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Activation of an IL-6:STAT3-dependent Transcriptome in Pediatric-onset Inflammatory Bowel Disease

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Abstract

Background: While activation of the IL-6-dependent transcription factor signal transducer and activator of transcription 3 (STAT3) has been implicated in the pathogenesis of inflammatory bowel disease (IBD), a direct effect on mucosal gene expression and inflammation has not been shown. We hypothesized that a proinflammatory IL-6:STAT3-dependent biological network would be up regulated in pediatric-onset IBD patients, and would be associated with the severity of mucosal inflammation.

Methods: Patients with pediatric-onset IBD were enrolled at diagnosis and during therapy. Serum cytokine analysis was performed using Bioplex. STAT3 phosphorylation (pSTAT3) in peripheral blood leukocytes (PBLs) was assessed by flow cytometry. Immunohistochemistry of colonic mucosa was used to localize pSTAT3 and STAT3 target genes. Microarray analysis was used to determine RNA expression profiles from colon biopsies.

Results: Circulating IL-6 was upregulated in active IBD patients at diagnosis and during therapy. STAT3 activation was increased in PB granulocytes, IL-6-stimulated CD3⁺/CD4⁺ lymphocytes, and affected colon biopsies of IBD patients. The frequency of pSTAT3+PB granulocytes and colon epithelial and lamina propria cells was highly correlated with the degree of mucosal inflammation. Microarray and Ingenuity Systems bioinformatics analysis identified IL-6:STAT3-dependent biological networks upregulated in IBD patients which control leukocyte recruitment, HLA expression, angiogenesis, and tissue remodeling.

Conclusions: A proinflammatory IL6:STAT3 biologic network is upregulated in active pediatric IBD patients at diagnosis and during therapy. Specific targeting of this network may be effective in reducing mucosal inflammation.

Keywords

interleukin-6; Crohn's disease; ulcerative colitis; chemokines; microarray

The etiology of inflammatory bowel disease (IBD) is unknown, but disease pathogenesis is believed to involve a complex interaction between susceptibility genes, the mucosal immune system, and the intestinal microbial environment. Both Crohn's disease (CD) and ulcerative

colitis (UC) are characterized by a cytokine-driven mixed inflammatory infiltrate in the intestinal mucosa. However, cytokine profiles of UC and CD patients reported to date have been complex and overlapping. For example, tumor necrosis factor- α (TNF α), interferon- γ (IFN- γ), and multiple interleukins including IL-6, IL-12, IL-17, and IL-23 have been implicated in the pathogenesis of CD. In contrast, IL-5, IL-6, IL-13,¹ and IL-17 have been found to be elevated in UC.

A critical cytokine signal transduction pathway involves a family of latent cytoplasmic transcription factors, the signal transducers and activators of transcription (STATs). STATs have been implicated in the pathogenesis of many human diseases, including multiple types of cancer, dilated cardiomyopathy, and liver disease.^{2,3} STAT3 has been shown to promote effector T cell resistance to apoptosis in chronic intestinal inflammation,⁴ and increased levels of the activated form of STAT3, tyrosine phosphorylated STAT3 (pSTAT3), have been demonstrated in lamina propria mononuclear cells (LPMCs) of patients with CD and UC.^{4,5} IL-6, a known activator of STAT3, has been found to be elevated in the serum of patients with IBD⁶ and to predict clinical relapse in CD. Moreover, a small placebo-controlled trial demonstrated that a humanized antibody against the IL-6 receptor reduced disease activity in patients with CD.⁷ Importantly, these studies have focused on adult IBD patients on therapy. What role IL-6 and STAT3 play in pediatric IBD patients or in patients prior to treatment at diagnosis is unknown. Moreover, pSTAT3 has not been well characterized in circulating peripheral blood leukocytes (PBLs).⁸

Cell type-specific STAT3-deficient mice have illustrated the pleiotropic effects of activated STAT3 in mucosal inflammation. Disruption of STAT3 in macrophages and neutrophils causes increased susceptibility to lipopolysaccharide-induced endotoxin shock and chronic enterocolitis.⁹ In contrast, mice with T-cell-specific deletion of STAT3 are healthy and have normal T-cell development. However T cells from these mice are resistant to IL-6 promotion of cell proliferation and survival.¹⁰ More recently, STAT3 activation via IL-6 has been implicated in the development of spontaneous ileitis and colitis in SAMP1/Yit and IL-10-deficient mice, respectively.^{11,12} Collectively, these animal studies demonstrate that STAT3 may function in proinflammatory pathways in certain immune cells (T cells) but be antiinflammatory in other cell types (macrophages), in a cytokine (IL-6 versus IL-10)-dependent manner.

