University of Cincinnati

Date: 2/7/2012

I, Tara A Wilson, hereby submit this original work as part of the requirements for
the degree of Doctor of Philosophy in Cell & Molecular Biology.

It is entitled:
STAT3 Regulation of Mucosal Inflammation in Pediatric Crohn’s Disease and
Murine Colitis

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Last Printed: 2/22/2012
STAT3 Regulation of Mucosal Inflammation in Pediatric Crohn’s Disease and Murine Colitis

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment for the requirements for the degree of

DOCTOR OF PHILOSOPHY (Ph.D)

In the Department of Cancer and Cell Biology of the College of Medicine
February 2012
by
Tara A. Willson

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Abstract

Significance: Signal Transducer and Activator of Transcription 3 (STAT3) is crucial for both innate and adaptive mucosal immune responses in human inflammatory bowel disease (IBD) and murine models of colitis. Background: Our lab has reported up-regulation of IL-6:STAT3-dependent biological networks in colonic mucosal biopsies from IBD patients. We identified increased frequency of STAT3 activated lamina propria and epithelial cells that correlated with histologic disease severity and epithelial injury. Recent genome-wide association studies (GWAS) have associated genetic variants in STAT3 with risk for IBD. Goals: Goals that are met in this dissertation are as follows: (1) Determine the functional effect of rs744166 STAT3 IBD risk SNP (STAT3 “A” risk allele) in pediatric Crohn’s disease phenotype, leukocyte recruitment to the gut, peripheral T lymphocyte and granulocyte STAT3 activation, and STAT3 signaling. (2) As no suitable mode for the study of in-vivo epithelial STAT3 specific affects exits, create and characterize an epithelial Stat3 deficient murine model (3) and determine if the loss of STAT3 in the epithelial compartment would promote the development of chronic colitis following acute dextran sodium sulfate (DSS) injury. Results: (1) The STAT3 “A” risk allele is associated with colonic up-regulation of chemokines IL-8, CXCL1, and CXCL3, which promote CXCR2+ neutrophil recruitment to the gut, and the neutrophil activation products, calprotectin (S100A8/S100A9) and S100A12. The frequency of neutrophils expressing CXCR2 was increased, and the frequency of pSTAT3+ or CXCR2+ neutrophils correlated with histologic severity in colonic biopsies from patients carrying the risk allele. Peripheral blood frequency of basal CD4+ lymphocytes and basal and IL-6/IL-6R stimulated granulocytes expressing pSTAT3 was increased in patients carrying the STAT3”A” risk allele. EBV-transformed lymphocytes from patients carrying the STAT3”A” risk allele exhibited increased basal and IL-6 stimulated STAT3
activation. Patient clinical and demographic characteristics did not differ based on STAT3 “A” risk allele genotype although the above-mentioned phenotypic differences seem to define a newly described sub-population of pediatric CD patients. (2-3) In our murine model of intestinal epithelial cell Stat3 deletion (Stat3\textsuperscript{AIEC}) chronic inflammation at day 28 was more severe following 7 days of acute injury with DSS which was not accounted for by a sustained defect in epithelial proliferation or apoptosis as compared to littermate control (Stat3\textsuperscript{Flx/Flx}) mice. Colonic lamina propria frequency of total pSTAT3+ cells was increased and correlated with histologic injury and F480+pSTAT3+ macrophages, and CD3+pSTAT3+ T-lymphocytes were increased in Stat3\textsuperscript{AIEC} mice compared to littermate controls. Colonic expression of Stat3 target genes \textit{Reg3β} and \textit{Reg3γ} that mediate epithelial restitution were significantly decreased in Stat3\textsuperscript{AIEC} mice. However colonic expression of \textit{Il-17a}, \textit{IFNγ}, \textit{Cxcl2}, \textit{Cxcl10}, \textit{Ccl2}, and \textit{Ccl4} were significantly increased and \textit{Il-17a} expression correlated with the increased lamina propria frequency of CD3+pSTAT3+ T-lymphocytes in Stat3\textsuperscript{AIEC} mice. \textbf{Conclusion:} The rs744166 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. The loss of STAT3 activation in the epithelial compartment ultimately leads to a more severe chronic inflammation, reduced epithelial restitution gene expression and expansion of pSTAT3+ lymphocytes and \textit{Il-17a} expression.
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Chapter 2 contains published original research: citation:

Willson, TA et al., STAT3 Genotypic Variation and Cellular STAT3 Activation and Colon Leukocyte Recruitment in Pediatric Crohn’s Disease, JPGN. 2011.

Doi: 10.1097/MPG.0b013e318246be78

PMID: 22197944

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Acknowledgments

I would first like to acknowledge my graduate mentor Dr. Lee A. Denson. His guidance and mentoring has developed and matured my scientific career. I have really enjoyed my time training in a cross functional environment with both translation and basic science research. I also thank Dr. Denson for serving as Chair of my thesis committee. I would like to thank my thesis committee Dr. David Hildeman, Dr. Nelson Horseman, Dr. Marshall (Chip) Montrose, and Dr. Yi Zheng.

I would like to thank all of the past and present members of the Denson Laboratory. Benjamin Kuhn, Charles Samson, Erin Molden, Sharon D’Mello, Xiaonan Han, and Shila Gilbert. A special thanks to Ingrid Jurickova, who has been instrumental in helping me see the light at the end of the tunnel, thank you for being so wonderful! Another special thanks to Erin Bonkowski, for keeping the lab running, we would be lost without you! Thanks to Kris Steinbrecher and Eleana Harmel-Laws for experimental support, scientific insight, and helping me remember to have fun and enjoy every day. Special thanks to Sandy Geideman for her kind words and helping me administratively. All of these people have not only been instrumental in my progression and development as a scientist, but also have become my friends.

Lastly and most importantly, I would like to thank my family and friends for their never-ending support, encouragement, and understanding especially my mother Carol Willson, my brother Kyle Willson, and my grandfather Glenn Chipps. I am so blessed to have your constant love and support. Thanks Mom for helping me believe in myself and giving me courage, I am so proud to be your daughter. I would also like to thank my grandmother, Mary Chipps, who is no longer with us for the ambition that she inspired in me. Finally, I would like to express my
utmost thankfulness for my best friend Brian Pan whose unwavering love, support, encouragement, and patience have given me the confidence to rise above and move forward.
Preface

This thesis dissertation is manuscript-based consisting of two independent research hypotheses, which resulted in one first author publication and one publication under review. There are four chapters herein:

1- Introduction and literature review
2- Manuscript one: preface, abstract, introduction, materials and methods, results, discussion, and bibliography
3- Manuscript two: preface, abstract, introduction, materials and methods, results, discussion, and bibliography
4- Overall Discussion, Alternative hypothesis and Future Directions
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Chapter 1

Literature Review
Introduction

Autoimmune diseases affect 50 million Americans, this in direct comparison to 81 million affected by heart disease and 11 million by cancer\(^1\). Autoimmune diseases are the second highest cause of chronic illness in the United States and Inflammatory Bowel Disease (IBD) ranks 3\(^{rd}\) in prevalence among the autoimmune diseases\(^2\). IBD, a chronic relapsing inflammation of gastrointestinal tract can arise in an otherwise healthy individual resulting in increased morbidity and decreased quality of life. Approximately four million people worldwide currently suffer from IBD frequently experiencing diarrhea, abdominal pain, fever, chronic fatigue, and weight loss. The clinical presentation, inflammatory location and the type of inflammation, is phenotypically characterized as Crohn’s Disease (CD), Ulcerative Colitis (UC), or IBD unspecified (IBD-U). While the precise etiology is unknown, IBD is thought to be the consequence of environmental factors triggering an inappropriate and continuing mucosal inflammatory immune responses to enteric bacteria in genetically susceptible individuals (Figure 1) \(^3\).

IBD is chronic, with relatively low mortality. Thus, medical management focuses on maintaining remission but refractory disease is highly likely and this creates high caseloads for
current gastroenterologists. The estimated annual disease-attributable direct cost of IBD is $6.3 billion (Crohn's disease accounting for $3.6 billion of that figure) and the cost is significantly higher for children with IBD compared with adults\(^4\). Moreover, the yearly inpatient burden of pediatric IBD is immense, $152.4 million (2010 US$) with 64,985 days spent in the hospital, evidence that improved therapeutics are needed to reduce the significant inpatient burden\(^5\).

Since 1972, hundreds of research grants have been funded for IBD research in areas expanding infectious pathogens, immunology, therapeutics, genetics and intestinal bacterial microflora. Private funding sources such as the Crohn’s and Colitis Foundation of America (CCFA) and the Eli and Edythe Broad Foundation have provided millions for IBD research within the United States. The National Institutes of Health (NIH) has increased its’ funding for IBD research each year during a period when overall funding for the NIH remained flat, this in part due to the quality of the applications, the interest of people at the NIH in IBD, and congressional recommendations encouraging the agency to develop its IBD research portfolio\(^6\). These efforts have translated into approximately 688 current IBD focused research projects sponsored by the NIH. As there is still no cure for IBD, clinicians and researchers have focused new technologic and scientific approaches to develop therapeutics and further our understanding of IBD etiology.
Epidemiology of IBD

**Incidence and Prevalence.** Population-based studies reveal the uneven distribution of IBD throughout the world. The highest disease rates occur in western or industrialized countries with globalization inducing increased incidence in previously low incidence areas, such as Southern Europe and Asia (Figure 2). The annual incidence of UC was highest in Europe (0.6 to 24.3 per 100,000 person-years) then North America (0 to 19.2 per 100,000 person-years) and finally Asia and the Middle East (0.1 to 6.3 per 100,000 person-years). The annual incidence of CD was highest in North America (0 to 20.2 per 100,000 person-years) followed by Europe (0.3 to 12.7 per 100,000 person-years) and then Asia and the Middle East (0.04 to 5.0 person-years). Molodecky et al. also reported that IBD has the highest reported prevalence in Europe (UC: 505 per 100,000 persons; CD: 322 per 100,000 persons) and North America (UC: 249 per 100,000 persons).
persons; CD: 319 per 100,000 persons). Overall, it is estimated that 2 million and 1.4 million individuals in Europe and the United States are suffering with IBD, respectfully.

**Age.** The descriptive epidemiology of IBD differs when comparing an adult population to a pediatric population. Recent reports suggest that pediatric-onset IBD is a sub population of IBD due to the varying phenotypic and natural history differences in young patients. Childhood IBD incidence are on the rise as the number of younger patients (younger than 18 years) has increased significantly. IBD is diagnosed 25% of time during childhood and estimates suggest 100,000 children in North America currently suffer from IBD with three CD cases for every new UC case. A recent comprehensive review of worldwide trends in epidemiology for pediatric-onset IBD suggested that the incidence of IBD is rising and the incidence of CD has risen significantly in several countries, while most studies have reported stable incidence of pediatric-onset UC (Figure 3).

**Sex.** Adult-onset IBD shows equal ratio of male to female disease with perhaps slightly more women having the disease. However, the incidence of UC verse CD in males and females varies among high and low-incidence rate areas. In high-incidence areas males are more affected by UC whereas CD is more frequent in females but incidence of CD is men has increased and is becoming equivalent to that of women. However, in pediatric-onset IBD, CD has a male predominance whereas UC has a female predominance and there has been no molecular elucidation for this difference in adult versus pediatric CD:UC gender ratio. Interestingly, the male to female ratio normalizes between 14 and 17 years.

**Ethnic Differences and Geographic Location.** Studies describing IBD characteristics in racial/ethnic groups are beginning to surface as most of the current studies have been conducted in primarily Caucasian populations. In the studies conducted it appears that Caucasian are at the
Crohn’s Disease

Ulcerative Colitis

Figure 3. Temporal trends of incidence rates for (A) Crohn's disease and (B) ulcerative colitis in studies reporting incidence at multiple time points. Where a year range is reported, incidence rate is reported for the final year in the range (e.g., if incidence is reported for 1990–1999, rate is plotted as incidence for 1999). Reprinted from Inflamm Bowel Disease, 17, Benchimol et al., Epidemiology of pediatric inflammatory bowel disease: a systematic review of international trends. #23-439, Copyright (2011), with permission form John Wiley and Sons.
of Caucasian with Asian Americans and Hispanics incidence lower but on the rise\textsuperscript{26,27}. The assessment of ethnic/racial variation of indigenous prevalence and incidence rates compared to that of the immigrated prevalence and incidence rates will be essential to identify the environmental factors that control IBD development in genetically susceptible individuals. The genes that incur disease susceptibility also seem to be ethnic-specific\textsuperscript{28,29}. In regards to geographical location, the further distance from the equator the higher the incidence of CD in both the Western Hemisphere and Western Europe\textsuperscript{27}. This trend is also evident within the same country as seen in the studies from the United States and in the south of France\textsuperscript{30,31}. Recently low sunlight exposure was associated with increased incidence of CD in France and may explain the reduced incidence with low degrees of latitude from the equator\textsuperscript{32}. Vitamin D is a fat soluble vitamin that is produced in the skin following UV exposure and can also be absorbed through the diet\textsuperscript{33}. Vitamin D status is low in patients with IBD and Genome Wide Association Studies (GWAS) show polymorphisms in the VDR gene are associated with increased susceptibility to CD and UC\textsuperscript{34,35,36}. However defining if vitamin D status or VDR genotype cause or effect the disease still remains to be elucidated.

**Defining Crohn’s Disease**

**Epidemiology.** Considering both pediatric and adult onset IBD, Crohn’s disease is less common than ulcerative colitis. Crohn’s disease is most commonly diagnosed between 15 and 30 years of age\textsuperscript{37}. CD has a particularly high incidence in Ashkenazi Jews reported to be associated with the IBD5 locus in a pediatric-onset population however other studies have failed to define this genetic susceptibility and hypothesize the existence of novel, yet unidentified, genetic variants unique to this population\textsuperscript{38,39}. The number of people with Crohn’s disease has been steadily increasing, particularly among children and in the non-Western world\textsuperscript{16}. In the
Olmsted County, Minnesota cohort, since 1991 the prevalence of CD has increased about 31% and in direct comparison UC has decreased 7%\textsuperscript{21}.

**Location and Intestinal Manifestation.** CD is commonly referred to as a “skip lesion” where inflammation is discontinuous or patchy and can affect any part of the gastrointestinal tract. The immune response can affect all layers of the gastrointestinal tract, (transmural), can extend to the serosa (penetrating disease) and can progress to fistulæ, an abnormal connection between different parts of the intestine, and stricturing. Expected histologic findings include small superficial ulcerations over a Peyer’s patch (aphthoid ulcer) and focal chronic inflammation extending to the submucosa, sometimes accompanied by noncaseating granuloma formation. Generally, CD mostly affects the distal ileum and colon. In pediatric patients the terminal ileum is the most common site of CD, with 60% having ileo-colonic disease and 20% to 30% having colon only disease\textsuperscript{24}. Unfortunately CD presentation can be highly variable leading to a delayed diagnosis with symptoms depending on location and extent and severity of involvement\textsuperscript{40}. Gastrointestinal symptoms depend on location and become symptomatic when lesions are extensive or distal, accompanied by systemic inflammatory reaction, or when stricture or abscesses and fistulas, are involved\textsuperscript{40,41}. No correlation exists with symptoms and progression of anatomical injury as stricture and fistulas can develop for years without any symptoms but ileitis alone can be associated with refractory abdominal pain and fatigue\textsuperscript{41}. About 1 in 4 patients present with abdominal pain, weight loss and diarrhea\textsuperscript{42}, evaluated individually 70 to 90% of patients present with diarrhea, 45 to 66% abdominal pain and/or 65 to 70% weight loss\textsuperscript{43}. In pediatric IBD, growth failure is a critical concern and may be the only initial indicator of CD even before gastrointestinal symptoms present\textsuperscript{44}. Clinical disease activity involves relapsing and remitting inflammation (flares) with varying duration of remission as 10-
15% of patients experience chronic continuous disease course. The major complications associated with CD are bowel obstruction resulting from inflammation induced increased thickness of the intestinal wall and often results in surgery to remove the diseased portion of the bowel. Ulcers or open sores can arise from chronic inflammation anywhere in the digestive tract and the worst circumstances extend completely through the intestinal wall to create fistulas. Fistulas can occur between the intestine and skin, or between the intestine and another organ, such as the bladder or vagina. Internal fistulas may result in food bypassing areas of the bowel that are necessary for absorption and ultimately cause retardation in growth and malnutrition. External fistula can cause continuous drainage of bowel contents to the skin and may become infected forming an abscess, which can be life threatening if left untreated. Perianal fistulas are the most common kind of fistula and referred to as an anal fissure or crack/cleft in the anus or in the skin around the anus where infections can occur and is often associated with painful bowel movements. The pathogenesis of fistulae formation is still unknown.

CD, unlike UC, has a wide spectrum of chronic intestinal inflammatory phenotypes categorized by the Montreal Classification developed in 2005 to sub-divide IBD patients by age of diagnosis, location of disease, and disease behavior (Table 1). The dynamic nature of pediatric CD disease phenotype was not sufficiently captured by the Montreal Classification particularity in disease behavior over time, disease location, and growth failure. Therefore this system was recently redefined for pediatric IBD in 2009 and is known as the Paris Classification (Table 1). This accurate phenotypic classification system was set up to be used in conjunction with genotypic data to better predict clinical course and streamline interpretation of genetic analysis.
**Table 1. Montreal Classification of Crohn’s Disease**

<table>
<thead>
<tr>
<th>Age at Diagnosis</th>
<th>Location</th>
<th>Behavior</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 = &lt; 16 years</td>
<td>L1 = terminal ileal + limited cecal disease</td>
<td>B1 = non-stricturing non-penetrating</td>
<td>N/A</td>
</tr>
<tr>
<td>A2 = 17 - 40 years</td>
<td>L2 = colonic</td>
<td>B2 = stricturing</td>
<td></td>
</tr>
<tr>
<td>A3 = &gt; 40 years</td>
<td>L3 = ileo-colonic</td>
<td>B3 = penetrating</td>
<td></td>
</tr>
</tbody>
</table>

*L4 = isolated upper GI disease
P = perianal disease

* is a modifier that can be added to L1, L2 or L3
* is a modifier that can be added to B1, B2 or B3

**Paris (Pediatric) Modification**

<table>
<thead>
<tr>
<th>Age at Diagnosis</th>
<th>Location</th>
<th>Behavior</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1a = 0-10 years</td>
<td>L1 = distal ileal 1/3 ileum + limited cecal disease</td>
<td>B1 = nonstricturing/nonpenetrating</td>
<td>G0: No evidence of growth delay</td>
</tr>
<tr>
<td>A1b = 10-17 years</td>
<td>L2 = colonic</td>
<td>B2 = stricturing</td>
<td>G1: Growth delay</td>
</tr>
<tr>
<td>A2 = 17-40 years</td>
<td>L3 = ileo-colonic</td>
<td>B3 = penetrating</td>
<td></td>
</tr>
</tbody>
</table>

L4a = upper disease proximal to ligament of Treitz.*
L4b = upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum.*

B2B3 = both penetrating and stricturing disease, either at the same or different times

| * is a modifier that can be added to B1, B2 or B3 |

*In both the Montreal and Paris Classification systems L4 and L4a/L4b may coexist with L1, L2, L3, respectively.

B1 - Nonstricturing, nonpenetrating disease: uncomplicated inflammatory disease without evidence of stricturing or penetrating disease.

B2 - Stricturing disease: the occurrence of constant luminal narrowing demonstrated by radiologic, endoscopic, or surgical examination combined with prestenotic dilation and/or obstructive signs or symptoms but without evidence of penetrating disease.

B3 - Penetrating disease: the occurrence of bowel perforation, intraabdominal fistulas, inflammatory masses and/or abscesses at any time in the course of the disease, and not secondary postoperative intra-abdominal complication (excludes isolated perianal or rectovaginal fistulae).

B2B3 – Stricturing and penetrating disease: the presence of both B2 and B3 phenotypes in the same patient, either at the same moment in time, or separately over a period of time. Adapted from Inflamm Bowel Dis 17, Levine et al., Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. 1314-1321, Copyright (2011), with permission from John Wiley and Sons.

**Disease Progression.** Disease progression (remission, relapse, and surgery rate) is rather challenging to assess within a single cohort therefore interpreting results from multiple studies is even more difficult as quantification of these parameters vary from study to study as do definitions of remission, relapse, and surgery. Thus many studies involving referral centers and population based cohorts have tried to extrapolate this data and the most reputable studies are prospective population based studies. These studies follow a cohort of individuals diagnosed within a population with a known incidence and prevalence, having proper exclusion and
inclusion criteria (migration), routine follow up in clinical course, and document surgical and or drug therapy and mortality. As difficult as these studies are, several have been completed for CD worldwide. In the Olmstead Country, Minnesota population based study (residents who were diagnosed with CD from 1970 to 2004) at diagnosis, 35% had pure ileitis (L1), 36% had colitis alone (L2), and 29% had ileocolitis (L3) and under a median follow-up of 8 to 9 years, 60% of patients had progressed to more extensive disease. Within this same cohort 13% had unremitting disease, 75% experienced a chronic intermittent course, 2% could not be adequately characterized, and 10% were “cured” (included in “cure” were those with intestinal resection).

In a retrospective study ileal only disease progressed to include the colon in less than 20% of patients and colon only disease extended to the small bowel in less than 20% of patients (Figure 4). About 3-5% of patients in L1, L2 or L3 classifications also have associated upper GI lesions. The Inflammatory Bowel South-Eastern Norway (IBSEN) population-based study reported that CD patients at diagnosis had 27% pure ileitis (L1), 45% colitis alone (L2), 26% ileocolitis (L3), and 4% upper

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Figure 4. Distribution (%) of affected sites initially (left panel) and maximally (right panel) among Olmsted County, Minnesota, residents diagnosed with CD, 1943-1982. Reproduced from Gut, 29, Gollop et al., The Epidemiologic aspects of Crohn's disease: a population based study in Olmsted County, Minnesota, 1943-1982, 49-56, Copyright (1988), with permission from BMJ Publishing Group Ltd.
gastrointestinal disease (L4). After 5 years of follow-up, the resultant figures were 23%, 37%, 38%, and 6%, respectively and overall changes in disease location observed in 14% of patients at 5 years\textsuperscript{52}. A European population-based study involving 358 patients with Crohn's disease showed 15% had ileitis, 42% had colitis, 37% had ileocolitis, and 6% had upper gastrointestinal disease at the time of diagnosis\textsuperscript{53}. Overall, these studies suggest an equal ratio of disease classification for L1, L2, or L3 upon diagnosis.

What has troubled gastroenterologists is how to accurately predict clinical disease course and severity based on the initial presentation and evolution of disease behavior. Recent reports involving the Olmsted Country and IBSEN population-based cohorts have elucidated changes in disease behavior over time. In the IBSEN study 200 Norwegian patients with Crohn's disease were compared at diagnosis and in a 5 year follow up where 61% had nonstricturing nonpenetrating disease compared to 47% (at 5 years), 28% had stricturing phenotype compared to 33% (at 5 years), and 12% had penetrating disease compared to 21% (at 5 years)\textsuperscript{52}. The Olmsted County, Minnesota cohort had almost 18.6% of the patients with evidence of a stricturing or penetrating intestinal complication at diagnosis or within 90 days of diagnosis, 50% experienced intestinal complications 20 years after diagnosis, and found that factors associated with development of CD complications were the presence of ileal involvement and perianal disease\textsuperscript{54,55}.

Perianal (anal tags, perirectal abscesses, nonhealing deep fissures, and fistula) and perirectal disease are complications of CD not found in UC. In a retrospective study on Stockholm County, Sweden patients with colonic or rectal Crohn's disease, perianal or rectal fistulas occurred in 37% of those followed between 1955 and 1989\textsuperscript{56}. In the Olmsted County cohort, the cumulative risk for developing a perianal fistula was 21% and 26% after 10 and 20
years, respectively with 75% of patients undergoing surgery and 24% managed medically\textsuperscript{57}. In pediatric-onset CD approximately 33% of patients will develop a perianal fistula or abscesses in their lifetime\textsuperscript{15}.

Medical management is focused on mucosal healing and clinical remission commonly referred to as less need for steroid therapy, and reduced rates of hospitalization and surgical resection\textsuperscript{58,41}. Clinical remission (including surgical resection if needed to achieve remission) and relapse rates in two Scandinavian population-based cohorts reported that the probability of a continuously active course without clinical remissions was 4% and 1% after 5 and 10 years\textsuperscript{45,59}. However, the probability of a relapse-free course decreased rapidly with time, from 22% after 5 years to 12% after 10 years, respectively\textsuperscript{45,59}.

**Surgery.** The removal of a diseased portion of the bowel is reserved for complications of disease such as obstructions, fistulas and/or abscesses, or if the disease does not respond to medical therapy. Surgery may not be curative as inflammation can reside at the site of the resection or can appear in other locations. Approximately 70%-80% of patients with CD will require intestinal surgery 20 years post diagnosis\textsuperscript{60}. Cumulative incidence of surgery at 10, 15, and 20 years was found to be 61%, 70%, and 82%\textsuperscript{61}. Within the first year of diagnosis the cumulative risks for bowel surgery, non-bowel surgery, and all CD-related surgeries was 3.4%, 1.4%, and 4.8%, and at 5 years was 13.8%, 4.5%, and 17.7%\textsuperscript{62}. Increased age at diagnosis, greater disease severity, and stricturing or penetrating disease increased the risk of bowel surgery\textsuperscript{62}. Schaefer et al. also reported that other factors such as initiation of immunomodulator therapy within 30 days of diagnosis, sex, race, and family history of inflammatory bowel disease did not influence the risk of bowel surgery. In a recent detailed analysis of a large multicenter cohort of pediatric CD patients, the incidence of surgery was 5.7% at 1 year, 17% at 5 years, and 28.4% at
10 years from the time of diagnosis\textsuperscript{63}. In this cohort, female gender, poor growth at presentation, initial diagnosis of UC, and development of an abscess, fistula, or stricture was associated with an increased risk of surgery\textsuperscript{63}.

An important clinical question is how medication affects the risk of surgery, which is difficult to interpret as the relationship may reflect a patient’s clinical disease severity or the efficacy of the drug(s). A recent publication by Bouguen and Peyrin-Biroulet evaluated all known data sets for randomized trial, referral centers and population based studies to compare surgical rates before and after the era of biologics. They were able to show in population based study that before the era of biologics the rate of surgical requirements ranged from 27\% to 61\% at 5 years and in the era of biologics the rate of surgery ranged from 25\% to 33\% within 5 years\textsuperscript{64}. They also showed that in a randomized control trial, 1 year of treatment to evaluate the efficacy of adalimumab (TNF Inhibitor), the rate of surgery was nine times greater in the placebo group than in the adalimumab group. In pediatric CD, treatment with infliximab or 5-aminosalicylic acid were associated with decreased risk for surgery\textsuperscript{63}.

Current studies dichotomize patients by medication exposure (exposed or not exposed). However, prospective investigations that utilize defined disease severity scores as clinical endpoints and collect uniform data for medications variables such as start, stop, and duration of exposure will clarify associations between medication use and surgical risk. These studies will ultimately lead to risk stratification during the course of CD and help to guide therapy that may improve disease course and decrease the need for surgery.

\textit{Mortality.} A meta-analysis on mortality in CD, was conducted in population-based studies and revealed a slight but significantly increased mortality in patients, especially women, with CD compared with the background population\textsuperscript{65}. The study identified an increased
mortality from pulmonary cancer, malignant melanoma, and potentially from intestinal cancer combined, but not from colorectal cancer. The pooled standardized mortality ratio (SMR) was 1.39 reduced from a previous meta-analysis (SMR 1.52) which included both population-based and referral center studies.66

**Defining Ulcerative Colitis**

*Epidemiology.* Long-term follow up studies can provide valuable contributions, however limitations exist when comparing across cohorts because information is not systematic or consistently reported in the same way. The most reliable data comes from population based studies, more specifically the IBSEN and Olmsted County, Minnesota cohorts. The yearly incidence of UC reported in the IBSEN (Norway) cohort was 12.8/100,000 in 1997.67 This is higher than the Olmsted County cohort incidence between 1940 and 1993 which was 7.0/100,000 person-years.68 The Olmstead cohort incidence increased in 1990–2000 to 8.8/100,000 person-years, however these increases were shown to be seen after 1940 and it is believed that UC incidence has remained stable over the past 30 years.21 When calculated for 10-year age groups, the highest incidence was among the 20–29 and 30–39 year age groups (13.9/100,000 and 12.0/100,000 person-years, respectively) and the lowest incidence was found in the 0–19 year age group (2.4/100,000 person-years).21 Since 1991 the prevalence of UC has decreased by 7%, while the prevalence of CD increased about 31%.21

*Location and Intestinal Manifestation.* UC involves the rectum in 95% of patients with variable proximal extension.40 Inflammatory lesions are generally limited to the mucosa and are continuous with variable severity of ulceration, edema, and hemorrhage. Inflammatory lesions do not progress to strictures or fistulae. Expected histologic finding are acute and chronic
mucosal inflammation with infiltration of polymorphonuclear leukocytes and mononuclear cells, crypt abscesses, distortion of the mucosal gland, and goblet cell depletion. At the time of presentation ulcerative colitis is equally distributed to either the rectum, the colorectum distal to the splenic flexure or proximal to the splenic flexure. UC has a wide spectrum of chronic intestinal inflammatory phenotypes categorized by the Montreal Classification developed in 2005 to sub-divide IBD patients by location of disease, and disease behavior (Table 2). As the features in pediatric disease phenotype are not sufficiently captured by the Montreal Classification, this system was recently redefined for pediatric IBD in 2009 and is known as the Paris Classification (Table 2).

