IFN- γ along with the transcription factors IRF-1 and T-bet and the chemokine IP-10, which are all associated with STAT1/4 signaling and Th1 immune response, were noticeably more elevated in active CD. Taken together, these data support earlier findings suggesting that UC is associated with a moderately polarized Th2 immune response and CD is associated with a Th1 immune response (12, 13, 17). Importantly, in addition to the NF- κ B-inducing and inducible cytokines, IL-12/23 p40, IFN- γ , IL-33, and IP-10 were also expressed at lower levels in either UC and CD contributing to a blurred dichotomy between Th1 and Th2 immune response.

In addition to inflammatory signaling genes we examined the expression of regulatory cytokines and transcription factors along with signaling regulators like kinases and phosphatases (Table II). The anti-inflammatory cytokine IL-10 that can mediate NF- κ B inhibition and the TGF- β 1 cytokine that induces Foxp3 expression in regulatory T-cells (Tregs), were slightly elevated in CD and significantly elevated in UC. Interestingly, the expression of Foxp3, a marker and necessary transcription factor for T-regulatory cells, was significantly reduced in normal appearing colon of UC patients, suggesting that this deficiency might allow for the heightened immune response in active UC and this deficiency might be masked by inflammation in active disease. Also, we examined several important members of the JAK-STAT signaling cascade including STAT1/3/4/6 and JAK1/2/3 and only saw a significant decrease of the STAT3 gene in active CD, which could represent a

compensatory mechanism of reducing IL-6/IL-10 signaling or deficiency in expression, especially in the light that STAT3 is genetically associated with CD. Although changes in mRNA levels of STAT genes are expected to have a notable effect on signal transduction, cytokines activate the JAK/STAT signaling cascade through phosphorylation and increased STAT phosphorylation is a prominent feature of active IBD (9, 29, 63).

We were also very interested in quantifying the expression of inhibitors of JAK/STAT and NF- κ B signaling because they have overlapping functions in inhibiting several signaling events and in turn effectively dampening the augmented inflammatory response in IBD. First, the cytokine-inducible SOCS1 and SOCS3 that inhibits STAT1/STAT3 activation through direct binding to signaling complexes were elevated in UC and CD thus establishing potential feedback inhibition to signal transduction (28, 36, 37). The tyrosine phosphatases PTPN1 and SHP-2 (PTPN11), along with the phosphatases PTPN2 and PTPN22 that are genetically linked to IBD, did not show significant difference in expression. In contrast, the protein tyrosine phosphatase SHP-1 was significantly decreased in active UC and CD and quiescent UC (Figure 3L). SHP-1 is a tumor suppressor gene and a master negative regulator of proinflammatory cytokine signaling shown to inhibit STAT1/3/6, NF- κ B, and T-cell receptor signaling (22, 64, 65). Decreased SHP-1 gene expression secondary to promoter hypermethylation has been documented in other autoimmune diseases, myeloproliferative disorders and colon cancer cell lines (41, 66, 67). More research is needed to characterize the expression and regulation of SHP-1 in UC and CD patients that might be at least partly responsible for the dysregulated and pronounced cytokine signaling seen in IBD.

In addition to characterizing genes regulating signaling, numerous downstream inflammatory genes were differentially regulated in UC and CD with emphasis on the role in tissue injury and transcriptional regulation (Table II and Figure 6). Chemokines were dramatically elevated in active UC and CD and the chemokine IP-10 was elevated in quiescent CD and eotaxin-1 was elevated in quiescent UC. It is important to note that histologically normal-appearing colon in IBD has substantial elevation in inflammatory gene expression suggesting that tissue lymphocytes and epithelial cells bathed in blood-borne and adjacent tissue cytokines are constantly activated and might have important implication in clinical relapse and remission as well as cancer progression (68-70). Furthermore, adhesion molecules that facilitate immune cell entry in the mucosa/submucosal layer were elevated in active UC and CD with ICAM-1 significantly elevated in aCD and VCAM-1 being significantly elevated in aUC (48, 49). This is important in light of potential and approved IBD treatments targeting the integrins that bind these adhesion molecules (7).

