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STAT3 Genotypic Variation and Cellular STAT3 Activation and Colon Leukocyte Recruitment in Pediatric Crohn Disease

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Abstract

Objectives—Genotypic variation in *STAT3* increases risk for IBD, and *STAT3* dependent inflammatory networks are induced in the colon in these patients. We **hypothesized** that *STAT3* “A” risk allele carriage would be associated with increased cellular *STAT3* activation and colon leukocyte recruitment.

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Methods—Colonic expression of genes regulating STAT3 signaling and leukocyte recruitment and function was measured in pediatric CD patients stratified by *STAT3* genotype. The frequency of colonic pSTAT3+ and CXCR2+ neutrophils was determined using immunohistochemistry. STAT3 tyrosine phosphorylation (pSTAT3) was measured in circulating leukocytes by flow cytometry, and mechanisms regulating STAT3 activation were tested in IBD EBV-transformed lymphocytes (EBL).

Results—Colonic expression of *IL-6*, the STAT3 target gene *SOCS3*, the neutrophil chemo-attractants *IL-8*, *CXCL1*, and *CXCL3*, and the neutrophil products *S100A8*, *S100A9* and *S100A12* were increased in patients carrying the *STAT3* “A” risk allele. The frequency of neutrophils expressing the cognate receptor for IL-8, CXCR2, was increased in colonic biopsies from patients carrying the risk allele, and the frequency of pSTAT3+ or CXCR2+ neutrophils correlated with histologic severity. The frequency of CD4+ lymphocytes and granulocytes expressing pSTAT3 was increased in patients carrying the *STAT3* “A” risk allele. EBL's from patients carrying the *STAT3* “A” risk allele exhibited increased basal and IL-6 stimulated STAT3 tyrosine phosphorylation, increased transcription of *STAT3* and *SOCS3* after IL-6 stimulation, and increased membrane localization of the IL-6 receptor, GP130, and JAK2.

Conclusions—The *STAT3* “A” risk allele is associated with increased cellular STAT3 activation and up-regulation of pathways which promote recruitment of CXCR2+ neutrophils to the gut.

Keywords

Pediatric Crohn's disease; Signal Transducer and Activator of Transcription 3 (STAT3); neutrophil recruitment; CXCR2

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing and remitting inflammatory disorders of the gastrointestinal tract commonly referred to as the inflammatory bowel diseases (IBDs). IBD pathogenesis is complex involving the gut flora, epithelial barrier function, and innate and adaptive immunity. The precise etiology remains unclear but evidence suggests that it involves dysregulation of the host immune response to luminal flora. Genome wide association studies (GWASs) have now identified multiple genetic factors that confer IBD susceptibility, with some unique to CD or UC, and some shared.[1–3]. Genotypic variation in *STAT3* has been linked to risk for both CD and UC [1]. Recently, newly described permutations to build protein-protein interaction (PPI) networks from GWAS identified risk associated genetic loci revealed that in CD the core candidate network involved JAK2 and STAT3 [4].

In IBD STAT3 activation has been well documented with several potential roles. In animal models, Stat3 activation in intestinal epithelial cells is required for acute wound healing responses, but also promotes development of colitis-associated cancer during chronic inflammation [5, 6]. Stat3 activation in myeloid cells mediates anti-inflammatory effects of IL-10; targeted deletion of Stat3 in this cell type leads to severe spontaneous entero-colitis [7]. Conversely, Stat3 activation in CD4+ T cells is required for differentiation of Th17 effector lymphocytes, and blockade of IL-6:Stat3 signaling ameliorates both ileitis and

colitis in animal models [8, 9]. Recent work by Nguyen et al. has shown that Stat3 is essential for the neutrophil migratory response to CXCR2 ligands such as CXCL2 via activation of G-CSF induced CXCR2 expression and modulation of CXCR2 signal transduction [10]. We have previously shown that STAT3 activation was increased in PB granulocytes, IL-6-stimulated CD3⁺/CD4⁺ lymphocytes, and affected colon biopsies of pediatric IBD patients at diagnosis and during therapy [11]. Furthermore, we identified an IL-6:STAT3 biological network that drives leukocyte recruitment and thereby mucosal inflammation in this setting.

We sought to define the functional consequences of the recently identified G>A intronic SNP (rs744166) within the *STAT3* gene in pediatric CD patients. We hypothesized that the *STAT3* risk allele “A” would be associated with increased cellular STAT3 activation, and differences in colonic expression of chemokines driving leukocyte recruitment. We found that carriage of the *STAT3* “A” risk allele is associated with increased cellular STAT3 activation and up-regulation of chemokines expressed on 4q12–13 which promote CXCR2+ neutrophil recruitment to the gut.

Materials and Methods

Materials

Human IL-6 and soluble IL-6 receptor (sIL-6R) were from R&D Systems (Minneapolis, MN). Tyrosine phosphorylation state specific STAT3 (pSTAT3) (SC-7993, Santa Cruz Biotechnology, Santa Cruz, CA), CXCR2 (CD182, BD Biosciences) and secondary antibodies (Vector Laboratories, West Grove, PA) were used for immunohistochemical (IHC) analysis. pSTAT3 antibodies and antibodies for CD3, CD4, IL6R, and GP130 for flow cytometry and ImageStream^x (amnis® Seattle, WA) analysis were from BD Biosciences (San Jose, CA). Western blot antibodies to pSTAT3 (SC-7993), STAT3, pSTAT1 (SC-135648), STAT1, GP130, IL-6 receptor alpha, JAK2, and SOCS3 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Study Subjects for microarray and interrogation of colonic biopsies

Colon biopsies were obtained from an area of active disease in the ascending colon in pediatric CD patients, and from the same segment of normal colon in healthy controls. The diagnosis of CD was made using established clinical, radiological, and histological criteria. The Pediatric Crohn's Disease Activity Index (PCDAI) was used to measure clinical severity, and the Crohn's Disease Histological Index of Severity (CDHIS) was used to measure mucosal severity [12, 13]. The Montreal system was used to classify disease location and behavior [14]. The mean age (range) for the healthy controls for the colon biopsy studies was 10 (6–18) years; 57% were male.