### Table 2. Montreal Classification for Ulcerative Colitis

<table>
<thead>
<tr>
<th>Extent</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 = ulcerative proctitis</td>
<td>SO = Clinical remission</td>
</tr>
<tr>
<td>E2 = Left sided UC (distal to splenic flexure)</td>
<td>S1 = Mild disease</td>
</tr>
<tr>
<td>E3 = extensive (proximal to splenic flexure)</td>
<td>S2 = moderate UC</td>
</tr>
<tr>
<td></td>
<td>S3 = Severe UC</td>
</tr>
</tbody>
</table>

### Paris (Pediatric) Modification

<table>
<thead>
<tr>
<th>Extent</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 = Ulcerative proctitis</td>
<td>SO = never severe *</td>
</tr>
<tr>
<td>E2 = Left sided UC (distal to splenic flexure)</td>
<td>S1 = ever severe *</td>
</tr>
<tr>
<td>E3 = Extensive (hepatic flexure distally)</td>
<td></td>
</tr>
<tr>
<td>E4 = Pancolitis (proximal to hepatic flexure)</td>
<td></td>
</tr>
</tbody>
</table>

* Severe defined by Pediatric Ulcerative Colitis Activity Index (PUCAI) ≥ 65

Adapted from Inflamm Bowel Dis 17, Levine et al., Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. 1314-1321, Copyright (2011), with permission from John Wiley and Sons.

**Disease progression.** A small fraction of UC patients have unremitting activity, while most bear relapsing inflammation with periods of remission. Flare severity and response to treatment is unpredictable and ranges from minor to life-threatening fulminant colitis that is non-responsive to treatment and requires colectomy. A recent report by Magro et al. included twenty-one studies from seven large cohorts. This summarized data revealed that overtime disease extends from proctitis to pancolitis with approximately 30% of patients having proctitis
upon diagnosis. A 5 and 10 year follow up revealed extension from initial location varied 10%–19% and 11%–28%, respectively. Preventing relapse is the most important goal for UC medical management however it appears that most patients will have a relapse in the first year of disease with cumulative relapse rates between 67%–83% after 10 years of diagnosis\textsuperscript{69}. In the IBSEN cohort within 5 years of diagnosis 78% of UC patients had at least one relapse and having the first relapse in the first year of UC was associated the fact that more relapses were imminent\textsuperscript{70}.

**Surgery.** Surgical procedures in UC patients are categorized as either emergency or elective. Emergency operations are due to perforation of the bowel and massive haemorrhage usually indicated by failed medical therapy and toxic megacolon\textsuperscript{71}. Elective surgery indications included chronic continuous symptoms, corticosteroid dependence, partial response to medical therapy and the presence of malignancy/dysplasia\textsuperscript{71}. In a comprehensive review of large cohorts, from 1962–2004 there was a decreasing trend in the proportion of colectomies\textsuperscript{69}. The major reasons for surgery were acute serious flares or chronic relapsing disease\textsuperscript{72}. The Olmsted County cohort (1949–2001), had a cumulative probability of proctocolectomy of 9.0% after 5 years, 18.7% after 15 years, and 20.8% after 25 years of disease\textsuperscript{73}. The IBSEN cohort from (1990–1993), had an excessive risk of surgery in patients with pancolitis at diagnosis compared to those patients with proctitis and left-sided UC at diagnosis\textsuperscript{67}. At 10-year follow up, the majority of colectomies were performed during the first 2 years of disease and in patients with extensive colitis, this was also similar in the European Collaborative Study IBD cohort\textsuperscript{67,72}. These patterns were also similar in the Danish cohort (1962-1987) where colectomy was generally higher in the first 2 years of disease and among patients with pancolitis at diagnosis. 10 years after diagnosis, patients older than 50 had a 70% reduced risk of colectomy than those less than 30 years of age in the IBSEN cohort\textsuperscript{72}.
Mortality. Risk factors associated with increased UC mortality are having a short time interval from UC onset and diagnosis within the first 5 years of disease, having systemic symptoms of weight loss and fever and having pancolitis at diagnosis. Langholz et al also reported that the median age at death for UC-related causes was 58 years for men and 76 years for women, and for other causes was 70 years for men and 75.5 years for women. In the IBSEN cohort at 1 year of disease, 0.2% and at 5 years of disease 6% of patient deaths were IBD-related (spontaneous colon perforation, cholangiocarcinoma and septic shock). At 10 years of disease, the 6% of deaths also resulted from complications associated with UC that included colorectal cancer. In the Olmsted County cohort the standardized mortality ratio (SMR) from 1940-1959 was 0.8 and from 1990-2001 was 0.5, while having pancolitis was associated with a higher risk of mortality. The overall survival was 81% at 20 years and 69% at 30 years of follow-up. In the 2004 update of the Olmsted County cohort, (median follow-up of 15 years) 19% of deaths related to UC were due to complications of severe UC (perforation, intestinal hemorrhage, and postoperative acute myocardial infarction or sepsis, primary sclerosing cholangitis, and metastatic colorectal cancer) and the median age at death was 81 years for men and 71 years for women.

Extraintestinal Features

Given the systemic and immune mediated nature of IBD, extraintestinal manifestations (EIMs) are commonly experienced and may often precede the development of gastrointestinal symptoms, delaying diagnosis (especially in CD) and contributing to the morbidity and mortality of IBD patients. Approximately 25% to 40% of patients will exhibit some EIM during their lifetime. Fever, usually low grade and chronic, malnutrition (due to reduced caloric intake in
response to abdominal pain), anemia, and fatigue are common non-specific symptoms. Weight loss can be seen in both adult and pediatric onset IBD but in pediatric onset failure to maintain a normal growth velocity is the most common systemic feature of IBD and is more frequent in CD than UC.\textsuperscript{40}

Growth delay (60-80\% in CD; 6 -12\% in UC) and delayed onset of puberty may be the initial presentation of CD in children\textsuperscript{44} and patients may experience delayed skeletal maturation\textsuperscript{40}. Growth delay in children with IBD may be the result of a number of factors, including nutritional status, activity of inflammation, disease severity, genotype (IBD5 - OCTN1/OCTN2\textsuperscript{79} and Card15 in a neutralizing GM-CSF antibody background\textsuperscript{80}) all of which may lead to a growth hormone resistant state\textsuperscript{80,81}. Growth failure is present at diagnosis in up to 80\% of children with Crohn's disease (CD), and persists into adulthood in 30\%.\textsuperscript{82} Growth failure is defined as either reduced growth velocity over a period of 3-4 months, in centimeters per year, for age or a fall in height percentile from the child’s previous level along with delayed skeletal maturation and delayed onset of puberty\textsuperscript{40,83}. Osteoporosis affects approximately 40\% of adult IBD patients and therefore decreased bone mass density is extremely important EIM in the pediatric population\textsuperscript{15}.

Other EIMs that are slightly less common but parallel disease activity are joint pain or swelling\textsuperscript{84}, clubbing of the distal phalanges which is rare but most frequent in children with CD\textsuperscript{40}, oral aphthous stomatitis (seen in 20-30 \% of CD and 5-10\% UC)\textsuperscript{85}, and characteristic skin lesions (described in 10-15\% of IBD patients)\textsuperscript{77} such as erythema nodosum or pyoderma gangrenosum. Other less frequent EIMs are ocular manifestations (episcleritis and uveitis) and the hepatobiliary complication sclerosing cholangitis\textsuperscript{15}. 
Diagnosis

Diagnosis of IBD is established on the basis of clinical, laboratory, bowel imaging, endoscopic assessment, and histologic findings. A full medical history should include onset of symptoms, recent travel, family history of IBD, drug history including antibiotics and non-steroidal anti-inflammatory drugs (both thought to be environmental triggers of IBD), prior appendectomy (appendicitis can mimic CD) and smoking. A proper physical exam including abdominal signs of tenderness and or palpable mass (abscess or inflammatory masses) should be carefully examined. Localized pain to the right lower quadrant is indicative of CD and diffuse tenderness associates with small bowel involvement and overall pain depends on extent of colonic involvement\textsuperscript{15,40}. UC patients may also have bloody and mucus mixed stool, along with lower abdominal cramping\textsuperscript{71}. Rectal bleeding without diarrhea can be due to fissure, polyp, rectal ulcer syndrome, or Meckel diverticulum\textsuperscript{15}. Perianal disease as seen in CD, presents with skin tags, fissures, fistulae and abscess. A thorough examination should be conducted for enteric pathogens often encountered during travel which may mimic IBD and should be ruled out before making a diagnosis\textsuperscript{40}. Laboratory testing is usually performed to identify patients at high risk, for whom definitive diagnostic procedures such as endoscopy and colonoscopy are warranted.

\textit{Hematologic Tests.} Initial tests should include full blood count, renal function, albumin and inflammatory markers (C-reactive protein (CRP) or erythrocyte sedimentation ratio (ESR)), and metabolic profile that includes liver enzymes\textsuperscript{40,86}. C-reactive protein (CRP) is an acute phase protein that is increased in nearly 100\% of patients with CD and approximately 50\% with UC\textsuperscript{87}. However CD patients with established active disease do not have increased levels of CRP\textsuperscript{88}. Elevated liver enzymes levels should prompt an evaluation for associated liver disease\textsuperscript{40}. 
**Fecal Markers.** The two most commonly utilized fecal markers with the most reliable read out for IBD are calprotectin and lactoferrin. The concentration of calprotectin (S100A8/S100A) in feces is an indirect measure of neutrophil infiltrate in the bowel mucosa and has been linked to disease activity and severity. At 50ug/g the estimated sensitivity and specificity for identification of patients with IBD from those without were 89% and 81%, respectively. Lactoferrin is an iron-binding protein found in neutrophil granules and serum and is secreted by mucosal membranes. Gisbert et al reported that the lactoferrin test identified IBD patients with a mean sensitivity of 80% and specificity of 82%. Lastly, these tests can help differentiate between IBD and irritable bowel syndrome (IBS) but can’t distinguish IBD from various other types of intestinal inflammation. S100A12 is a S100 protein and is a marker for neutrophil activation that correlates with disease activity and may be more specific to IBD. In a pediatric cohort, fecal levels of S100A12 greater than 10 mg/kg identified IBD with a sensitivity of 96% and a specificity 92%. In adults, Kaiser et al reported that S100A12 distinguished patients with IBD from those with IBS and viral enteritis with high sensitivity and specificity, and correlated better with intestinal inflammation than fecal calprotectin or other biomarkers. They also concluded that S100A12 reflects inflammatory activity of chronic IBD.

**Serologic Markers.** Anti-Saccharomyces cerevisiae antibodies (ASCA) and perinuclear antineutrophil cytoplasmic autoantibodies (pANCA) are serologic markers that can be used in combination with other diagnostics for diagnosis of IBD. Increased titers of ASCA was found to identify patients with CD by high specificity (96%–100%) but low sensitivity (approximately 50%) while increased levels of pANCA was more common in patients with UC or those with CD who had UC-like pancolitis. In a meta-analysis comprising 3,841 UC and 4,019 CD patients the ASCA+ with pANCA− test offered the best sensitivity for CD (54.6%) with 92.8%
specificity), pANCA+ sensitivity and specificity for UC were 55.3% and 88.5%, respectively and in a pediatric subgroup sensitivity and specificity were improved to 70.3% and 93.4% when combined with an ASCA-97. However diagnostic precision decreased for ASCA discrimination between CD and UC in isolated colonic CD97. Newer additions to serological evaluations include antibodies to *Escherichia coli* outer membrane porin (OmpC), *Pseudomonas fluorescens*-associated sequence (I2), and flagellin (CBir1) however the sensitivity of these individual markers for identifying patients with IBD is low, ranging from 11% to 83% and thus only offer modest contributions98. Individual assays are merged into panels to maximize the sensitivity and specificity of these serological tests and the IBD Serology 7 (IBD7) panel (Prometheus Laboratories, San Diego, CA), is the most widely used in the United States and has been available since 200699. However in a head to head competition, in a population of children referred for initial evaluation of suspected IBD, the predictive value of routine blood tests (anemia, thrombocytosis, and increased erythrocyte sedimentation rate (ESR)) had better sensitivity and specificity than the Prometheus IBD7 panel99.

Other serologic markers in the early stages of investigation for utility in IBD phenotyping, clinical course and need for surgery are neutralizing intrinsically produced antibodies against specific cytokines. TGF-β and IL-10 are two cytokines important in peripheral tolerance and development of T-regulatory cells. Ebert el al. reported that anti- TGF-β antibodies were significantly higher in UC patients and anti-IL-10 antibodies significantly increased in CD patients compared to controls100. They also noted that anti- IL-2, IL-6, IL-8, IL-12, IL-18, IFN-α and IFN-γ antibodies were not different between UC and CD patients compared to controls100. Our lab has reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies, which decrease neutrophil antimicrobial function, are increased in the
serum of pediatric and adult CD patients. The anti-GMCSF antibody levels are associated with ileal involvement (P < .001), ileal location, duration of disease, and stricturing/penetrating behavior (odds ratio, 2.2; P = .018). These studies suggest that a cytokine deficient state may promote disease by inhibiting the cytokine action. This brings forth the idea that rather than cytokine production, cytokine action would be a true measure of its deficiency or excess in IBD. Auto-antibody studies are still in their infancy and larger prospective studies are needed to elucidate the association with disease phenotype, clinical course and therapy.

**Genetic Markers.** Genome-wide association studies (GWAS) have identified 100 genetic risk loci of which 28 associate with both UC and CD. These loci reside in genes involved in innate and adaptive immunity, autophagy, and intestinal homeostasis. Researchers are avidly seeking to define how these loci affect disease phenotypic expression, clinical course, and treatment modalities. Experts predict that genetic testing will have a very limited role in diagnosing patients. However, recent studies from our lab and others justify the importance of genetic markers in an integrated prognostic approach. Prometheus has just released the first genetic diagnostic for IBD, the PROMETHEUS® IBD sgi Diagnostic™ test. This is a 4th-generation IBD diagnostic test, the first and only test to combine serologic, genetic, and inflammation markers, (“sgi”), in the proprietary Smart Diagnostic Algorithm for added diagnostic clarity. This assay includes evaluation for single nucleotide polymorphisms associated with disease risk in ATG16L1(Autophagy), STAT3(IL-6/IL-10/IL-23/IL-17 signaling pathway), NKX2-3(intestinal differentiation), and ECM1(involved in angiogenesis, and tumor biology).

Recent studies have tried to associate genetic variation with disease phenotype. Giving only a modest capacity to predict clinical outcomes (AUC .59 to .76) NOD2, JAK2 and IL23R
along with smoking habit and age at onset were associated with disease phenotype in CD patients with 10 years of follow-up \(^{102}\). A large cohort of CD patients from Belgium were genotyped for 50 SNPs (including the Wellcome Case Control Consortium GWAS SNPs and 13 SNPs from confirmed susceptibility loci [IL23R, ATG16L1, STAT3, TNFSF15, IBD5 (5q31 and 10q21)] and homozygosity at the minor allele of the rs1363670, in close proximity to IL12B gene, was found to be associated with stricturing disease\(^{103}\). In that same cohort any IBD-related surgery was associated with homozygosity of the minor allele of rs1363670 and carriage of a NOD2 variant\(^{103}\). In abstract form at Digestive Disease Week 2011, Dubinsky et al. presented a predictive model of surgery by extrapolating genetic markers (the IL12B locus and Chr. 4q31), small bowel disease location, age at onset, and a serologic antibody sum in a cohort of 1115 CD patients with an AUC of .77 \(^{104}\). In a cohort of 385 CD patients Lichtenstein et al utilized serologic and NOD2 genetic markers to achieved an AUC of 0.80 (95\% CI, 0.757–0.846)\(^{105}\).

These studies are the future of IBD research and illustrate the need for researchers and clinicians to work cohesively to aggregate larger patient cohorts complete with full genotypic profiles for multivariate analysis of genotype-phenotype (disease location, progression, behavior, treatment response) associations in the context of environmental factors (smoking, geographic location, ethnicity, diet, appendectomy, intestinal microbiota). The Immunochip project of the International IBD Genetics consortium has utilized a custom-designed chip to provide genotypic data of 200,000 markers (IBD loci and additional candidate genes based on functional and genomic positional data) in a large cohort (15,000 CD, 12,000 UC, 21,000 healthy controls). The first preliminary results were presented in October 2011 at the United European Gastroenterology week and will be presented at Crohn’s and Colitis Foundation of American meeting this December 2011. These studies promise great insight into the complexity of IBD.
pathogenesis. Lastly, newer models of IBD pathogenesis are even moving beyond genetic advances toward RNA biology and protein-protein interactions. Furthermore, the utility of biomarkers in defining disease activity, mucosal healing, predicting disease course and relapse, and response the therapy are of great interest for the clinician in defining disease management. The current status of applying biomarkers beyond clinical diagnosis of IBD and distinguishing CD vs UC can be reviewed elsewhere.

**Endoscopic Evaluation.** Endoscopic examination with biopsies establishes the diagnosis of IBD. Endoscopy and histologic assessment of biopsies is the most perceptive and specific evaluation to aid in assessing the extent and severity of disease and differentiate a diagnosis of UC or CD. Therefore, Ileo-colonoscopy with multiple biopsies taken from all segments of the GI tract is the first-line procedure. A newer recognized endoscopic technique for complete small bowel interrogation is wireless capsule endoscopy (WCE). The capsule is the size of a pill and contains a tiny camera to allow excellent small-bowel visualization. Published studies have demonstrated that this technique is superior to standard endoscopic and radiographic modalities in diagnosing small bowel CD, is a first-line imaging modality for suspected CD in adults, and approved for use in children aged 2 years and older. WCE has also been indicated for reclassification of IBD-U patients and in previously diagnosed CD patients allows greater detection of the overall burden of small bowel disease resulting in more targeted and effective therapy. WCE has been reported to be non-specific (10% of healthy individuals have mucosal breaks and erosions in the small bowel) and contraindications include intestinal obstruction, stenosis or strictures, and pacemakers. However, capsule retention occurs with a frequency of 1-2% and is a major complication.
**Radiologic Evaluation.** Radiologic/Enterography allows the clinician to see beyond the mucosa to understand the full transmural view and assess for extra-enteric complications such as fistulas or abscesses and obstruction. Radiographic evidence is usually needed for patients with CD to assess extent, location, and severity of disease and as of 2002, the “Gold Standard” was small bowel follow-through. However, newer technologies such as magnetic resonance imaging (MRI) computed tomography scans (CT), and ultrasound (US) are the most successful widely used to date. CT and MR enterography involve oral luminal contrast and have a high diagnostic sensitivity in detecting small bowel involvement and extra luminal complication of CD, including fistula and abscess. Plain abdominal X-rays can be non-specific, however if toxic megacolon is suspected or obstruction plain films should be used before any contrast.

Since most children with IBD will receive multiple radiologic exams, their total radiation exposure is of concern and MRI, the modality with the least ionizing radiation, is the imaging modality of choice in children. Ultrasound is also a non-ionizing imaging modality to evaluate the site, extent, activity, and complications such as abscess formation in Crohn’s disease. It is widely available and inexpensive but operator dependent and has wide inter-observer variability.

A white blood cell scan can help in distinguishing CD and UC. The patient’s leukocytes are isolated and labeled with a radioactive substance, then infused back into the patient and watched for uptake in areas of infection or inflammation within the body. Positron emission tomography (PET) for IBD patients involves administering of radio-actively labeled glucose ($^{18}$F-FDG), that is more readily taken up by cells that are inflamed or cancerous, allowing clinicians to see areas of active inflammation. PET has not been shown to be a better diagnostic than CT or MR enterography in the detection and anatomic characterization of
intestinal disease but is more useful in accurate and non-invasive quantification of disease activity by assessing the presence and degree of inflammation.\(^{114}\) PET can also be combined with CT known as “co-registration” of CT however a recent study in children with IBD found that the co-registration of CT had no additional benefit in diagnosis.\(^{115}\)

**Clinical Disease Activity.** Clinical disease activity indices should include grading and weighting of specific symptoms and intestinal manifestations, with defined cutoff scores corresponding to clinically important disease states from remission to severe disease activity that is then clinically validated for reliability, responsiveness (improvement or worsening) and feasibility.\(^{116,117}\) Inflammatory bowel disease indices include composite evaluation of symptom severity, quality of life, laboratory tests and endoscopic findings.

The Crohn’s disease activity index (CDAI) was developed in 1976 and evaluates eight disease variables including liquid stools, abdominal pain, general well-being, extra intestinal

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Multiplier</th>
</tr>
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<tbody>
<tr>
<td>Number of liquid stools</td>
<td>Sum of 7 days</td>
<td>x 2</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Sum of 7 days rating (graded from 0-3 on severity)</td>
<td>x 5</td>
</tr>
<tr>
<td>General well-being, subjectively assessed</td>
<td>Sum of 7 days (graded as 0 = well to 4 = terrible)</td>
<td>x 7</td>
</tr>
<tr>
<td>Extra intestinal complications</td>
<td>Arthritis/arthritis, iritis/uveitis, erythema nodosum, pyoderma gangrenosum, aphthous stomatitis, anal fissure/fistula/abscess, fever &gt; 37.8 °C</td>
<td>x 20</td>
</tr>
<tr>
<td>Antidiarrheal drugs ( 0 = no, 1 = yes)</td>
<td>Use in the previous 7 days</td>
<td>x 30</td>
</tr>
<tr>
<td>Abdominal mass</td>
<td>(0 = none, 2 = questionable, 5 = definite)</td>
<td>x 10</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>Males:&lt;0.47 Women&lt;0.42</td>
<td>x 6</td>
</tr>
<tr>
<td>Body weight, % deviation from standard weight</td>
<td>[1- (ideal/observed)] x100</td>
<td>x 1</td>
</tr>
</tbody>
</table>

*Table 3. Crohn’s Disease Activity Index (CDAI)*

complications, antidiarrheal drugs, abdominal mass, hematocrit, and body weight (Table 3). These variables are weighted by using an individual multiplier for each score and complied to give the CDAI score that can range from 0 to approximately 600. Investigators have arbitrarily labeled CDAI scores of 150–219 as mildly active disease and scores of 220–450 as moderately active disease however clinical studies continually determine cutoffs for a defined population or specific clinical endpoint. The Harvey-Bradshaw (HBI) is a simplified version of CDAI and the correlation between CDAI and HBI scores are high however the CDAI is the most widely used in clinical trials for CD. The Pediatric Crohn’s disease activity index (PCDAI) was modified from the CDAI and is the preferred method for assessing disease activity in clinical trials of pediatric Crohn’s disease (Table 4). PCDAI modifications disregarded the use of antidiarrheal agents, decreased weighting of subjectively reported abdominal pain and general well-being, however assess limitations to participate in normal activities and laboratory parameters of linear growth, serum albumin, and erythrocyte sedimentation rate (ESR). Therefore, this index functions as a noninvasiveness, valid, response index used as a primary outcome measure to reflect disease activity (remission, severe disease, moderate disease) for prognosis and treatment decisions. Another measurement of clinical disease activity is the Crohn’s disease endoscopic index of severity (CDEIS), developed in 1989, is used to assess the severity of mucosal inflammation or mucosal healing. Parameters for the CDEIS include healed ulceration, erythema, mucosal oedema, aphthoid ulcerations, superficial and deep ulcerations and stenosis. A simplified endoscopic severity index for CD (SES-CD) was developed in 2004 to evaluate the size and penetration of ulceration as a reflection of disease severity in CD.
<table>
<thead>
<tr>
<th>Table 4. Pediatric Crohn’s Disease Activity Index (PCDAI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td>Abdominal pain</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Stools / day</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Patient Functioning—General Well-being</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Weight</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Height at diagnosis</td>
</tr>
<tr>
<td>* is previous percentile</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Height Velocity</td>
</tr>
<tr>
<td>* is deviation from normal</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Abdomen</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Peri-rectal disease</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Extra-intestinal Manifestations</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Hematocrit (%) M=Male and F=Female</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>ESR mm/hr</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

**Sum PCDAI**

Several disease scores for measuring disease activity in UC patients have been developed but still there is no clinically defined gold standard\textsuperscript{125,126}. The two methods most readily used in clinical trials are the Ulcerative colitis disease activity index (UCDAI) and the Mayo Disease Activity Index (MDAI) which are a composite of clinical and endoscopic disease activity (Table 5, UCDAI)\textsuperscript{127,128}.

In children, the Pediatric Ulcerative Colitis Activity Index (PUCAI) is a noninvasive evaluation of UC disease activity that has been shown as a valid, highly reliable, and a responsive index with which to assess disease activity(Table 6)\textsuperscript{129,130}. Turner et al demonstrated a very strong correlation of the PUCAI with macroscopic mucosal inflammation supporting the use of this score as a measure of disease activity in children without endoscopic assessment. The development of and endoscopic assessment of has recently been reported for UC by Travis et al. They report that the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) accurately predicts overall assessment of endoscopic severity of UC however they the validity and responsiveness need further testing before it can be applied as an outcome measure in clinical trials or clinical practice\textsuperscript{131}.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool frequency</td>
<td>1–2 Stools/day=normal</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3–4 Stools/day=normal</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;4 Stools/day=normal</td>
<td>3</td>
</tr>
<tr>
<td>Rectal Bleeding</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Streaks of blood</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Obvious blood</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Mostly blood</td>
<td>3</td>
</tr>
<tr>
<td>Mucosal appearance</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild friability</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Moderate friability</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Exudation, spontaneous bleeding</td>
<td>3</td>
</tr>
<tr>
<td>Physician’s rating of disease activity * Includes endoscopic assessment</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>3</td>
</tr>
</tbody>
</table>

Sum UCDAI

Each variable is scored from 0–3 so that the total index score ranges from 0–12; 0–2: remission; 3–6: mild; 7–10: moderate; >10: severe UC. Adapted from N Engl J Med. 317, Schroeder et al. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. 1625-1629. 1987.
### Table 6. Pediatric Ulcerative Colitis Activity Index (PUCDAI)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal pain</td>
<td>No pain</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pain can be ignored</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Pain cannot be ignored</td>
<td>10</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Small amount only, in less than 50% of stools</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Small amount with most stools</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Large amount (&gt;50% of the stool content)</td>
<td>30</td>
</tr>
<tr>
<td>Stool consistency of most stools</td>
<td>Formed</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Partially formed</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Completely unformed</td>
<td>10</td>
</tr>
<tr>
<td>Number of stools per 24 hours</td>
<td>0–2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3–5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6–8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>&gt;8</td>
<td>15</td>
</tr>
<tr>
<td>Nocturnal stools (any episode causing wakening)</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>10</td>
</tr>
<tr>
<td>Activity level</td>
<td>No limitation of activity</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Occasional limitation of activity</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Severe restricted activity</td>
<td>10</td>
</tr>
</tbody>
</table>

**Sum of PUCAI (0–85)**


### Histologic Disease Activity / Mucosal Healing

The histopathologic features of IBD are variable and depend on the type of disease (CD or UC), the duration of disease and the severity of bowel inflammation. In newly diagnosed CD patients, the earliest lesions are apthous ulcers\textsuperscript{132}. The apthous ulcers occur in the small and large bowel, and are associated with submucosal lymphoid aggregates covered by specialized antigen-transporting cells called membranous (M) cells. CD histology reveals neutrophilic infiltration of surface epithelial cells and crypts leading to crypt abscesses, crypt destruction and mucosal ulceration. A hallmark of CD is the presence of noncaseating granuloma which can be
identified in 15% of endoscopic biopsies and up to 70% of operative specimens\textsuperscript{133}. UC is histopathologic features include a granular mucosa with areas of superficial ulceration, neutrophilic infiltration of crypts and the development of crypt abscesses. Contrary to CD, inflammation is largely mucosal and depletion of goblet cells is more prominent. In the chronic phase histologic features of IBD included distorted crypt architecture, crypt atrophy, increased numbers of round cells and neutrophils in the lamina propria, giant cells and basal lymphoid aggregates\textsuperscript{133}.

<table>
<thead>
<tr>
<th>Table 7. Crohn’s Disease Histologic Index of Severity (CDHIS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td>Epithelial Damage</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Architectural Changes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Infiltration of mononuclear cells in the lamina propria</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Infiltration of polymorphonuclear cells in the lamina propria</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Polymorphonuclear cells in epithelium</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Presence of erosion and/or ulcers</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Presence of granuloma</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>No. of biopsy specimens affected</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Sum CDHIS</strong></td>
</tr>
</tbody>
</table>

Each topic is scored independently. Moderate increase, up to twice the number of cells that can normally be expected; severe increase, more than twice the normal number of cells. Reprinted from Gastroenterology, 114, D’Haens et al. Early lesions of recurrent Crohn’s Disease caused by infusion of intestinal contents in excluded ileum, 262-267, Copyright (1998), with permission from Elsevier.