Another source of tissue injury and antimicrobial activity is the production of reactive oxygen species (ROS) and free radicals generated by COX2, NADPH subunit Nox2, iNOS, and Arginase I (50, 51, 53, 71). Also, ROS have been shown to regulate the autophagy pathway and to lead to proliferative changes (72). All of the above enzymes were upregulated in active CD and UC, with the exception of the STAT6-inducible arginase-1 that was elevated in aUC and showed a significantly reduced expression in active CD. Arginase-1 is highly expressed by myeloid suppressor cells and epithelial cells and has important roles in gastrointestinal defense against parasites and modulates innate immunity through L-arginine availability and polyamine production (55, 73-75). The decreased arginase-1 expression in aCD might be secondary to a polarized Th1 response seen in CD, which in turn might result in a decreased number of myeloid suppressor cells in CD. Other genes upregulated in inflamed mucosa of UC and CD patients included caspase-1 that promotes secretion of IL-1, IL-18, and IL-33 and the proteases MMP3 and ADAM8, Mucin 5ac, Defensin β -2 and Bcl-2.

Many of the downstream genes are characterized by extensive transcriptional regulation, where their promoter has several active sites for different transcription factors including NF- κ B, STATs, IRFs, and AP-1, thus contributing to the redundancy of the inflammatory response and widespread expression of these genes in inflamed tissue. In addition, infectious ligands stimulating innate immune receptors like TLRs and NODs and secreted cytokines have considerable overlap in signaling through the same receptor subunits and transcription factors effectively activating similar signaling pathways (Figure 6). Consequently, an amplified immune response is seen in IBD and thus it may be difficult to delineate and therapeutically inhibit distinct pathways in UC versus CD patients.

Although these results are potentially applicable to IBD pathogenesis, there are some inherent limitations of this study. Disease severity was based on strict histopathologic criteria allowing for defined and homogeneous groups regarding tissue inflammation, but the study lacks direct clinical correlation of disease severity and sub-classification to gene expression levels. Alternately, mucosal inflammation as used in this study might be a better marker of disease activity allowing more analogous study groups (76). Furthermore, this study utilized whole colon biopsy tissue limiting our ability to examine the cellular localization and expression of genes. On the other hand, this study, using whole tissue has the advantage of becoming a practical diagnostic tool that requires minimal expertise, cost and time to perform. Importantly, this is the first study demonstrating that quantifying gene expression in FFPE colon biopsies is a feasible and practical approach to develop diagnostic and prognostic markers in IBD. In prospective studies we plan to correlate our current findings with several clinical parameters, allelic polymorphisms in IBD, and cellular localization of novel genes mostly focusing on novel genes and genes that are highly modulated or differentially expressed in IBD (Figure 7). GWAS analysis has uncovered important novel genes and pathways implicated in IBD, necessitating knowledge of the transcriptional regulation and understanding of the signaling networks. Moreover, several biologic agents targeting TNF- α , IL-12/23 p40, IL-6, α 4 integrins, ICAM-1, CCR9, CD40, MyD88 are current or promising IBD treatments and this study provides a potential approach that can be used to identify molecular markers to help select and predict responses to individual therapies. This study might form the basis for providing a new outline in understanding, categorizing, and treating IBD.

Acknowledgments

We would like to extend our deepest gratitude to Dr. Themistocles Dassopoulos, Director of Inflammatory Bowel Diseases Program, Gastroenterology Division at Washington University School of Medicine for his encouragement and critical manuscript revisions and data interpretation. We would like to thank Dr. Isobel Scarisbrick at the Mayo Clinic for providing the cDNA plasmids used in performing real time RT-PCR, Dr. Ross Gruber at Albert Einstein College of Medicine for assisting with primer design and optimization and figures, Dr. Frank Middleton at Upstate Graduate School for providing equipment and helping with statistical analysis, and Chiharu Kumagai for helping with mRNA quality control and cDNA conversion. This work was supported in part by research grants from an NIH grant (NS041593) to Dr. Paul Massa and institutional funding to Dr. George Christophi and Dr. Steve Landas. None of the authors has any potential financial conflict of interest related to this manuscript and the results of this report have been generated, analyzed, and interpreted independently of any outside participation or influence.