Gene array studies have begun to elucidate potential STAT3 target genes that may be regulated by pSTAT3 after translocation to the nucleus and DNA binding. However, previous gene array analyses have focused on global gene transcriptomes that differentiate treated adult IBD patients¹³ from healthy controls or on specific immune regulatory genes such as chemokines. To date, gene array analysis has not been used to identify specific cytokine:STAT-dependent biologic networks that are dysregulated in pediatric IBD or prior to treatment. We hypothesized that IL-6:STAT3-dependent biologic networks regulate mucosal inflammation in pediatric IBD. We have identified an IL-6:STAT3-dependent network that is upregulated in IBD patients and promotes granulocyte and effector T-cell recruitment and epithelial cell injury.

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) antibodies and chemokine antibodies for immunohistochemistry were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were from Santa Cruz Biotechnology. pSTAT3 antibodies and antibodies for CD4, CD3, CD8, and CD14 for flow cytometry analysis were from BD Biosciences (San

Jose, CA). Fecal calprotectin was measured by a commercial laboratory (Genova Labs), and CRP was measured in the CCHMC clinical laboratory.

Patient-Based Studies

Colon biopsies and blood samples were obtained from children with IBD and healthy controls following informed consent during colonoscopy performed for clinical care. For CD patients, biopsies were obtained from an area of active disease in the ascending colon or the most proximal area of active disease if the ascending colon was endoscopically normal. For UC patients, biopsies were taken from the ascending colon if affected and from the most proximal affected area otherwise. The diagnosis of IBD was made using established clinical, radiological, and histological criteria. Patients with indeterminate colitis were excluded. The Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Clinical Activity Index (PUCAI) were used as measures of clinical severity of IBD.^{14,15} The Montreal Classification was used to classify patients by age at diagnosis and by disease location and behavior.¹⁶ Serum cytokine levels were measured using the Bioplex assay.

Flow Cytometry

Whole blood was collected in sodium heparin tubes at the time of colonoscopy and placed directly on ice. Surface staining was done in aliquots of whole blood for 30 minutes at room temperature (RT) in the dark. Red blood cells (RBCs) were lysed in 0.15 M ammonium chloride buffer (ACK) for 15 minutes at RT. PBLs were pelleted (5 min at 500g), washed in 4% fetal bovine serum (FBS) in DPBS, and stored at 4°C until flow cytometry. PBLs were prepared for intracellular phosphotyrosine analysis using a method modified from Nolan.¹⁷ Briefly, RBCs were lysed in ACK for 15 minutes at RT, pelleted, and washed twice with DPBS. Pellets were resuspended in serum-free DMEM and stimulated for 30 minutes at 37°C with IL-6 and sIL6-R or control medium. Immediately after stimulation paraformaldehyde was added to the culture medium to a final concentration of 2% and PBLs were stored overnight at 4°C. To permeabilize the cell membranes PBLs were pelleted, washed in PBS, resuspended in ice-cold 100% methanol, and incubated on ice for 20 minutes. PBLs were then washed twice in PBS, cell surface and anti-pSTAT3 antibodies were added, and incubated for 30 minutes at RT in the dark. Pellets were washed in 4% FBS-DPBS and stored in 4°C until flow cytometry.

Colon Histology Analysis and Immunohistochemistry

Paraffin-embedded hematoxylin-stained colon biopsies were scored in a blinded manner by an experienced pediatric pathologist (E.B.) using the Crohn's Disease Histologic Index of Severity (CDHIS) and the Ulcerative Colitis Histologic Index of Severity (UCHIS). The CDHIS is a validated histologic score of acute and chronic inflammation in CD.¹⁸ The UCHIS is a validated histologic score of inflammation for UC.¹⁹ For immunohistochemistry, paraffin-embedded slides were deparaffinized and antigen unmasking was done at boiling for 10 minutes with 10 mM sodium citrate (pH 6). Endogenous peroxide was quenched with 10% hydrogen peroxide for 5 minutes at RT. Slides were subsequently blocked with 10% serum and then incubated overnight at 4°C with primary antibodies as follows: rabbit anti-pSTAT3 and anti-CXCL10 and anti-CXCL11. Sections were incubated with 10% rabbit IgG as negative control. Biotinylated goat antirabbit secondary antibody and streptavidin was applied sequentially for 35 minutes at RT after washing in PBS. Hematoxylin was used for nuclear counterstaining following peroxidase (DAB) development. Images were captured using a Zeiss microscope and Axioviewer image analysis software v. 4.5 (Deutsland, Carl Zeiss, Germany). Seven fields at 100× were selected at random to quantify the number of pSTAT3-positive cells per high-powered field (HPF).