Genotyping

Genomic DNA was extracted from whole blood using the PureGene Kit (Gentra System, Minneapolis, MN). Patients were genotyped for the *STAT3* G>A (rs744166) single nucleotide polymorphism (SNP) using the TaqMan system [1].

Gene Array Analysis

Two colon biopsies from the ascending colon were placed in RNAlater™ (Qiagen, Valencia, CA) at 4°C. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Samples were submitted to the CCHMC Digestive Health Center Microarray Core where the quality and concentration of RNA was measured and the global pattern of gene expression was determined using Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 arrays as previously reported [11]. These data were previously published in a study which examined overall biologic networks induced in the colon in pediatric IBD, without stratification by *STAT3* genotype [11]. Data were normalized to allow for array-to-array comparisons, and differences between groups were detected in Genespring with a significance at the 0.05 level and mean fold change relative to healthy control samples. The complete dataset is available at the NCBI gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) accession number GSE9686.

Gene expression by real-time quantitative reverse transcription- PCR (RT-PCR)

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) or an RNeasy Plus Kit (Qiagen, Valencia, CA) and reverse transcribed using Accuscript High Fidelity First-Strand Synthesis System (Stratagene, Cedar Creek, TX). Brilliant SYBR Green based detection (Stratagene) utilizing the Stratagene Mx3000P PCR machine was used to determine gene expression. The mRNA levels of the gene of interest and that of the internal standard, hypoxanthine phosphoribosyltransferase (*HPRT*) or glyceraldehyde phosphate dehydrogenase (*GAPDH*), were measured and expressed as a ratio to *HPRT* or *GAPDH*. Primer sequences are as follows: *STAT3* forward 5'-ATG GAA GAA TCC AAC AAC GGC AGC-3'; *STAT3* reverse 5'-AGG TCA ATC TTG AGG CCT TGG TGA-3'; *CXCL3* forward 5'-AGC ACC AAC TGA CAG GAG AGA AGT -3'; *CXCL3* reverse 5'- AGT CCT TTC CAG CTG TCC CTA GAA-3'; *IL-8* forward 5'-AGA AAC CAC CGG AAG GAA CCA TCT -3'; *IL-8* reverse 5'- AGA GCT GCA GAA ATC AGG AAG GCT- 3'; *SOCS3* forward 5'- ATT CGC CTT AAA TGC TCC CTG TCC- 3'; *SOCS3* reverse 5' TGG CCA ATA CTT ACT GGG CTG ACA-3'.

Colon Histology and Immunohistochemistry (IHC)

Paraffin-embedded hematoxylin-stained colon biopsies were scored in a blinded manner by a pediatric pathologist (M.C.) using the CDHIS [13]. For IHC, paraffin-embedded slides were deparaffinized and antigen unmasking was done by boiling for 10 minutes 10mM sodium citrate (pH 6) for *CXCR2* and 1mM EDTA (pH 8) for p*STAT3*. Endogenous peroxide was quenched with 3% hydrogen peroxide for 15min at RT and tissue was permeabilized with .3% Triton X-100 for 15 min at RT. Slides were subsequently blocked with 3% serum and then incubated overnight at 4°C with primary antibodies. Detection and visualization of stained cells was achieved using the R.T.U kit (Vector Laboratories, West Grove, PA) with DAB (diaminobenzidine) as the chromogen.

Enzyme-Linked Immunosorbent Assay (ELISA) for measurement of serum IL-6

IBD patient sera were tested using sandwich ELISAs for the presence of human IL-6 as previously reported [11, 15]. Media from EBL cultures was analyzed for IL-6 concentration by ELISA at baseline prior to stimulation.

Flow Cytometry

Whole blood was collected in sodium heparin tubes at the time of colonoscopy and placed directly on ice. Samples were stimulated with IL-6/sIL-6R and surface and intracellular staining was performed as described [11].

EBV-transformed lymphoblastoid cell lines (EBLs) culture and stimulation

Peripheral blood samples were obtained from 18 IBD patients, and cells were isolated by gradient centrifugation. These cells were then transfected with Epstein-Barr Virus (EBV) to create immortalized EBLs. EBLs were cultured overnight in serum-free media with 1×10^6 cells/mL and then stimulated with 100 ng/mL IL-6 and 50ng/mL IL-6 receptor for 10 minutes prior to protein isolation and for 3 hours prior to RNA isolation.

Preparation of EBL Cytosolic, Membrane, and Nuclear Proteins

Nuclear and Cytosolic protein fractions were obtained using NE-PER Nuclear and Cytoplasmic Extraction Kit according to manufacturer's recommendations (Thermo Scientific® Waltham, MA). Membrane protein fraction was obtained using MEM-PER Eukaryotic Membrane Protein Extraction Reagent Kit according to manufacturer's recommendations (Thermo Scientific® Waltham, MA).