REFERENCES

1. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med*. 2009; 361:2066–2078. [PubMed: 19923578]
2. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol*. 2010; 28:573–621. [PubMed: 20192811]
3. McGovern DP, Gardet A, Torkvist L, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet*. 2010; 42:332–337. [PubMed: 20228799]

4. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008; 40:955–962. [PubMed: 18587394]
5. Budarf ML, Labbe C, David G, et al. GWA studies: rewriting the story of IBD. *Trends Genet.* 2009; 25:137–146. [PubMed: 19217684]
6. Thompson AI, Lees CW. Genetics of ulcerative colitis. *Inflamm Bowel Dis.* 2010
7. Melmed GY, Targan SR. Future biologic targets for IBD: potentials and pitfalls. *Nat Rev Gastroenterol Hepatol.* 2010; 7:110–117. [PubMed: 20134493]
8. Atreya I, Atreya R, Neurath MF. NF-kappaB in inflammatory bowel disease. *J Intern Med.* 2008; 263:591–596. [PubMed: 18479258]
9. O'Shea JJ, Lahesmaa R, Vahedi G, et al. Genomic views of STAT function in CD4+ T helper cell differentiation. *Nat Rev Immunol.* 2011; 11:239–250. [PubMed: 21436836]
10. Neurath MF, Weigmann B, Finotto S, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med.* 2002; 195:1129–1143. [PubMed: 11994418]
11. Zhu J, Yamane H, Cote-Sierra J, et al. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res.* 2006; 16:3–10. [PubMed: 16467870]
12. Camoglio L, Te Velde AA, Tigges AJ, et al. Altered expression of interferon-gamma and interleukin-4 in inflammatory bowel disease. *Inflamm Bowel Dis.* 1998; 4:285–290. [PubMed: 9836081]
13. Parronchi P, Romagnani P, Annunziato F, et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol.* 1997; 150:823–832. [PubMed: 9060820]
14. Scaldaferrri F, Correale C, Gasbarrini A, et al. Mucosal biomarkers in inflammatory bowel disease: key pathogenic players or disease predictors? *World J Gastroenterol.* 2010; 16:2616–2625. [PubMed: 20518083]
15. Liu ZJ, Yadav PK, Su JL, et al. Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol.* 2009; 15:5784–5788. [PubMed: 19998498]
16. Rogler G, Brand K, Vogl D, et al. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology.* 1998; 115:357–369. [PubMed: 9679041]
17. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol.* 2008; 14:4280–4288. [PubMed: 18666314]
18. Lodenkemper C. Diagnostic standards in the pathology of inflammatory bowel disease. *Dig Dis.* 2009; 27:576–583. [PubMed: 19897978]
19. Odze R. Diagnostic problems and advances in inflammatory bowel disease. *Mod Pathol.* 2003; 16:347–358. [PubMed: 12692200]
20. Antica M, Paradzik M, Novak S, et al. Gene expression in formalin-fixed paraffin-embedded lymph nodes. *J Immunol Methods.* 2010; 359:42–46. [PubMed: 20570676]
21. Christophi GP, Isackson PJ, Blaber S, et al. Distinct promoters regulate tissue-specific and differential expression of kallikrein 6 in CNS demyelinating disease. *J Neurochem.* 2004; 91:1439–1449. [PubMed: 15584920]
22. Christophi GP, Panos M, Hudson CA, et al. Macrophages of multiple sclerosis patients display deficient SHP-1 expression and enhanced inflammatory phenotype. *Lab Invest.* 2009; 89:742–759. [PubMed: 19398961]
23. Christophi GP, Massa PT. Central neuroinvasion and demyelination by inflammatory macrophages after peripheral virus infection is controlled by SHP-1. *Viral Immunol.* 2010; 22:371–387. [PubMed: 19951174]
24. Clark-Langone KM, Sangli C, Krishnakumar J, et al. Translating tumor biology into personalized treatment planning: analytical performance characteristics of the Oncotype DX(R) Colon Cancer Assay. *BMC Cancer.* 2010; 10:691. [PubMed: 21176237]
25. Sanchez-Navarro I, Gamez-Pozo A, Gonzalez-Baron M, et al. Comparison of gene expression profiling by reverse transcription quantitative PCR between fresh frozen and formalin-fixed, paraffin-embedded breast cancer tissues. *Biotechniques.* 2010; 48:389–397. [PubMed: 20569212]