Gene Array Analysis

Two colon biopsies from the ascending colon for CD (>90%) and from the most proximal affected colon for UC were obtained at the time of colonoscopy and immediately placed in RNA later (Qiagen, Germany) at 4°C. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and stored at -80°C. Samples were then submitted to the CCHMC Digestive Health Center Microarray Core where the quality and concentration of RNA was measured by the Agilent Bioanalyzer 2100 (Hewlett Packard, Corvallis, OR) using the RNA 6000 Nano Assay to confirm a 28S/18S ratio of 1.6–2.0. 100 ng of total RNA was amplified using Target 1-round Aminoallyl-aRNA Amplification Kit 101 (Epicentre, WI). The biotinylated cRNA was hybridized to Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 arrays, containing probes for ~22,634 genes. The images were captured using Affymetrix Genechip Scanner 3000. The complete dataset is available at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) accession number GSE9686. Genespring software was used in the CCHMC Digestive Health Center Bioinformatics core to analyze fold changes in gene expression between disease types. 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 of 1.5 relative to healthy control samples. Ingenuity Systems software was used to group the differentially expressed genes into biologically relevant networks.

Statistical Analysis

A Fisher's exact test was used to analyze categorical variables and a Mann–Whitney *t*-test for single comparisons or a Kruskal–Wallis with Dunn's multiple comparison was used to analyze continuous variables. Spearman's linear regression analysis was used to correlate values and area under the curve receiver operator characteristic (AUROC) analysis was used to calculate the specificity and sensitivity of pSTAT3 positive PB granulocytes in differentiating IBD patients from healthy controls. All graphs and statistical analysis were done using Prism 4 from GraphPad (San Diego, CA) software. A *P*-value <0.05 was considered statistically significant.

Ethical Considerations

The study was approved by the CCHMC Institutional Review Board and CCHMC General Clinical Research Center Scientific Advisory Committee, and informed consent and, as applicable, assent, was obtained from all study participants and their parents.

RESULTS

Patient Demographics

A total of 74 subjects were recruited for the initial FACS and gene array studies. This included 17 patients with CD, 11 patients with UC, and 20 healthy controls who were enrolled at the time of the initial diagnostic endoscopy, and 26 patients with active treated CD (CD TR) who were enrolled at the time of a subsequent endoscopy performed for clinical care. Subsequently, an additional 42 patients attending the CCHMC IBD clinic for routine clinical care were recruited in order to define differences in the PBL FACS assay between patients in remission and those with clinically active disease. Table 1 provides demographic and clinical data on the patient cohort. There were no statistically significant differences for age at diagnosis, gender, or race between the groups. Disease activity as measured by the PCDAI for CD and CD TR and the PUCAI for UC did not differ between the groups. Most subjects had moderately active disease. Demographic and clinical data including age, sex, race, medications, and duration of disease for CD TR, PCDAI, or PUCAI at study entry, and Montreal Classification of disease location and behavior is given for each IBD patient in Tables 2, 3.¹⁶ The mean length of follow-up for the patients enrolled at diagnosis was 1.5 years (range 0–2.8 years).

Serum Cytokines and STAT3 Tyrosine Phosphorylation in Peripheral Blood Leukocytes

Previous studies have shown that serum IL-6 and sIL-2R are elevated in active IBD, and that persistently elevated IL-6 is associated with relapse. We found that serum IL-6 was upregulated to a comparable degree in CD and UC at diagnosis, as well as CD TR, compared to healthy controls. By comparison, sIL-2R was upregulated in CD and UC, but not CD TR. In healthy controls the serum IL-6 level was 15 ± 3 pg/mL compared to CD 36 ± 6 pg/mL, CD TR 41 ± 7 pg/mL and UC 85 ± 47 pg/mL ($P < 0.05$ versus control). Similarly, the healthy controls had sIL-2R levels of 549 ± 65 pg/mL compared to CD 850 ± 116 pg/mL, CD TR 631 ± 94 pg/mL and UC 1133 ± 137 pg/mL ($P < 0.05$ for CD and UC versus control).