Mucosal healing or endoscopic remission as a therapeutic endpoint in research trials and clinical practice is effective in optimizing long-term outcomes\textsuperscript{134}. Histopathologic analysis of CD and
UC as it relates to biologic disease activity and predictions of future outcomes remains largely unknown. The current method for assessment of histologic outcomes is a reduction in the CD or UC Endoscopic Index of Severity (CDEIS/UCEIS) and the CD or UC Histologic Index of Severity (CDHIS/UCHIS) (Table 7 and 8).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphonuclear cells in epithelium</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chronic inflammation only</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mild active cryptitis (but no abscesses)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Moderate Active (few crypt abscess)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Severe active inflammation (numerous crypt abscesses)</td>
<td>4</td>
</tr>
<tr>
<td>No. of biopsy specimens affected</td>
<td>None (0 of 6)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(\leq 33%) (1 or 2 of 6)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(33% \text{--} 66%) (3 or 4 of 6)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(\geq 66%) (5 or 6 of 6)</td>
<td>3</td>
</tr>
<tr>
<td>Sum UCHIS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each topic is scored independently. Moderate increase, up to twice the number of cells that can normally be expected; severe increase, more than twice the normal number of cells. Reprinted from Gastroenterology, 126, Rutter et al. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis, , 451-459, Copyright (2004), with permission from Elsevier.

**Treatment**

Medical therapy relies on classic anti-inflammatory and immunosuppressant drugs: corticosteroids, mesaline compounds, corticosteroids, azathioprine and the like. Biologic drugs such as TNF-\(\alpha\), have added greatly to the ability to control IBD while newly described mechanisms of controlling the gut microbiome through probiotics, antibiotics and fecal transplant are on the horizon. In April 2011 the American College of Gastroenterology IBD Task Force, consisting of selected experts in epidemiology and therapy of IBD, released an evidence-based systematic review on medical therapies for IBD in the American Journal of
Gastroenterology. They developed a protocol for systematically reviewing the data on currently available therapies for UC and CD pertaining to both inducing remission and in preventing relapse of the disease. The group reported evidence-based statements with strength of recommendation graded according to standard criteria. The following pages are summarized drug description and listed recommendations from this report.

**5-ASA Therapy.** 5-ASA was the first drug used for UC and combines the antibacterial properties of sulfonamides with the anti-inflammatory effects of salicylates. Also known as mesalamine (free, unconjugated 5-ASA) and mesalamine prodrugs (azo-bonded 5-ASA) have similar modes of action. Sulfasalazine, the standard azo-bonded 5-ASA designer drug, is engineered to release free 5-ASA in the colon, protecting it from proximal absorption. Newer topical and oral 5-ASA agents are delivered to different anatomic sites, corresponding to the distribution of disease thus having minimal systemic side effects. The precise mode of action is unclear, but the effectiveness of the compound is related to its mucosal concentration and it is absorbed by colonic epithelial cells. 5-ASA is believed to be anti-inflammatory by modulating inflammatory cytokine production along with nuclear factor kappa B RelA/p65 phosphorylation and inhibiting the biosynthesis of prostaglandins and leukotriene’s. 5-ASA is also thought to be an antioxidant that traps free radicals, which are potentially damaging byproducts of metabolism. Although very rare, serious adverse effects of 5-ASA are interstitial nephritis, pancreatitis, pneumonitis, pericarditis, and hepatitis. **Recommendations from the American College of Gastroenterology IBD Task Force (Talley et al., 2011) for treatment of IBD with 5-ASA are as follows:** 5-ASA therapies are effective at inducing remission in mild-to moderately-active UC with a strong recommendation and moderate quality of evidence. In preventing relapse of quiescent UC 5-ASA is very effective with strong
recommendation and high quality of evidence. 5-ASA therapies are not recommended for inducing remission in active CD with a weak recommendation and low quality of evidence. In preventing relapse of quiescent CD 5-ASA therapies are not recommended with a weak recommendation and low quality of evidence.

Figure 5. 5-ASA treatment recommendations for IBD. QOE, Quality of evidence. Reprinted by permission with Macmillan Publishers Ltd: Talley, N.J., et al. An evidence-based systematic review on medical therapies for inflammatory bowel disease. Am J Gastroenterol 106 Suppl 1, S2-25; (2011)

Corticosteroid Therapy. The first Randomized Controlled Trial of cortisone in UC was 1954. Since then corticosteroids have been widely used for UC and CD but in only acute exacerbations due to the short-term adverse effects (risk of infection and psychiatric disorders) and long term adverse effects (loss of bone mineral density and diabetes mellitus). Therefore budesonide was synthetically derived to achieve enhanced corticosteroid concentration to the distal ileum and proximal colon while limiting systemic bioavailability to minimize systemic adverse effects. Corticosteroids inhibit the immune system through modulation of glucocorticoid receptor interaction in the nucleus, trafficking of inflammatory cells to the intestine, inducing apoptosis of activated lymphocytes, and decreasing inflammatory cytokine
Corticosteroids were reviewed only for treatment of acute CD and UC and maintenance therapy of CD with budesonide was evaluated since the systemic adverse effects are lower with this preparation. Recommendations from the American College of Gastroenterology IBD Task Force (Talley et al., 2011) for treatment of IBD with corticosteroids are as follows: Corticosteroid therapies are effective at inducing remission in active UC and CD with a strong recommendation and low quality of evidence. Budesonide therapy is effective at inducing remission in mild-to-moderately-active CD with a strong recommendation and loquacity of evidence. Standard corticosteroids are more effective than budesonide at inducing remission in mild-to-moderately-active CD with a weak recommendation and low quality of evidence. Budesonide is not recommended for preventing relapse in quiescent CD with weak recommendation and low quality of evidence.

**Imunosuppressant Therapy.** Thiopurine analogs (6-MP and its pro-drug azathioprine), methotrexate, cyclosporin and more recently tacrolimus are immunosuppressants. Azathioprine and 6-MP are purine anti-metabolites which impede DNA synthesis and inhibit the proliferation of rapidly dividing cells such as lymphocytes\(^{157}\). These drugs are associated with nausea, allergic reactions, acute pancreatitis, hepatitis, increased risk of infection, malignancy, and bone marrow suppression\(^{158}\). Methotrexate acts by inhibiting the metabolism of folic acid and decreasing the availability of folate needed to synthesize DNA in rapidly dividing lymphocytes. The adverse effects of methotrexate include hepatotoxicity, pneumonitis, increased risk of infection, malignancy, alopecia, stomatitis, and myelosuppression. Cyclosporin and tacrolimus both inhibit calcineurin which impedes T-lymphocyte signal transduction and IL-2 transcription leading to reduced effector T-cell responses. The main adverse effect of these drugs is renal toxicity and drug levels should be monitored\(^{158}\). Other side effects include hypertension, hirsuitism, headache, opportunistic infections, seizures, and paresthesia\(^{158}\). **Recommendations from the American College of Gastroenterology IBD Task Force (Talley et al., 2011) for treatment of IBD with Immunosuppressant are as follows:** Azathioprine and 6-MP are not recommended for inducing remission in active UC but are effective at preventing relapse in quiescent UC with weak recommendation and low quality of evidence. Methotrexate is not recommended for inducing remission in active UC or for preventing relapse in quiescent UC with a weak recommendation and low quality of evidence. IV cyclosporin is effective at improving symptoms in hospitalized patients with severely active UC not responding to corticosteroids. Tacrolimus is not recommended in mild-to-moderately active UC with weak recommendation and very low quality of evidence. Azathioprine and 6-MP are not recommended for inducing remission in active CD but are effective at preventing relapse in quiescent CD with
a weak recommendation and low quality of evidence. Intramuscular methotrexate is effective at inducing remission in active CD with weak recommendation and low quality of evidence. Cyclosporin is not recommended for inducing remission in active CD or for preventing relapse in quiescent CD with weak recommendation and very low quality of evidence. Methotrexate is effective at preventing relapse in quiescent CD with weak recommendation and low quality of evidence.
**Biologic Therapy.** Treatment of IBD started to include biologics in 1998 with current therapy guidelines devised from composite evidence of clinical trials, clinical series, and expert opinions. Thus the IBD Task Force conducted a systematic review and meta-analysis of randomized control trials (RCTs) to estimate the effectiveness and safety of these drugs in IBD. Overall there were no statistically significant differences between adverse events in patients randomized to biological therapy compared with placebo but the risk of infection increases if these agents are used in combination and concerns that the biological therapies may increase the risk of lymphoma have been evaluated. Most biologics target tumor necrosis factor alpha (TNF-α) a pleiotropic inflammatory cytokine shown to be linked to many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis, hidradenitis suppurativa and refractory asthma. Therapies used in CD inhibit TNF using a monoclonal antibody such as infliximab (Remicade), adalimumab (Humira), certolizumab pegol (Cimzia). Another biologic used for IBD is anti-α 4 integrin antibodies (natalizumab) which works by blockade of leucocyte adhesion to
endothelium, inhibiting extravasation and the inflammatory process. **Recommendations from the American College of Gastroenterology IBD Task Force (Talley et al., 2011) for treatment of IBD with biologics are as follows:** Infliximab is effective at inducing remission in ambulatory patients with moderate-to-severely-active UC with a strong recommendation and moderate quality of evidence. Infliximab is effective at improving symptoms in hospitalized patients with severely active UC with a weak recommendation and very low quality of evidence. Anti-TNF antibody therapies (infliximab, adalimumab, and certolizumab pegol) are effective at inducing remission in ambulatory patients with moderate-to-severely-active CD with a strong recommendation and moderate quality of evidence. Anti-α 4 integrin antibodies (natalizumab) are effective at inducing remission in ambulatory patients with moderately-to-severely-active CD with a weak recommendation and moderate quality of evidence. Anti-TNF α antibodies (infliximab, adalimumab, certolizumab pegol) are effective in cessation of fistula drainage in CD with a strong recommendation and low quality of evidence. Anti-TNF α antibodies (infliximab, adalimumab, certolizumab pegol) are effective at preventing relapse in quiescent CD with a strong recommendation and high quality of evidence. Anti-α 4 integrin antibodies (natalizumab) are effective in prevention of relapse in quiescent CD with a weak recommendation and low quality of evidence. Infliximab is effective in prevention of relapse in healed fistulizing CD with a weak recommendation and low quality of evidence.
Antibiotics. Modulating the gut flora using probiotics, transplanting the entire intestinal microbiota, or using antibiotics to inhibit harmful bacterial luminal antigens are currently being evaluated as treatment modalities for IBD. Since the substantial clinical efficacy of probiotics in IBD is less evident and fecal transplant is in its infancy, these studies were not considered. Antibiotics were evaluated showing promise but had no recommendation for a particular class of drug. Recommendations from the American College of Gastroenterology IBD Task Force (Talley et al., 2011) for treatment of IBD are as follows: Antibiotic therapies have a statistically significant effect at inducing remission in UC and CD but are not recommended as no particular class of drug could be recommended in clinical practice with a weak recommendation very low quality of evidence. Antibiotic therapies are effective at reducing fistula drainage in CD with a weak recommendation and low quality of evidence. Antibiotic therapies shows a statistically significant effect at preventing relapse in quiescent CD but these are not recommended as no
particular class of drug can be recommended in clinical practice with a weak recommendation and very low quality of evidence.

**Biologic Treatment options.** Other potential biologic treatment options under active investigation involve inhibition of inflammatory cell recruitment and adhesion, recombinant anti-inflammatory cytokines and cytokine inhibition, inhibitory T-cell antibodies, growth factors, and hormones\textsuperscript{160,161}. Biologics with clinical efficacy in IBD are reviewed in the proceeding paragraphs.

The development of anti-adhesion drugs that can selectively inhibit recruitment and inappropriate retention of leukocytes has shown promise for the treatment of chronic inflammation. A monoclonal antibody directed against the \( \alpha_4 \)-integrin, Natalizumab, binds both \( \alpha_4 \)-\( \beta_1 \) and \( \alpha_4 \)-\( \beta_7 \) integrins to block lymphocyte adhesion and is approved for use in CD\textsuperscript{162}. Unfortunately \( \alpha_4 \)-\( \beta_1 \) interacts with vascular cellular adhesion molecule-1 (VCAM-1), which is present on every vascular bed in the body and this immunosuppression can reactivate the latent JC virus inducing a progressive multifocal leukoencephalopathy (PML), that could be fatal\textsuperscript{163}. The monoclonal antibody directed against \( \alpha_4 \beta_7 \), Vedolizumab (MLN-02), selectively inhibits Madcam-1-mediated leukocyte gut mucosal adhesion and was found to be effective in a dose dependent manner for both CD and UC\textsuperscript{164,165}. Vedolizumab alters gut inflammation without inducing systemic immunosuppression by exclusively binding to \( \alpha_4 \beta_7 \) integrin and inhibiting adhesion of \( \alpha_4 \beta_7 \)-expressing cells to both Madcam-1 and fibronectin but not Vacam-1\textsuperscript{166}. Vedolizumab’s binding capacity was the highest for subset of human peripheral blood memory CD4(+) T lymphocytes (25%) that included gut-homing interleukin 17 T-helper lymphocytes with no binding in the majority of memory CD4(+) T lymphocytes (60%), neutrophils, and most
monocytes\textsuperscript{166}. Vedolizumab is currently in phase III clinical trials (GEMINI) for both UC and CD\textsuperscript{167-169}.

Another anti-adhesion drug in phase III clinical trials is an orally bioavailable small molecule CCX282-B (GSK-1605786, Traficet-ENTM or anti-CCR9) that selectively blocks the human CCR9 receptor, a small bowel specific chemokine receptor found on the majority of lamina propria T-cells that binds CCL25\textsuperscript{170}. The Phase II (PROTECT-1) and Phase III (SHIELD-3) clinical trials showed promising end point reductions in CDAI and maintained remission rates however the results are still not fully published. GlaxoSmithKline has reformulated CCX-282 for bioavailability and GSK1605786A is currently recruiting for Phase III clinical trials to investigate the efficacy and safety in patients with moderate to severe Crohn’s disease with completion expected in 2014\textsuperscript{171}.

Blockade of lymphocyte-vascular gut endothelium interactions with monoclonal antibodies seem to show promise in patients with UC. Visilizumab, an IgG2 monoclonal anti-CD3 antibody, was shown to be clinically beneficial in UC patients not responding to iv corticosteroids and decreased circulating CD4(+) T-cell in all patients\textsuperscript{172}. Basiliximab, an IgG1 monoclonal anti-CD25(IL-2 receptor) antibody, promoted prolonged remission after a single treatment, in moderate steroid-resistant UC\textsuperscript{173}.

Cytokine inhibition of IL-6 and CD T-helper1 polarizing cyotkines IL-12, IL-18, and IFN\textsubscript{\gamma} have also been designed and tested for efficacy\textsuperscript{174}. The humanized anti-IL-6 receptor monoclonal antibody, Tocilizumab, blocks both the membrane-bound and the soluble form of IL-6R and has been studied for potential use in the treatment of CD. A pilot study showed promising results with 80\% of patients given a biweekly infusion for 12 weeks showing a significantly higher clinical response rate then the placebo group, however only two patients
receiving tocilizumab achieved remission and endoscopic and histologic examination showed no difference between tocilizumab and placebo groups.\textsuperscript{175,176} A humanized anti-IFNγ antibody, fontolizumab, showed a biologic effect in a recent randomized clinical trial by reducing CRP levels in all fontolizumab groups compared to placebo\textsuperscript{177}. The anti-IL-12 antibody, ustekinumab, targeted against the p40 subunit of two important cytokines in CD pathophysiology, IL-12 and IL-23 has shown effects in CD patients. Treatment of either moderate-to-severe CD disease, CD patients previously given infliximab, and CD non-responders to infliximab with ustekinumab improved clinical response compared to placebo and reduced serum CRP levels\textsuperscript{178,179}. The anti-inflammatory cytokine, recombinant human IL-10 was not successful at inducing remission in patients with active CD showing no benefit for treatment of active disease\textsuperscript{180}.

The immunostimulator and growth factor, sargramostim (recominant human granulocyte-macrophage colony-stimulating factor) and filgrastin (granulocyte-colony stimulating factor) have shown promising results for inducing CD remission and reducing clinical disease activity but there are some inconsistencies across studies and these drugs need further investigation\textsuperscript{181-183}. The growth factor, growth hormone (GH), has been shown to modulate intestinal inflammation and mucosal repair in murine models of colitis\textsuperscript{184,185,186}. In clinical trials GH has improved linear growth, remission rates, and clinical disease activity in pediatric CD patients but larger clinical trials are needed to investigate GH’s potential in mucosal healing and repair in human colitis\textsuperscript{187,188,189}. 
The Fundamental Basis of Inflammatory bowel disease

The pathogenesis of IBD is complex and still not completely understood. Basic concepts are conserved in human and murine colitis and in spite of different genetic predispositions and environmental triggers, the common mediator of disease is still an altered immune response \(^{190,191}\). Under normal homeostatic conditions, the intestine has an immunologic tone of tolerance toward commensal flora and dietary antigens that is referred to as a state of “nonpathogenic inflammation” that keeps the immune response conditioned and has the ability to induce an immune response to pathogens\(^{192}\). This also establishes a hypo-responsive phenotype to pathogenic organisms upon infection that can be quickly controlled and is effective. IBD constitutes a loss of tolerance toward commensal flora, dietary antigens, and components of the actual immune response as evidenced by serological antibodies to a patient’s own neutrophils, colonizing yeast, E. coli, flagella, and cytokines (autoantibodies) that are routinely found in the serum of IBD patients\(^{193,194}\). Regulatory immune cells mediate tolerance and in IBD these cells are inadequately stimulated or maintained allowing an inflammatory response to either commensal or pathogenic to be over stimulated or persist and develop into a destructive inflammatory reaction\(^{195,196}\). The characterization of this immune dysfunction has been associated with T-cell activation and for over two decades the pattern of helper T-cell dysfunction has been traditionally distinguished as Th1 and Th2. It is clear that CD and UC are immunologically distinct as CD patients overproduce cytokines within the lamina propria associated with a T helper (Th) 1 response, such as IL-12 and interferon-\(\gamma\)^{197}. Conversely, UC patients overproduce cytokines associated with the Th2 response, such as IL-5 and IL-13\(^{198}\). Murine models of innate and adaptive mucosal inflammation also follow this Th1/Th2 paradigm and advance our understanding of CD4 T-cell biology. In 2006, T-cell mediated mucosal
immunology became more complex with the discovery that a subset of T cells, Th17 cells, induce the inflammatory pathology originally believed to be mediated by Th1 cells and IL-12 that require the IL-12 family member IL-23 as a growth factor\textsuperscript{199}. Furthermore, new advances in uncovering this complex mucosal immunology has revealed that in various settings of mucosal inflammation, Th1, Th2, and Th17 cells have pro- or anti-inflammatory properties\textsuperscript{200,201}. Recent data also suggests that neutrophils thought to be the final effector cell in acute inflammatory response also have the ability to crosstalk in chronic mucosal inflammation\textsuperscript{202}. Thus altered immune responses in both innate and adaptive immunity are considered to be key elements related to the pathogenesis of IBD.

**Hypothesized Pathogenesis in IBD**

IBD pathogenesis studies involve multiple basic scientific disciplines, population and social studies, psychology, genetics, microbiology, immunology, biochemistry, cellular and molecular biology, drug discovery, and DNA engineering. What we currently understand is that both CD and UC are the result of the combined effects of four basic components: environmental factors, the input of multiple genetic variations, alterations in the intestinal microbiota, and aberrations of innate and adaptive immune responses. It has become clear that none of these four components can, by itself, trigger or maintain intestinal inflammation but that the combination of these various factors is needed to bring about CD or UC in individual patients. Unfortunately there is no smoking gun in IBD pathogenesis and of substantial proof is that the concordance rate in monozygotic twins is 10-15\% in UC and 30-35\% in CD\textsuperscript{203}. Moreover genetic factors shown to have high penetrance in IBD including NOD2, IL-23R and ATG16L1 do not account for the striking rise in the incidence of CD and UC seen in the 20th century implicating the strong influence of environmental factors\textsuperscript{204}. Because patients have various combinations of these
disease susceptibility factors it creates diverse mechanisms of pathogenesis, clinical course, and treatment response. As the awareness of individual variability increases, so does the observation that we face the perplexity of elucidating these already complex mechanisms involved in chronic intestinal inflammation within the setting of each patient's individual (environmental, genetic, inflammatory, and gut microbial) variability. So as we move forward clinicians and researchers have to reformulate study designs that incorporate and control for these complex factors with the hope of a more tailored approach for medical therapy. There are several hypotheses for the pathogenesis of IBD which are still evolving.

**Hygiene Hypothesis.** The increased incidence of IBD and other autoimmune diseases parallels globalization and is thought to be the result of better hygiene. Improved hygienic conditions deplete organisms and their antigens that are crucial in developing normal levels of immunoregulation. The first to recognize this environmental effect was Charles Harrison Blackely in the 1870’s when he noticed that aristocrats and city-dwellers were more likely to get hay fever than were farmers. Microorganisms are thought to be necessary for programming the immune system particularly in the gut and damping future inflammatory responses by helping to establish the immunological balance between pro-inflammatory T-cell subsets and tolerance-inducing regulatory T cells. This is referred to as the “hygiene hypothesis” and is centered on the belief that a child overprotected from exposure to common environmental infectious agents then becomes infected with the pathogenic agents later in life (delayed exposure) and induces an inappropriate immunologic response leading to the development of an abnormal or ineffective inflammatory process. A literature review conducted by Koloski et al. found that helminthic infection, Helicobacter pylori exposure, antibiotic use, breastfeeding and sibship represented factors potentially associated with development of IBD supporting the
hygiene hypothesis in IBD\textsuperscript{209}. Furthermore, helminth Trichuris suis ova therapy in patients with active CD and active UC has shown improved patient response and remission rates with no adverse side effects in just 12 weeks of therapy\textsuperscript{210,211}.

**Environmental Risk Factors.** Environmental risk factors are highlighted by the fact that twins studies do not show a 100\% concordance rate and that increased IBD prevalence in immigrant populations from low IBD risk areas have a similar or higher risk of developing IBD when compared to the native population\textsuperscript{212-214}. Recent review of studies investigating the impact of environmental factors in familial IBD reported that the increased frequency of smoking habit, appendectomy, fecal-oral transmitted infections, eating habits, and pets were associated with the development of IBD\textsuperscript{215}. The most well characterized environmental risk factors involve the breakdown of intestinal barrier function, dysbiosis (an imbalance between “healthy” and “unhealthy” bacteria) and/or the presence of a pathogenic agent.

*The intestinal Microbiota.* The intestinal Microbiota in patients with IBD reveals a dysbiosis selection toward mucosal associated bacteria and reduced commensal species diversity compared to healthy controls\textsuperscript{216-219,220}. Reduced bacteria with anti-inflammatory properties and/or more bacteria with proinflammatory properties are found in IBD patients compared to healthy controls\textsuperscript{216}. Experimental models of colitis have shown that the intestinal microbiota is a major contributor to chronic inflammation and antibiotic treatment or housing in germ free conditions suppresses the colitis phenotype. In humans, fecal stream diversion resolves downstream inflammation in CD but reappears in postoperative exposure\textsuperscript{136,221}. Several studies have demonstrated increased risk of developing IBD following gastroenteritis\textsuperscript{222,223}. In a Danish, population-based cohort, long-term follow-up study showed that gastroenteritis caused by nontyphoid *Salmonella* or *thermophilic Campylobacter* increased the risk of developing IBD\textsuperscript{222}.
In CD patient’s colonization with a pathogenic adherent-invasive Escherichia coli (AIEC) is increased compared to controls and AIEC adheres to the brush border of primary ileal enterocytes isolated from CD patients but not from individuals without IBD. AIEC infected macrophages release large amounts of TNFα and CD patient AIEC infected macrophages have been shown to induce granulomas in vitro that can recruit lymphocytes. Many other infectious organisms have been suggest to be involved in IBD pathogenesis such as *mycobacteria paratuberculosis*, *Listeria*, *Pseudomonas fluorescens*, *Bacteroides vulgatus* but warrant further investigation.

**Smoking.** Smoking is an indisputable environmental factor in IBD. Smoking contributes to initiation of CD but has a protective effect in the development of UC and passive smoking has also been shown to be a novel risk factor in both CD and UC. In addition to the impact on disease susceptibility, smoking habit also modifies the disease clinical course increasing the risk of experiencing a relapse and the need for surgery. Smokers have luminal microbiota that consist of significantly higher bacteroides and investigation of whether this is a mechanism for the negative effects of smoking on CD is necessary.

**Nonsteroidal anti-inflammatory drugs (NSAID)s.** NSAIDs are analgesic, anti-inflammatory drugs readily available over the counter. NSAID acute and chronic ingestion promotes altered intestinal barrier dysfunction by inhibition of protective prostaglandins. The link between NSAIDs use and IBD activity is strong, however confounding factors make it difficult to establish cause and effect.

**Genetic Risk Factors with a focus on STAT3.** Genome-wide association studies examine common genetic variants in different individuals to see if any variant is associated with a trait. These variants are single-nucleotide polymorphisms (SNPs) and studies compare
millions of genetic variants using SNP arrays to DNA from patients with disease (cases) and similar people without the disease (controls). If one type of the variant (one allele) is more frequent in people with the disease, the SNP is thus "associated" with the disease and considered to mark a region of the human genome that influences the risk of disease. Results are presented as allele frequency with the fundamental unit for reporting effect sizes being the odds ratio. The odds ratio is the proportion of individuals in the case group having a specific allele, and the proportions of individuals in the control group having the same allele. When the allele frequency in the case group is much higher than in the control group, the odds ratio will be higher than 1.

GWAS has identified 99 non-overlapping genetic risk loci, including 28 that are shared by CD and UC. Loci implicated in IBD reveal many pathways important in intestinal homeostasis and barrier function, epithelial restitution, autophagy, innate and adaptive immune regulation, reactive oxygen species (ROS) generation, and endoplasmic reticulum (ER) stress (Figure 11). Interestingly, loci in multiple genes that involve STAT3 signaling are associated with susceptibility to CD as well as UC. These include TYK2, JAK2, STAT3, interleukin-10 (IL-10), interleukin-23 (IL-23), interleukin-23 receptor (IL-23R), interleukin-12 B precursor (IL-12B), and interleukin 12 receptor (IL-12R). The tyrosine kinases TYK2 and JAK2 directly phosphorylate STAT3 in response to both the IL-6 and IL-10 family of cytokines. IL-10 is an anti-inflammatory cytokine that directly activated STAT3 and is involved in regulatory T cell generation and maintenance. IL-23 and the IL23R induce STAT3 activation and are involved in Th17 cell generation whereas IL-12B and the IL-12R help mediate Th1 cell lineage commitment. The association of these loci with IBD implicate that STAT3 plays a central role in the pathogenesis of IBD and functions as a key conductor of innate and adaptive inflammatory responses.
Functional studies to determine causation have focused on coding variants however, non-coding SNPs can also modify gene expression through alternative splicing, transcription factor binding, locus accessibility, translational efficiency, and trans-regulators such as non-coding RNAs and micro RNAs\textsuperscript{237}. The rare IL10RA polymorphisms were identified by fine mapping and are associated with the loss of IL-10 signaling and development of early-onset IBD\textsuperscript{245}.
Recent data has shown that the IL23R R381Q gene variant protects against immune-mediated diseases by impairing IL-23-induced Th17 effector response. Additionally, the functional consequence of the non-coding STAT3 rs744166 “A” risk allele is investigated in a cohort of pediatric CD patients and EBV-transformed lymphocytes in Chapter 2.

**Signal Transducer Activator of Transcription-3 (STAT3) in IBD**

Cytokine responses are involved in the pathogenesis more specifically the initiation, dysregulation, perpetuation and resolution of the immune response in IBD. Current research has focused on the role of STAT mediated cytokine response in IBD. Among STATs, the role of STAT3 is the most well documented in human IBD studies and experimental IBD models. Once in the nucleus, STAT3 induces gene expression of molecules with diverse biological functions including cell growth, survival and apoptosis, cell motility, inflammation, and negative feedback. Therefore, STAT3 activation is highly context-dependent with distinct roles in innate and acquired immune cells that differ in acute and chronic inflammation and ultimately dependent on STAT3’s mode of action.

**STAT3’s Mode of Action.** The JAK–STAT paradigm is employed by most cytokine receptors to transmit chemical signals outside the cell, through the cell membrane, and into gene promoters on the DNA to induce rapid gene expression. The mammalian Janus Kinase family, are intracellular, non-receptor tyrosine kinases, that transduce cytokine-mediated signals and have four members: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). There are seven mammalian STAT family members identified: STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6 that function as latent cytoplasmic transcription factors and mediate cytokine and growth factor signaling pathways.
other STATs has a conserved amino-terminus for tetramerization, a DNA binding domain with a sequence specificity for a palindromic IFN-\(\gamma\)-activated sequence (GAS) element analogous to STAT1, an SH2 domain involved in receptor recruitment along with STAT protein dimerization, and a carboxy-terminal trans-activation domain. Cytoplasmic membrane receptor tails not only serves as a docking sites for JAKs but certain residues have been identified to be crucial for JAK activation. Ligand binding at the cell surface induces receptor chain dimerization activating receptor associated JAKs that in turn phosphorylate STATs at a single conserved carboxy-terminally located tyrosine residue. This induces reciprocal phospho-tyrosine SH2 domain interactions allowing STATs to form stable homo and heterodimers that traverse the nuclear pore and bind GAS elements in conjunction with other cofactors to induce transcriptional regulation of target genes. The most prominent mechanism utilized to negatively regulative JAK/STAT signaling is the suppressor of cytokine signaling (SOCS) proteins. The SOCS family members modulate signaling by several mechanisms shown to include binding and inactivation of JAKs, blocking access of STATs to receptor binding sites, and ubiquitination of signaling proteins and their subsequent targeting to the proteasome.