Immunoblot analysis

40 µg cytoplasmic protein was separated by NuPAGE® Novex® 4–12% Bis-Tris gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were independently stained for rabbit-anti-human antibodies against STAT3, STAT1, IL-6 receptor, GP130, JAK2, SOCS3, and β-Actin loading control. 20 µg of nuclear protein was separated by gel electrophoresis in a similar fashion and stained for rabbit-anti-human antibodies against pSTAT3, pSTAT1, and TFIIβ loading control. 40 µg membrane protein was separated by gel electrophoresis in a similar fashion and stained for rabbit-anti-human antibodies against IL-6 receptor, GP130, JAK2, and β-Tubulin loading control. Protein bands were quantified by normalized chemoluminescent arbitrary units (AU) via LAS Image Reader and MultiGauge Software (Fujifilm®).

ImageStream^x analysis

EBLs were stimulated as described above and then stained with antibodies for pSTAT3, IL-6 receptor, and DRAQ5 nuclear marker. 5×10^4 cells were analyzed by IDEAS application © Version 4.0.779.0 for nuclear co-localization of pSTAT3.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM© Version 4.01. Continuous variables were analyzed using unpaired t test, two sample t test with Welch's correction, or

Kruskal-Wallis with Dunn's test for multiple comparisons. Discrete variables were analyzed using Fisher's exact test. A p-value <0.05 was considered significant.

Ethical Considerations

The patient-based studies were approved by the CCHMC and University of Utah Institutional Review Boards, and consent was obtained from parents and assent from subjects age 11 and above.

Results

Clinical and Demographic Characteristics

The clinical and demographic data for the CD patient cohort stratified by *STAT3* "A" risk allele utilized for the FACS and colon biopsy studies are provided in Table 1. There were no differences for age, gender, disease location, medication exposure, or clinical or histologic disease activity in patients stratified by the *STAT3*"A" risk allele.

Colon Expression of Genes Regulating Leukocyte Recruitment and Function is Up-regulated in CD Patients Carrying the *STAT3* "A" Risk Allele

We first asked if the *STAT3* "A" risk allele would be associated with differences in colonic expression of genes we had previously reported to be up-regulated in active colonic IBD and involved in leukocyte recruitment. We stratified gene expression determined by microarray as a function of *STAT3*"A" risk allele (Table 2). We found that patients carrying the *STAT3* "A" risk allele exhibited a significant increase in colonic expression of *IL-6*, the *IL-6*:*STAT3* target gene *SOCS3*, leukocyte recruitment genes expressed on 4q12-q13, and *S100A8*, *S100A9* and *S100A12*. Serum and mucosal S100 proteins, calprotectin (*S100A8*/*S100A9*) and *S100A12*, are elevated in children with active IBD, have enhanced expression in pathological conditions of chronic inflammation, and are involved in phagocyte chemotaxis and function [16]. By comparison, expression of *CXCL9-11* or *CCL11* did not differ between the groups. Importantly differences in colon gene expression were not accounted for by differences in overall histologic severity, epithelial injury or lamina propria cellularity (mononuclear or polymorphonuclear cells), as measured by the CDHIS sub-scores (Table 1).

Since *SOCS3* expression is an indicator of *STAT3* activity and *IL-8* and *CXCL3* play a critical role in neutrophil chemotaxis we elected to confirm their up-regulation by quantitative real time PCR. *SOCS3* expression was induced five-fold in CD patients carrying the *STAT3* "A" risk allele (p = 0.03, Figure 1A). *IL-8* expression trended towards a three-fold higher value in CD patients carrying the *STAT3* "A" risk allele (p = 0.07, Figure 1B). *CXCL3* mRNA expression was induced twelve-fold in CD patients carrying the *STAT3*"A" risk allele (p = 0.01, Figure 1C). Collectively, these results demonstrated that the *STAT3* "A" risk allele is associated with increased expression of genes mediating leukocyte recruitment located on 4q12-q13 and phagocyte S100 products.

The Frequency of Neutrophils Expressing CXCR2 is Increased in the Colon of CD Patients Carrying the *STAT3* "A" Risk Allele

The frequency of total neutrophils in non-risk allele patients was 5.5 ± 1.3 per hpf compared to 7.5 ± 1.9 per hpf in CD patients carrying the *STAT3* "A" risk allele which was not significantly different. However, both were significantly increased compared to 2.1 ± 0.3 neutrophils per hpf in controls ($p < 0.05$, data not shown). We then asked if there would be an increase in the frequency of neutrophils expressing activated *STAT3* (p*STAT3*) or the cognate receptor for CXCL3 and IL-8, CXCR2, a *STAT3* target gene, within colon samples stratified by *STAT3* "A" risk allele. In disease controls lacking the risk allele the frequency of p*STAT3*⁺ neutrophils was equal to 1.8 ± 0.5 per hpf, compared to 3.6 ± 0.8 per hpf in CD patients carrying the *STAT3* "A" risk allele ($p = 0.09$, Figure 2A and 2B). The frequency of p*STAT3*-positive neutrophils per hpf was positively associated with the overall histologic index of severity ($r = 0.65$, $p = 0.02$; Figure 2C). CD patients carrying the *STAT3* "A" risk allele exhibited an increased frequency of neutrophils expressing CXCR2 in the colon compared to disease controls ($p = 0.03$; Figure 2D & 2E). The frequency of CXCR2⁺ cells per hpf was highly related to the overall histologic index of severity within the colon biopsy ($r = 0.68$, $p = 0.01$; Figure 2F).