26. April C, Klotzle B, Royce T, et al. Whole-genome gene expression profiling of formalin-fixed, paraffin-embedded tissue samples. *PLoS One*. 2009; 4:e8162. [PubMed: 19997620]
27. Green TM, de Stricker K, Moller MB. Validation of putative reference genes for normalization of Q-RT-PCR data from paraffin-embedded lymphoid tissue. *Diagn Mol Pathol*. 2009; 18:243–249. [PubMed: 19861891]
28. Leon AJ, Gomez E, Garrote JA, et al. High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas from patients with IBD. *Mediators Inflamm*. 2009; 2009:580450. [PubMed: 19657406]
29. Rosen MJ, Frey MR, Washington MK, et al. STAT6 activation in ulcerative colitis: A new target for prevention of IL-13-induced colon epithelial cell dysfunction. *Inflamm Bowel Dis*. 2011
30. Pastorelli L, Garg RR, Hoang SB, et al. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc Natl Acad Sci U S A*. 2010; 107:8017–8022. [PubMed: 20385815]
31. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol*. 2010; 10:103–110. [PubMed: 20081870]
32. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010; 10:170–181. [PubMed: 20154735]
33. Taylor AW. Review of the activation of TGF-beta in immunity. *J Leukoc Biol*. 2009; 85:29–33. [PubMed: 18818372]
34. Yoshimura A, Wakabayashi Y, Mori T. Cellular and molecular basis for the regulation of inflammation by TGF-beta. *J Biochem*. 2010; 147:781–792. [PubMed: 20410014]
35. Yu QT, Saruta M, Avanesyan A, et al. Expression and functional characterization of FOXP3+ CD4+ regulatory T cells in ulcerative colitis. *Inflamm Bowel Dis*. 2007; 13:191–199. [PubMed: 17206665]
36. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol*. 2007; 7:454–465. [PubMed: 17525754]
37. Horino J, Fujimoto M, Terabe F, et al. Suppressor of cytokine signaling-1 ameliorates dextran sulfate sodium-induced colitis in mice. *Int Immunol*. 2008; 20:753–762. [PubMed: 18381351]
38. Xu D, Qu CK. Protein tyrosine phosphatases in the JAK/STAT pathway. *Front Biosci*. 2008; 13:4925–4932. [PubMed: 18508557]
39. Scharl M, McCole DF, Weber A, et al. Protein tyrosine phosphatase N2 regulates TNFalpha-induced signalling and cytokine secretion in human intestinal epithelial cells. *Gut*. 2011; 60:189–197. [PubMed: 21115548]
40. Doody KM, Bourdeau A, Tremblay ML. T-cell protein tyrosine phosphatase is a key regulator in immune cell signaling: lessons from the knockout mouse model and implications in human disease. *Immunol Rev*. 2009; 228:325–341. [PubMed: 19290937]
41. Christophi GP, Hudson CA, Gruber RC, et al. SHP-1 deficiency and increased inflammatory gene expression in PBMCs of multiple sclerosis patients. *Lab Invest*. 2008; 88:243–255. [PubMed: 18209728]
42. Zimmerman NP, Vongsa RA, Wendt MK, et al. Chemokines and chemokine receptors in mucosal homeostasis at the intestinal epithelial barrier in inflammatory bowel disease. *Inflamm Bowel Dis*. 2008; 14:1000–1011. [PubMed: 18452220]
43. Arijis I, De Hertogh G, Machiels K, et al. Mucosal gene expression of cell adhesion molecules, chemokines, and chemokine receptors in patients with inflammatory bowel disease before and after infliximab treatment. *Am J Gastroenterol*. 2011; 106:748–761. [PubMed: 21326222]
44. Palmieri O, Latiano A, Salvatori E, et al. The -A2518G polymorphism of monocyte chemoattractant protein-1 is associated with Crohn's disease. *Am J Gastroenterol*. 2010; 105:1586–1594. [PubMed: 20125127]
45. Christophi GP, Massa PT. Central neuroinvasion and demyelination by inflammatory macrophages after peripheral virus infection is controlled by SHP-1. *Viral Immunol*. 2009; 22:371–387. [PubMed: 19951174]
46. Rodriguez-Bores L, Fonseca GC, Villeda MA, et al. Novel genetic markers in inflammatory bowel disease. *World J Gastroenterol*. 2007; 13:5560–5570. [PubMed: 17948929]