Different STAT proteins show preferred specificity for individual cytokine families.
STAT3 is the most pleiotropic member activated by a variety of cytokines and certain growth factors, including the interleukin-6 (IL-6) and Interleukin-10 (IL-10) cytokine family, and epithelial growth factor (EGF) \(^{270}\). In human and murine intestinal inflammation the most important STAT3 regulators are IL-6 considered a pro-inflammatory cytokine and IL-10 an anti-inflammatory cytokine that both activate STAT3 by different mechanisms with different gene expression profiles \(^{271}\).

**The IL-6-STAT3 Signaling Pathway.** The IL-6 family of cytokines (IL-6, IL-11, IL-27, IL-31, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor (CNTF), and CT-1 are characterized by the use of the IL-6 signal transducing subunit, gp130 receptor β (GP130). IL-6 and IL-11 are restricted to GP130 homodimerisation whereas IL-27, IL-31, LIF, OncostatinM, CNTF, and CT-1 induce heterodimeric GP130 receptor complexes \(^{272}\). IL-6 binding the IL-6 receptor-α subunit (CD126) triggers engagement and dimerization of GP130 that activates receptor associated JAK1, JAK2 and Tyk2 \(^{273}\). The receptor associated Jak’s mediate subsequent tyrosine phosphorylation of the GP130 receptor allowing docking sites for STAT3 SH2 domains to create the close proximity needed for JAK1/2 to phosphorylate STAT3 \(^{274-276}\). STAT3 activation seems to be induced by JAK2 and to a lesser extent JAK1 in response to IL-6 and in human colorectal cancer cell lines JAK2 and to a lesser extent JAK1 but not JAK3 induced STAT3 phosphorylation and subsequent activation involved in survival, cell cycle, and tumor cell invasion \(^{277,278}\). JAK mediated tyrosine phosphorylation at a single site (Y705) in the carboxy-terminus of STAT3 is required for dimerization, nuclear translocation, and DNA binding \(^{279}\). STAT3 can also be activated by serine phosphorylation at a site within the transactivation domain (S727) thought to be involved in maximal transcriptional activity by enhanced recruitment of necessary transcriptional cofactors \(^{280}\). Cancer associated cytosolic and
Figure 13. Regulation of intracellular STAT3 signaling  Stat3 signaling is induced by various kinases (green) in a phosphorylation dependent manner (green arrows), and counteracted by several regulatory proteins (red). Stat3 activation occurs in response to gp130 hetero or homodimerization following binding IL6 type cytokines (blue) to their specific transmembrane receptor α-subunits (white). Phosphorylation of four membrane distal tyrosine (Y, black) residues by constitutively associated JAK-family tyrosine kinases (TK) is sufficient to enable src-homology domain (SH)-2 mediated binding of STAT3 and, to a lesser extent, of STAT1. Once tyrosine phosphorylated, STAT3/1 form homo- and heterodimers, which translocate and transactivate target genes, including the negative regulator SOCS3. STAT3 can also be phosphorylated by certain cytosolic TKs and receptor TK. The serine threonine kinases (STK) mediate serine-phosphorylation that maximizes the transcriptional activity. Gp130 also engages the Ras/ERK pathway through binding of the tyrosine phosphatase SHP2 to the membrane proximal phospho-Y (Y757 in mouse and Y759 in humans). This phosphor-tyrosine also provides the binding site for SOCS3 to mediate proteasomal degradation of ligand-occupied receptor complexes. Gp130 signaling is also attenuated by the Y-phosphatase activity of SHP2, while cytoplasmic PIAS3 protein sequesters Y-phosphorylated STAT3 from homodimerization, nuclear translocation and target gene activation. Adapted from Jarnicki and Ernst. Stat3: linking inflammation to epithelial cancer—more than a “gut” feeling. Cell Division 2010 5:14, Copyright (2010) with permission from BioMed Central Ltd.

receptor tyrosine kinases can also activate STAT3. Elegant genetically engineered murine models have shown that the cytoplasmic tail of GP130 contains four tyrosine residues that are required and sufficient for activation of STAT3 and STAT1, with STAT3 having the predominant role in vitro and in vivo. Additionally in the cytoplasmic tail of GP130 another tyrosine residue (Y757 in mouse, Y759 in humans) is responsible for engagement of the
cytoplasmic Src homology tyrosine phosphatase (SHP2) and subsequent activation of the Ras-ERK pathway to transduce activities regulating mitogenesis (Figure 13)\textsuperscript{273}.

**Negative Regulators of the IL-6 signal transducing subunit GP130.** The Suppressor of cytokine signaling-3 (SOCS3), is a negative regulator of GP130 induced STAT3/STAT1 activation and functions as a classic negative feedback loop via direct transcription by STAT3 activation\textsuperscript{282}. SOCS3 binds to the same phospho-tyrosine that enables the activation of SHP2 (Y757 in mouse and Y759 in humans), and inhibits further binding of STAT3 SH2 domains to the GP130 receptor and binds the receptor associated JAKs to restrain their activity\textsuperscript{283,284}. Binding of SOCS3 to activated GP130 has been shown to induce SOCS3 mediated JAK associated proteosomal degradation maintaining the transient nature of STAT3\textsuperscript{285}. The tyrosine phosphotase activity of SHP2 can also inhibit GP130 signaling and cytoplasmic protein inhibitor of activated STAT, 3 (PIAS) can sequester tyrosine-phosphorylated STAT3 from homodimerization, nuclear translocation and target gene activation\textsuperscript{283,286}.

**The IL-10-STAT3 Signaling Pathway.** The IL-10 family of cytokines IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 all share the common IL-10 receptor-\(\beta\) subunit. In the case of the IL-10R, the IL-10R \(\alpha\)-chain has two defined docking sites for STAT3, whereas GP130 provides four docking sites and STAT3 activation via IL-10 mediates an anti-inflammatory response (AIR) in cells expressing the IL-10 receptor \(\alpha\) chain.\textsuperscript{287,288} Interleukin-10 is a pleiotropic cytokine, whose main function is the limitation and ultimately termination of immune responses in IBD. The primary role of IL-10 shown in vitro and in vivo is to inhibit the powerful stimulatory effects of TLR agonists such as LPS that act on macrophages and dendritic cells\textsuperscript{289}. IL-10 acts as a key mediator for maintaining gut homeostasis and mice lacking IL-10 are characterized by excessive cytokine release and development of enterocolitis\textsuperscript{290}. The IL-10
SHP2 is a ubiquitously expressed cytoplasmic PTP, all tyrosine-phosphorylated signaling components are potential substrates for the SHP2 phosphatase. PIAS3 specifically inhibits STAT3-mediated gene expression (after IL-6 stimulation). SOCS3 is functionally most related and inhibits IL-6-type cytokine signaling by acting on JAKs and thereby inhibiting the phosphorylation of gp130, STATs and the JAKs themselves. The balance of protein synthesis and degradation also affects IL-6-type cytokine signal transduction and the half-lives of the various players involved in IL-6 signaling differ substantially. The feedback inhibitor SOCS3 is very short-lived, STAT3 and SHP2 have slow turnover rates while Janus kinases JAK1, JAK2, TYK2 and gp130 show intermediate half-lives. (Representation of the negative regulators and their sites of action (red arrows). Reprinted with permission from Biochem. J. 374, Heinrich et al. Principles of interleukin (IL)-6-type cytokine signalling and its regulation, 1-20, Copyright (2003) the Biochemical Society.)

family receptor subunits lack SOCS3 binding sites, thus IL-10 receptor engagement results in sustained STAT3 activation due to the loss of SOCS3 inhibition, this mediates the difference in effector properties of IL-6 versus IL-10 induced STAT3 activation (Figure 15). Importantly, heterologous cytokine receptor systems that activate STAT3 but are naturally refractory such as the IL-22R, or an engineered IL-6R to be refractory to SOCS3 mediated inhibition activate an AIR indistinguishable from IL-10. Thus transient STAT3 activation by IL-6 in wild-type
macrophages promotes inflammatory responses. However, macrophages lacking the SOCS3 gene or carrying a mutation in the SOCS3-binding site of GP130 have both IL-10 and IL-6 suppression of lipopolysaccharide-induced tumor necrosis factor (TNF) and IL-12\textsuperscript{292}. In vitro, SOCS3-deficient CD4\textsuperscript{+} T cells produced more transforming growth factor (TGF)-beta1 and interleukin (IL)-10 and chromatin immunoprecipitation assay revealed that more STAT3 was recruited to the TGF-beta1 promoter in SOCS3-deficient T cells than in control T cells\textsuperscript{293}. SOCS3 also has a significant role in constraining the generation of Th17 cells by attenuating the phosphorylation of STAT3 and IL-23 induced Stat3 phosphorylation is enhanced in the absence of SOCS3\textsuperscript{294}. However the AIR response induced by IL-10-STAT3 is still not completely understood. The AIR is selective, targeting subsets of inflammatory genes accounting for \~{}10–15\% of the genes induced by TLR signaling after LPS stimulation. IL-10 mediated STAT3 activation induces the transcriptional repressor, ETV3 and a transcriptional corepressor, Strawberry notch homologue 2 (SBNO2) which has strong repressive activity for NF-\kappa B\textsuperscript{295}. Therefore, perhaps unrecognized or undetectable additional signals other that the IL-10R can potentially act in conjunction with an IL-10-STAT3 response in an anti-inflammatory manner to regulate inflammation. This could explain divergent effects of STAT3 activation in effector T-cell function as STAT3 via IL-6 and IL-23, in combination with factors like IL-1\beta, TGF-\beta, and RoR\gammat, induce T helper 17 (Th17) formation\textsuperscript{296}. Whereas IL-2-induced STAT5 activation is required for the development of FOXP3\textsuperscript{+} regulatory T cells (Tregs) and STAT3 activation by IL-10 is important for the function of Tregs such as their ability to suppress pathogenic Th17 responses\textsuperscript{297,298}. 
Figure 15. Strength and duration of STAT3 activation determines cellular responses. STAT3 activation patterns and associated outcomes differ between IL6 and IL10 family cytokine-induced signaling. In response to binding of ligand (blue), signaling from gp130 is transient, because the phosphorylated, membrane proximal tyrosine residue (Y, red) provides a binding site for the negative regulator SOCS3. Although SOCS3 is also induced in response to activation of the IL10 receptor family, STAT3 signaling remains sustained, because the IL10 receptor chains lack the corresponding YxxV motif. Similarly, signaling from the mutant gp130Y757F receptor is sustained due to phenylalanine (F) substitution of the Y757 residue (Y759 in human GP130), resulting in exaggerated activation of its down-stream signaling molecules. The range of target genes activated differs between acute and sustained STAT3 activation, most evident in macrophages where the former promotes and the latter suppresses inflammatory response. Adapted from Jarnicki and Ernst. Stat3: linking inflammation to epithelial cancer-more than a “gut” feeling. Cell Division 2010 5:14, Copyright (2010) with permission from BioMed Central Ltd.
**STAT3 in Human IBD**

Many studies have shown that compared to other STAT proteins, STAT3 is heavily involved in the pathogenesis of IBD. In IBD there have been no known activating mutations in the STAT3 gene but it is well known that the pro-inflammatory overproduction of IL-6 is the main mediator of constitutive STAT3 activation while the regulatory and anti-inflammatory action of IL-10 induced STAT3 activation is lost\(^{271}\). IL-6 is believed to regulate mucosal inflammation in large part via tyrosine phosphorylation of STAT3 in effector lymphocytes and granulocytes. Some of the first studies to better highlight STAT3 activation in mucosal inflammation were by Suzuki et al. who detected phosphorylation of STAT3 but not STAT1, STAT5, or STAT6 in whole colonic tissue extracts from DSS treated mice and mucosal biopsies of UC and CD patients\(^{253}\). These studies also found STAT3 activation in all colitis models examined including DSS treatment, TCR-\(\alpha\) gene knockout, CD4\(^+\) T cell transfer, IL-10 knockout, macrophage- and neutrophil specific STAT3 conditional knockout as well as TNBS treated\(^{253}\). However, these studies were done from whole colonic extracts whereas Lovato et al. reported constitutive activation of STAT3 and SOCS3 in colonic mucosal CD3\(^+\) and CD4\(^+\) T cells of patients with CD but not healthy controls\(^{248}\). One criticism of these experiments was the use of intestinal and peripheral T-cell lines grown with high doses of IL-2 before assessing STAT3 activation. Musso et al. went on to demonstrate that activated STAT3 was detected by immunobloting of freshly isolated lamina propria mononuclear cells from inflamed IBD tissues, but not in peripheral blood mononuclear cells from control subjects or IBD patients\(^{249}\). They also demonstrated that activation of STAT3 is confined to actively inflamed areas of the mucosa in IBD, where phosphorylated STAT3 is found exclusively in infiltrating CD3\(^+\) T lymphocytes and CD68\(^+\) macrophages but not neutrophils\(^{249}\). However more recent studies have suggested
that STAT3 is also activated in peripheral and lamina propria neutrophils. Mudter et al. reported an increased amount of total STAT3 protein in both UC and CD compared to non-inflammatory control cells, and total STAT3 correlated with increased STAT3 activation in tissue sections from both UC and CD. These studies also revealed that the increased frequency of pSTAT3+ cells directly correlated with the histological degree of inflammation and that activated T lymphocytes and innate colonic epithelial cells demonstrated STAT3 activation.

Neurath et al. evaluated cytokine production of stimulated T cell and macrophage enriched lamina propria cells in patients with CD compared to control. Using ELISA they described an upregulation of IL-6 compared to IL-4, IL-5, IL-10 and IL-11. Furthermore, lamina propria T cells in CD patients activated by IL-6 induced STAT3 dependent induction of anti-apoptotic genes such as bcl-2 and bcl-xl. Mitsuyama et al. established that colonic mucosa from patients with active disease contains larger amounts of IL-6 than colonic mucosa from patients with inactive disease or normal controls. RT-PCR analysis of mucosal specimens showed that of the inflammatory cytokines IL-1β, TNFα and IL-6, IL-6 mRNA levels were the highest in active IBD patients. Furthermore, spontaneous production of IL-6 by peripheral blood and lamina propria mononuclear cells was increased in patients with IBD. These were the first studies to propose that IL-6 mediated STAT3 could foster T-cell survival and aggravate chronic colitis.

These above mentioned studies have all involved adult patients receiving immunomodulatory therapies with longstanding chronic disease. Brown et al. revealed that in intestinal biopsies from newly diagnosed untreated pediatric IBD patients the level of IL-6 secretion significantly correlated with the graded severity of histological inflammation. Carey et al. was the first to identify a pro-inflammatory IL-6:STAT3 biological network that drives mucosal inflammation in pediatric onset CD both at diagnosis and during therapy. Serum IL-6
was significantly increased in CD and UC patients at diagnosis and treated CD patients with active disease. In the peripheral blood, CD3+CD4+ lymphocytes were hyper-responsiveness to IL-6 stimulation and the basal frequency of pSTAT3-positive granulocytes was elevated in IBD patients compared to healthy controls. In colonic biopsies, the frequency of total pSTAT3-positive cells (lamina propria and epithelial) correlated with overall mucosal inflammation and increased epithelial injury and the frequency of pSTAT3+ peripheral blood granulocytes correlated with the severity of mucosal inflammation. In addition, Carey et al. identified biologically relevant networks present where IL-6 and STAT3 functioned as central regulatory nodes with genes involved in immune and inflammatory mediators or chemokines, cytokines, and growth factors. Recent studies indicate that serum IL-6 has excellent diagnostic accuracy for detecting patients that have active disease as evaluated by CDEIS. These studies have been seminal in developing a role for STAT3 activation more specifically IL-6 induced STAT3 activation within the intestinal mucosa of pediatric-onset and adult-onset IBD. Importantly, this activation is observed in both acquired and innate immune cells such as T-lymphocytes and the intestinal epithelial cells. Additionally, in a novel murine model of CD, the SAMP/YIT mouse, IL-6 induced STAT3 activation contributes to the development of this spontaneous chronic ileitis and colitis model. These data implicate that IL-6:STAT3 signaling mediates pro-inflammatory gene expression within the mucosa of IBD patients.

**IBD Patients with True Acute Colitis?** To understand the function of STAT3 during intestinal inflammation, it is helpful to divide inflammation into three distinct stages (1) acute involving the innate immune response, (2) chronic, involving the adaptive immune response and (3) recovery or wound healing and resolution of the adaptive immune response also referred to as clinical remission in human subjects. Sophisticated tissue specific conditional transgenic and
gene deletion murine models have allowed the investigation of STAT3 in innate and adaptive colitis. However, murine models are often difficult to interpret in comparison to human studies especially when a patient diagnosed with IBD may have the disease for several months and is ultimately in the chronic phase. Therefore in human studies the acute phase and timing of inflammation is hard to decipher and study design may involve dichotomized patient populations of newly diagnosed untreated versus treated and patients in remission. The only true acute IBD lesion is the postoperative recurrence model of CD where an ileocolic resection with anastomosis to remove diseased bowel induces an immediate disease remission. However even with diseased bowel removed the endoscopic recurrence of disease arises in the neoterminal ileum in 30% of patients after 3 months and in up to 80% of patients after 1 year\textsuperscript{309}. In these patients that subsequently developed recurrence, ileal mucosal IL-1\(\beta\), IL-6 and TNF\(\alpha\) levels at the time of remission postoperatively were significantly higher than in the group who remained in remission at 1 year (IL-1\(\beta\), \(p=0.02\); IL-6, \(p=0.003\); TNF\(\alpha\), \(p=0.03\)) on univariate analysis\textsuperscript{310}. In the same study a multivariate analysis revealed that only elevated mucosal IL-6 levels were shown to be an independent risk factor for postoperative disease recurrence. In this same model, patients with low IL-10 mRNA levels in ileal biopsies preoperatively were found at 3 months to have higher rates of endoscopic recurrence than those with high IL-10 mRNA levels (80% vs 40%, \(p=0.02\))\textsuperscript{311}. These data highlight the pro-inflammatory role of mucosal IL-6 along with a deficiency in mucosal IL-10 in disease initiation or acute colitis. Another important factor shown to be involved in disease initiation is the bacterial flora and/or other components of the faecal stream. Interestingly, postoperative recurrence patients with diversion of faecal stream proximal to an ileocolic anastomosis had no endoscopic evidence of recurrence in the neoterminal ileum 6 months after surgery however, after restoring bowel continuity, essentially
all patients had rapid endoscopic recurrence in the neoterminal ileum\textsuperscript{221}. The aforementioned studies point out the importance of mucosal IL-6 and IL-10 as well as the bacterial flora and/or other components of the fecal stream in initiation of IBD. Moreover, the postoperative recurrence model is the closest human model to deduce initiating events in IBD.

**STAT3 in Innate and Adaptive Immunity, One Cell at a Time**

The innate immune response is the first line of defense against invading pathogens. The innate immune system consists of physical and chemical barriers, cells and molecules that recognize pathogen-associated molecular patterns (PAMPs), and effector systems that facilitate destruction of pathogens. PAMPs are recognized by pattern-recognition receptors, such as Toll-like receptors (TLRs) on the cell surface and intracellular Nod-like receptors (NOD) of innate immune cells\textsuperscript{312}. Dysfunction in the innate immune system and/or inappropriate adaptive immune response can mediate chronic intestinal inflammation\textsuperscript{313}. IBD is more associated with a defective adaptive immune system as inflamed mucosa from CD and UC patients consistently shows the presence of infiltrating activated CD4\textsuperscript{+} T lymphocytes. Abnormalities in regulatory pathways ultimately control both the innate and the adaptive response whereas dendritic cells (DCs) and various cytokines, important in the innate immune system determine the type of adaptive T cell response that predominates during the inflammatory process\textsuperscript{314}.

The gut associated lymphoid tissue (GALT), is the largest reservoir of lymphoid tissue in the body separated by a single layer of epithelial cells and mucus from an immense amount of luminal bacterial (more than 1000 species) and dietary antigens\textsuperscript{315}. Luminal bacteria aid in digestion of food, protection against pathogens, and help maintain immune system conditioning. The immunologic tone within the GALT is regulated by unique mechanisms that involve
development and maintenance of Preyer’s patches, isolated lymphoid follicles and connection to the mesenteric lymph node and spleen. A key cell type within the GALT that regulates tolerance and antigen presentation is the dendritic cell (DC).

**Dendritic Cells.** DCs have a unique function in sensing and then reacting to the gut microenvironment. DCs shape the immune response by responding with tolerance to normal commensal microbiota and luminal antigens or generate protective immune responses against pathogens without causing pathology\(^{316}\). DCs directly sense the luminal content, extending dendrites between epithelial cells, and recognize luminal antigens in the lamina propria through apoptosis of epithelial cells or transported by specialized epithelial cells present in the follicles of Peyer’s patches, microfold (M) cells \(^{317}\). DCs migrate from the lamina propria to the draining mesenteric lymph nodes (MLNs) constitutively delivering antigens to present to B and T cells initiating either a tolerogenic or an inflammatory response\(^{318} \ 319\). In the periphery regulatory T cells (Tregs) induced by DCs can suppress the function of effector T cells diverting naïve T cells that are reactive to inoffensive(self) antigen \(^{316}\). DCs also mediate the protective immune response involved in effector cell induction and determine whether a T helper (Th) 1 (IFN-\(\gamma\)), Th2(IL-4), or Th17(IL-17) response will prevail while the inflammatory milieu, containing cytokines such as IFN-Is, TNF, IL-6, IL-12, and IL-23, may also regulate DC activity and direct effector T cell differentiation\(^{320}\). Murine models of colitis have shown accumulation of activated DCs throughout the lamina propria and MLNs \(^{247}\). Targeted deletion of the Stat3 gene with a DC-specific CD11c-cre (Stat3 CKO) impairs weight gain and mice develop ileocolitis that is associated with impaired mucosal tolerance \(^{321}\). Moreover, Stat3 CKO DCs develop normally, but are resistant to IL-10 suppression and exhibit both an enhanced ability to secrete inflammatory cytokines and stimulate T cells. These experiments emphasize an important role
for Stat3 in antagonizing DC activity and directly link DC hyperactivity with inflammatory disease.

**Macrophages.** Another innate immune cell type in the intestine is the resident macrophage. Studies in human and animal models have shown a role for abnormal macrophage immune responses to the intestinal microbiota in the pathogenesis of IBD. Resident macrophages vary greatly compared to newly recruited inflammatory monocytes/macrophages during an infection. Resident macrophages reside underneath the epithelium in the lamina propria, waiting to phagocytose luminal microbes that breach the epithelial barrier. These macrophages are efficient at eliminating bacteria within the lamina propria but are not professional antigen-presenting cells and have low expression of surface costimulatory molecules, and do not initiate and immune response. Resident macrophages maintain homeostasis in the intestine by clearing apoptotic cells and debris, promoting epithelial repair and producing IL-10 that is shown to maintain expression of FOXP3 in colonic Tregs. Resident macrophage function is determined by cytokines in the microenvironment such as TGF-β, IL-10, and IL-6 and they mediate tolerance by inducing differentiation or inhibition of T cells and influence the adaptive immune response. In the midst of tissue injury or infection newly recruited macrophages exhibit an inflammatory phenotype and secrete pro-inflammatory mediators such as TNFα, nitric oxide (NO), and IL-1 that are involved in the activation of various microbial defense mechanisms. In patients with IBD, macrophages are increased in the inflamed mucosa and are functionally different than resident macrophages in that they can mount a robust immune response to luminal microbes and express T costimulatory molecules (CD40, CD80, and CD86), PAMP receptors (TLR2, TLR4, CD89, TREM1, and CD14), and become an important source of TNFα. Deletion of STAT3 in the myeloid cell lineage
induces spontaneous colitis due to impaired IL-10 signaling in macrophages and neutrophils\textsuperscript{259}. Similarly, mice lacking expression of IL-10 spontaneously develop colitis, which is a consequence of macrophage differentiation into pro-inflammatory subsets that produce large amounts of IL-12 and IL-23 and depletion of macrophages in IL-10 KO mice prevents development of colitis\textsuperscript{322}. STAT3-deficient macrophages also demonstrated enhanced CD8 T cell cross presentation with antigen\textsuperscript{330}. Thus IL-10 induced STAT3 activation in the myeloid cell lineage helps suppress a pro-inflammatory phenotype and maintains gut homeostasis.

\textbf{Neutrophils.} Neutrophils were regarded as short-lived innate immune effector cells that engulf and kill invading pathogens however new evidence suggests that neutrophils also play a part in effector and regulatory circuits in both the innate and adaptive immune systems\textsuperscript{331}. Neutrophils express a vast array of pattern recognition receptors (PPRs) including TLRs and NLRs (Nod-like-receptors) and are characterized as phagocytic cells that release noxious enzymes from their granules and produce reactive oxygen intermediates (ROI) with antimicrobial potential\textsuperscript{332}. Neutrophils also either spontaneously or following appropriate stimulation secrete multiple cytokines and chemokines that enhance phagocytosis, activate complement and regulate inflammation\textsuperscript{333}. Other non-classical forms of neutrophil effector function include release of extracellular fibrillary networks termed neutrophil extracellular traps (NETs) that snare microorganisms and soluble pattern recognition molecules (PRMs) with antibody-like properties\textsuperscript{334}.

The production of neutrophils is extensive in steady state and the major activity in hematopoiesis is devoted to the production of monocytes and granulocytes (myelopoiesis). Granulocyte colony-stimulating factor (G-CSF), promotes myeloid progenitor cell proliferation, neutrophil differentiation, and neutrophil mobilization from the bone marrow into the circulating
blood. G-CSF binds the homodimeric G-CSF receptor (G-CSFR), which activates associated Jak1 and Jak2 protein tyrosine kinases whereby experiments in vitro demonstrate that STAT3 is strongly activated and to a lesser extent so are STAT1 and STAT5. The STAT3 receptor recruitment site within the G-CSF receptor is critical for controlling G-CSF-responsive neutrophil production/mobilization and SOCS3 has been shown to be important in terminating signals from the G-CSF receptor. STAT3 controls macrophage inflammatory protein-2 MIP-2(CXCL2) responsive neutrophil mobilization from the bone marrow and STAT3-deficient neutrophils have defective migration in response to CXCR2 ligands, the main receptor for CXCL2. G-CSF-stimulated CXCR2(II8rβ) transcription occurs by direct binding of STAT3 to the II8rβ promoter in immature neutrophils (Gr-1lo) that enhances their responsiveness to CXCL2(MIP-2). Furthermore, in mice deletion of STAT3 in the bone marrow causes death within 4–6 weeks after birth with Crohn’s disease-like pathogenesis in both the small and large intestine inducing inflammatory cell infiltration, ulceration, bowel wall thickening, and granuloma formation. Interestingly these STAT3 deficient mice have neutrophilia with a pseudoactivated innate immune responses with high levels of TNFα and IFNγ (Th1-type response), inhibition of the NAPDH oxidase activity, and increased NFκB activity after LPS stimulation. Therefore STAT3 has critical roles in the development, regulation and tolerance of innate immunity, and deletion of STAT3 during hematopoiesis results in abnormalities in myeloid cells that causes Crohn’s disease-like pathogenesis.

Once neutrophils leave the bone marrow they are recruited to sites of infection through the help of endothelial cell adhesion molecules. Removal of circulating neutrophils from the blood ameliorates inflammation in IBD patients suggesting that they play a major role in the course of inflammation. Microbes and resident macrophages generate signals as sites of
infection that activate local endothelial cells to provide signals for neutrophil to gain access to
tissues. Bypassing neutrophils are captured and guided across the endothelial cell lining where
chemokines, cytokines and products generated by live microorganisms guide neutrophils toward
the microbes. Neutrophil cell surface expression of PSG-1(CD162), L-selectin(CD62 ligand),
ESL-1, and CD44 engages endothelial cell P-selectin/E-selectin to mediate rolling and activation
of the neutrophil integrins that interact with ICAM-1 and VCAM-1 expressed on endothelial
cells. LFA-1(αLβ2) mediates stop of rolling and firm adhesion together with Mac-1(αMβ2) after which paracellular or transcellular migration occurs. Concurrently
endothelial cells activated by TNFα, IL-1β, and IL-17 produce IL-8 and MIP-2 that activates
neutrophils. IL-8 and the murine homologues KC(CXCL1) and MIP-2(CXCL2), are
neutrophil chemoattractants that orchestrate neutrophil activation and recruitment from the blood
into sites of infection, inflammation and injury by promoting endothelial adhesion and
transmigration. Their functional effects are mediated by binding CXCR1 and CXCR2. CXCR2 has proved to be a potent mediator of neutrophil recruitment in murine models of
autoimmune diseases including IBD and increased mucosal expression of these chemokine
receptors and their ligands in IBD may explain the massive influx of leucocytes in active
disease. Acute induction of human IL-8 production by the intestinal epithelium triggered
neutrophil infiltration and in a model of dextran sulphate sodium (DSS) induced acute colitis
there is a marked increase in KC(CXCL1) and MIP-2(CXCL3) within the colon. Furthermore, the up-regulation of IL-8 in the colonic mucosa of IBD patients correlates with the
histological degree of inflammation and chemokine mRNA expression. Interestingly, calprotectin (S100A8/S100A9) a neutrophilic cytosolic protein can be detected in stool and is
thought to be a valid biomarker for intestinal inflammation. In pediatric IBD, faecal
calprotectin was a direct measure of intestinal inflammation and a good marker of the risk of histological relapse. Therefore, targeting neutrophil influx is a potential therapeutic strategy for IBD. Additionally, Chapter 2 of this PhD thesis reveals, that pediatric CD patients carrying the STAT3 rs744166 “A” risk allele have increased frequency of peripheral blood pSTAT3 positive leukocytes, increased STAT3 activation, increased colonic expression of IL-8, CXCL1, CXCL3, calprotectin (S100A8/S100A9), S100A12, and increased frequency of colonic CXCR2 positive neutrophils. Therefore, the rs744166 SNP may be a biomarker for a new cohort of pediatric CD patients in which blockade of CXCR2 would potentially be therapeutically relevant.