Peripheral Blood Lymphocyte and Granulocyte *STAT3* Tyrosine Phosphorylation are increased in CD Patients Carrying the *STAT3* "A" Risk Allele

We then asked whether differences in the frequency of p*STAT3* neutrophils observed in the mucosa would be reflected in differences in circulating granulocytes. We have previously shown that *STAT3* activation was increased in PB granulocytes, IL-6-stimulated CD3⁺/CD4⁺ lymphocytes, and affected colon biopsies of pediatric IBD patients [11]. We therefore asked whether carriage of the *STAT3* "A" risk allele would be associated with increased cellular *STAT3* tyrosine phosphorylation. We measured intracellular CD3⁺/CD4⁺ lymphocyte and granulocyte *STAT3* tyrosine phosphorylation (p*STAT3*) before and after stimulation with IL-6/IL-6R by flow cytometry [17]. The cell surface markers CD3 and CD4 were used to identify the lymphocyte population and granulocytes were identified based upon scatter properties (Figure 3A). Patients carrying the *STAT3* "A" risk allele exhibited a significantly higher basal frequency of p*STAT3*⁺CD3⁺/CD4⁺ lymphocytes ($p = 0.01$, Figure 3B) and p*STAT3*⁺granulocytes ($p = 0.0004$, Figure 3C) compared to non-risk allele patients. Moreover, patients carrying the *STAT3* "A" risk allele also exhibited a significantly higher frequency of p*STAT3*⁺ granulocytes after IL-6/IL-6R stimulation ($p = 0.001$, Figure 3C) compared to non-risk allele patients. Comparison of un-stimulated and stimulated cells within the same genotype revealed that only samples from patients carrying the risk allele exhibited a significantly higher frequency of p*STAT3*⁺ lymphocytes ($p = .003$, Figure 3B) or granulocytes ($p = .02$, Figure 3C) following IL-6/IL-6R stimulation. This was specific as the frequency of p*STAT5*⁺CD3⁺/CD4⁺ lymphocytes was not different in *STAT3* "A" risk allele patients at 13 ± 8 ($n = 21$) compared to 9.4 ± 8 ($n = 5$) in non-risk allele patients. Similarly, the frequency of p*STAT5*⁺ granulocytes was not different in *STAT3* "A" risk allele patients at 48 ± 34 ($n = 21$) compared to 36 ± 22 ($n = 5$) in non-risk allele patients. We also measured the circulating concentration of the *STAT3* activating cytokine IL-6 and found that there was significantly less in patients carrying the *STAT3* "A" risk allele at 34 ± 5 pg/ml ($n = 22$).

compared to non-risk allele patients 86 ± 25 pg/ml ($n=3$, $p<0.05$). Collectively, these data demonstrated that the *STAT3* "A" risk allele is associated with increased *STAT3* activation in primary peripheral blood leukocytes.

IL-6 stimulated *STAT3* activation and membrane localization of the IL-6 Signaling Complex are increased in Immortalized B-cell lines from IBD patients carrying the *STAT3* risk allele

To investigate the mechanism by which the *STAT3* "A" risk allele could be mediating differences in cellular *STAT3* activation we utilized EBV transformed B-cell lines (EBL) from IBD patients genotyped for the *STAT3* risk allele. Table 3 provides the clinical and demographic data for the EBL patient cohort stratified by *STAT3* "A" risk allele carriage, while Table 4 provides B cell phenotyping stratified by the *STAT3* "A" risk allele. There were no differences by genotype for age at diagnosis, gender, IBD phenotype, or medication exposures at the time of sample collection. Moreover, the B cell phenotype did not vary by IBD phenotype, so results were stratified by *STAT3* "A" risk allele within the entire IBD cohort.

We first tested whether the *STAT3* "A" risk allele would be associated with differences in cytosolic abundance of the IL-6:*STAT3* signaling complex, or *STAT3* itself. Neither cytosolic protein abundance of *STAT3*, *STAT1*, IL-6 receptor, *JAK2*, *GP130*, nor *SOCS3* varied by *STAT3* "A" risk allele (Figure 4). However, nuclear protein abundance of tyrosine phosphorylated *STAT3* was significantly increased at baseline in EBLs from *STAT3* "A" risk allele patients to 4 ± 1 normalized chemoluminescent arbitrary units (AU), compared to 1 ± 0.4 AU in non-risk allele patients ($p=0.04$, Figure 5A and 5B). Following IL-6 stimulation, the nuclear protein abundance of phosphorylated *STAT3* was also significantly increased to 32 ± 4 AU in EBLs from *STAT3* "A" risk allele patients compared to 19 ± 4 AU in non-risk allele patients ($p=0.04$). Conversely, analysis of the alternative IL-6:*STAT1* pathway determined that the nuclear protein abundance of phosphorylated *STAT1* was significantly decreased following IL-6 stimulation in EBLs from *STAT3* "A" risk allele patients compared to non-risk allele patients ($p=0.001$, Figure 5C and 5D). Since nuclear *STAT3* accumulation was increased we then asked if transcription of *STAT3* target genes including *STAT3* and *SOCS3* would also be increased. Neither *STAT3* nor *SOCS3* basal mRNA expression differed by *STAT3* risk allele carriage; however, following IL-6 stimulation, *STAT3* and *SOCS3* mRNA expression were significantly increased in EBLs from *STAT3* "A" risk allele patients compared to non-risk allele patients ($p=0.003$ and $p=0.04$, Figure 5E and 5F).