47. Kaser A, Ludwiczek O, Holzmann S, et al. Increased expression of CCL20 in human inflammatory bowel disease. *J Clin Immunol.* 2004; 24:74–85. [PubMed: 14997037]
48. Ghosh S, Panaccione R. Anti-adhesion molecule therapy for inflammatory bowel disease. *Therap Adv Gastroenterol.* 2010; 3:239–258.
49. Wang C, Hanly EK, Wheeler LW, et al. Effect of alpha4beta7 blockade on intestinal lymphocyte subsets and lymphoid tissue development. *Inflamm Bowel Dis.* 2010; 16:1751–1762. [PubMed: 20848481]
50. Rezaie A, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci.* 2007; 52:2015–2021. [PubMed: 17404859]
51. Singer II, Kawka DW, Schloemann S, et al. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology.* 1998; 115:297–306. [PubMed: 9679035]
52. Cox DG, Crusius JB, Peeters PH, et al. Haplotype of prostaglandin synthase 2/cyclooxygenase 2 is involved in the susceptibility to inflammatory bowel disease. *World J Gastroenterol.* 2005; 11:6003–6008. [PubMed: 16273614]
53. Rokutan K, Kawahara T, Kuwano Y, et al. Nox enzymes and oxidative stress in the immunopathology of the gastrointestinal tract. *Semin Immunopathol.* 2008; 30:315–327. [PubMed: 18521607]
54. Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology.* 2004; 113:427–437. [PubMed: 15554920]
55. Hong SK, Maltz BE, Coburn LA, et al. Increased serum levels of L-arginine in ulcerative colitis and correlation with disease severity. *Inflamm Bowel Dis.* 2010; 16:105–111. [PubMed: 19637336]
56. Siegmund B. Interleukin-1beta converting enzyme (caspase-1) in intestinal inflammation. *Biochem Pharmacol.* 2002; 64:1–8. [PubMed: 12106600]
57. von Lampe B, Barthel B, Coupland SE, et al. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut.* 2000; 47:63–73. [PubMed: 10861266]
58. Knolle MD, Owen CA. ADAM8: a new therapeutic target for asthma. *Expert Opin Ther Targets.* 2009; 13:523–540. [PubMed: 19397475]
59. Corfield AP, Carroll D, Myerscough N, et al. Mucins in the gastrointestinal tract in health and disease. *Front Biosci.* 2001; 6:D1321–1357. [PubMed: 11578958]
60. Taupin DR, Kinoshita K, Podolsky DK. Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proc Natl Acad Sci U S A.* 2000; 97:799–804. [PubMed: 10639160]
61. Dossinger V, Kayadmir T, Blin N, et al. Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors. *Cell Physiol Biochem.* 2002; 12:197–206. [PubMed: 12297725]
62. Wehkamp J, Fellermann K, Herrlinger KR, et al. Human beta-defensin 2 but not beta-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. *Eur J Gastroenterol Hepatol.* 2002; 14:745–752. [PubMed: 12169983]
63. Mudter J, Weigmann B, Bartsch B, et al. Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am J Gastroenterol.* 2005; 100:64–72. [PubMed: 15654782]
64. Lopez-Ruiz P, Rodriguez-Ubreva J, Cariaga AE, et al. SHP-1 in cell-cycle regulation. *Anticancer Agents Med Chem.* 2011; 11:89–98. [PubMed: 21291405]
65. Massa PT, Wu C. Increased inducible activation of NF-kappaB and responsive genes in astrocytes deficient in the protein tyrosine phosphatase SHP-1. *J Interferon Cytokine Res.* 1998; 18:499–507. [PubMed: 9712366]
66. Xu SB, Liu XH, Li BH, et al. DNA methylation regulates constitutive expression of Stat6 regulatory genes SOCS-1 and SHP-1 in colon cancer cells. *J Cancer Res Clin Oncol.* 2009; 135:1791–1798. [PubMed: 19551406]
67. Nakase K, Cheng J, Zhu Q, et al. Mechanisms of SHP-1 P2 promoter regulation in hematopoietic cells and its silencing in HTLV-1-transformed T cells. *J Leukoc Biol.* 2009; 85:165–174. [PubMed: 18948549]