IL-6 is believed to regulate mucosal inflammation in large part via tyrosine phosphorylation of the STAT3 transcription factor in effector lymphocytes and granulocytes. We therefore asked whether basal or IL-6-dependent STAT3 tyrosine phosphorylation in peripheral blood lymphocytes or granulocytes would be increased in active IBD. We measured basal and IL-6-stimulated intracellular PBL STAT3 tyrosine phosphorylation (pSTAT3) by flow cytometry using a method modified from Nolan.¹⁷ Granulocytes were identified based on negative staining for CD3 (lymphocytes) and CD14 (monocytes) and the side and forward scatter properties of this population, as shown in Figure 1A. The cell surface markers CD3 and CD4 were used to identify the CD4⁺ lymphocyte population (data not shown). The percent pSTAT3-positive granulocytes or CD4⁺/CD3⁺ lymphocytes were then determined relative to isotype controls as illustrated in Figure 1A.

We found that IBD patients at diagnosis (combined CD and UC) and with active treated CD (CD TR) had a significantly higher basal frequency of pSTAT3-positive granulocytes compared to healthy controls (Fig. 1B). In healthy controls the percent positive pSTAT3 granulocytes was $5.8 \pm 1.4\%$, compared to IBD $23.6 \pm 6.1\%$ and CD TR $26.1 \pm 5.8\%$. By comparison, the basal frequency of pSTAT3+ CD3⁺/CD4⁺ lymphocytes did not differ between the groups (Fig. 1C, control $1.0 \pm 0.4\%$, IBD $3.0 \pm 0.8\%$ and CD TR $2.8 \pm 0.7\%$, $P > 0.5$).

We then asked whether IL-6-stimulated STAT3 tyrosine phosphorylation in peripheral blood lymphocytes or granulocytes would differ between active IBD and controls. Control granulocytes exhibited a 2.4-fold increase in the frequency of pSTAT3-positive cells after stimulation with IL-6 (from $5.8 \pm 1.4\%$ to $13.9 \pm 3.9\%$), which was not statistically different from the relative increase observed in IBD patients (IBD basal $23.6 \pm 6.1\%$, IL-6-stimulated $39.0 \pm 7.4\%$; CD TR basal $26.2 \pm 5.8\%$, IL-6-stimulated $38.7 \pm 5.6\%$, $P > 0.5$). IL-6 stimulation increased the frequency of pSTAT3-positive CD3⁺/CD4⁺ lymphocytes in healthy controls from $1.2 \pm 0.4\%$ to $5.1 \pm 1.4\%$ ($P < 0.01$). Patients with IBD at diagnosis and CD TR exhibited a statistically significant increase in the frequency of pSTAT3-positive CD3⁺/CD4⁺ lymphocytes with IL-6 stimulation compared to healthy controls (Fig. 1C). In IBD and CD TR patients respectively, the basal pSTAT3-positive CD3⁺/CD4⁺ lymphocytes were $3.0 \pm 0.8\%$ and $2.8 \pm 0.7\%$, and increased to $15.3 \pm 4.4\%$ and $18.9 \pm 4.4\%$ after IL-6 stimulation. Consistent with this, the serum IL-6 level was highly correlated with the basal frequency of pSTAT3-positive granulocytes, and the IL-6-stimulated frequency of CD3⁺/CD4⁺ lymphocytes, with a Spearman's coefficient of $r = 0.81$ ($P < 0.05$, $n = 45$) and $r = 0.85$ ($P < 0.05$, $n = 45$) for granulocytes and lymphocytes, respectively. Taken together, these data demonstrated that the IL-6-dependent transcription factor STAT3 is activated in peripheral blood granulocytes and lymphocytes of patients with active IBD, and that IBD lymphocytes exhibit an increased responsiveness to IL-6, relative to healthy controls.

Peripheral Blood Granulocyte STAT3 Tyrosine Phosphorylation and Mucosal Inflammation

We then asked whether the frequency of pSTAT3-positive PB granulocytes would correlate with clinical disease activity and the degree of mucosal inflammation in active IBD patients.

The frequency of pSTAT3-positive PB granulocytes was increased 2-fold in patients with clinically active IBD, relative to those in remission (Fig. 2A). The severity of mucosal inflammation was determined by computing the CDHIS for the CD patients and the UCHIS for the UC patients.¹⁸ Linear regression analysis using Spearman's correlation demonstrated that the frequency of pSTAT3+ peripheral blood granulocytes was highly related to the degree of mucosal inflammation measured by the CDHIS or UCHIS, with $r = 0.76$ ($P < 0.0001$, $n = 33$; Fig. 2B).