Membrane protein abundance of the IL-6 receptor under basal conditions was increased two-fold in EBLs from *STAT3* "A" risk allele patients compared to non-risk allele patients ($p=0.04$, Figure 6A and 6B). The membrane protein abundance of *GP130* was increased two-fold in EBLs from *STAT3* "A" risk allele patients compared to non-risk allele patients ($p=0.003$, Figure 6C and 6D). The membrane protein abundance of *JAK2* was increased three-fold in EBLs from *STAT3* "A" risk allele patients compared to non-risk allele patients ($p=0.008$, Figure 6E and 6F). Importantly, IL-6 did not appear to be acting in an autocrine fashion to regulate the IL-6:*STAT3* signaling complex as EBL supernatants measured by ELISA demonstrated no difference in IL-6 concentration between *STAT3* "A" risk allele

patients at 22pg/ml compared to non-risk allele patients at 20 pg/ml. Additionally, analysis of EBLs by ImageStream^x (Amnis®) demonstrated increased nuclear co-localization of pSTAT3 following IL-6 stimulation in the STAT3 “A” risk allele EBL (10.8 fold increase) compared to the non-risk allele EBL (Figure 7). Collectively, these data demonstrated that targeting of the IL-6:STAT3 signaling complex to the membrane, and IL-6:STAT3 signaling was enhanced in EBLs from patients carrying the *STAT3* “A” risk allele.

Discussion

Recently identified IBD risk loci encode candidate genes involved in maintenance of the epithelial barrier, innate responses to microbial products, and differentiation and function of effector and regulatory lymphocytes. STAT3 activation has been well documented in these processes in both human and murine colitis where transient activation induces protective mechanisms but persistent activation furthers disease progression and ultimately malignant transformation. The aim of the current study was to delineate how the recently identified intronic G>A *STAT3* SNP (rs744166) is associated with specific pathways involved in the pathogenesis of CD. We found that the *STAT3* “A” risk allele is associated with increased cellular STAT3 activation, and induction of pathways regulating leukocyte recruitment and function in the affected colon in the patient sub-group with this genotype.

The mechanism of enhanced cellular STAT3 responsiveness was not known and to dissect the biochemical pathway we utilized EBLs created from IBD patients. We demonstrated increased IL-6 dependent *STAT3* tyrosine phosphorylation in EBLs from *STAT3* “A” risk allele patients compared to non-risk allele patients. This mirrored our findings regarding increased peripheral blood leukocyte STAT3 tyrosine phosphorylation in patients carrying the *STAT3* “A” risk allele. We found that EBLs carrying the *STAT3* “A” risk allele possess increased membrane protein abundance of the IL-6:STAT3 receptor complex (IL-6 receptor, GP130, and JAK2), in the absence of a difference in autocrine IL-6 exposure. The enhanced membrane accumulation of the IL6R signaling complex likely accounts for the increased cellular responses to IL-6 via STAT3 activation. We feel this is specific to the STAT3 pathway in that we found a decrease in STAT1 activation in EBLs carrying the *STAT3* “A” risk allele. These differences in cell signaling may drive disease in the sub-group of patients who carry the *STAT3* risk allele via STAT3 dependent effects upon T-lymphocyte and granulocyte differentiation, activation and survival.

The *STAT3* risk SNP (rs744166) is located within the intron between exon 1 and exon 2 and might be predicted to regulate gene expression; however, we did not observe association between the *STAT3* “A” risk allele and mRNA expression. Future work will ultimately require sequencing of the entire gene and its surrounding genomic sequence to delineate the genetic basis for differences in cellular STAT3 responses. The IL-6:STAT3 biologic network was the focus for our work and we realize our limitation in that we did not interpret responses to other STAT3 IBD effector cytokines such as IL-10, IL-11, IL-17, IL-22, IL-23, and IL-27 or the expression level of their cognate receptors. We utilized the EBV-transformed B cell lines as a model system for testing the effect of *STAT3* genotype upon JAK:STAT signaling. While the B cell signaling responses may have been influenced by the EBV-transformation, we observed a similar enhancement in lymphocyte STAT3 activation

in primary cells from IBD patients. Future studies will be required to characterize the mechanistic basis for differences in cell signaling in IL-6 stimulated T-cells and granulocytes from the peripheral blood of IBD patients.

Patients carrying the *STAT3* “A” risk allele exhibited increased colonic expression of chemokines located on 4q12-q13 (*IL-8*, *CXCL2*, and *CXCL3*) and *S100A8*, *S100A9* and *S100A12*. Serum and mucosal S100 proteins, calprotectin (S100A8/S100A9) and S100A12, known as damage associated molecular patterns, are found at high concentrations in inflamed tissue and have been shown to be involved in neutrophil chemotaxis [19]. Increased mucosal release correlates with fecal markers of IBD disease activity, and in myeloid progenitor cells up-regulation of S100A8 and S100A9 was shown by direct binding of STAT3 to the gene promoter via chromatin immunoprecipitation [18] [20]. Here we demonstrate that S100A8, S100A9 and S100A12 are up-regulated in CD patients carrying the *STAT3* “A” risk allele, in the absence of an overall difference in clinical or mucosal disease activity. Furthermore, we did not find differences in the expression of genes classified in other pathways such as immune and inflammatory mediators, cancer and cell proliferation, ECM tissue remodeling, or metabolism. This suggests that patients carrying the *STAT3* “A” risk allele may have underlying biology that involves increased neutrophil chemotaxis and activation. However, future studies which directly measure neutrophil chemotaxis will be required to test this.

We did investigate pathways which are involved in neutrophil mobilization since they are closely associated with the outcome of inflammation.[21, 22]. It has been shown that STAT3 regulates CXCR2 expression during mobilization responses and CXCR2 binds IL-8 and CXCL6 to promote neutrophil migration while CXCL1, -2, -3, and -5 enhance neutrophil chemoattractant activity.[10, 23, 24]. In murine models of chemically induced colitis small molecule antagonism of the CXCR2 receptor or genetic deletion reduces MPO (neutrophil) activity, colonic damage and clinical symptoms [25, 26]. Thus we evaluated the frequency of neutrophils expressing pSTAT3, and the cognate receptor for IL-8, CXCR2+, and found them to be increased in colonic biopsies from CD patients carrying the *STAT3* “A” risk allele. Consistent with the murine studies, we found that the frequency of pSTAT3+ or CXCR2+ neutrophils was highly correlated with histologic severity. Importantly, overall lamina propria cellularity measured within the same biopsies from which we scored the above parameters did not vary for mononuclear or polymorphonuclear sub-scores. These data demonstrate that current clinical scoring systems are not able to distinguish between these differential pathways driving disease and also suggest the utility of fecal calprotectin as a plausible biomarker for this sub-population of CD.