68. Rouleau J, MacLeod AR, Szyf M. Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway. *J Biol Chem*. 1995; 270:1595–1601. [PubMed: 7829490]
69. Valinluck V, Sowers LC. Inflammation-mediated cytosine damage: a mechanistic link between inflammation and the epigenetic alterations in human cancers. *Cancer Res*. 2007; 67:5583–5586. [PubMed: 17575120]
70. Valinluck V, Sowers LC. Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. *Cancer Res*. 2007; 67:946–950. [PubMed: 17283125]
71. Deshane J, Zmijewski JW, Luther R, et al. Free radical-producing myeloid-derived regulatory cells: potent activators and suppressors of lung inflammation and airway hyperresponsiveness. *Mucosal Immunol*. 2011
72. Huang J, Brumell JH. NADPH oxidases contribute to autophagy regulation. *Autophagy*. 2009; 5:887–889. [PubMed: 19550142]
73. Bowcutt R, Bell LV, Little M, et al. Arginase-1 Expressing Macrophages are Dispensable for Resistance to Infection with the Gastrointestinal Helminth *Trichuris muris*. *Parasite Immunol*. 2011
74. Peranzoni E, Marigo I, Dolcetti L, et al. Role of arginine metabolism in immunity and immunopathology. *Immunobiology*. 2007; 212:795–812. [PubMed: 18086380]
75. Bonaparte KL, Hudson CA, Wu C, et al. Inverse regulation of inducible nitric oxide synthase (iNOS) and arginase I by the protein tyrosine phosphatase SHP-1 in CNS glia. *Glia*. 2006; 53:827–835. [PubMed: 16565987]
76. Stidham RW, Higgins PD. Value of mucosal assessment and biomarkers in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol*. 2010; 4:285–291. [PubMed: 20528116]

Figure 1. Histology with Hematoxylin and Eosin staining representative of the colon biopsy tissue samples used in the study

Colon biopsy samples from IBD patients based on clinical history, endoscopic findings, and histopathological classification were used in the study. Disease severity was only determined by histopathologic criteria determined by two independent pathologists. **A.** Normal appearing colon from normal subjects (NS) **B.** Quiescent ulcerative colitis (qUC) – histologically normal-appearing colonic mucosa from a patient having documented UC based on both clinical and pathologic criteria, **C.** Severe inflammation in active UC (aUC), **D.** Quiescent Chron’s Disease (qCD) - histologically normal-appearing colonic mucosa from a patient having documented CD based on both clinical and pathologic criteria. The magnification in the pictures is 40 with the scale on the images showing 100 microns.

Figure 2. Cytokine gene expression in IBD

FFPE Colon biopsy samples from normal subjects (NS), active ulcerative colitis (aUC), quiescent UC (qUC), active Crohn's Disease (aCD), and quiescent CD (qCD) were used to quantify gene expression using real time RT-PCR. Gene expression was normalized to NS, which was set to be 1. In histograms, * demonstrates a p value of 0.05-0.005 between the group and normal subjects, ** if $p < 0.005$ between group and normal subjects, and δ if the $p < 0.05$ between aUC and aCD.

Figure 3. Gene expression of mediators and modulators of cytokine signaling including transcription factors and phosphatase in IBD

FFPE Colon biopsy samples from normal subjects (NS), active ulcerative colitis (aUC), quiescent UC (qUC), active Crohn's Disease (aCD), and quiescent CD (qCD) were used to quantify gene expression using real time RT-PCR. Gene expression was normalized to NS, which was set to be 1. In histograms, * demonstrates a p value of 0.05-0.005 between the group and normal subjects, ** if p<0.005 between group and normal subjects, and δ if the p<0.05 between aUC and aCD.