Chemokine-directed leukocyte trafficking can also be directed by IL-6. IL-6 stimulates myelopoiesis and causes accompanying changes in the numbers of peripheral neutrophils due to increased demargination of intravascular neutrophils. IL-6 participates in neutrophil migration by inducing production of chemokines such as IL-8 and CCL2(MCP-1/Monocyte chemoattractant protein-1) and by inducing expression of adhesion molecules such as ICAM-1 on endothelial cells. Moreover, increased mRNA levels in colonic mucosal biopsies and enhanced lamina propria mononuclear cell IL-6 production has been shown in both CD and UC. In CD patients circulating IL-6 was considered to correlate with the inflammatory activity and intestinal IL-6 production was highly correlated with the severity of endoscopic and histopathological signs of inflammation. Thus, elevated serum IL-6 levels in CD patients with steroid induced remission were highly predictive for a following relapse. Studies show that lamina propria T cells, macrophages (CD68+), and epithelial cells are the main cell types responsible for elevated intestinal IL-6 concentration. Increased circulating levels can be detected in both pediatric(early-onset) and adult IBD patients. Studies show
that the transition from acute to sustained or chronic response involves enhanced leukocyte activation, differentiation of T-cell subsets and T-cell proliferation mediated by STAT3 and controlled by a mechanism known as IL-6 trans-signaling\textsuperscript{251,369}.

**Neutrophils hold the key in transition from acute to chronic Inflammation.** In the lamina propria, classical IL-6 signaling is restricted to neutrophils, monocytes/macrophages and epithelial cells since they express the IL-6R\textsubscript{α} (Rose-John et al, 2006). IL-6 trans-signaling is initiated by shedding of the IL-6R from the cell surface of the initial neutrophil infiltrate, resulting in soluble IL-6R (sIL-6R) (Figure 16). This sIL-6R has been shown to induce CXC and CC chemokine expression of IL-8(CXCL8), CCL2(MCP-1), CCL20(MCP-3) and in acute inflammation inhibit neutrophil recruitment while promoting the attraction of monocytes and lymphocytes\textsuperscript{370}. This induces IL-6-s-IL-6R complexes that can signal via the GP130 receptor. Gp130 is ubiquitously expressed on all cell types whereas the expression of the IL-6R is restricted to some lymphoid cells including monocytes, macrophages, neutrophils, B-cells, subpopulations of T-cells and non-lymphoid cells such as hepatocytes\textsuperscript{371-373}. Atreya et al. demonstrated that the IL-6–s-IL-6R system modulates T cell resistance against apoptosis and that a pathway of T-cell activation driven by IL-6–sIL-6R strongly contributes to the perpetuation of chronic intestinal inflammation\textsuperscript{251}. In CD patients, isolated lamina propria mononuclear cells and T-cells secreted elevated levels of IL-6 and sIL-6R that then form IL-6-sIL-6R complexes\textsuperscript{251}. These complexes then stimulate gp130 on the surface of lamina propria T cells in the gut, causing a STAT3-dependent up regulation of intestinal T-cell resistance to apoptosis through induction of the anti-apoptotic genes Bcl-2 and Bcl-xl whereas the expression of the STAT3 independent pro-apoptotic gene Bax is unaffected\textsuperscript{251,374}. The increased Bcl-xl/Bax ratio has been
shown to mediate resistance against intestinal T-cell apoptosis in experimental models of colitis as in IBD patients. Two, three, and six.

Figure 16 Schematic model of IL-6 trans-signaling in inflammatory bowel disease. There is increased production of IL-6 by macrophages, lymphocytes and intestinal epithelial cells in response to continuous stimuli by intestinal microflora. The soluble IL-6 receptor (sIL-6R) is generated through shedding of the membrane-bound receptor from the surface of macrophages or neutrophils by proteolytic cleavage. This process can occur under stimulation by the acute-phase protein CRP in macrophages and by microbial metalloproteases in human monocytes. The increased formation of IL-6/IL-6R complexes that interact with gp130 on the membrane of T cells lacking membrane-bound IL-6R through trans-signaling leads to an increased expression and nuclear translocation of STAT3. This causes the induction of anti-apoptotic genes, such as Bcl-xl and Bcl-2 and resulting in an augmented resistance of T cells to apoptosis. The ensuing T-cell expansion contributes to the perpetuation of intestinal inflammation. Reprinted from Cytokine and Growth Factor Reviews, 17, Mitsuyama et al. Interleukin-6 trans-signaling in Inflammatory Bowel Disease. 251-261. Copyright (2006) with permission from Elsevier.
T-Lymphocytes. CD4+ T cells play a major role in initiating and shaping the chronic immunopathologic process of IBD. The inflamed mucosa of IBD patients is characterized by the heavy infiltration of activated CD4+ T lymphocytes and blockade of the activation or activity of these CD4+ T cells can reduce the ongoing mucosal inflammation in both murine models of colitis as well as in patients with IBD. It is commonly accepted that pathogenic T cells constantly stimulated by commensal enteric bacterial antigens cause chronic intestinal injury. IL-6:STAT3 signaling is an important inflammatory pathway that promotes T-cell survival, proliferation and effector function. Both CD and UC are characterized as an exaggerated immune response in genetically susceptible individuals. However, they differ by the nature of the inflammation and subsets of T helper cells that mediate the perpetual recruitment and/or commitment of naïve T cells and granulocytes. Most experimental models of chronic intestinal inflammation are dependent on lamina propria T-cells even if there is a primary epithelial defect, solidifying the importance of mucosal T-cells during chronic inflammation. T-cell effector lineage is influenced by the cues derived from the microenvironment, particularly cytokines. CD4+ T cells can diverge into four main subgroups, three sets of helper T cells Th1, Th2, Th17 and the regulatory Treg cells that all secrete characteristic types of cytokines. Tregs suppress excessive immune responses and this regulation is lost in CD and UC patients. CD is driven by a Th1/Th17 response in which IL-12(p34/p40) and IL-23(p19/p40) play key roles whereas the Th2-like response in which NKT cells producing IL-13 and IL-5 is the major response in UC (Figure 17). However, recent murine and human studies from several labs also implicate a role for the IL-17 producing Th17 lineage in UC. Thus, the unique adaptive immune response differentiates CD from UC.
STAT3 plays a critical role in T-cell apoptosis, proliferation and differentiation however, these STAT3 functions ultimately depends on the microenvironment and the activation status of the T-cell. In the initial studies regarding T-cell STAT3 deficiency, IL-6-induced proliferation was severely impaired and did not prevent apoptosis as compared to wild type STAT3 T-cells, which was independent of the anti-apoptotic protein Bcl-2. Teague et al. found a difference in the response of T cells to IL-6, depending on the activation status of the cells whereby IL-6 inhibited the death of naive T cells but had no effect on the death of either superantigen-activated T cells, or T cells bearing memory markers. Activated T cells had a lower level of surface IL-6
receptors but there was a similar level of STAT3 phosphorylation (Y705/S727) in both naïve and activated T-cells treated with IL-6. Interestingly, they did however observe a profound inhibition of IL-6–induced Stat1 phosphorylation in activated T cells compared with resting T cells. In this model system IL-6 was not sufficient to induce T-cell survival in activated and memory T-cells. In the aforementioned studies STAT3 induced by IL-6 trans-signaling enhanced mucosal T-cell survival in murine models of colitis and human IBD and blockage of this STAT3 induced signal with an antibody against the IL-6R enhanced T-cell apoptosis and had the downstream effect of inhibiting mucosal inflammation. In a T cell-transfer colitis model of lymphopenic Rag2−/− mice reconstituted with naive T cells from control (Stat3flx/flx) or Stat3−/− (CD4-Cre;Stat3flx/flx) mice resulted in protection of CD4+ T cell STAT3 deficient mice. Further experiments revealed that STAT3 in CD4+ cells was essential for differentiation of Th17 cells and in its absence T cells were nonpathogenic and tended to divert toward a Treg cell fate. Chip-Seq in T-cells from these mice identified that almost all the genes known to regulate Th17 cell differentiation as well as genes that promote survival and proliferation were direct targets of STAT3. In the opposing T cell lineage Chaundhry et al. utilized Foxp3-Cre:Stat3flx/flx mice to delete STAT3 in Treg cells and demonstrated a selective dysregulation of TH17 responses upon Treg-specific ablation of Stat3 and identify Treg dependent control of TH17 responses as an essential component of immune homeostasis. Finally, certain Th17 cytokines such as IL22 induce epithelial barrier protective properties such as increasing defensin secretion by paneth cells, enhancing mucin production by goblet cells, and promoting IEC migration, thereby limiting inflammation. These studies in entirety demonstrate that action of STAT3 in T lymphocytes is highly context-dependent and may mediate anti or pro-inflammatory effector function.
**Intestinal Epithelial Cells.** The intestinal epithelium is the primary barrier between large quantities of commensal and potentially pathogenic bacterial species from the underlying gut associated lymphoid tissue (GALT). Thus intestinal epithelial cells in addition to digestion and absorption of nutrients also perform indispensable barrier functions that mediate homeostasis and inhibits host immune-microbial interactions. The intestinal epithelial cells (IECs) maintain this important homeostatic barrier through intracellular tight junctions that maintain a selective and polarized epithelium. This physical barrier is reinforced by mucin and antimicrobial peptides secreted to the apical surface by goblet and paneth cells \(^{378,385}\). Epithelial cells themselves can also secrete antimicrobial peptides including defensins, cathelicidins, and calprotectin \(^{386,387}\). IECs also express TLRs and NLRs, at low levels to help combat an innate or adaptive immune response \(^{388,389}\). Tonic signaling through PRRs on IEC seems to be tolerogenic as IEC-specific deletion of components in the IKK complex (inhibitor of NfκB (IkB) kinase), p65/RelA, or murine transgenic expression of the human NOD2 gene increase susceptibility to intestinal inflammation in multiple disease models \(^{390-392}\).

Murine models and human genetic and environmental linkage studies in IBD reveal that increased epithelial permeability and bacterial immune activation as the major contributor in the development of acute and chronic intestinal inflammation \(^{377,393}\). Almost all experimental mouse models of inflammatory bowel disease can be inhibited in mice devoid of intestinal flora \(^{394-396}\). A recent report revealed that overexpression of CD97, an adhesion G-protein-coupled receptor in intestinal epithelial cells of transgenic mice attenuates colitis by strengthening adherens junctions \(^{397}\). The epithelial barrier is an important consideration in all phases of murine colitis and human IBD.
Another mechanism that mediates IEC homeostasis is efficient epithelial wound healing and restoration of the epithelial barrier in response to acute mucosal insults. Non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, enteric or other systemic infections, and stress are all reported potential triggers of IBD and are plausible inducers of gut injury. As the actual primary cause of IBD remains unclear it is suspected to be the result of either an initial defect in the intestinal barrier or a breakdown of immune tolerance. In dextran sodium sulfate (DSS) induced murine colitis, IEC STAT3 activation has a crucial role in the maintenance of the intestinal epithelial barrier and homeostasis via proliferation, survival, and wound healing.

DSS exerts direct toxic effects on epithelial cells independently of the underlying gut immune system and its transient administration provides an experimental system to assess the critical first phase of wound healing. Murine models in which gp-130-STAT1/3 signaling is abolished (gp130\(^{\Delta STAT}\)) or in IL-6 deficient mice (IL-6/-/-), there is exaggerated acute intestinal colitis in response to DSS as compared to wild type. This was due to impaired epithelial wound healing with consistently more severe epithelial erosion. This suggests that IL-6 induced STAT3 activation promotes epithelial restitution and wound repair important during the innate immune response. However, these models evaluated STAT3 contribution to innate immune response by inactivating STAT3 in multiple cells types. Bollrath et al. and Grivennikov et al. went on to show that ablation of STAT3 signaling in IEC resulted in greater susceptibility to DSS induced epithelial damage and mucosal inflammation. However, this protected animals from the colitis associated cancer tumorigenesis, whereby hyper-activation of GP130 mediated STAT3 promoted tumor incidence and growth. Gene chip analysis of IECs isolated from DSS treated IEC STAT3 deficient mice compared to controls demonstrated a reduction in
the expression of genes involved in cellular stress response, apoptosis, and pathways associated with IEC wound healing specifically, Reg3 family members\textsuperscript{398}. Additionally, IL-22 knockout animals were also more susceptible to DSS induced colitis and showed reduced in-vivo wound closure rate compared to IL-6KO and control\textsuperscript{398}. Thus epithelial cell STAT3 seems to regulate survival, proliferation, and wound healing events during acute colitis and colitis-associated cancer. However, these studies have investigated the contribution of IEC STAT3 in acute injury or during tumorigenic chronic colitis conditions. Thus loss of STAT3 within the epithelial compartment would likely increase risk for the development of chronic inflammation following an acute self-limited gut injury, which has not been formally tested. A model of acute injury and either resolution or development of chronic colitis in a genetically susceptible host would be expected to be more applicable to the current concept of IBD pathogenesis in humans. Therefore, chapter 3 of this PhD thesis investigates the functional consequences of IEC STAT3 deletion in a model of chronic colitis in which mice are exposed to DSS for seven days, and then assessed during recovery over the next twenty-one days. These studies reveal that deletion of IEC STAT3 promotes T-lymphocyte STAT3 activation and chronic colitis.

**Summary**

STAT3 is a master regulator of mucosal inflammation with activation occurring in multiple cell types in a highly context dependent manner. Transient STAT3 activation is essential for gut homeostasis while constitutive activation leads to chronic inflammation. During the innate immune response, IL-6 induced STAT3 activation results in effector function of DCs, macrophages, and polymorphonuclear cells to mediate robust clearance of infection while IL-10 induced STAT3 activation leads to inhibition of these cell types. The function of regulatory T
cells mediated by IL-10 induced STAT3 activation and Th17 cells mediated by IL-6 induced STAT3 activation allows the mucosal immune system to fight infection and return to steady state. In IEC’s during acute models of colitis IL-6 induced STAT3 activation mediates proliferation and survival while IL-22 induced STAT3 activation mediates wound healing responses. Importantly, the loss of STAT3 within the epithelial compartment following an acute self-limited gut injury, has not been formally investigated during chronic phases of colitis.

In IBD there is loss of IL-10 induced regulatory pathways and the pro-inflammatory overproduction of IL-6 drives STAT3 activation and continued mucosal inflammation. Thus, with STAT3 being such an integral part of the mucosal immune response it is not surprising that recent GWAS studies have associated the genetic variant rs744166 in STAT3 with risk for IBD.

Chapter 2 of this PhD thesis builds upon the understanding that newly diagnosed pediatric CD patients have up-regulation of mucosal IL-6:STAT3 dependent biologic networks that drive disease and stratifies these patients by carriage of the STAT3 rs744166 “A” risk allele. These studies show that the rs744166 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. Additionally, Chapter 3 reveals that the loss of Stat3 activation in the epithelial compartment ultimately leads to more severe chronic colitis with reduced epithelial restitution gene expression and expansion of pSTAT3+ lymphocytes and IL-17a expression. These studies as a whole reveal the kaleidoscopic nature of STAT3 activation in the context of IBD and suggest that downstream mediators of disease may be more relevant in therapeutic intervention.
References

7. remember to add citation for IBD review


Chapter 2

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

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Doi: 10.1097/MPG.0b013e318246be78

PMID: 22197944

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License # 2847790442225

Preface

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Abstract: 250 words

Text: 4164 words (excludes abstract, keywords, references, and figure legends)

Figures: 7

Tables: 4

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


Writing assistance: not applicable

Competing Interest: None to declare

Grant Support: This work was supported by the Bioinformatics, Gene Expression, Integrative Morphology and Flow Cytometry cores of the National Institutes of Health (NIH)-supported Cincinnati Children’s Hospital Research Foundation Digestive Health Center (1P30DK078392-01), and NIH grants R01 DK078683 (LAD), R01 DK068164 (LAD), T32 DK007727 (BK & RC), and DK069513 and the Primary Children’s Medical Center Foundation (SLG). This investigation was supported by Public Health Service research grant UL1-RR025764 and CO6-RR11234 from the National Center for Research Resources.
Author Contributions:

Study concept and design: TW, BK, SG, LD

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Obtained funding: SG, LD

Administrative, technical, or material support: IJ, SG, EB, RC, HX

Study supervision: SG, LD
Abstract & Key Words

**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.

**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.

**Key words:** 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® (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 (Stratgene) 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 TTG 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 ACT-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].
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 pSTAT3. Endogenous peroxide was quenched with 3% hydrogen peroxide for 15 min at RT and tissue was permeabilized with 0.3% 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 1x10^6 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® analysis.** EBLs were stimulated as described above and then stained with antibodies for pSTAT3, IL-6 receptor, and DRAQ5 nuclear marker. 5x10⁴ 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 subscores (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.** We then asked if there would be an increase in the frequency of neutrophils expressing activated STAT3 (pSTAT3) 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 pSTAT3+ 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 pSTAT3-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).

Moreover, 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+.3 neutrophils per hpf in controls (data not shown).

**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 pSTAT3 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 (pSTAT3) 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 pSTAT3\(^+\)CD3\(^+\)/CD4\(^+\) lymphocytes (\(p=0.01\), Figure 3B) and pSTAT3\(^+\)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 pSTAT3\(^+\) 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 pSTAT3\(^+\) lymphocytes (\(p=.003\), Figure 3B) or granulocytes (\(p=.02\), Figure 3C) following IL-6/IL-6R stimulation. This was specific as the frequency of pSTAT5\(^+\)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 pSTAT5\(^+\) 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+5pg/ml (\(n=22\)) compared to non-risk allele patients 86+25pg/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® (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.
Acknowledgment

This work was supported by the Bioinformatics, Gene Expression, Integrative Morphology and Flow Cytometry cores of the National Institutes of Health (NIH)-supported Cincinnati Children’s Hospital Research Foundation Digestive Health Center (1P30DK078392-01), and NIH grants R01 DK078683 (LAD), R01 DK068164 (LAD), T32 DK007727 (BK & RC), and DK069513 and the Primary Children’s Medical Center Foundation (SLG). This investigation was supported by Public Health Service research grant UL1-RR025764 and CO6-RR11234 from the National Center for Research Resources. Ramona Bezold, Kathleen Lake, and Ann Rutherford provided outstanding support with subject recruitment.
Figures and Tables

Figure 1

A. SOC3

\[
\begin{align*}
\text{colon mRNA} \quad \text{relative expression} \\
\text{GG} & \quad \text{AG AA} \\
\hline
0 & \quad 1 & \quad 2 & \quad 3 & \quad 4 & \quad 5 \\
\end{align*}
\]

\[ p = .03 \]

B. IL-8

\[
\begin{align*}
\text{colon mRNA} \quad \text{relative expression} \\
\text{GG} & \quad \text{AG AA} \\
\hline
0 & \quad 5 & \quad 10 & \quad 15 \\
\end{align*}
\]

\[ p = .07 \]

C. CXCL3

\[
\begin{align*}
\text{colon mRNA} \quad \text{relative expression} \\
\text{GG} & \quad \text{AG AA} \\
\hline
0 & \quad 5 & \quad 10 & \quad 15 & \quad 20 \\
\end{align*}
\]

\[ p = .01 \]
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 ± 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

A.

[Images of histological sections labeled CTR, GG, AA, with a scatter plot showing pSTAT3+ Neutrophils per HPF and significance levels marked with p = .09 for GG and p = .02 for AG_AA.]

D.

[Additional images and graphs showing CXCR2+ Neutrophils per HPF and significant correlation (r = 0.68, p = 0.007) with CDH1S.]
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; 400x 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; 400x 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

A

B

C

Frequency of pSTAT3+CD4+CD3+

p = 0.01

p = 0.003

Frequency of pSTAT3+ Granulocytes

p = 0.004

p = 0.02
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 ± 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

A. STAT3

B. STAT1

C. JAK2

D. IL-6r

E. GP130

F. SOCS3
Figure 4. Cytosolic protein abundance of STAT3, STAT1, IL-6 receptor, JAK2, GP130, nor SOCS3 varied by STAT”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

A

B

C

D

E

F

STAT3

SOCS3

EBV cell line mRNA
relative expression

EBV cell line mRNA
relative expression

p = 0.04

p = 0.04

p = 0.01

p = 0.001

p = 0.002

p = 0.04
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 TFIIB 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 ± 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

A

B

C

D

E

F

2-32
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

Fold Change in pSTAT3 Nuclear Co-Localization

GG

GG +IL-6

Fold Change in pSTAT3 Nuclear Co-Localization 0

AA

AA +IL-6

Fold Change in pSTAT3 Nuclear Co-Localization 10.8
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® (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

<table>
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<tr>
<th>STAT3 Genotype</th>
<th>Age (n=8)</th>
<th>Male Gender</th>
<th>Colon-only Location</th>
<th>STER</th>
<th>6-MP</th>
<th>5ASA</th>
<th>PCDAI</th>
<th>CDHIS</th>
<th>CDHIS Sub-Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>12(3)</td>
<td>88%</td>
<td>38%</td>
<td>38%</td>
<td>25%</td>
<td>25%</td>
<td>36(16)</td>
<td>5(4)</td>
<td>4.5 (2) 2.3(.5) 4.3(1)</td>
</tr>
<tr>
<td>AA or AG</td>
<td>13(3)</td>
<td>75%</td>
<td>40%</td>
<td>39%</td>
<td>25%</td>
<td>25%</td>
<td>36(17)</td>
<td>7(4)</td>
<td>4.5 (3) 2.8(2) 4.8(3)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Cytoband</th>
<th>FOLD CHANGE</th>
<th>STAT3 Genotype:</th>
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<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>AG/AA</td>
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<td><strong>JAK/STAT Signaling</strong></td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
<td>7p21</td>
<td>1.4</td>
<td>2.8*</td>
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<tr>
<td>IL-6R</td>
<td>Interleukin 6 Receptor</td>
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<td>IL-6ST</td>
<td>Interleukin 6 Signal Transducer</td>
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<td>JAK2</td>
<td>Janus Kinase 2</td>
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<td>1.3</td>
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<td>Signal Transducer and Activator of Transcription 1</td>
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<td>Signal Transducer and Activator of Transcription 5A</td>
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<td>CCL4</td>
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<td>CXCL3</td>
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<td>S100 Calcium Binding Protein A12</td>
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<td>5.8*</td>
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</table>

GG, AG/AA: CD null, heterozygous or homozygous for the STAT3 “A” risk allele, fold-change is relative to healthy control colon, *p<0.05 or **p<.01 versus GG by unpaired t test with Welch’s correction.
<table>
<thead>
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<th>Genotype</th>
<th>Age</th>
<th>Male</th>
<th>CD</th>
<th>UC</th>
<th>5-ASA</th>
<th>Azathioprine</th>
<th>STER</th>
<th>Infliximab</th>
<th>Methotrexate</th>
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<tr>
<td>GG (n=8)</td>
<td>16(8-35)</td>
<td>25%</td>
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<td>75%</td>
<td>88%</td>
<td>38%</td>
<td>63%</td>
<td>13%</td>
<td>0%</td>
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<td>AA (n=10)</td>
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<td>50%</td>
<td>90%</td>
<td>20%</td>
<td>11%</td>
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</tbody>
</table>

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


Chapter 3

Deletion of Intestinal Epithelial Cell STAT3 Promotes T Lymphocyte STAT3 Activation and Chronic Colitis Following Acute Dextran Sodium Sulfate Injury in Mice

Tara A. Willson BS\textsuperscript{1,4}, Ingrid Jurickova MD\textsuperscript{1}, Margaret Collins MD\textsuperscript{2}, and Lee A. Denson MD\textsuperscript{1,4*}
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Preface

Abstract: 251 words

Text: 5,763 words

Abbreviations: Dextran Sodium Sulfate (DSS) Chronic Colitis, Signal Transducer and Activator of Transcription 3 (STAT3), Interleukin-17 (IL-17), Azoxymethane (AOM), macrophage (MΦ)

Financial disclosures: The authors have no financial arrangement(s) with a company whose product figures prominently in the submitted manuscript or with a company making a competing product.

Writing assistance: not applicable

Competing Interest: None to declare

Grant Support: This work was supported by the Cincinnati Children’s Hospital Research Foundation Digestive Health Center (1P30DK078392-01), and NIH grants R01 DK078683 and DK068164.

Author Contributions:

Study concept and design: TW, LD
Acquisition of data: TW
Analysis and interpretation of data: TW, MC, LD
Drafting of the manuscript: TW, LD
Critical revision of the manuscript for important intellectual content: TW, LD
Statistical analysis: TW
Obtained funding: LD
Administrative, technical, or material support: IJ
Study supervision: LD
Abstract and key words

Background: Intestinal epithelial cell (IEC) Stat3 is required for wound healing following acute Dextran Sodium Sulfate (DSS) injury, and we have reported that the IBD JAK2 risk polymorphism is associated with reduced cellular STAT3 activation. We hypothesized that loss of IEC STAT3 would promote the development of chronic colitis following acute DSS injury. Methods: Colitis was induced in IEC-specific Stat3 deficient mice (Stat3\(^{\DeltaIEC}\)) and littermate controls (Stat3\(^{Flx/Flx}\)) with 4%DSS for 7 days, followed by water consumption for 21 days. Epithelial and immune mediators and severity of colitis were determined. Results: Survival, colon length, and histologic injury were significantly worse at day 28 in Stat3\(^{\DeltaIEC}\) mice. IEC proliferation and apoptosis did not vary by genotype at day 28. The colonic lamina propria frequency of pSTAT3+ cells was increased at day 28 and correlated with histologic injury in Stat3\(^{\DeltaIEC}\) mice. The frequency of colonic F480+pSTAT3+ macrophages and CD3+pSTAT3+ T-lymphocytes were increased in Stat3\(^{\DeltaIEC}\) mice as compared to Stat3\(^{Flx/Flx}\) controls. In Stat3\(^{\DeltaIEC}\) mice, colonic expression of Stat3 target genes Reg3\(\beta\) and Reg3\(\gamma\) which mediate epithelial restitution were significantly decreased, while expression of IL-17a, IFN\(\gamma\), CXCL2, CXCL10, CCL2, and CCL4 were significantly increased and correlated with the increase in histologic severity at Day 28 (p<.05). IL-17a expression also correlated with the increased lamina propria frequency of CD3+pSTAT3+ T-lymphocytes. Conclusions: Loss of intestinal epithelial Stat3 leads to more severe chronic inflammation following acute injury which is not accounted for by a sustained defect in epithelial proliferation or apoptosis but rather expansion of pSTAT3+ lymphocytes and IL-17a expression.

Key words: Dextran Sodium Sulfate (DSS), Chronic Colitis, Signal Transducer and Activator of Transcription 3 (STAT3), and Interleukin 17A
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). The precise etiology remains unclear but evidence suggests that it involves the deregulation of the host immune response to luminal flora. Large scale genome wide association studies (GWASs) have now identified multiple genetic factors that confer IBD susceptibility.\(^1\)\(^-\)\(^3\) Genotypic variation in \textit{STAT3} and \textit{JAK2} has been linked to IBD risk.\(^1\) We have recently reported that carriage of the \textit{STAT3} risk allele is associated with increased cellular STAT3 activation and up-regulation of chemokines expressed on 4q12-13 which promote neutrophil recruitment to the gut\(^4\). Conversely, we have found that patients carrying the \textit{JAK2} risk allele have decreased STAT3 activation in IL-6-stimulated PB CD3+/CD4+ lymphocytes and reduced colonic expression of the STAT3 target gene \textit{REG3a} suggesting that reduced STAT3 activation may also be associated with increased risk for IBD\(^5\).

Signal transducers and activators of transcription (STATs) mediate cytokine signaling. Constitutive activation of STATs, especially STAT3, has been reported in several inflammatory and malignant disorders. STAT3 is a pleiotropic transcription factor that displays tissue specific differences in function. STAT3 is activated by multiple cytokines and growth factors and roles for IL-6, IL-11, IL-17, IL-22, IL-27, growth hormone and leptin in experimental colitis have been elucidated.\(^6\)\(^-\)\(^{15}\) In animal models, Stat3 activation in intestinal epithelial cells is required for acute wound healing responses via induction of \textit{Reg3} family members, but also promotes development of colitis-associated cancer during chronic inflammation.\(^16\),\(^17\) Stat3 activation in myeloid cells mediates anti-inflammatory effects of IL-10; targeted deletion of Stat3 in this cell type leads to severe entero-colitis.\(^18\) 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.\(^6\),\(^9\) Tyrosine phosphorylation
of STAT3 induced by IL-6 has also been linked to effector lymphocyte and granulocyte activation in IBD.\textsuperscript{19} 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.\textsuperscript{20} We identified an IL-6:STAT3 biological network that drives leukocyte recruitment and thereby mucosal inflammation in this setting.