STAT3 Activation and Mucosal Inflammation in Colon Biopsies

We then asked whether a similar increase in the frequency of pSTAT3-positive cells would be observed in colon biopsy samples obtained from patients with active IBD.

Immunohistochemistry (IHC) was used to identify pSTAT3-positive cells in colon biopsy sections obtained from patients with active IBD and healthy controls. Healthy controls demonstrated a modest number of pSTAT3-positive cells primarily in the subepithelial lamina propria (see arrow, Fig. 3A). The mean number of pSTAT3-positive cells per HPF in controls was equal to 21.1 ± 1.9 . Patients with IBD demonstrated a significantly higher number of pSTAT3-positive cells per HPF (Fig. 3B). In addition, whereas epithelial cells were rarely positive in controls, IBD patients consistently exhibited an increased frequency of pSTAT3-positive epithelial cells (see arrowheads, Fig. 3A). Linear regression analysis using a Spearman correlation demonstrated that the mean number of pSTAT3-positive cells per HPF was highly related to the overall histological index of severity for both CD and UC ($r = 0.69$, $P < 0.0001$; Fig. 3C). This relationship was quite similar to that observed for the frequency of pSTAT3-positive peripheral blood granulocytes and the CDHIS/UCHIS (Fig. 2B). The frequency of pSTAT3-positive cells per HPF was also related to the epithelial injury subscore of the CDHIS (score for epithelial damage, architectural distortion, and erosion/ulcers), as shown in Figure 3D ($r = 0.52$, $P = 0.0007$).

IL-6 and STAT3-dependent Gene Expression Profiles in IBD Colonic Mucosa

While several studies have reported genomic expression signatures for active and inactive IBD compared to control, no study to date has determined whether specific IL-6 and/or STAT3-dependent biologic networks are upregulated in active IBD. We therefore determined the global pattern of colonic gene expression in patients with CD and UC at diagnosis, CD TR, and healthy controls. In all, 545 genes were found to be differentially regulated between controls and active CD. The majority of these genes were also differentially expressed between UC and normal. Supervised hierarchical cluster analysis was used to sort expression profiles for each subject within the 4 groups, using the genes that were differentially expressed between active untreated CD and healthy controls (Fig. 4).

Ingenuity Systems software was then used to identify biologically relevant networks present within the CD genomic signature. Four networks were identified for which all of the components were also present within the CD genomic signature. This is equivalent to a P -value of 10^{-52} that these would be found within the CD genomic signature by chance. For 2 of these networks, IL-6 or STAT3 appear to function as the central regulatory nodes (Fig. 5A,B). The combined IL-6 and STAT3-dependent networks contain 70 genes that are listed in detail in Table 4. The majority of these genes are immune and inflammatory mediators or chemokines, cytokines, and growth factors. The relative expression of these genes between the 3 IBD groups was remarkably similar, supporting a common mode of transcriptional regulation.

CXCL10 and CXCL11 Localization by Immunohistochemistry

RNA expression profiles generated from colonic mucosal biopsies do not allow for cell-specific localization of gene products. The chemokines CXCL10 and 11, which are known to promote tissue recruitment of CXC3R+ effector T lymphocytes in IBD, were identified as IL-6/STAT3-

dependent genes in the microarray analysis (Fig. 5A). We therefore asked whether these would be upregulated at the protein level in epithelial or lamina propria cells in active IBD. Using IHC, both chemokines were found to be expressed at a low level in healthy controls, primarily within surface epithelial cells (Fig. 6). By comparison, in active IBD we identified increased expression within both lamina propria mononuclear cells and the epithelium. Qualitatively, this expression seemed to be highest in UC patients (Fig. 6). Taken together, these data have identified a proinflammatory IL-6:STAT3-dependent biological network that appears to drive mucosal inflammation both at diagnosis of IBD and in active treated CD patients.

DISCUSSION

While several studies have implicated IL-6:STAT3 signaling in the pathogenesis of IBD, a comprehensive assessment of STAT3 activation and target gene expression in both treated and untreated patients has not previously been performed. The aims of the current study were to determine whether IL-6:STAT3 activation does correlate with mucosal inflammation in active pediatric-onset IBD, and to define the relevant biological networks that mediate this effect. We found that serum IL-6 and activated STAT3 were increased in both CD and UC patients at diagnosis, and remained elevated to a very similar degree in treated CD patients with active disease. These data suggest that IL-6:STAT3 signaling is likely to play a critical role in both early and later stages of the disease. The frequency of pSTAT3-positive cells in colon biopsies correlated well with both overall histological indices of severity, and the components of the CDHIS related specifically to epithelial injury. This may simply reflect that the majority of activated immune cells in the gut in IBD contain pSTAT3. However, the relationship with epithelial features including erosions and architectural changes suggests that the increased frequency of pSTAT3 cells is directly related to ongoing injury and repair.