Figure 4. Gene expression of chemokines, chemokine receptors, and adhesion molecules in IBD FFPE Colon biopsy samples from normal subjects (NS), active ulcerative colitis (aUC), quiescent UC (qUC), active Crohn's Disease (aCD), and quiescent CD (qCD) were used to quantify gene expression using real time RT-PCR. Gene expression was normalized to NS, which was set to be 1. In histograms, * demonstrates a p value of 0.05-0.005 between the group and normal subjects, ** if p<0.005 between group and normal subjects, and δ if the p<0.05 between aUC and aCD.

Figure 5. Gene expression of cytokine-induced inflammatory genes that can mediate tissue injury in IBD

FFPE Colon biopsy samples from normal subjects (NS), active ulcerative colitis (aUC), quiescent UC (qUC), active Crohn's Disease (aCD), and quiescent CD (qCD) were used to quantify gene expression using real time RT-PCR. Gene expression was normalized to NS, which was set to be 1. In histograms, * demonstrates a p value of 0.05-0.005 between the group and normal subjects, ** if p<0.005 between group and normal subjects, and δ if the p<0.05 between aUC and aCD.

Figure 6. Schematic summarizing the cytokine signaling pathways involved in IBD pathogenesis that were quantified in this study

* Designates that there is documented genetic association of the genes with IBD.

Figure 7. Venn diagram demonstrating gene expression in active or quiescent ulcerative colitis and Crohn's disease

Potential genes that could be used in prospective studies to differentiate between UC and CD and correlate to disease severity.

Table I
Biometric data of controls (normal subjects) and IBD patients used in the study

The data are shown in mean value $\pm$ SD. The age of the subjects during biopsy is shown in years. F stands for females and M stands for males. The disease duration in years at the time of biopsy is shown for each group as the mean value $\pm$ SD. The average onset of disease can be calculated by subtracting the disease duration from the average age of the subjects.

Patient Category	Number of Subjects	Age (Years)	Gender	Disease Duration
Controls	25	34 $\pm$ 15	12F, 13M	-----
Active UC	21	35 $\pm$ 14	9F, 12M	6.7 $\pm$ 5.2
Inactive UC	17	38 $\pm$ 12	8F, 9M	7.8 $\pm$ 6.8
Active CD	20	29 $\pm$ 13	9F, 11M	5.7 $\pm$ 6.3
Inactive CD	15	34 $\pm$ 16	6F, 9M	5.3 $\pm$ 5.7

Table II
Summary of gene expression in IBD along with functions and transcriptional regulation

Genes were quantified in this study with real time RT-PCR using FFPE colon biopsy samples from active UC (aUC), active CD (aCD), quiescent/histologically normal-appearing colon from UC (qUC), and quiescent CD (qCD).

Category	Transcription Regulation	Function	Gene	Active UC	Quiescent UC	Active CD	Quiescent CD
Cytokines	TLR/NFκB	+NF-κB/Apoptosis	TNF-α	++	NC	++	NC/+
	NF-κB/API	+Neutroph/CXCR1/2	IL-8	+++	NC	+++	NC
	NF-κB/STAT3	+NF-κB	IL-17	++++	NC	++++	NC
	*NF-κB	+STAT3, gp130, RAS	IL-6	+++	NC	++++	NC
	*TLRs, STAT1, IRF	+STAT4, Th1/Th17	IL-12/23 p40	+	NC	+++	NC
	*STAT4	+STAT1/STAT2	IFN-γ	NC/+	NC	++	NC/+
	Sp1/3, Antigen	+STAT6/PI3K	IL-13	+++	+	NC	NC
	TRAF6/NF-κB/API	+NF-κB/sI2/MAPK	IL-33	++	+	+	NC
	*Multiple I	+STAT3, +Treg	IL-10	+++	NC	NC/+	NC
	Activation/Secretion	+SMADs, +Treg	TGF-β1	+	NC	NC/+	NC
Transcription Factors	STAT1/NFκB	+IFNγ, Th1	IRF-1	NC	NC	+++	NC
	STAT4/STAT1	+Th1 Phenotype	T-bet	NC	NC	+	NC
	STAT6	+Th2 Phenotype	GATA3	+	NC	↓	NC
	*NA	Multiple	STAT3	NC	NC	↓	NC
	SMAD1-7/STAT5	+Tregs	FOXP3	NC/+	↓	NC/+	NC
	*NA	Kinase+ STAT1/3/4/6	JAK2	NC	NC	NC	NC
Regulators of Signaling	STAT3/STAT1	(-) JAK1/2/3, STAT1	SOCS1	NC/+	NC	++	NC
	STAT3/STAT1	(-) JAK2/STAT3	SOCS3	+++	NC	+	NC
	*NFκB	(-)STAT1, ERK, p38	PTPN2	NC/+	NC	NC/+	NC
	Methylation, STATs	(-) STAT1/3/6/NF-κB	SHP-1	↓	↓	↓	NC
	NA	(-) STAT1/3, +STAT5	SHP-2	NC	NC	NC	NC
	*NFκB	+ MΦs/CCR2	MCP-1	+++	NC/+	+++	NC/+
Chemokines	MΦ activation	MCP-1 Receptor	CCR2	++	NC	+++	NC