Previous studies in IEC Stat3 deficient mice have examined the effects of either acute DSS induced colon injury, or multiple rounds of DSS administration together with injection of the carcinogen Azoxymethane (AOM) to induce colitis associated cancer. These studies have shown that while Stat3 activation promotes survival and proliferation of IECs in response to acute injury, during chronic colitis under tumorigenic conditions epithelial Stat3 drives the development of colitis associated cancer\textsuperscript{16, 17}. While these prior studies suggested that loss of Stat3 within the epithelial compartment would likely increase risk for the development of chronic inflammation following an acute self-limited gut injury, this had not been formally tested. This model of acute injury and either resolution or development of chronic colitis in a genetically susceptible host would be expected to be more applicable to the current concept of IBD pathogenesis in humans. We therefore sought to define the functional consequences of intestinal epithelial specific deletion of Stat3 in a model of chronic colitis in which mice are exposed to DSS for seven days, and then assessed during recovery over the next twenty-one days. We hypothesized that loss of IEC Stat3 would promote the development of chronic colitis following acute DSS injury. We found that loss of intestinal epithelial Stat3 leads to more severe chronic inflammation following acute injury which is not accounted for by a sustained defect in epithelial proliferation or apoptosis but rather expansion of pSTAT3\(^+\) lymphocytes and IL-17A expression.
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Material and Methods

Animals
To delete Stat3 in intestinal epithelial cells (IEC), we crossed Stat3<sup>lox/lox</sup> mice (termed Stat3<sup>Flx/Flx</sup>) obtained with permission from Takeda via Dr. Jeffrey Whitsett at CCHMC and backcrossed for five generations on a C57BL/6 background before intercrossing to villin-Cre (B6.SJL-Tg(Vil-Cre)997Gum/J, Jackson Laboratories, Bar Harbor, Maine) to generate compound mutant villin-Cre/Stat3<sup>lox/lox</sup> mice (termed Stat3<sup>Flx/Flx</sup>). Cre-negative littermates were used as controls (termed Stat3<sup>Flx/Flx</sup>). We confirmed deletion of intestinal epithelial STAT3 using an in-vivo model of LPS exposure and PCR for total STAT3 in isolated enterocytes (Supplemental Figure 1). Stat3<sup>Flx/Flx</sup> mice were born in mendelian ratios, developed normally and showed no obvious spontaneous phenotype as has been previously published<sup>10, 17</sup>. All mice were maintained in conventional housing at CCHMC and all experiments were performed in accordance with CCHMC and Institutional Animal Care and Use Committees of CCHMC.

DSS Colitis
Intestinal inflammation was induced by 4% DSS water (m.w. 36,000-50,000):MP Biomedical, LLC, Solon, OH) for 7 days in acute studies designated (day 7) or 7 days followed by 21 days of water designated (day 28) to study progression from acute phase to chronic inflammation and recovery. Healthy control animals received water only designated (day 0). The dosing of DSS was established by initial experiments within our colony to induce moderate to severe colitis while minimizing mortality. Body weight change during DSS protocol was calculated by dividing body weight on the specified day by body weight at day 0 (starting body weight) and expressed in percentage and recorded on Day 0, 3, 5, 7, 10, 12, 18, 20, 22, 26, and 28. In all experiments, littermate controls were used to assure comparison of mice on the same genetic background. DSS injury occurs throughout the colon at varying levels and
the area involved increases from the proximal to the distal colon\textsuperscript{22}. Therefore we utilized the anal verge as the most distal reference point from which to perform histopathological analysis.

**Tissue sampling**

Mice were sacrificed and the colon from the ileocecal valve to the anal verge was removed. Colon length was determined from the cecocolic junction to the anal verge. The cecum was removed at the cecocolic junction and the whole colon flushed with ice-cold PBS. The colon was then cut into two equal segments one labeled as distal and the other as proximal. The distal and proximal segments were filed open and cut into two equal portions the length of the segment. One portion of distal and proximal colon was laid flat and fixed in 10\% neutral buffered formalin overnight, transferred to 70\% ethanol, processed and embedded, sectioned at 5-µm, and utilized for immunohistochemistry. The remaining distal and proximal portions were Swiss-rolled at the base of a vinyl cryomold that was subsequently filled with O.C.T compound (Tissue Tek\textsuperscript{®}, Sakura Finetek USA, Inc) frozen on dry ice, and stored at -80°C for later use in immunohistochemical analyses and RNA extraction\textsuperscript{23}. All sections from paraffin embedded and Swiss-rolled OCT fixed colons were processed so that the two segments had continual villus crypt architecture from the anal verge to the cecocolic junction for representation of the whole colon.

**Histo-pathological assessment of DSS-induced colitis.**

After staining with hematoxylin-eosin colon sections were scored as previously described with the following alternations\textsuperscript{24}. It is known that C57BL/6J mice upon DSS administration develop distal and middle colonic disease with little disease in the most proximal portion\textsuperscript{22}. To orient a starting point for analysis we developed a scoring system to account for total colonic disease by using the anal verge to mark the most distal starting point for scoring and the following four consecutive longitudinal 5x High Powered Fields (HPF) scored as the distal colon and the following four, 5x HPF scored as the proximal
colon. With the observer blinded to genotype, a disease score was determined separately for the distal and proximal portions by adding the scores for area involved, erosion/ulceration, crypt loss, edema, and infiltration of immune cells into the mucosa. The total disease score was calculated as the sum of distal and proximal disease score divided by two.

**Immunohistochemical Analyses (IHC)**

For IHC, paraffin-embedded slides were deparaffinized and antigen unmasking was done by boiling for 10 minutes in 10mM sodium citrate (pH 6) for cleaved caspase-3 (Cell Signaling, Danvers, MA), and 1mM EDTA (pH 8) for tyrosine phosphorylation state specific (Tyr705) STAT3 (pSTAT3-XP) (Cell Signaling, Danvers, MA). 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 4ºC with primary antibodies. Biotinylated secondary anti-rabbit antibodies (Vector Laboratories, Burlingame, CA) were added and incubated at room temperature for 1hr. 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.

Quantification of cleaved caspase-3 staining in epithelial cells of the distal and proximal colon was performed, with observer blinded to genotype, by counting positive cells in three 400x high-powered fields (HPFs) in the mucosa that bordered ulcers of the distal and proximal colon or in the corresponding area of the colon in untreated controls. BrdU staining was performed as suggested by the manufacturer (BrdU staining kit; Invitrogen, Carlsbad, CA). Mice were injected with BrdU labeling reagent (Zymed®, Invitrogen, Carlsbad, CA) per manufacturer instructions 2hr before sacrifice and processed for immunohistochemistry as above. Quantification of pSTAT3 or BrDU positive epithelial cells from properly oriented crypts defined as those with clear cell column lining both sides of an open luminal area, were counted within .5cm (or one 50x HPF) of the mucosa that bordered ulcers in the distal and
proximal colon or in the corresponding segment of the colon in untreated controls. The number of pSTAT3 or BrdU-positive cells per crypt divided by the total number of cells per crypt was determined with the observer blinded to genotype and data are expressed as a labeling index. Histological analysis and microphotographs were attained on an Olympus BX51 microscope utilizing the Olympus DP71 digital camera system and analyzed using DP-BSW ver .03.02 software.

**Immuno-fluorescence**

5-µm-thick colonic sections were fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 min and washed with phosphate-buffered saline once followed by permeabilization with ice-cold methanol at -20 for 10 min. The cells were then blocked with 5% bovine serum albumin for 60 min. To double label, sections were incubated with rabbit anti-mouse pSTAT3-XP antibody overnight at 4°C. The following day sections were incubated with Rhodamine (TRITC) conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and then sequentially incubated with either rat anti-mouse F4/80 (eBioscience, San Diego, CA) to stain macrophages, or goat anti-mouse CD3-ε (Santa Cruz Biotechnology, Santa Cruz, CA.) to stain T lymphocytes for 1 hour at room temperature and finally incubated with DyLight™ 488-conjugated anti-goat or Fluorescein (FITC)-conjugated anti-Rat secondary antibodies (Jackson ImmunoResearch Laboratories Inc.). Cell nuclei were labeled with DAPI diluted in the aqueous mounting media, Fluoromount-G (Southern Biotech), to prevent fluorochrome quenching during analysis and for a semi-permanent seal. Images were captured using Olympus BX51 microscope utilizing the Olympus DP71 digital camera system and analyzed using DP-BSW ver .03.02 software.

**Real-time RT-PCR**

Swiss Rolled colonic tissues embedded in O.C.T compound were cross sectioned into four to five 30-µm-thick sections, giving approximately 20mg of total colonic tissue. The sections were rinsed in
RNA later® RNA stabilization reagent (Ambion, Austin, TX) to remove O.C.T medium and RNA was extracted using RNeasy Plus Kit (Qiagen, Valencia, CA). RNA integrity was validated on a denaturing gel with proper 2:1 ratio (28S:18S) and quantified using NanoDrop® 1000 Spectrophotometer (ThermoScientific, Wilmington, DE). After DNase I treatment (Invitrogen, Carlsbad, CA), RNA was reverse transcribed with Oligo-dT using SuperScript III 1st Strand cDNA Synthesis kit. SYBR Green based detection (Stratgene) utilizing the Stratagene Mx3000P PCR machine was used to determine gene expression. Expression data were normalized to β-actin mRNA expression and presented as relative message level. Primer sequences are listed in Table 1.

**Statistical analysis**

All values are presented as mean ± SEM. Total histology scores, colon length, and histology sub-scores were compared using an analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons. %Body Weight was compared using a 2 way Analysis of Variance (ANOVA). Comparisons were made between Stat3^{Flx/Flx} littermate controls and Stat3^{ΔIEC} mice at day 0, day 7 or day 28 using un-paired Student t test. Association between two variables was measured by Pearson's correlation coefficient. Statistical analyses were performed using GraphPad PRISM© Version 4.01. A p-value <0.05 was considered statistically significant.
Results

Stat3^ΔIEC mice exhibit more severe acute and chronic colitis following DSS administration. Stat3^{Flx/Flx} littermate controls and Stat3^ΔIEC mice were administered 4% DSS for 7 days to induce acute colitis (day 7) followed by water consumption alone for 21 days (day 28) with untreated mice used as controls (day 0) (Figure 1A). Colitis was not apparent at day 0 in either genotype. Stat3^ΔIEC mice at day 7 exhibited a more severe total histology score of 12.6 ± 0.55 compared to 9.1 ± 1.5 for Stat3^{Flx/Flx} littermate controls (p<.0001) (Figure 1B). Day 28 analysis revealed that both genotypes had sustained chronic colitis however the Stat3^ΔIEC mice had more total histology scores of 8.6 ± 0.44 compared to 5.3 ± 0.38 for Stat3^{Flx/Flx} littermate controls (p<.0001) (Figure 1B). Kaplan-Meyer analysis demonstrated increased mortality in Stat3^ΔIEC mice which became apparent by day 12 (Figure 1C). Both genotypes began to lose weight after the 7th day of DSS administration and by day 10 the body weight of the Stat3^ΔIEC mice was reduced by 10.6% compared with 4.3% in Stat3^{Flx/Flx} littermate controls (p<.001) (Figure 1D). Colon length was shortened to a similar extent in both genotypes at day 7, but the length of the colon was significantly reduced at day 28 to 8 ± 0.2 cm in Stat3^ΔIEC mice compared to 9 ± 0.2 cm in Stat3^{Flx/Flx} littermate controls (p<.005) (Figure 1E).

The histologic sub-scores for overall area involved at day 7 and day 28 were significantly worse in Stat3^ΔIEC mice at 3.4 ± 0.2 and 2.8 ± 0.2 compared to 2.6 ± 0.4 and 2.0 ± 0.2 in Stat3^{Flx/Flx} littermate controls (p<.001) (Figure 2B). There was substantial specific injury to the epithelial cell layer as measured by ulceration and crypt loss that persisted within the Stat3^ΔIEC deficient animals. At day 7 Stat3^ΔIEC mice had almost twice the amount of ulceration with a score of 1.7 ± 0.2 compared to .8 ± 0.3 in Stat3^{Flx/Flx} littermate controls (p<.0001) (Figure 2C). The ulceration persisted in the Stat3^ΔIEC mice at day 28 and was almost four times more severe in at .73 ± 0.1 compared to .15 ± 0.1 in the Stat3^{Flx/Flx} littermate controls (p<.0001) (Figure 2C). Crypt loss at day 7 was not significantly different between genotypes but at day
28 Stat3\textsuperscript{AIEC} mice had almost double the amount of crypt loss at 2.46±.2 compared to 1.7±.13 in the Stat3\textsuperscript{Flx/Flx} littermate controls (p<.05)(Figure 2D). The immune cell sub-score at day 7 in Stat3\textsuperscript{AIEC} mice was also significantly higher at 2.4±.1 compared to 1.5±.3 in Stat3\textsuperscript{Flx/Flx} littermate controls (p<-001) (Figure 2E). At day 28 both genotypes still had infiltration of immune cells but in Stat3\textsuperscript{AIEC} mice it was more significant at 2.1±.1 compared to 1.3±.1 in Stat3\textsuperscript{Flx/Flx} littermate controls (p<.001) (Figure 2E).

**IEC proliferation and survival does not differ from controls at Day 28 in Stat3\textsuperscript{AIEC} mice.**

IEC Stat3 deficiency in DSS induced acute colitis has shown that Stat3 functions to induce intestinal wound healing responses, proliferation, and protection from apoptosis during acute injury\textsuperscript{10,17,16}. We confirmed Stat3 activation in response to DSS in colonic enterocytes at day 7 in the littermate controls (Figure 3A). The frequency of nuclear pSTAT3 positive IEC per crypt was equal to 4.8±1.6 percent at day 0, which was significantly increased to 15.2±6 percent at day 7, and returned to basal levels of 1.6±.77 percent at day 28 (p<0.05) (Figure 3B). Stat3\textsuperscript{AIEC} mice as expected had no nuclear IEC pSTAT3 staining. To examine proliferation of IECs we injected untreated control, acute and chronic phase mice with BrdU, and sacrificed the animals 2 hours later. At Day 7 we found a significant reduction in basal crypt proliferation rates as the percentage of BrdU positive cells per crypt was 3.8±.6 in Stat3\textsuperscript{AIEC} mice compared to 7.3±.6 in Stat3\textsuperscript{Flx/Flx} littermate controls (p<.005) (Figure 3D). There was no difference at day 28 between genotypes. To examine whether IEC-specific deletion of Stat3 renders enterocytes more susceptible to apoptosis at baseline and during acute and chronic phases of colitis we quantified immunohistochemical staining for cleaved caspase-3 positive cells per HPF at day 0, day 7 and day 28. We found that at day 7 Stat3\textsuperscript{AIEC} mice had a trend for increased caspase-3 positive IECs per HPF but this comparison was not significant and there was no difference between genotypes at day 0 or day 28 (Figure 3F).
We therefore asked if other signaling pathways that control the expression of anti-apoptotic, pro-proliferative and immune response genes known to overlap and have redundant functions to Stat3, such as the NFkB signaling pathway, might mediate wound healing, proliferation and enhanced survival in the IEC Stat3 deficient animals. We stained for nuclear phosphorylated Rela(p65) but did not observe a difference in the percentage of nuclear RelA(p65) positive IEC per crypt at day 0, day 7 or day 28 between the genotypes (Suppl. Figure 2B). As expected, the frequency of RelA(p65) positive IEC per crypt was increased in both genotypes at day 7. We also did not observe a difference in the expression of IkBa, which is direct downstream target of activated RelA(p65), between the genotypes at day 28 (Suppl. Figure 2C). STAT1 or STAT5 activation might also promote a survival signal which could compensate for the loss of STAT3. Immuno-histochemical analysis of consecutive colonic sections for pSTAT1 and pSTAT3 showed no activation of pSTAT1 in the epithelial compartment of either genotype at day 0, day 7 (data not shown) or day 28 (Suppl. Figure 2D). Similarly, we observed no nuclear IEC staining for pSTAT5 in either genotype at day 28 (Suppl. Figure 2E). Collectively, these data suggested that the increased severity of colitis at day 28 in Stat3^ΔIEC^ mice was not accounted for by differences in IEC survival or proliferation, or induction of compensatory IEC signaling pathways.

**Development of chronic colitis involves Stat3 activation in the non-epithelial compartment in Stat3^ΔIEC^ mice.**

In order to identify the lamina propria cellular populations involved during DSS induced chronic colitis, colonic sections of control (day 0) and DSS-induced chronic colitis (day 28) mice were examined for the frequency of pSTAT3 positive enterocytes and lamina propria cells, and double positive pSTAT3⁺/F4/80⁺ MΦ and pSTAT3⁺/CD3⁺ T-lymphocytes per high powered field (HPF). In Stat3^Flx/Flx^
littermate controls the number of IEC that were positive for nuclear pSTAT3 staining at day 28 was equal to $4.5 \pm 2$ cells per HPF with no IEC nuclear pSTAT3 detectable in the Stat3$^{\Delta IEC}$ mice (Figure 4A). In the lamina propria, the Stat3$^{\Delta IEC}$ mice exhibited a significant increase in pSTAT3$^+$ cells per HPF at $23.5 \pm 3$, compared to $9.2 \pm 2$ cells per HPF in the Stat3$^{Flx/Flx}$ littermate controls ($p<.001$) (Figure 4B). The frequency of cells in which surface staining for F4/80 co-localized with nuclear pSTAT3 was significantly increased as well from $2 \pm 3$ cells per HPF in Stat3$^{Flx/Flx}$ mice to $8.7 \pm 1$ cell per HPF in Stat3$^{\Delta IEC}$ mice ($p<.001$) (Figure 4C). The frequency of total F4/80$^+$ MΦ per HPF was also significantly increased in Stat3$^{\Delta IEC}$ compared to Stat3$^{Flx/Flx}$ littermate controls (data not shown). The frequency of cells in which surface staining for CD3$^+$ co-localized with nuclear pSTAT3 was also significantly increased in Stat3$^{\Delta IEC}$ mice at $1 \pm 3$ cells per HPF compared to $.1 \pm .02$ cells per HPF in Stat3$^{Flx/Flx}$ mice ($p<.05$). The frequency of total CD3$^+$ cells per HPF was also significantly increased in Stat3$^{\Delta IEC}$ compared to Stat3$^{Flx/Flx}$ littermate controls (data not shown). Neutrophils did not seem to be playing a major role in the chronic colitis at day 28. We assessed mucosal S100 proteins, S100A8/S100A9, involved in phagocyte chemotaxis and function and found only S100A9 increased in STAT3$^{\Delta IEC}$ mice compared to STAT3$^{Flx/Flx}$ littermate controls (Suppl. Figure 3A&B). However, when we stained for neutrophil elastase, secreted by neutrophils during inflammation, the frequency of lamina propria neutrophil elastase positive cells per HPF was decreased in Stat3$^{\Delta IEC}$ mice compared to Stat3$^{Flx/Flx}$ littermate controls at day 28 (Suppl. Figure 3C). We also examined the colonic expression of CXCR2, a receptor known to mediates neutrophil migration to sites of inflammation, in Stat3$^{Flx/Flx}$ littermate controls and Stat3$^{\Delta IEC}$ mice at day 0 and day 28 and found no differences (Suppl. Figure 3E). Finally, the overall frequency of lamina propria cells expressing nuclear pSTAT3 was significantly associated with histology scores at day 28 in the Stat3$^{\Delta IEC}$ mice, but not in littermate controls ($r=.77$, $p =.03$) (Figure 4E).
Colonic expression of Stat3 target genes that mediate epithelial restitution is significantly decreased while SOCS3, IL-6, IFN-γ and IL-17A expression are increased in Stat3\textsuperscript{\textAE} mice at day 28. Since mice deficient in IEC Stat3 exhibited a more significant lamina propria infiltrate at day 28 we next investigated colonic expression of genes involved in epithelial restitution and mucosal inflammation. We performed the expression analysis from the same colonic sections for which we determined the composition of cellular infiltrate, which allowed us to correlate colonic gene expression with immune cell population frequencies and histology scores. Using quantitative RT-PCR we found that mRNA expression for the Stat3 dependent epithelial restitution genes Reg\textbeta and Reg\textgamma was significantly decreased at both day 0 and day 28 in Stat3\textsuperscript{\textAE} mice compared to Stat3\textsuperscript{\textFlx/Flx} littermate controls (p<.005) (Figure 5A&5B). Conversely, colonic expression of SoCS3, a direct downstream target of Stat3 activation, was significantly increased in Stat3\textsuperscript{\textAE} mice at day 28, likely reflecting the increased lamina propria Stat3 activation observed at this time point (p<.04) (Figure 5C). Expression of IL-6 was not different between genotypes at day 0 or day 28 (Figure 5D). IFNγ expression was significantly increased at day 0 and day 28 in Stat3\textsuperscript{\textAE} mice compared to Stat3\textsuperscript{\textFlx/Flx} littermate controls (p<.01) (Figure 5E), while IL-17A expression was also increased at day 28 with Stat3\textsuperscript{\textAE} mice expressing 163.6±67 relative units (RU) compared to 33.9±7 RU in Stat3\textsuperscript{\textFlx/Flx} littermate controls (p<.02) (Figure 5F). By comparison, we did not observe a significant difference in colonic expression of IL-10, IL-4, TNFα, or IL-1β between genotypes at day 28 (Suppl. Figure 4A,B,C&D), suggesting that the differences in IFNγ and IL-17A expression were specific.

Colonic expression of chemokines involved in leukocyte recruitment is increased in Stat3\textsuperscript{\textAE} mice at Day 28. We have found that the frequency of colonic pSTAT3\textsuperscript{+}/F4/80\textsuperscript{+}MΦ, pSTAT3\textsuperscript{+}/CD3\textsuperscript{+} T-lymphocytes, and colonic expression of IFNγ and IL-17A were increased at day 28 in Stat3\textsuperscript{\textAE} mice compared to littermate controls. We therefore next asked whether there would be differences in
expression of chemokines which mediate trafficking of these cells. Colonic expression of CXCL5 was increased in both genotypes at day 28 (Figure 6A). By comparison, day 28 CXCL2 expression was increased significantly more in Stat3\textsuperscript{ΔIEC} mice at 54.3±21 RU compared to 11.6±2 RU in Stat3\textsuperscript{Flx/Flx} littermate controls (p<.03) (Figure 6B). The day 28 expression of CXCL10 was significantly increased in Stat3\textsuperscript{ΔIEC} mice at 77±21 RU compared to 20±3 RU in Stat3\textsuperscript{Flx/Flx} littermate controls (p<.001) (Figure 6C). CCL2 expression was also significantly increased at day 28 in Stat3\textsuperscript{ΔIEC} mice at 3.4±.6 RU compared to 2.1±.63 RU in Stat3\textsuperscript{Flx/Flx} littermate controls (p<.05) (Figure 6D). Lastly, the expression of CCL4 was significantly increased at day 28 in Stat3\textsuperscript{ΔIEC} at 2.5±.1 RU compared to 1.1±.1 RU in Stat3\textsuperscript{Flx/Flx} littermate controls (p<.05) (Figure 6E). By comparison, we did not observe differences between genotypes at day 28 for colonic expression of CXCL1, CCL5, or CCL20, supporting the specificity of the differences observed (Suppl. Figure 4E, F&G).

Colonic expression of IL-6, IL-10, IFNγ and IL-17A correlates with histologic severity and colonic expression of IL-17A correlates with the frequency of pSTAT3\textsuperscript{+}CD3\textsuperscript{+} T-lymphocytes in Stat3\textsuperscript{ΔIEC} mice at day 28. We then used linear regression analysis to determine whether the colonic expression of IL-6, IL-10, IFNγ, and/or IL-17A was associated with the histology scores. We found that colonic expression of IL-6 was associated with increased histologic score in Stat3\textsuperscript{ΔIEC} mice (r=.76, p=.03) as was IL-10 (r=.77, p=.02), IFNγ (r=.82, p=.01), and IL-17A (r=.77, p=.03). However there was no significant correlation in the Stat3\textsuperscript{Flx/Flx} littermate controls for any of these cytokines. We next asked whether the frequency of CD3\textsuperscript{+}/pSTAT3\textsuperscript{+} T-lymphocytes per HPF correlated with IL-17A colonic mRNA expression, and found that this was the case in the Stat3\textsuperscript{ΔIEC} mice (r=.90, p=.002) but not in the Stat3\textsuperscript{Flx/Flx} littermate controls.
Discussion

In the present study we have demonstrated that the loss of IEC Stat3 promotes the development of chronic colitis after administration of DSS, in terms of increased degree of histologic severity, reduced survival, and reduced colon length. Body weight was significantly reduced in Stat3<sup>ΔIEC</sup> mice as compared to Stat3<sup>Flx/Flx</sup> littermate controls after removal of DSS at day 10 which was followed by a rapid recovery by day 12 in both genotypes. This is not surprising in that C57BL/6J mouse strain has been shown to develop chronic colitis while returning to their original weight 3 to 4 weeks after removal of DSS<sup>42</sup>. Since some of the Stat3<sup>ΔIEC</sup> mice died around day 12 it is likely that those animals would have had a significant negative impact on the body weight, colon length, and histological score parameters, however we nevertheless observed significant differences at day 28 in the Stat3<sup>ΔIEC</sup> cohort.

IEC Stat3 deficient mice exhibited more severe histologic injury at day 7, recapitulating what has previously been published, and here we show that they continue to exhibit more severe disease through three weeks of water recovery at day 28. The severity was worse in all areas scored including area involved, ulceration, crypt loss, and inflammatory infiltrate. Over the course of the 21 day water consumption Stat3<sup>Flx/Flx</sup> littermate controls and Stat3<sup>ΔIEC</sup> mice were able to significantly reduce ulceration and crypt loss when comparing day7 to day 28 within genotypes. This is thought to be mediated by intestinal epithelial cell proliferation, survival and wound healing responses. However, the immune cell score remained high in both genotypes from day 7 to day 28. This suggests that those Stat3<sup>ΔIEC</sup> mice that survived were able to induced regenerative responses to the initial DSS induced injury regardless of a loss in Stat3 activation within the epithelium but however these mice still had more severe colonic injury compared to Stat3<sup>Flx/Flx</sup> littermate controls at day 28.

Upon evaluation of pSTAT3 within the epithelial crypts we observed that at day 7 more Stat3 activation was induced than at day 28 in the Stat3<sup>Flx/Flx</sup> littermate controls. We did not evaluate this at
the biochemical level, as epithelial preps from ulcerated and colitic tissue can be contaminated by other cell populations. Secondly, we elected to stain for the activated phosphorylation site of Stat3 within the IEC cellular niche. This allowed us to gain better insight into the relative area in which Stat3 was induced and how it relates to active or disease free areas of the colon and the patchy nature of the DSS colitis model. Thus we concentrated our efforts within 0.5cm from the ulcer border or similar areas of the colon if no ulcer was present. Our studies at day 7 revealed, as previously published, that the Stat3^{Flx/Flx} mice had a greater epithelial proliferative response however at day 28 there was no difference between genotypes. Epithelial cell survival was not reduced in Stat3^{AlEC} mice at day 7 however it has been suggested that the most significant portion of apoptosis occurs within the first 2 or 3 days of DSS administration and therefore we may have missed this difference between genotypes at day 7 \textsuperscript{43}. Furthermore, there was also no difference for IEC apoptosis at day 28 between genotypes. Both genotypes exhibited an improvement in crypt loss and ulceration between day 7 and 28, although these remained more severe in the Stat3^{AlEC} mice. We did not observe a compensatory increase in the frequency of crypt epithelial cells expressing the activated forms of p65, pSTAT1, or pSTAT5 at day 7 or day 28 in the Stat3^{AlEC} mice. However, we did observe a persistent defect in expression of the Stat3 dependent epithelial restitution genes Reg3β and Reg3γ in the Stat3^{AlEC} mice, which likely contributed to the relative defect in wound healing\textsuperscript{10}. This may also explain why patients carrying the JAK2 risk allele have reduced colonic expression of REG3α and why they may be at greater risk for development of IBD\textsuperscript{5}. Furthermore, recent data in UC patients associated the level of SOCS3 activation within the epithelium with a shorter time to relapse and more severe inflammation during relapse hypothesizing that the increased SOCS3 may make the epithelial cells more susceptible in various conditions.\textsuperscript{44}

Chemokines are small chemotactic cytokines that regulate the trafficking and migration of leukocytes \textsuperscript{45}. Previous reports in C57BL/6J mice have determined that expression of CXCL1, CXCL2,
CXCL10, CCL2, CCL3 and CCL4 remain elevated during the chronic phase of DSS induced colitis. Our results were consistent with these prior reports in that we observed increased expression of CXCL2, CXCL10, CCL2 and CCL4 in Stat3IEC mice in the setting of more severe chronic colitis which is likely contributing to the differences in the inflammatory infiltrate observed at day 28. It will be important in future studies to determine whether the Stat3 deficient epithelium is the source of these chemokines, and if so, whether Stat3 directly regulates their expression. The cellular infiltrate in Stat3IEC mice remarkable showed an increased frequency within the lamina propria of pSTAT3+ cells including pSTAT3+ MΦ and T-lymphocytes along with increased frequency of total MΦ and T-lymphocytes (data not shown) compared to Stat3Flx/Flx littermate controls. The frequency of pSTAT3+ cells within the lamina propria was positively correlated with a more severe histologic score suggesting that these cells are either induced in response to the healing process or are directly mediating worse disease.