We did not observe an association between STAT3 risk allele carriage and disease location or severity within our cohort. However, recent work from Ferguson et. al confirmed increased risk for CD associated with the *STAT3* “A” risk allele and demonstrated associations with clinical phenotypes[Ferguson, 2010 #1236]. In that study the frequency of CD patients with *STAT3* “GG” homozygous allele carriage was equal to 12.3%. They reported a significantly increased frequency of extra-intestinal manifestations, inflammatory disease behavior, and colonic involvement in individuals who have the *STAT3* “A” risk allele. This is consistent with our studies regarding mechanisms of colonic disease in

patients carrying the STAT3 “A” risk allele, although patients homozygous for the “G” allele were at very low numbers in our patient population, ultimately limited the power of our analyses. Future studies with greater power to detect clinical associations will be needed to elucidate associations with disease behavior, response to therapy, and rates of colorectal cancer and surgery.

Lastly, data from human and murine models of colitis indicate that STAT3 may be an important target for the treatment of IBD. Recent clinical trials reported in abstract form have shown that the oral Janus Kinase inhibitor, CP-690,550 (CP), is effective in moderate-to-severe UC patients in a dose-dependent manner with improvements in clinical response and remission rates[28]. However CP was not effective in CD[29]. The specificity of CP is for JAK1 and JAK3 over JAK2, and JAK3 is restricted to hematopoietic cells whereas JAK1 and JAK2 are ubiquitously expressed [30, 31]. The divergent result in CD versus UC may reflect differences in the underlying pathogenesis and supports further study of specific JAK:STAT signaling pathways in these disorders.

It is likely that there are several immunogenetic forms of IBD, with CD and UC representing the broadest clinical classifications. While therapeutic options have increased over the past decade, our ability to target newer biologic therapies to specific subgroups of patients has lagged behind. Our data suggests that inhibition of JAK:STAT3 signaling warrants further clinical investigation, and that stratification of CD patients by the STAT3 “A” risk allele may define patient populations that have varying clinical efficacy to investigational agents including the oral Janus Kinase inhibitor, CP-690,550. Furthermore, activation of STAT3 occurs during innate and acquired immune responses in multiple cell types having both pro and anti-inflammatory functions [7, 32–35]. Thus STAT3 activation has been referred to as a double-edged sword and investigating factors which mediate inflammation downstream of STAT3 may lead to more targeted approaches. Collectively, our studies demonstrate that the *STAT3* IBD “A” risk allele (rs744166) is associated with increased cellular STAT3 activation and up-regulation of chemokines which promote CXCR2+ neutrophil recruitment to the gut in a newly described sub-population of CD patients.

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Abbreviations

(CDHIS)	Crohn's Disease Histologic Index of Severity
(EBL)	Epstein Barr Virus transformed lymphocytes
(GM-CS Ab)	Granulocyte-Macrophage Stimulating Factor auto-antibodies
(IBD)	Inflammatory Bowel Disease
(IL-6)	Interleukin 6
(IL6R)	IL-6 receptor
(JAK2)	Janus-associated Kinase 2
(PCDAI)	Pediatric Crohn's Disease Activity Index
(SNP)	Single Nucleotide Polymorphism
(STAT3)	Signal Transducer and Activator of Transcription 3
(UC)	Ulcerative Colitis

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Figure 1. Colon Expression of the STAT3 Target Gene *SOCS3* and Chemokines Expressed on 4q12-q13 are Increased in CD patients Carrying the *STAT3* “A” Risk Allele

Colon mRNA expression was measured by quantitative real time PCR for (A) *SOCS3*, (B) *IL-8*, and (C) *CXCL3* in biopsies taken from active areas of disease. Data are expressed relative to hypoxanthine phosphoribosyltransferase (HPRT) expression as the mean $\pm$ SEM. GG (n=4) and AG/AA (n= 10): Crohn's Disease at diagnosis null, heterozygous or homozygous for the *STAT3* “A” risk allele. Differences were tested by unpaired t-test with Welch's correction.

Figure 2. The Frequency of CXCR2+Neutrophils is Increased in Colon Biopsies from CD Patients Carrying the STAT3 "A" Risk Allele and the Frequency of pSTAT3+ and CXCR2+ Neutrophils Correlates with Histological Severity

Immunohistochemistry for (A) pSTAT3 was performed in colon biopsies taken at diagnosis from active areas of disease and (B) the frequency of pSTAT3+ neutrophils in 10 random high-powered fields (HPF; 400 $\times$ magnification) was determined. CTR: healthy control (n= 6); GG (n=3), AG/AA (n=12): CD at diagnosis null, heterozygous or homozygous for the *STAT3* "A" risk allele. Differences were tested by unpaired t-test with Welch's correction. (C) Pearson's regression analysis was used to test for an association between the frequency of pSTAT3+ neutrophils per HPF and histological severity measured by the CDHIS ($r = .65$, $p < 0.02$). Line arrows depict pSTAT3 positive neutrophils; block arrows depict non stained neutrophils.