Other groups have investigated the cell populations that express activated STAT3 in IBD patients. Musso et al⁸ examined treated adult IBD patients and detected pSTAT3 in LPMCs by Western blot and localized pSTAT3 to lamina propria CD68⁺ macrophages and CD3⁺ T cells by immunofluorescence, but did not detect pSTAT3 in PBMCs. Moreover, colocalization of pSTAT3 with tissue neutrophils identified by neutrophil elastase staining was not observed. In contrast, Lovato et al²⁰ detected pSTAT3 by Western blot in cultured peripheral and intestinal CD4⁺ T cell lines derived from treated adult CD patients. We found that basal pSTAT3 was detectable by flow cytometry in peripheral granulocytes, but only very low levels were found in peripheral CD4⁺/CD3⁺ lymphocytes from IBD patients. Differences in the techniques used and the study populations may account for the different results. However, similar to Lovato et al, we detected increased pSTAT3 in peripheral CD3⁺CD4⁺ lymphocytes from IBD patients after stimulation with IL-6. Ongoing studies will adapt this technique to tissue samples to more precisely define activation of STAT3 within the lamina propria immune cell populations.

The basis for CD3⁺CD4⁺ lymphocyte hyper-responsiveness to IL-6 stimulation in IBD is not yet known. Holub et al²¹ demonstrated that peripheral lymphocytes from CD patients have elevated RNA expression of the 2 components of the IL-6 receptor, the 80-kDa binding chain and gp130. Ongoing studies in our group will determine whether IBD patients have increased cell surface IL-6 receptor or gp130 expression that could potentially mediate hyper-responsiveness of CD3⁺/CD4⁺ lymphocytes to IL-6. Whereas activated STAT3 in lymphocytes has been shown to mediate resistance to apoptosis⁴ and therefore effector T cell survival, its role in granulocyte function is less clear. Our patients demonstrated elevated levels of activated STAT3 in granulocytes, and this correlated very well with the severity of mucosal inflammation. Potentially, IL-6:STAT3 regulates granulocyte survival and recruitment to the intestinal mucosa in a manner similar to that evoked for effector T cells. Future studies will test this mechanism.

Previous gene array analyses have used surgical resections or pinch biopsies from adult patients with known IBD on treatment.¹³ Our work is the first study to assess genome-wide expression patterns in pediatric IBD patients at diagnosis before receiving treatment. Compared to other published gene arrays we identified a number of common genes but also many newly identified genes. For example, other groups have also identified upregulation of primarily immune-related genes including the major histocompatibility genes, interferon inducible genes, chemokines including *CXCL1-3*, and the transcription factors *STAT1* and *3*.¹³ We have expanded the number of immune-related genes upregulated in IBD and related these to upstream IL-6:STAT3 activation. Importantly, we have identified coordinate upregulation of the CXCR3+ effector T cell chemokines CXCL9-11 in pediatric-onset patients; these were not identified in prior studies of adult patients at diagnosis or on therapy. Combined with upregulation of sIL2-R, this suggests a prominent activation of CXCR3+ T cell responses at diagnosis in these patients. An intriguing report recently identified CXCL10 as a nonredundant regulator of the balance of recruitment between effector and regulatory T cells to atherosclerotic lesions.²² If this is supported by studies in the gut, blockade of this pathway may provide an effective, highly targeted therapeutic approach to reduce mucosal inflammation.

Targeting of the dysregulated STAT3 pathway in IBD may be of benefit in inhibiting survival and recruitment of effector T lymphocytes and neutrophils. Moreover, as IL-6 has been implicated as a direct suppressor of linear growth and bone formation in children with CD, specific targeting of this cytokine may yield additional anabolic benefits in this population.^{23,24} Constitutive STAT3 activation has been implicated in the pathogenesis of colon cancer secondary to its ability to inhibit apoptosis and induce cell proliferation; therapy targeting STAT3 may also decrease the risk of carcinoma in IBD. However, as STAT3 may have antiinflammatory effects in macrophages and dendritic cells, and be required for epithelial wound healing, care needs to be taken to develop a selective inhibitor of STAT3 activation. Taken together, our studies have identified a proinflammatory IL-6:STAT3 biological network that drives mucosal inflammation in pediatric-onset CD both at diagnosis and during therapy, and which will likely provide new targets for more specific therapeutic approaches.