SOCS3, a direct downstream target of activated Stat3, was increased in expression in the Stat3IEC mice which we believe is consistent with the pronounced pSTAT3+ infiltrate within the lamina propria. Colonic IFNγ expression was increased at both day 0 and day 28 in the Stat3IEC mice. We did not observe qualitative differences in pSTAT1 activation in the epithelial cell compartment at these time points. However, as IFNγ is produced by Th1 cell types, and IL-4 expression did not differ between genotypes, this likely represents a basal Th1 skewing in the Stat3IEC mice. Colonic IL-17A expression was increased only at day 28 in Stat3IEC mice, in the setting of chronic colitis. IL-17A is released by Th17 inflammatory T-cells, shown to involve direct binding of Stat3 to the IL-17A promoter, and known to be involved in many chronic autoimmune disorders. We did not detect a difference between genotypes for expression of IL-10, IL-6, IL-1β or TNFα, suggesting that there was not a global difference in activation of mucosal inflammatory pathways in the Stat3IEC mice. Consistent with an effector function, we observed a strong correlation between the frequency of
pSTAT3+ T lymphocytes and IL-17A expression, and between IL-17A expression and histologic injury. Future studies employing Th17 blockade will be required to determine whether this will ameliorate chronic colitis in this setting. In summary, we have shown that loss of IEC Stat3 leads to more severe chronic inflammation following acute injury which is not accounted for by a sustained defect in epithelial proliferation or apoptosis, but rather expansion of pSTAT3+ lymphocytes and up-regulation of *IL-17A* expression.
Acknowledgements

We would like to thank Eleana Laws, MS and Erin Molden, MS for excellent technical assistance. This work was supported by Cincinnati Children’s Hospital Research Foundation Digestive Health Center (1P30DK078392-01), and NIH grants R01 DK078683 and DK068164 (LAD).
Figures and Tables

Figure 1

A. DSS induced Murine Colitis

B. Histology score vs. Day

C. Percent survival vs. Treatment Day

D. % Body Weight vs. Days

E. Colon length (cm) vs. Day

F. Images of Day 28 samples
Figure 1. Stat3^{AIIEC} mice exhibit more severe acute and chronic colitis after DSS administration.

(A) Schematic representation of the DSS-induced colitis model. Mice lacking intestinal epithelial Stat3 (Stat3^{AIIEC}) and littermate controls were given 4% DSS for 7 days followed by consumption of water alone for 21 days. (B) Colon histologic injury was determined as shown. (C) Survival (n ≥ 21 mice per genotype) and (D) weight loss were determined and are shown. (E) Colon length was measured as shown with (F) representative images of the gross appearance. Data are shown as mean ± SEM. Differences between genotypes were analyzed by (B and E) one-way ANOVA or (D) two-way ANOVA, with Bonferroni post-test (*, p < .05; ** p < .005; *** p < .0001).
Figure 2

A Day 0

Day 28

B

C

D

E

Area Involved Score

Ulceration Score

Crypt Loss Score

Immune Cell Score

Days 0, 7, 28
Figure 2. All histologic sub-scores are increased in Stat3$^{\Delta IE C}$ mice compared to Stat3$^{\text{Fli/Fli}}$ littermate controls at day 28. (A) Representative images from the same segment of the colon at day 0 and day 28 utilizing the anal verge as an anatomical landmark. Original magnification was 100x. (B) Area involved, (C) epithelial monolayer ulceration (D) crypt loss and (E) extent of immune cell infiltration are shown at day 7 and day 28 ($n$$\geq$5 mice per group, per genotype). Data are shown as mean ± SEM. Differences within (dotted line) and between (solid line) genotypes were analyzed by one-way ANOVA with Bonferroni post-test (* $p < .05$; ** $p < .005$; *** $p < .001$).
Figure 3

A

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B

% pSTAT3 Pos. IEC per Crypt

C

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D

Proliferation Index

E

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<td>Stat3 ΔIEC</td>
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F

Cleaved Caspase 3 Pos. IEC/HPF
Figure 3. IEC proliferation and apoptosis do not differ at day 28 between Stat3^AIEC^ mice and littermate controls. Imuno-histochemical analysis of (A) phophos-STAT3 quantified as (B) IECs positive per crypt and proliferation measured by BrDU incorporation shown in (C) expressed as a proliferation index (D) (average of 12 properly oriented crypts/mouse n≥5 mice per genotype) and apoptosis measured by (E) cleaved caspase-3 quantified as (F) positive cells per HPF (n≥5 mice per genotype and three fields counted per mouse) in colons from Stat3^{Flx/Flx} mice and STAT3^{AIEC} mice is shown. Analysis was performed in .5 cm area of mucosa that bordered ulcers in the distal and middle colon or in corresponding areas of the colon if no ulcer was found. Data are shown as mean ± SEM. Differences within and between genotypes were compared by un-paired t-test (*p < .05 and ** p < .005).
Figure 4

A) Day 28

Epithelial pSTAT3+ cells per HPF

Stat3   STAT3 ΔIEC

B) Day 28

Lamina Propria pSTAT3+ cells per HPF

Stat3   STAT3 ΔIEC

C) Day 28

F480/ pSTAT3

Stat3   STAT3 ΔIEC

D) Day 28

CD3/ pSTAT3

Stat3   STAT3 ΔIEC

E) Day 28

Histology Score

Stat3 ΔIEC

r = .77, p = .03

Stat3 ΔIEC

r = -17, p = .67
Figure 4. Chronic colitis in Stat3^{ΔIEC} mice involves Stat3 activation in the non-epithelial compartment. Colon sections were quantified by fluorescent microscopy for phosphorylated-Stat3 and the frequency of (A) enterocyte pSTAT3 positive cells and (B) lamina propria pSTAT3 positive cells in 10 random high powered fields (HPF; 400x magnification) was determined in Stat3^{Flx/Flx} littermate controls and Stat3^{ΔIEC} mice at day 28 (n > 6 mice per group). Immunofluorescent labeling of phosphorylated-Stat3 and (C) (F4/80{	extsuperscript{+}}) macrophages and (D) (CD3{	extsuperscript{+}}) T-lymphocytes was used to determine the frequency of double positive cells in 10 random high powered fields (HPF; 400x magnification) of Stat3^{Flx/Flx} littermate controls and Stat3^{ΔIEC} mice at day 28 (n ≥ 6 mice per group, positive cells are marked by arrowhead). (E) Pearson’s regression analysis was used to test for an association between the frequency of lamina propria pSTAT3{	extsuperscript{+}} cells per HPF and histological severity measured by the histology score at day 28 in Stat3^{Flx/Flx} littermate controls (r = .17, p = .67) and Stat3^{ΔIEC} mice (r = .77, p = .03). Data are shown as mean ± SEM. Differences between genotypes were compared by un-paired t-test (*, p < .05; ** p < .005; *** p < .0001).
Figure 5

A

B

C

D

E

F

RegIIIβ Colonic mRNA

RegIIIγ Colonic mRNA

SOCS3 colonic mRNA

IL-6 Colonic mRNA

IFN-γ Colonic mRNA

IL-17A Colonic mRNA

Day 0

Day 28

Day 0

Day 28

Day 0

Day 28

Day 0

Day 28

Day 0

Day 28

Day 0

Day 28

Day 0

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Day 0

Day 28

Day 0

Day 28
Figure 5. Colonic expression of Stat3 target genes that mediate epithelial restitution is decreased while SOCS3, IFN-γ, and IL-17A expression are increased in Stat3ΔIEC mice at Day 28. Real-time RT-PCR was performed on mRNA isolated from whole colonic serial sections for (A) Reg3β (B) Reg3γ (C) SOCS3 (D) IL-6 (E) IFN-γ and (F) IL-17A expression in Stat3Flx/Flx littermate controls and Stat3ΔIEC mice at day 0 and day 28 (n≥7 mice per genotype). Data are normalized to β-actin mRNA expression and shown as mean ± SEM. Differences between genotypes at day 0 and day 28 were compared by unpaired t-test (*, p < .05; ** p < .005; *** p < .0001).
Figure 6. Colonic expression of \( CXCL2, CXCL10, CCL2, \) and \( CCL4 \) is significantly increased in Stat3\(^{AI/E}\) mice at Day 28. Colon mRNA expression was measured by quantitative real time PCR for (A) \( CXCL5 \) (B) \( CXCL2 \) (C) \( CXCL10 \) (D) \( CCL2 \) and (E) \( CCL4 \) in Stat3\(^{Flx/Flx}\) littermate controls and Stat3\(^{AI/E}\) mice at day 0 and day 28 of the colitis model (n>7 mice per genotype). Data were normalized to \( \beta\)-actin mRNA expression and are shown as the mean \pm\ SEM (*, p < .05; ** p < .005; *** p < .0001 by unpaired t-test).
Figure 7

A

Day 28

Histology Score vs. IL-6 Colonic mRNA

- Stat3^ΔIEC
  - r = .78, p = .03
- Stat3^Flx/Flx
  - r = .12, p = .75

B

Day 28

Histology Score vs. IL-10 Colonic mRNA

- Stat3^ΔIEC
  - r = .77, p = .02
- Stat3^Flx/Flx
  - r = .12, p = .78

C

Day 28

Histology Score vs. IFNg Colonic mRNA

- Stat3^ΔIEC
  - r = .82, p = .01
- Stat3^Flx/Flx
  - r = .06, p = .87

D

Day 28

Histology Score vs. IL-17A Colonic mRNA

- Stat3^ΔIEC
  - r = .77, p = .03
- Stat3^Flx/Flx
  - r = .05, p = .91

E

Day 28

IL-17A Colonic mRNA vs. CD3^+/pSTAT3^+ cells per HPF

- Stat3^ΔIEC
  - r = .90, p = .002
- Stat3^Flx/Flx
  - r = .61, p = .11
Figure 7. Colonic expression of IL-6, IL-10, IFN-γ and IL-17A correlates with histologic severity and IL-17A expression correlates with the frequency of colon pSTAT3⁺CD3⁺ T-lymphocytes in Stat3ΔIEC mice at day 28. Pearson regression analysis was used to test for an association between normalized colonic expression of (A) IL-6 (B) IL-10 (C) IFNγ and (D) IL-17A and histologic severity score. E) Pearson regression analysis was used to test for an association between IL-17A colonic expression and the frequency of CD3⁺/pSTAT3⁺ T-lymphocytes per 400x high-powered field (r=.90, p<.002) (n>7 mice per genotype). Data are shown as mean ± SEM (*, p < .05; ** p < .005; *** p < .0001 by unpaired t-test).
### Table 1. Real-time quantitative reverse transcription-PCR (RT-PCR) Primers

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<tr>
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<th>Forward</th>
<th>Reverse</th>
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Supplemental Figures

Supplemental Figure 1

A  

4 hr LPS

Stat3^Fli/Fli  Stat3^ΔIEC

B  

4 hr LPS

Stat3^ΔIEC  Stat3^ΔIEC  Flx/Flx  Flx/Flx

pSTAT3  Total STAT3  TFIIβ

C

ileum  colon

Stat3  Stat3 ΔIEC  Stat3 ΔIEC  Stat3 ΔIEC

Stat3  β-actin
Supplemental Figure 1. Characterization of Stat3 Intestinal Epithelial cell deficient mice (Stat3\textsuperscript{ΔIEC}) compared to littermate controls (Stat3\textsuperscript{Flx/Flx}). (A) Representative immunofluorescence images of phospho-STAT3 (Tyr705) staining in the colon of mice injected for 4hrs with 200mg/kg endotoxin showing robust epithelial STAT3 activation in Stat3\textsuperscript{Flx/Flx} littermate controls and only STAT3 activation is the lamina propria of Stat3\textsuperscript{ΔIEC} mice. (B) Western blot analysis of phophos-STAT3 (Tyr705) in isolated colonic enterocytes 4hrs after injection of 200mg/kg endotoxin showing enhanced Stat3 activation in Stat3\textsuperscript{Flx/Flx} littermate controls and loss of Stat3 activation in Stat3\textsuperscript{ΔIEC} mice. (C) IECs from the ileum and colon of Stat3\textsuperscript{Flx/Flx} and Stat3\textsuperscript{ΔIEC} mice were isolated and assessed for expression of total Stat3 by PCR, β-actin shown as a reaction control.
Supplemental Figure 2

A

Stat3 Flx/Flx

Stat3 Δ IEC

B

% p65 pos. IEC per Crypt

Day 0  Day 7  Day 28

* * *

C

IκBα Colonic mRNA

Day 0  Day 28

* * *

D

pSTAT1  pSTAT3

E

pSTAT5

STAT3 Flx/Flx

STAT3 Δ IEC
Supplemental Figure 2. Activation of NFκB, STAT1 and STAT5, plausible compensatory mechanisms for protection of IECs during colitis in a STAT3 deficient epithelium, did not vary qualitatively in Stat3ΔIEC mice compared to Stat3Flx/Flx littermate controls. Immunohistochemical analysis of (A) phospho-p65 (Ser276) quantified as (B) IECs positive per crypt within .5cm of disease area expressed as a labeling index (average of 12 properly oriented crypts/mouse n≥5 mice per genotype). (C) Real-time RT-PCR was performed on mRNA isolated from whole colonic serial sections for IκBα, a direct downstream target of activated RelA/p65, and the expression in Stat3Flx/Flx littermate controls and Stat3ΔIEC mice at day 0 and day 28 (n≥7 mice per genotype). Data are normalized to β-actin mRNA expression. (D) Representative immunohistochemical images of consecutive fields in colonic tissue stained for phospho-STAT1 and phospho-STAT3 at day 28 showing little to no STAT1 activation in the epithelium or lamina propria in both genotypes. (E) Representative image of colonic tissue stained for phospho-STAT5 (Tyr694) at day 28 in Stat3Flx/Flx littermate controls and STAT3ΔIEC mice. Analysis was performed in .5 cm area of mucosa that bordered ulcers in the distal and middle colon or in corresponding areas of the colon if no ulcer was found. Data are shown as mean ± SEM. Differences within and between genotypes were compared by un-paired t-test (*p < .05 and ** p < .005).
Supplemental Figure 3

A

B

C

D

E

Willson et al.
Supplemental Figure 3. Neutrophil mediated responses during chronic colitis in Stat3\(^{Flx/Flx}\) littermate controls and Stat3\(^{AIEC}\) mice. (A) Real-time RT-PCR was performed on mRNA isolated from whole colonic serial sections for phagocytic products (A) S100A8 and (B) S100A9, known to be involved in neutrophil chemotaxis and function, in Stat3\(^{Flx/Flx}\) littermate controls and Stat3\(^{AIEC}\) mice at day 0 and day 28 (n>7 mice per genotype). Colon sections were quantified by fluorescent microscopy of neutrophil elastase which is secreted by neutrophils during inflammation. The frequency of (C) lamina propria neutrophil elastase positive cells in 10 random high powered fields (HPF; 400x magnification) was determined in Stat3\(^{Flx/Flx}\) littermate controls and Stat3\(^{AIEC}\) mice at day 28 (n > 6 mice per group). (D) Representative image showing neutrophil elastase positive cells marked by arrow head. (E) Real-time RT-PCR was performed on mRNA isolated from whole colonic serial sections for CXCR2, a receptor that mediates neutrophil migration to sites of inflammation, in Stat3\(^{Flx/Flx}\) littermate controls and Stat3\(^{AIEC}\) mice at day 0 and day 28 (n>7 mice per genotype). All Real-time PCR data are normalized to β-actin mRNA expression. Data are shown as mean ± SEM. Differences between genotypes were compared by un-paired t-test (*, p < .05).
Supplemental Figure 4. Colonic expression of IL-10, Th2 cytokine IL-4, TNFα, IL-1β, CXCL1, CCL5 and CCL20 expression do not vary between genotypes at Day 0 or Day 28. Real-time RT-PCR was performed on mRNA isolated from whole colonic serial sections for (A) IL-10 (B) IL-4 (C) TNFα (D) IL-1β (E) CXCL1 (F) CCL5 and (G) CCL20 expression in Stat3^Flx/Flx littermate controls and Stat3^ΔIEC mice at day 0 and day 28 (n≥7 mice per genotype). Data are normalized to β-actin mRNA expression and shown as mean ± SEM. Differences between genotypes at day 0 and day 28 were compared by unpaired t-test (*, p < .05).
References


44. Li Y, de Haar C, Nuij VJ, et al. SOCS3 Expression is a Predictive Factor of Relapse of Mucosal Inflammation in Chronic UC. Gastroenterology 2011;140:S-1.


Chapter 4

Overall Discussion, Alternative Hypotheses and Future Directions
Introduction

Estimates suggest that 1.4 million individuals in the United States are suffering from IBD. IBD pathogenesis is multifactorial 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. Augmented STAT3 activation has been well documented in both human and murine colitis. Transient activation induces protective mechanisms but persistent activation furthers disease progression and ultimately malignant transformation. Therefore, deciphering the cellular, temporal and transcriptional context of STAT3 activation as it relates to mucosal inflammation is of great interest. In murine models, Stat3 activation in intestinal epithelial cells is required for acute wound healing responses via induction of Reg3 family members, but also promotes development of colitis-associated cancer during chronic inflammation\(^1,2\). Stat3 activation in myeloid cells mediates anti-inflammatory effects of IL-10; targeted deletion of Stat3 in this cell type leads to severe entero-colitis\(^3\). 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\(^4,5\). Tyrosine phosphorylation of Stat3 induced by IL-6 has also been linked to effector lymphocyte and granulocyte activation in IBD. We had 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\(^4,5\). We were the first to identify an IL-6:STAT3 biological network that drives leukocyte recruitment and thereby mucosal inflammation in this setting.
Large-scale genome-wide association studies (GWAS) have determined that 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. Genotypic variation in STAT3 is linked to risk for both CD and UC and there are now 99 genetic factors that confer IBD susceptibility, with 30% shared between CD and UC (Figure 1)\textsuperscript{6-9}. Several of the other shared susceptibility loci (IL-10, JAK2, TYK2, and IL-23) also utilize STAT3 as the downstream signaling effector. Newly described permutations to build protein-protein interaction (PPI) networks from GWAS identified risk-associated loci, revealed that in CD the core candidate network involved Janus-associated kinase 2 (JAK2) and STAT3 \textsuperscript{10}. 

Figure 1. Inflammatory bowel disease susceptibility loci. The loci (depicted by lead gene name) attaining genome-wide significance are shown for CD (red), UC (blue) and IBD (black where $p<5\times10^{-8}$ in CD and UC; red where $p<5\times10^{-8}$ in CD and $5\times10^{-4}$ in CD). Reproduced from Gut, Lee et al., 60, New IBD genetics: common pathways with other disease, 1739-1753, Copyright (2011) with permission from BMJ Publishing Group Ltd.
Waterman et al. evaluated the overlap between CD and UC genetic loci stratified by pathogenic pathways and by disease location and found that gene variants associated with immune response amplification and perpetuation such as IL12B (p40), IL-23R, and STAT3 associated with Th17 differentiation, and genes involved in barrier function, were similar in their prevalence between UC and CD\textsuperscript{11}. This data supports the hypothesis that host immune response to luminal flora seem to differentiate CD and UC and highlight the pivotal role STAT3 has in the overall course of intestinal inflammation and mucosal pathology. Evidence suggests that IBD disease course that starts earlier in life may have a stronger genetic influence however, 11 GWAS have been performed in adult IBD, compared to only two GWAS performed exclusively in early-onset disease\textsuperscript{12-15}. These studies however have revealed that early-onset and adult disease share the majority of the identified risk loci and that the STAT3 rs744166 is associated with risk in both early-onset and adult IBD.

**Pediatric versus Adult Onset IBD**

IBD is diagnosed 25\% of the time during childhood and estimates suggest 100,000 children in North America currently suffer from IBD with three CD cases for every new UC case\textsuperscript{16,17}. Childhood IBD incidence ranges from 0.3–10.9/100,000/year and recent reports suggest that pediatric-onset IBD is a sub population of IBD due to the varying phenotypic and natural history differences in young patients\textsuperscript{18,19}. Childhood IBD incidence is on the rise as the number of younger patients (younger than 18 years) has increased significantly\textsuperscript{20-22}. Adult-onset IBD shows equal ratio of male to female disease with perhaps more women having the disease\textsuperscript{17}. However, in pediatric-onset IBD, CD has a male predominance whereas UC has a female predominance and there has been no molecular elucidation for this difference in adult versus
pediatric CD:UC gender ratio\textsuperscript{17,23}. In early-onset disease, presentation has a more extensive anatomical involvement compared with adult disease with major lesions scattered throughout the gastrointestinal tract in CD and a pancolitis in UC\textsuperscript{16,23}. Disease behavior is similar to adult disease, with progression from inflammatory disease to stricturing/penetrating complications however surgical need is earlier in early-onset UC but later for CD\textsuperscript{23-25}. Amre et al. recently reported in a Canadian pediatric population that the STAT3 rs744166 SNP was significantly associated with overall susceptibility for CD and ileal disease with or without colonic involvement at diagnosis\textsuperscript{26}. Ultimately we did not find differences for age, sex, disease location, medication exposure, or clinical or histologic disease activity at diagnosis in patients stratified by the STAT3 "A" risk allele in our pediatric cohort. We ultimately wanted to investigate the underlying functional consequences that could potentially infer risk for development of IBD in association with the STAT3 rs744166.

**Biochemical interrogation of the IL-6:STAT3 signaling pathway**

We found that the STAT3 "A" risk allele is associated with increased cellular STAT3 activation in peripheral blood leukocytes. To dissect the mechanism of enhanced cellular STAT3 responsiveness we utilized EBLs (Epstein–Barr virus (EBV) transformed lymphoblastoid cell lines) created from patients with IBD. These cell lines are a readily available and renewable resource for functional genotype-phenotype studies in humans. However, aberrant gene expression induced by EBV transformation leads to skepticism for the usefulness of EBLs as an alternate model to primary tissues.

Caliskan et al. compared global gene expression and methylation profiles in primary B cells and transformed EBLs and revealed that most of the differences in gene expression levels
are of small magnitude, and that EBLs recapitulate the naturally occurring gene expression variation in primary B cells\textsuperscript{27}. This suggests that EBLs are a sufficient model for interrogating genetic architecture that underlies regulatory variation\textsuperscript{27}. They did find inter-individual variation in EBV copy number that was significantly greater than the intra-individual variation, as suggested by previous studies showing that differences in EBV copy numbers may significantly contribute to regulatory variation across cell lines\textsuperscript{28}. However when Caliskan et al. investigated gene expression data they identified only 160 genes whose expression levels were significantly associated with EBV copy numbers and found that most of these genes were involved in the anti-apoptotic role of NFkB signaling.

Furthermore, only 33 genes were differentially expressed between primary B cells and EBLs with a fold change greater than 1.5. Therefore, EBLs are an important in-vitro tool to assess the biochemical differences of IL-6 induced STAT3 activation in patients carrying the STAT3 risk allele. Furthermore, 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
subgroup of patients who carry the STAT3 risk allele via STAT3-dependent effects upon T-lymphocyte and granulocyte differentiation, activation, and survival.

**Alternative hypothesis and Future Directions**

**Membrane localization of IL-6R/GP130/JAK2.** The increased membrane localization of the IL-6R and GP130 as it relates to the STAT3 “A” risk allele suggest increased levels of synthesis and targeting to the membrane. The receptor complex via endocytosis is removed from the membrane after IL-6 induced signaling therefore, experiments to investigate rates of receptor internalization, degradation or retargeting to the membrane can be interpreted with pulse chase methodology in conjunction with cycloheximide to prevent de novo protein synthesis. Alternatively, JAK2 has been shown to mediate increased stabilization and surface expression of IFN-γR2 in Th17 cells as well as the erythropoietin receptor through favoring Golgi processing and cell surface expression29,30. We could investigate this mechanism of JAK2 mediated receptor stabilization and surface expression in EBLs utilizing SiRNA technology to reduce JAK2 expression and then quantify the abundance of membrane IL-6R/GP130 receptor in patients with and without the STAT3 risk allele. Furthermore, if the STAT3 risk allele is associated with JAK2 mediated IL-6/GP130 receptor stability then heterozygous patients should have intermediate levels of membrane receptor compared to non-risk allele and homozygous risk allele patients.

**The STAT3 splice variant - STAT3β.** Functional studies to determine causation have focused on coding variants, however the STAT3 risk SNP (rs744166) is located within the intron between exon 1 and exon 2 and may be predicted to regulate gene expression. We did not observe association between the STAT3 “A” risk allele and mRNA expression in colonic biopsies but did find an increase in unstimulated EBLs that could not be explained by IL-6
induced autocrine activation. STAT3 has two distinct isoforms, STAT3α and STAT3β. The splicing variant STAT3β uses an alternative acceptor site within exon 23 that leads to a truncated isoform lacking the C-terminal transactivation domain that acts as a dominant negative inhibiting STAT3 activation. Recently the antitumorigenic potential of STAT3β was investigated and induction of endogenous STAT3β lead to apoptosis and cell-cycle arrest in cell lines with persistent STAT3 tyrosine phosphorylation\textsuperscript{31}. This group also found down-regulation of STAT1β the proliferative antagonist isoform of STAT1 which is thought to primarily promotes growth arrest, apoptosis, and anti-tumor immunity downstream of type I and II interferons\textsuperscript{32} \textsuperscript{31}. This not only infers that a differences in the ratio of STAT3α and β isoforms could have profound effects on gene expression but also indicates that the STAT3β isoform could mediate levels of STAT1α by controlling STAT1β expression. This could have profound effects on T lymphocyte and granulocyte activation and gene expression. Primers utilized for EBL experiments and the Affymetrix probe set for STAT3 in the Human Gene U1333 Plus 2.0 array for colonic biopsy mRNA expression do not distinguish between the different isoforms for STAT3. Therefore, it is plausible that the STAT3 “A” risk allele could be associated with a difference in the ratio of the STAT3α and the dominant negative form STAT3β that would inhibit STAT3 activation by binding the available STAT3α isoform after stimulation. This could also explain why patient EBLs null for the STAT3 risk allele have higher levels of pSTAT1 activation after IL-6 stimulation in that the STAT3β isoform downregulates the STAT1β isoform allowing increased STAT1α signaling. The Utilization of RNA-Seq with Next generation sequencing technology from both EBLs and colonic biopsies would allow the assessment of total STAT3 transcripts (STAT3α, STAT3β or an unknown mutant) between STAT3 risk allele and non-risk allele patients. Moreover there are no known constitutively activation mutations in
STAT3, and 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.

**Phenotypic outcomes in STAT3 risk allele 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. 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. 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 leads to 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. 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. 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. 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.

**Alternative Hypothesis and Future Directions**

**IL-8 as a marker of colonic disease.** In UC a colon only disease, IL-8 serum concentration has been related to endoscopic and histological severity and IL-8 seems to be a reliable biomarker closely related to disease activity. CD patient neutrophil migration to the gut lumen has been shown to be a feature of colonic disease irrespective of associated ileal lesions. Utilizing whole gut lavage, twenty-seven of 36 patients with isolated colonic Crohn's disease had detectable neutrophil elastase, a neutrophil-bound enzyme, whereas 3 of 15 with small-bowel involvement alone had detectable neutrophil elastase. This supports our results in that IL-8 seems to drive neutrophil mediated migration in colonic mucosal inflammation of which we interrogated in colonic biopsies from our pediatric CD patient cohort. Our patient population
had approximately 40% colon only disease in both the non-risk allele and risk allele genotypes. Therefore differences we see in colonic expression of IL-8, CXCL2, and CXCL3 and frequency of neutrophils expressing pSTAT3, and the cognate receptor for IL-8, CXCR2+ may be even more enhanced in CD with colon only or severe colonic involvement at diagnosis. We did not investigate UC patients but it would be predicted that UC has an even greater difference in gene expression for these neutrophil mediated pathways with carriage of the STAT3 “A” risk allele.

**IL-6 classical or trans-signaling?** One of the most intriguing and unexplainable findings was that the serum level of IL-6 was significantly reduced in patients carrying the STAT3”A” risk allele (Figure 2). Compared to other circulating cytokines known to be upregulated in IBD including soluble IL-2R (sIL-2R), tumor necrosis factor alpha (TNFα) and interleukin-5(IL-5), IL-6 was the only one that was increased in our CD patient cohort at diagnosis. This supports our previous publication showing that in newly diagnosed CD patients, a pro-inflammatory IL-6:STAT3 induced biologic network drives mucosal disease. One explanation could be that we only evaluated circulating IL-6 in a small number of patients or that patients carrying the STAT3 “A” risk allele have increased STAT3 activation that enhances negative feedback mechanisms. However the former conclusion it is hard to connect with overall reduced circulating IL-6. Even more interesting is that with suppressed circulating IL-6 levels we still found an increased frequency of peripheral blood leukocytes with basal STAT3 activation in patients carrying the STAT3 “A” risk allele. An alternative hypothesis would be that classical and trans-signaling mechanisms of IL-6 induced STAT3 activation vary with carriage of the STAT3 risk allele. Thus patients with the STAT3 risk allele may have increased IL-6/sIL-6R(soluble IL-6) complexes that also induce STAT3 activation but have bound the free IL-6 making it
undetectable by the IL-6 ELISA. Therefore suggesting the STAT3 risk allele patients have increased basal levels of trans-signaling.