Category	Transcription Regulation	Function	Gene	Active UC	Quiescent UC	Active CD	Quiescent CD
	* NF-κB	T-cells /MΦ/CCR6	CCL20	+++	NC	++	NC/+
	* DC & T-cells	CCL20 Receptor	CCR6	+	NC	↓	NC
	IRF1/STAT1/NF-κB	+Th1/CXCR10	IP-10	++	NC	+++	++
	STAT6/ NF-κB	+Th2/CCR4	TARC	+++	NC	NC	NC
	STAT6/NF-κB	Monocyte/Eos/CCR3	Eotaxin 1	++	+++	NC	NC
Adhesion Molecules	STAT1/NF-κB/AP1	Binds Integ β2/LFA-1	ICAM-1	NC/+	NC	++	NC/+
	STAT6/ NF-κB	Binds Integ β1/VLA-4	VCAM-1	++	+	NC/+	NC
Oxidative Stress Inducers	* NF-κB	+PGH2/TXA2	COX2	++++	NC	+++	NC
	STAT1/NF-κB, Rac	+H2O2	Nox2	+	NC	++	NC/+
	STAT1/NF-κB	NO production	iNOS	+	NC	++	NC/+
	STAT6	Polyamine+, -Arg	Arginase1	+	NC	↓	NC
Proteases	STAT1/NF-κB	IL-1b procession	Caspase 1	NC/+	NC	+	NC
	NF-κB/APC	Mucosal degradation	MMP3	+++	NC	+++	NC
	STAT6/NF-κB	Mucosal degradation	ADAM8	+	NC/↓	NC	NC
	STAT3/Sp1	Mucus barrier	Mucin1	NC/+	NC	NC	++
Mucosal Defense	NF-κB /SP1	Mucus barrier	Mucin5aC	++++	NC	++	NC/+
	-NF-κB/CEBPβ	Repair/anti-apoptotic	TTF3	↓	NC	NC	NC
	NF-κB	Anti-microbial	Defensin β2	++	NC	NC/+	++
	NF-κB	anti-apoptosis	BCL2	+	NC	NC/+	NC
Control	None	GAPDH, β-Actin, S18 rRNA		NC	NC	NC	NC

Expression differences are shown in relation to colon biopsies from normal subjects and an arbitrary scale was set to represent those differences: NC = no change, NC/+ = notable change that did not reach statistical significance, + = significant change under 5-fold, ++ = 5-20 fold change, +++ = 20-100 fold change, ++++ = 100-1000-fold change, +++++ = more than 1000-fold change.

* Designates that there is documented genetic association of the genes with IBD. The transcriptional regulation of genes is described here showing transcription factors that have been documented to modulate specific gene expression and is based on reference in the text. Also the function of each gene is briefly noted. Furthermore, other genes that have been quantified and did not show significant differences include: TLR4*, CARD 9*, IFN-γ receptor 1, IL-4Rα, IL-12rb, IL-17Ar, CCR4, CCR8, CXCR3, STAT1, STAT4, STAT6*, JAK1, JAK2*, JAK3, PTPN22*.

[†] Antigen stimulation of TCR, GATAT3, NFAT, AP-1, chromatin remodeling (32).