Immunohistochemistry for (D) CXCR2 was performed in colon biopsies taken at diagnosis from active areas of disease and the frequency of CXCR2+ (E) neutrophils in 10 random high-powered fields (HPF; 400 $\times$ magnification) was determined. CTR: healthy control (n=6); GG (n=3), AG/AA (n=14): Crohn's Disease at diagnosis null, heterozygous or homozygous for the *STAT3* "A" risk allele. Differences were tested by unpaired t-test with Welch's correction. (F) Pearson's regression analysis was used to test for an association between the frequency of CXCR2+ neutrophils per HPF and histological severity measured by the CDHIS ($r = 0.68$, $p < 0.01$). Line arrows depict CXCR2 positive neutrophils; block arrows depict non stained neutrophils.

Figure 3. Peripheral Blood Lymphocyte and Granulocyte STAT3 Tyrosine Phosphorylation is Increased in CD Patients Carrying the *STAT3* "A" Risk Allele

Intracellular STAT3 tyrosine phosphorylation (pSTAT3) was measured by flow cytometry in washed peripheral blood leukocytes before and after stimulation with IL-6/IL-6R. (PBLs). T helper lymphocytes were identified as the CD3⁺CD4⁺ population and granulocytes were identified based upon forward and side scatter properties. (A) Representative scatter grams for the frequency of CD3⁺CD4⁺ lymphocytes or granulocytes expressing pSTAT3 is shown, with and without IL-6/IL-6R stimulation. Frequency of (B) CD3⁺CD4⁺ lymphocytes and (C) granulocytes expressing pSTAT3 are shown as the mean $\pm$ SEM. GG (n=5), AG/AA (n=20–22): Crohn's Disease at diagnosis null, heterozygous or homozygous for the *STAT3* "A" risk allele. Differences were tested by unpaired t-test with Welch's correction.

Figure 4. Cytosolic protein abundance of STAT3, STAT1, IL-6 receptor, JAK2, GP130, nor SOCS3 varied by *STAT3* "A" risk allele
EBL cytosolic protein abundance of (A) STAT3, (B) STAT1, (C) JAK2, (D) IL-6 receptor, (E) GP130, and (F) SOCS3 were measured by immunoblot analysis. Data are expressed as normalized chemoluminescent arbitrary units (AU) to loading control β -actin via LAS Image Reader and MultiGauge Software (Fujifilm®). Representative immunoblot for each are shown. GG (n=6) and AA (n=9) : EBLs null or homozygous for the *STAT3* "A" risk allele. Differences were tested by unpaired t-test.

Figure 5. IL-6 Stimulated STAT3 Activation and Expression of *STAT3* and *SOCS3* are Increased in Immortalized B-cell lines from IBD Patients Carrying the *STAT3* "A" Risk Allele

EBL nuclear protein abundance of (A) tyrosine phosphorylated STAT3 and (C) tyrosine phosphorylated STAT1 before and after IL-6/IL-6R stimulation was measured by immunoblot analysis. Data are expressed as normalized chemoluminescent arbitrary units (AU) to loading control TFII β via LAS Image Reader and MultiGauge Software (Fujifilm®). Representative immunoblot for (B) pSTAT3 and (D) pSTAT1. EBL mRNA expression was measured by quantitative real time PCR for (E) STAT3 and (F) SOCS3 before and after stimulation with IL-6/ IL-6R. Data are expressed relative to glyceraldehyde phosphate dehydrogenase (GAPDH) as mean $\pm$ SEM. GG (n=6) and AA (n=6–9) : EBLs null or homozygous for *STAT3* "A" risk allele. Differences were tested by A, C, and E) unpaired t-test with Welch's correction or F) unpaired t-test.

Figure 6. Membrane Localization of the IL-6 receptor, GP130, and JAK2 are Increased in Immortalized B-cell lines from IBD Patients Carrying the *STAT3* "A" Risk Allele

EBL Membrane protein abundance of (A) IL-6 receptor (C) GP130 and (E) JAK2 were measured by immunoblot analysis. Data are expressed as normalized chemoluminescent arbitrary units (AU) to loading control β -Tubulin via LAS Image Reader and MultiGauge Software (Fujifilm®). Representative immunoblot for (B) IL-6 receptor, (D) GP130, and (F) JAK2. GG (n=7) and AA (n=10) : EBLs null or homozygous for the *STAT3* "A" risk allele. Differences were tested by unpaired t-test.

Figure 7. IL-6 Stimulated Nuclear Localization of STAT3 is Increased in EBL Carrying the *STAT3*"A" Risk Allele
EBLs from IBD patients carrying the *STAT3* "A" risk allele or disease controls carrying the "G" non-risk allele were examined under basal conditions and following IL-6 stimulation. ImageStream^x (Amnis®) analysis demonstrated increased nuclear co-localization of pSTAT3 following IL-6 stimulation in the *STAT3* "A" risk allele EBL (10.8 fold increase) compared to no-change in the non-risk "G" allele EBL.

Table 1

Clinical and Demographic Characteristics

STAT3 Genotype	Age	Male Gender	Colon-only Location	STER	6-MP	5ASA	PCDAI	CDHIS	CDHIS Sub-Scores		
									Epithelial	Mono	Poly
GG (n=8)	12(3)	88%	38%	38%	25%	25%	36(16)	5(4)	4.5 (2)	2.3(.5)	4.3(1)
AA or AG (n=29)	13(3)	75%	40%	39%	25%	25%	36(17)	7(4)	4.5 (3)	2.8(2)	4.8(3)

STER: corticosteroid, 6-MP: 6-mercaptopurine, 5ASA: 5-aminosalicylate, PCDAI: Pediatric Crohn's Disease Activity Index, CDHIS: Crohn's Disease Histological Index of Severity at the time of sample collection for FACS or microarray. Data are shown as the mean (SD) or frequency. CDHIS Sub-Scores (Epithelial, score for epithelial damage, architectural distortion, and erosion/ulcers; Mono, mononuclear cell sub-score; Poly, polymorphonuclear cell sub-score). Data are shown as the mean (SEM) or frequency. Data are shown as the mean (SEM).