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FIGURE 1.

Basal and IL-6-dependent STAT3 tyrosine phosphorylation is increased in peripheral blood leukocytes (PBLs). PBLs were purified from whole blood and intracellular STAT3 tyrosine phosphorylation (pSTAT3) was measured by flow cytometry before and after stimulation with IL-6/sIL-6R. A: Granulocytes were identified as CD3-negative cells in the granulocyte gate (arrow) of the scattergram as shown. The percent pSTAT3-positive granulocytes compared to isotype control are shown. Basal and IL-6-stimulated percent pSTAT3 positive (B) granulocytes and (C) CD3⁺/CD4⁺ lymphocytes are shown as scatterplots with means as indicated. NI, healthy control; IBD, Crohn's disease and ulcerative colitis at diagnosis; CD TR,

active treated CD. Kruskal–Wallis test with Dunn's multiple comparison test was used to determine differences between groups (** $P < 0.01$).

FIGURE 2.

STAT3 tyrosine phosphorylation in peripheral blood granulocytes correlates with mucosal inflammation. Peripheral blood leukocytes (PBLs) were purified from whole blood and granulocyte intracellular STAT3 tyrosine phosphorylation (pSTAT3) was measured by flow cytometry. The CDHIS or UCHIS were determined using biopsy specimens obtained concurrently with the blood sample for pSTAT3. A: The frequency of pSTAT3+ PB granulocytes is shown for IBD patients in remission and with active disease, with the means as indicated (* $P < 0.05$). B: Spearman's linear regression analysis was used to correlate the percent pSTAT3 positive granulocytes and the CDHIS or UCHIS ($P < 0.0001$).

FIGURE 3.

Mucosal STAT3 tyrosine phosphorylation correlates with histologic disease activity. A: Immunohistochemistry for tyrosine phosphorylated STAT3 (pSTAT3) was performed as shown in colon biopsies from patients with newly diagnosed Crohn's disease (CD) and ulcerative colitis (UC) and active treated CD (CD TR). The isotype control is shown in the inset. B: The mean number of pSTAT3-positive epithelial and lamina propria cells per 7 high-powered fields (HPF) was determined as shown. Kruskal–Wallis test with Dunn's multiple comparison test was used to determine differences between groups (** $P < 0.01$). C: The Crohn's Disease Histologic Index of Severity (CDHIS) and Ulcerative Colitis Histologic Index of Severity (UCHIS) were determined for the biopsy samples for which pSTAT3 was assayed, and related to the pSTAT3 value by Spearman's linear regression analysis ($P < 0.001$). D: The epithelial subscore of the CDHIS (score for epithelial damage, architectural distortion, and erosion/ulcers) was related to the pSTAT3 value by Spearman's linear regression analysis ($P = 0.0007$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 4.

Colonic genomic signature for genes differentially expressed in active Crohn's disease. RNA was prepared from colon biopsies obtained from healthy controls ($n = 8$), Crohn's disease at diagnosis (CD, $n = 11$), active treated CD (CD TR, $n = 9$), and ulcerative colitis at diagnosis (UC, $n = 5$) and subjected to microarray analysis using the Human Genome HG-U133 Plus 2.0 array from Affymetrix. Gene expression was analyzed by GeneSpring software to identify genes differentially expressed in both CD and CD TR compared to normal colon. Hierarchical cluster analysis was used to sort expression profiles for the 4 groups as shown. Each column displays the genomic signature for 1 subject. Genes upregulated are displayed in red, whereas downregulated genes are displayed in blue, as indicated on the accompanying expression bar. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 5. Proinflammatory IL-6 and STAT3-dependent biologic networks are upregulated in active Crohn's disease. The Ingenuity software application was used to identify biologically relevant networks present within the Crohn's disease (CD) genomic signature illustrated in Figure 4. A: IL-6 and (B) STAT3-dependent networks are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 6.

CXCL10 and CXCL11 are upregulated in colonic lamina propria mononuclear cells and epithelium in active disease. Immunohistochemistry for CXCL10 and CXCL11 was performed as shown in colon biopsies from patients with newly diagnosed Crohn's disease (CD) and ulcerative colitis (UC) and active treated CD (CD TR). The isotype controls are shown in the insets. Images representative of 3 subjects per group are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1

Clinical and Demographic Data

Disease	n	Male(%)	Age at Dx (Years; Range)	PCDAI/PUCAI (Range)	Early Relapse %
Control	20	57	10.3 (5.7–18.1)	—	—
CD	17	53	12.4 (5.4–17.3)	42.5 (15–65)	67
CD TR	26	74	12.0 (6.4–15.6)	37.5 (15–55)	N/A
UC	11	45	12.3 (6.6–18.3)	40.0 (10–75)	50

PCDAI, Pediatric Crohn's Disease Activity Index, PUCAI, Pediatric Ulcerative Colitis Clinical Activity Index.
Early relapse defines patients that clinically relapse within 1 year of diagnosis.