Not all cell types express the IL-6R, however GP130 is expressed ubiquitously in the body. In chronic intestinal inflammation the IL-6R is cleaved through ectodomain shedding from the cell surface of innate immune cells and is thus free to bind free IL-6 and induces IL-6-sIL-6R trans-signaling in cells devoid of the IL-6R\textsuperscript{43}. This mechanism has been shown to enhance lymphocytes proliferation and survival\textsuperscript{44}. We stimulated our cells with IL-6 and the sIL-6R to make sure we induced similar levels of STAT3 activation regardless of the cellular receptor expression. We also investigated the frequency of CD4+ T-lymphocytes and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Circulating levels of Inflammatory cytokines. Serum cytokine levels were measured by ELISA and shown as the mean ± SEM. CTL, healthy control; GG, AG_A, CD at diagnosis null, heterozygous and homozygous for the STAT3 risk allele. (n = 3 - 17), *p<.05; **p<.01; ***p<.001 by t-test.}
\end{figure}
granulocytes expressing the IL-6(CD126) and GP130(CD130) receptor by flow cytometric analysis and found no difference with carriage of the STAT3 risk allele (data not shown). However, this method may not be sensitive enough to detect differences of the receptor at the protein level. Therefore, quantification by western blot analysis of the receptor complex in the membrane and cytosolic fractions along with the comparison of STAT3 activation in response to IL-6 alone or in combination with the IL-6R would help decipher classical or trans-signaling mechanisms in peripheral blood leukocytes. Trans-signaling is also thought to be highly regulated by sGP130 (soluble gp130), that exist in high concentrations within the blood\textsuperscript{45}. sGP130 binds to the IL-6–sIL-6R complex and thereby inhibits the binding of the IL-6–sIL-6R complex to the membrane bound GP130\textsuperscript{45,46}. Interestingly there are no reports on circulating levels of this sGP130 in human IBD. Administration of a synthetic soluble GP130 (gp130-Fc) inhibits Stat3 phosphorylation and ameliorates colitis in the SAMP1/Yit murine model\textsuperscript{47}. The SAMP1/Yit murine model is the most closely related model of human CD as these mice spontaneously develop ileal-colonic disease very similar to CD. IL-6 trans-signaling has been shown to mediate chronic murine colitis and development of colonic cancer\textsuperscript{4,43,48,49}. However much less is known about this mechanism in human IBD. It has been shown that sIL-6R levels are significantly elevated in patients with active IBD compared to inactive IBD and increased IL-6 and sIL-6R production was also observed in the lamina propria during active IBD\textsuperscript{4,50}. Thus, sIL-6R molecules are able to bind free IL-6 and interpreting circulating free IL-6, sIL-6 and sGP130 in IBD patients is a necessity. A very recent publication has elegantly shown by in-vitro culture systems and mice that depending on the molar excess of IL-6 over sIL-6R, sgp130Fc plays a role in blocking trans-signaling because free IL-6 will not or will only partially be trapped in IL-6·sIL-6R·sgp130Fc complexes\textsuperscript{51}. We currently do not yet have a clear
understanding of these concepts in IBD patients and further studies are warranted. This may be why the humanized anti-IL-6 receptor monoclonal antibody, Tocilizumab that blocks both the membrane-bound and the soluble form of IL-6R has marginal clinical efficacy in CD patients\textsuperscript{52,53}. We ultimately did find increased circulating IL-6 in both STAT3 risk allele and non-risk allele genotypes compared to control. However, since we encountered such dramatic differences as a function of genotype it may be very interesting to investigate the level of free IL-6, free sIL-R, IL-6-sIL-6R and sGP130 in patients stratified by the STAT3 “A” risk allele.

**The JAK2/STAT3 axis as a biomarker for disease.** Researchers are avidly trying to define how these loci affect disease phenotypic expression, clinical course, and treatment modalities. The increase in relative risk conferred by individual risk alleles may only be moderate, however the combined associated risk may be substantial. Therefore, studies in patients carrying multiple risk alleles assessed and related to disease location, disease behavior, medical therapy, and remission will ultimately delineate a more personalized medical approach.

Combined genetic variants in genes that utilize STAT3 as a downstream effector include IL-6, JAK2, IL-10, IL-23, and IL-27. Associations with clinical disease phenotype and gene expression profiles of biopsies from inflamed and non-inflamed tissue for the same location as it relates to CD or UC at diagnosis and during treatment would allow interpretation of this genetic JAK2/STAT3 axis in disease behavior. We have interrogated only one of these SNPs, STAT3 rs744166. The complexity is immense, exemplified by our studies that show individual JAK2 or STAT3 alleles can mediate signaling and gene expression outcomes in combined JAK2/STAT3 analysis (data not shown). This indicates a matrix of possibilities in interpreting multiple SNPs and warrants that future studies assessing risk loci as biomarkers be as coherent as
possible in defining known IBD risk loci in patient demographics for enhanced interpretation of the combined associated risk.

**Therapeutic targeting of STAT3.** While therapeutic options have increased over the past decade, our ability to target newer biologic therapies to specific subgroups of patients has lagged behind. Data from human and murine models of colitis indicate that STAT3 may be an important target for the treatment of IBD. However, considering that STAT3 mediates both IL-10 induced anti-inflammatory and IL-6 induced pro-inflammatory pathways at various times within the disease course of both murine and human IBD it seems unlikely that an oral inhibitor of STAT3 would be feasible. 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. However CP was not effective in CD. 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. 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. 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. 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.

**STAT3 a double-edged sword**

As mentioned above STAT3 activation is a double-edged sword. Studies suggest that IEC STAT3 activation has a crucial role in the maintenance of intestinal epithelial barrier and homeostasis. In Chapter III we examined this dichotomy as loss of IEC Stat3 promoted the development of chronic colitis after administration of DSS. IEC Stat3 deficient mice had increased degree of histologic severity, reduced survival, and reduced colon length. Body weight was significantly reduced in Stat3^ΔIEC^ mice as compared to Stat3^Flx/Flx^ littermate controls after removal of DSS at day 10 which was followed by a rapid recovery by day 12 in both genotypes. Murine models in which gp-130-STAT1/3 signaling is abolished (gp130^{-STAT}) or in IL-6 deficient mice (IL-6^{-/-}), there is exaggerated acute intestinal colitis in response to DSS as compared to wild type^{58}. This was due to impaired epithelial wound healing with consistently more severe epithelial erosion^{58}. This suggests that IL-6 induced STAT3 activation promotes epithelial restitution and wound repair important during the innate immune response. However, these models evaluated STAT3 contribution to innate immune response by inactivating STAT3 in multiple cells types. Thus no suitable model for the study of in-vivo epithelial STAT3 specific affects in colitis existed. During creation and characterization of our epithelial Stat3 deficient murine model Bollrath et al. and Grivennikov et al. published that IEC ablation of STAT3 signaling resulted in greater susceptibility to DSS induced epithelial damage and mucosal inflammation but protected animals from the colitis associated cancer tumorigenesis. Moreover, hyperactivation of GP130 mediated STAT3 promoted tumor incidence and growth^{1,2}. Gene chip analysis of IECs isolated from DSS treated IEC STAT3 deficient mice compared to
controls demonstrated a reduction in the expression of genes involved in cellular stress response, apoptosis, and pathways associated with IEC wound healing specifically, Reg3 family members\textsuperscript{59}. Additionally recent publications have shown that loss of IL-22 mediated STAT3 activation in IL-22 knockout animals induces greater susceptibility to DSS induced colitis and showed reduced in-vivo wound closure rate compared to IL-6KO and control\textsuperscript{59}. Thus epithelial cell STAT3 seems to regulate survival, proliferation, and wound healing events during acute colitis and colitis-associated cancer. However, these studies have investigated the contribution of IEC STAT3 in acute injury or during tumorigenic chronic colitis conditions. Thus loss of STAT3 within the epithelial compartment would likely increase risk for the development of chronic inflammation following an acute self-limited gut injury, which has not been formally tested. A model of acute injury and either resolution or development of chronic colitis in a genetically susceptible host would be expected to be more applicable to the current concept of IBD pathogenesis in humans. Therefore, we defined the functional consequences of IEC STAT3 deletion in a model of chronic colitis in which mice are exposed to DSS for seven days, and then assessed during recovery over the next twenty-one days.

**Selecting the appropriate animal model of IBD**

Alternatively, we could have utilized other chemically induced murine models of IBD. Trinitro Benzene Sulfonic acid (TNBS) dissolved in ethanol and given intra-rectally induces an IL-12 mediated immune response immunologically accepted as a Th1 model with increased levels of TNF\(\alpha\), IL-6, IL-12, IL-17, and IFN\(\gamma\). Ethanol, required to break the mucosal barrier, and TNBS haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system\textsuperscript{60}. The conversion rate for colitis in a C57BL/6 background, of which
are mice are on, is only about 15% and large numbers of mice are needed to assure statistical
significance due to variability. Pilot experiments inducing TNBS colitis assessed 3 and 7 days
after administration did not show differences in weight, histologic score, or proliferation in
Stat3\textsuperscript{ΔIEC} compared to Stat3\textsuperscript{Flx/Flx} littermate controls (Figure 4). We also tested Piroxicam
induced ileitis that mimics an NSAID induced environmental trigger reported in human CD.
This model has been shown to induce focal transmural ileitis in CARD15/-/ mice with GM-CSF
Ab injection that ultimately induces defects in innate immunity and leads to development of
ileitis\textsuperscript{61}. We found no differences in weight after 2 weeks of Piroxicam exposure and did not
find disease in the ileum or colon of Stat3\textsuperscript{Flx/Flx} or Stat3\textsuperscript{ΔIEC} mice (Figure 5). These studies
suggested that we utilize an approach more effective in mediating epithelial responses.
Figure 3. TNBS colitis model in IEC STAT3 deficient mice. STAT3ΔIEC mice are not susceptible to TNBS induced colitis at day 3 or day 7. Mice lacking intestinal epithelial Stat3 (Stat3ΔIEC) and littermate controls were intrarectally given TNBS resuspended in 50% ethanol or 50% ethanol as control. (A-D) % Weight gain (g) (B-E) Histologic damage did not differ at day 3 or day 7. (C) Proliferation did not vary at day 3.
Dextran Sodium Sulfate (DSS) exerts direct toxic effects on epithelial cells independent of the underlying gut immune system and its transient administration provides an experimental system to assess the critical first phase of wound healing. In murine models DSS induced colitis follows a predictable time course, is very reproducible, and has a similar pathogenesis and therapeutic response to human IBD. Initially, DSS impacts the epithelial cell with loss of the basal one-third of the crypt by day 3. This imparts a breakdown in the epithelial barrier causing normal mucosal microflora substances to be exposed to the underlying lamina propria innate immune cell populations, which in turn provokes and adaptive immune response. Severe combined immunodeficient mice also develop severe intestinal inflammation and suggests that the adaptive immune system does not play a major part in the acute phase of this model.

DSS induced acute colitis is a 5-7 day treatment that results in inflammation driven by polymorphonuclear cells and macrophages, resulting in bloody diarrhea, ulceration, and infiltration with granulocytes. Importantly this model has a 95% conversion rate and in pilot experiments, we were able to induce a consistent acute colitis in both Stat3^Flx/Flx and Stat3^△IEC.
mice. This model induces initial Th1 response however in the chronic phase it is mixed Th1/Th2 and also has inflammatory features of both UC and CD pathology. In the C57BL/6 background it has been shown that one initial dose of DSS for 5-7 days induces a chronic colitis characterized with a significant inflammatory response, progressive production of IL-1, active IL-12, and IL-17 and increased production of chemokines CXCL1, CXCL2/3, CXCL10, CCL2, CCL3, CCL4. In C57BL/6 mice DSS-induced colitis treated with current clinical therapeutic agents, cyclosporine A, methotrexate, IL-12p40 and agonistic anti-CD3 antibody have given sufficient evidence for the models use in translation of mouse and human disease. Ultimately, there are many other murine models of IBD however in the C57BL/6 DSS was the perfect model to test epithelial dependent loss of Stat3 function in acute injury and evolution into chronic colitis.

Additionally, murine models of intestinal inflammation are greatly affected by the resident enteric bacteria. The gut microbiota is a key component in intestinal homeostasis and in humans a dysbiosis or imbalance of healthy bacteria has been linked to the development of IBD. Almost all experimental mouse models of inflammatory bowel disease can be inhibited in mice devoid of intestinal flora. The development of spontaneous colitis and immune system activation in IL-10-deficient mice is lost if animals are housed in germ-free conditions thus resident enteric bacteria are necessary for development of spontaneous colitis in these mice. Conventional housing unlike specific pathogen free barrier facilities increase the incidence and severity of the colitis phenotype and is more indicative of human disease. Moreover, variability in animal responses, particularly to immune-related stimuli, when assessed in the context of conventional versus barrier conditions deserves consideration. The murine studies in chapter 3 include animals housed in conventional facilities and this is likely to enhance
differences in immune phenotype and pathology. Thus, phenotypes may be more elusive in barrier facilities. Furthermore, understanding the role enteric bacteria may play in acute and chronic colitis as a function of epithelial dependent loss of Stat3 was outside the scope of this thesis but warrants further investigation.

**Location, Location, Location**

In our murine model of intestinal epithelial cell *Stat3* deletion (*Stat3*ΔIEC) chronic inflammation at day 28 was more severe following 7 days of acute injury with DSS which was not accounted for by a sustained defect in epithelial proliferation or apoptosis as compared to littermate control (*Stat3*Flx/Flx) mice. We concentrated our efforts within 0.5cm from the ulcer border or similar areas of the colon if no ulcer was present. Analysis from areas that were unaffected at day 7 and day 28 in both *Stat3*Flx/Flx and *Stat3*ΔIEC mice were very similar for proliferation and apoptosis (data not shown). Even more striking were the differences between distal and proximal disease between genotypes (data not shown). Distal disease was very similar between genotypes however proximal disease varied and thus we had to be very consistent with our approach for harvesting the colon and the overall analysis of disease histology, proliferation, apoptosis, and immunohistochemistry for activated p65/RelA, pSTAT1, pSTAT5. To orient a starting point for analysis we developed a scoring system to account for total colonic disease by using the anal verge to mark the most distal starting point for scoring and the following four consecutive longitudinal 5x High Powered Fields (HPF) scored as the distal colon and the following four, 5x HPF scored as the middle colon. We did not observe in any of the animals, regardless of genotype, inflammation past the middle/proximal portion that was scored in our developed system and thus we feel we were able to score total colonic disease. We did not find
patchy disease we had very consistent distal and middle disease, with the most proximal middle
disease having greater variation between genotypes. We reported our data as a total colonic
score, but observed very different levels of disease in the middle colon suggesting differential
regulation of STAT3 depending on colonic location. This may facilitate differences in
investigator interpretation of disease severity depending on how stringent one is in evaluation of
total colonic disease. Therefore we feel we have devised a very stringent and important scoring
system to evaluate total histologic disease severity.

**Interpretation of immunofluorescence and gene expression data**

In the same mice that we interrogated proliferation and apoptosis, we had residual
Swiss Rolled colonic tissues embedded in O.C.T compound. We cross sectioned these tissues
into four to five 30-µm-thick sections, giving approximately 20mg of total colonic tissue that we
could extract RNA from using RNA later to wash away the O.C.T compound. This was helpful
as we could also assess the frequency of different cell populations from subsequent portions of the
same tissue while also interpreting gene expression. We ultimately found that colonic lamina
propria frequency of total pSTAT3+ cells was increased and correlated with histologic injury and
F480+pSTAT3+ macrophages, and CD3+pSTAT3+ T-lymphocytes were increased in Stat3^{AIAC}
mice compared to littermate controls. Colonic expression of Stat3 target genes Reg3β and Reg3γ
that mediate epithelial restitution were significantly decreased in Stat3^{AIAC} mice. However
colonic expression of Il-17a, IFNγ, Cxcl2, Cxcl10, Ccl2, and Ccl4 were significantly increased
and Il-17a expression correlated with the increased lamina propria frequency of CD3+pSTAT3+
T-lymphocytes in Stat3^{AIAC} mice. Our results were consistent with prior reports in that we
observed increased expression of CXCL2, CXCL10, CCL2 and CCL4 in Stat3^{AIAC} mice in the
setting of more severe chronic colitis which is likely contributing to the differences in the inflammatory infiltrate observed at day 28. SOCS3, a direct downstream target of activated Stat3, was increased in expression in the Stat3$^{ΔIEC}$ mice which we believe is consistent with the pronounced pSTAT3+ infiltrate within the lamina propria. Moreover, this infiltrate most likely was responding to IL-6/GP130 receptor activation as SOCS3 is not a direct target of IL-10 induced STAT3 activation. Colonic IFNγ expression was increased at both day 0 and day 28 in the Stat3$^{ΔIEC}$ mice. We did not observe qualitative differences in pSTAT1 activation in the epithelial cell compartment at these time points. However, as IFNγ is produced by Th1 cell types, and IL-4 expression did not differ between genotypes, this likely represents a basal Th1 skewing in the Stat3$^{ΔIEC}$ mice $^{75}$. Colonic $IL-17A$ expression was increased only at day 28 in Stat3$^{ΔIEC}$ mice, in the setting of chronic colitis. IL-17A is released by Th17 inflammatory T-cells, shown to involve direct binding of Stat3 to the IL-17A promoter, and known to be involved in many chronic autoimmune disorders$^{76-78}$. Although there was a trend, we did not detect a difference between genotypes for expression of $IL-10$, $IL-6$, $IL-1β$ or $TNFα$, suggesting that there was not a global difference in activation of mucosal inflammatory pathways in the Stat3$^{ΔIEC}$ mice. Consistent with an effector function, we observed a strong correlation between the frequency of pSTAT3+ T lymphocytes and IL-17A expression, and between IL-17A expression and histologic injury. Future studies employing Th17 blockade will be required to determine whether this will ameliorate chronic colitis in this setting. In summary, we have shown that loss of IEC Stat3 leads to more severe chronic inflammation following acute injury which is not accounted for by a sustained defect in epithelial proliferation or apoptosis, but rather expansion of pSTAT3+ lymphocytes and up-regulation of $IL-17A$ expression.
Alternative Hypothesis

**Loss of IEC STAT3 results in enhanced bacterial sensing.** Is it possible that the loss of STAT3 in the epithelial compartment enhances microbial sensing by intestinal epithelial cells after activation or inflammation. The intestinal epithelium provides a critical interface between luminal bacteria and the mucosal immune system. Whereas normal commensal flora do not trigger acute inflammation, pathogenic bacteria trigger a potent inflammatory response. IFN-γ has been shown to positively regulated MD-2 promoter activity in IEC and co-expression of SOCS3 blocked IFN-γ-mediated MD-2 promoter activation\(^7^9\). In our model IFNγ expression is increased at day 0 and day 28 in Stat3\(^{AIEC}\) mice compared to Stat3\(^{Flx/Flx}\) littermate controls. Therefore in the epithelial compartment of Stat3\(^{AIEC}\) mice there would be reduced transcription of SOCS3 allowing for enhanced expression of the critical TLR4 co-receptor MD-2 which could alter toll-like receptor expression and bacterial reactivity.

**SHP2 is protective in mice devoid of IEC STAT3.** Recent data in abstract form suggests that knockdown of the Src homology tyrosine phosphatase (SHP2) in the human colonic cell line Caco-2 inhibited the ability of the cells to migrate and adhere compared to control cells. Additionally upon IL-6 induced GP130 activation SHP2 mediated activation induces Ras-ERK pathway seeming to transduce activities regulating mitogenesis\(^8^0\). The SHP-2 inducing site in the cytoplasmic tail of GP130 is the binding site for SOCS3 inhibition. In our Stat3\(^{AIEC}\) mice reduced levels of SOCS3 would therefore allow enhanced SHP2/Ras/ERK activation. This maybe an important mechanism in wound healing that is mediated at the early stages of acute colitis in response to IL-6. Thus may also be the alternative protective mechanism used to promote the wound healing and proliferation that allowed Stat3\(^{AIEC}\) mice to recover after day 7 compared to littermate controls and may be involved in epithelial proliferation from day 7 to day
28. We did not see other compensatory mechanisms including pSTAT1, pSTAT5, or p65 upregulated in the epithelium at day 7, or day 28. Therefore an increase in SHP2 activity may be extremely protective in the model and evaluating downstream effectors important in proliferation and survival induced by Ras and ERK activation would be important.

Future Directions

A need to define the STAT3/Th17/IL22 axis. The cytokine IL-23 is one of the key components involved in the induction and maintenance of Th17 cells through STAT3 activation. Recent data has shown that the IL23R R381Q gene variant protects against immune-mediated diseases by impairing IL-23-induced Th17 effector response\(^8\). Th17 cells secrete IL-22 shown to induce STAT3-dependent IEC survival, regeneration, and the production of antimicrobial compounds that directly limit the bacterial and/or the inflammatory T-cell component of the inflammatory response following inflammatory insult specifically directed by Th17 cells\(^8\). However, IL-22-induced STAT3 activation can also induce the production and release of IL-8 and TNFα, further enhancing IL-17A-driven inflammation\(^8\). Specifically IL-6, IL21, and IL23 are the cytokines known to drive the differentiation and survival of these cells. We did not find an increase in IL-6 expression but we did not investigate the level of expression for IL-21, IL-23, or IL-22. Transforming growth factor beta (TGF-β) expression has been demonstrated throughout wound healing and shown to regulate many processes involved in tissue repair, including production of ECM, migration, chemotaxis, and proliferation of macrophages, fibroblasts of the granulation tissue, epithelial and capillary endothelial cells\(^8\). Moreover TGFβ along with IL-10 are involved in mediating regulatory T-cell expansion as IL-10-induced STAT3 is important for the regulatory function of Tregs on Th17 cells\(^8\). We did not find an
increase in *IL-10* expression between our genotypes at day 28 however, the level of TGFβ may give insight into the overall regenerative capacity of the colon between genotypes. Th17 mediated IL-22 dependent STAT3 expression of membrane-bound mucin referred to as Muc1, functions as a lubricant and a physiological barrier between luminal contents and mucosal surface. Muc1 deficient mice develop more severe forms of Th1- and Th2-induced colitis than controls with increased colonic permeability and Th17-cells. This data suggests that IL-22 induced Muc1 expression functions as a negative feedback pathway that prevents an excessive Th17-cell response in inflamed colons of mice. In our model we did not evaluate Muc1 but at day 28 STAT3 induced Muc1 expression is probably substantially lower in the Stat3<sup>IEC</sup> mice compared to controls. We did find an increase in IL17A is probably mediated by increased number of Th17 cells that are also secreting IL-22 to which Stat3<sup>IEC</sup> mice cannot respond reducing the regenerative capacity and barrier function that is defective at day 28. Ultimately, we would want to define the level of expression of IL-21, IL-22, IL-23, TGBβ and Muc1 in our murine model at day 0 and day 28.

**IEC STAT3 in the chronic phase.** These studies highlight the protective and important role of STAT3 in maintaining barrier function and terminating inflammatory reaction during the chronic phase of colitis. This has only recently been appreciated as loss of STAT3 in the chronic phase has only been associated with a reduced colonic cancer phenotype. This data supports our hypothesis that loss of STAT3 in the epithelial compartment mediates more severe colitis. The specific mu-opioid receptor agonist DALDA induces STAT3 activation-dependent proliferation and migration of IEC, and data from DSS induced colitis models suggest that targeting epithelial STAT3 activation with this compound could be a potential therapeutic. This also supports recent data from humans showing that high expression of SOCS3 could conceivably interfere
with regenerative responses and epithelial cell homoeostasis enhancing the sensitivity of SOCS3 expressing epithelial cells to inflammatory damage. The high SOCS3 expression in IEC in UC remission is associated with a shorter time till relapse and an increased severity of inflammation during relapse\textsuperscript{88}. This supports other preliminary data from our lab in that patients carrying the JAK2 risk allele have decreased STAT3 activation reduced colonic expression of the STAT3 target gene \textit{REG3a} suggesting that reduced STAT3 activation may also be associated with increased risk for IBD\textsuperscript{89}. The JAK2 risk allele has also been associated with increased intestinal permeability and we would suggest that this is probably mediated by reduced epithelial STAT3 activation\textsuperscript{90}. These data and our own suggest that epithelial STAT3 activation is needed for protection from acute colitis through IL-6 induced proliferation and survival and that during the chronic phase STAT3 helps maintain mucosal homeostasis by inducing greater barrier function and wound healing/regeneration responses. This could be tested utilizing an IEC STAT3 deficient mouse with the tissue-specific and inducible Cre-mediated (vil-Cre-ER\textsuperscript{T2}) line\textsuperscript{91}. Thus, we could mediate loss of STAT3 in a time and phase dependent to more elegantly explain how STAT3 deficiency in the epithelial compartment impacts colitis.

\textbf{Defining the Cellular Source.} Ultimately, defining the cellular source of IL-17A and IFN\gamma and the total frequency of these cells within the lamina propria using flow cytometric analysis would be informative. However, one could also quantify these cells within the lamina propria by immunofluorescence double staining for the surface marker CD3/IL-17A and CD3/IFN. Interestingly, gamma delta T-cells have also been shown to be an important source of IL-17\textsuperscript{92}. Therefore comparison of double staining for the gamma delta TCR or CD4 with IL-17A would help further interpret which T-cells are producing the IL-17A. Additionally, the significant increase in F4/80+/pSTAT3+ macrophages should be characterize as an M1 or M2
phenotype. M1 is classically activated, generally inflammatory and mediates protection against invading pathogens with activation of STAT3 and STAT1 and induction of SOCS3 for release of IL23 and IL12. Alternatively, M2 an anti-inflammatory phenotype known to regulate wound healing, activates STAT6 and induces SOCS1 expression\(^9\). Based on our gene expression and immunofluorescence data we assume that these macrophages are M1 inducing pro-inflammatory antimicrobial functions. Epithelial expression of *Muc1*, *Reg3\(\beta\)*, *Reg3\(\gamma\)*, and *\(\beta\)-defensin-2* (epithelial restitution/barrier function genes) *Il-6*, *Il-10*, *IFN\(\gamma\)*, *Cxcl2*, *Cxcl5*, *Cxcl10*, *ccl2*, *ccl4*, *ccl20* could be quantified using laser capture micro-dissection of epithelial cells within colonic areas of active disease and inactive disease in both Stat3\(^{Flx/Flx}\) and Stat3\(^{\Delta IE/C}\) mice. The ability to define gene expression within the same animal in regards to epithelial crypts undergoing wound healing compared to adjacent non disease epithelial crypts would be extremely interesting in and of itself. We observed in histologic assessment differences in BrdU and caspase-3 in cells closest to the ulcer verses adjacent healthy tissue. This suggests that there are completely different gene expression profiles depending on colonic location and disease involvement. Therefore, it would be interesting to decipher epithelial genes important in wound healing and extracellular matrix interactions that are STAT3 dependent utilizing laser capture micro-dissection of epithelial cells form the colonic niche in regards to diseased and healthy tissue from Stat3\(^{\Delta IE/C}\) mice compared to Stat3\(^{Flx/Flx}\) littermate controls. Lastly, the lamina propria could also be interrogated utilizing laser capture micro-dissection protocol to help define the gene expression coming from the lamina propria infiltrate verse the epithelial barrier.
Summary

The STAT3 transcription factor is important in almost all cell types in the intestine. In the epithelial compartment STAT3 mediates proliferation and survival, wound healing, and regeneration. In the innate immune system, STAT3 induces proper protective and highly regulated immunity and in the adaptive immune system STAT3 mediates the highly aggressive pro-inflammatory Th17 cell type and the opposing regulatory T cell phenotype. Moreover, inflammation per se is both protective yet can be detrimental in a chronic setting to the host. Therefore, as STAT3 is involved in protection, perpetuation, and ultimately resolution and regeneration, it is a master regulator of mucosal inflammation. The studies herein have shown that loss of IEC Stat3 leads to more severe chronic inflammation following acute injury which is not accounted for by a sustained defect in epithelial proliferation or apoptosis, but rather expansion of pSTAT3+ lymphocytes and up-regulation of IL-17A expression. Further studies employing Th17 blockade will be required to determine whether this will ameliorate chronic colitis in this setting.

Lastly, in trying to define how the STAT3 rs744166 is associated with risk for IBD we revealed that the STAT3 IBD “A” risk allele 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. It is likely that there are several immunogenetic forms of IBD, with CD and UC representing the broadest clinical classifications, our studies implicate the potential of defining subpopulations in regards to improved therapeutic efficacy. Even though we did not observe differences in overall clinical demographics our patients homozygous for the “G” allele were at very low numbers in our patient population which ultimately limited the power of our analyses. Therefore, 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.
References