Table 2Colonic Expression of JAK/STAT Signaling and Leukocyte Recruitment Genes Stratified by *STAT3* Genotype

Gene Symbol	Gene Name	Cytoband	FOLD CHANGE	
			<i>STAT3</i> Genotype:	
			GG	AG/AA
JAK/STAT Signaling				
IL-6	Interleukin 6	7p21	1.4	2.8*
IL-6R	Interleukin 6 Receptor	1q21	.83	.84
IL-6ST	Interleukin 6 Signal Transducer	5q11	1.3	1.4
JAK2	Janus Kinase 2	9p24	1.3	1.9
STAT1	Signal Transducer and Activator of Transcription 1	2q32.2–q32.3	1.4	1.6
STAT3	Signal Transducer and Activator of Transcription 3	17q21	1.1	1.2
STAT5A	Signal Transducer and Activator of Transcription 5A	17q11.2	1.3	1.3
STAT5B	Signal Transducer and Activator of Transcription 5B	17q11.2	1.1	1.1
SOCS2	Suppressor of Cytokine Signaling 2	12q	1.0	.90
SOCS3	Suppressor of Cytokine Signaling 3	17q25.3	1.0	2.0**
Chemokines / Cytokines				
CCL4	Chemokine Ligand 4 (CC Motif)	17q12	1.6	2.4
CCL5	Chemokine Ligand 5 (CC Motif)	17q11.2–q12	1	.83
CCL11	Chemokine Ligand 11 (CC Motif)	17q21.1–q21.2	3.7	3.5
CCL17	Chemokine Ligand 17 (CC Motif)	16q13	0.83	.92
CCL25	Chemokine Ligand 25 (CC Motif)	19p13.2	0.73	.70
CCR9	Chemokine Receptor 9 (CC Motif)	3p21.3	1.2	1.0
CSF1R	Colony Stimulating Factor 1 Receptor	5q33.2–q33.3	1.8	1.6
CSF2RA	Colony Stimulating Factor 2 Receptor Alpha	Xp22.32	1.6	1.6
CXCR3	Chemokine Receptor 3 (CXC Motif)	Xq13	1.0	1.0
CXCL1	Chemokine Ligand 1 (CXC Motif)	4q12–q13	3.3	8.0*
CXCL2	Chemokine Ligand 2 (CXC Motif)	4q12–q13	2	4.8
CXCL3	Chemokine Ligand 3 (CXC Motif)	4q12–q13	2.1	5.5*
CXCL5	Chemokine Ligand 5 (CXC Motif)	4q12–q13	4.3	12.3
CXCL6	Chemokine Ligand 6 (CXC Motif)	4q12–q13	2.4	6.3
IL-8	Interleukin 8	4q12–q13	1.7	7.4*
CXCL9	Chemokine Ligand 9 (CXC Motif)	4q21	3.6	5.1
CXCL10	Chemokine Ligand 10 (CXC Motif)	4q21	3.6	6.3
CXCL11	Chemokine Ligand 11 (CXC Motif)	4q21.2	7.7	17.5
CXCL12	Chemokine Ligand 12 (CXC Motif)	10q11.1	1.5	1.6
IL-8RA	Interleukin 8 Receptor Alpha	2q35	1	1.1
IL-8RB	Interleukin 8 Receptor Beta	2q35	1.2	5.0
S100 Family Members				
S100A8	S100 Calcium Binding Protein A8	1q21	1.6	5.6*
S100A9	S100 Calcium Binding Protein A9	1q21	1.3	3.1*

Gene Symbol	Gene Name	Cytoband	FOLD CHANGE	
			STAT3 Genotype:	
			GG	AG/AA
S100A12	S100 Calcium Binding Protein A12	1q21	1.1	5.8*

GG, AG/AA: CD null, heterozygous or homozygous for the *STAT3* "A" risk allele, fold-change is relative to healthy control colon, GG by unpaired t test with Welch's correction.

*
p<0.05

**
p<.01 versus

Table 3

EBV Cell line Patient Demographics and Medication Exposures

<i>ST/AT3</i> Genotype	Age	Male Gender	CD	UC	5-ASA	Azathioprine	STER	Infliximab	Methotrexate
GG (n=8)	16(8–35)	25%	25%	75%	88%	38%	63%	13%	0%
AA (n=10)	15(9–19)	50%	20%	80%	80%	50%	90%	20%	11%

5-ASA: 5-aminosalicylate, STER: prednisone. Data are shown as the mean(range) or frequency.

Table 4**B Cell Surface Markers**

<i>STAT3</i> Genotype	CD19+	CD40+	CD10+	CD21+ CD23+	CD5+ B220+	CD5- B220+	CD27+ B220+	CD27- B220-	CD27- B220+	CD38- CD138+	CD40+ CD27+
GG (n=8)	100	97.5	15	42	0	18	2	8.5	15.5*	94	21*
AA (n=9)	100	97	1	62	0	41	1	1	42	84	4

Flow cytometry B Cell Surface Markers. Summary of findings indicate EBLs are mature B Cells with an increased resting state in EBLs without the *STAT3* risk allele "A". Data are shown as the median percent positive.

* $p < 0.05$