TABLE 2
Demographic and Clinical Data for Pediatric IBD at Diagnosis

Patient No.	Diagnosis	Age Dx. (yr)	Gender	Race	PCDAI/ PUCAI	Montreal Class*
1	CD	11.5	M	W	55	A1 L3 B1
2	CD	9.2	M	W	52.5	A1 L3 B1
3	CD	14.9	M	W	30	A1 L3 B1
4	CD	15.1	M	W	50	A1 L2 B1 P
5	CD	14.3	M	W	65	A1 L2 B1 P
6	CD	13.4	M	W	17.5	A1 L3 B1 P
7	CD	14.2	F	W	32.5	A1 L3 B1 P
8	CD	9.2	F	W	37.5	A1 L2 B1
9	CD	9.3	F	W	42.5	A1 L2 B1 P
10	CD	7.8	F	W	45	A1 L1 B1
11	CD	14.3	F	W	15	A1 L1 B1
12	CD	13.9	M	W	37.5	A1 L3 B3
13	CD	11.4	F	W	42.5	A1 L1 B1 P
14	CD	12.4	M	W	62.5	A1 L3 B1 P
15	CD	9.9	F	W	20	A1 L2 B1 P
16	CD	5.4	F	AA	No data	No data
17	CD	17.3	M	W	47.5	A2 L3 B1
18	UC	7.9	M	W	10	E1
19	UC	6.6	F	W	45	E3
20	UC	16.4	M	W	25	E3
21	UC	14.8	F	W	40	E3
22	UC	8.0	F	AA	50	E3
23	UC	15.0	M	W	50	E3
24	UC	11.9	F	W	75	E3
25	UC	18.3	M	W	15	E3
26	UC	14.3	M	AA	70	E2
27	UC	10.7	F	W	10	E3
28	UC	12.7	F	W	30	E3

W, White race; AA, African American race; PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Clinical Activity Index.

* A1 age ≤16 years old, A2 between 17 and 40 years old; L1 ileal location, L2 colonic, L3 ileocolonic, B1 nonstricturing, B2 stricturing, B3 penetrating, P perianal disease modifier. E1 ulcerative proctitis, E2 left-sided UC, E3 extensive UC (extends proximal to splenic flexure).

TABLE 3
Demographic and Clinical Data of Pediatric Treatment Refractory CD

Patient No.	Age Dx. (yr)	Gender	Duration CD (yr)	PCDAI	Montreal Class* (All Patients AI)	Medications
1	14.7	M	0.4	42.5	L3 B1	Pred, 6-MP
2	10.4	M	3.6	30	L3 B2	Pred, 6-MP
3	8.8	M	3.5	37.5	L2 B1	5-ASA, 6-MP
4	9.7	F	0.1	35	L3 B1	Pred, 6-MP
5	8.6	F	3.0	30	L3 B1 P	Pred, Mtx
6	7.2	M	0.5	55	L3 B1 P	Pred, 5-ASA, 6-MP
7	13.7	F	0.1	42.5	L3 B1	Pred, 6-MP
8	15.4	M	2.9	37.5	L2 B1	Budesonide, 5-ASA, 6-MP
9	7.6	M	2.8	30	L2 B1	Budesonide, 5-ASA, 6-MP
10	10.3	M	3.4	42.5	L2 B1	Budesonide, 5-ASA, 6-MP
11	12.6	M	0.1	30	L3 B1	Pred, 5-ASA
12	5.6	M	3.7	42.5	L2 B1	Pred, 5-ASA
13	15.3	M	1.3	35	L3 B1	Pred, 5-ASA, 6-MP
14	15.6	M	0.1	42.5	L2 B1	Pred, 6-MP
15	15.6	M	0.1	30	L3 B1	Pred, 5-ASA
16	11.4	F	0.7	37.5	L3 B2	Pred, 6-MP
17	15.3	M	0.1	45	L3 B1	Pred, 5-ASA
18	11.1	M	1.6	35	L1 B1	Mtx, Infliximab
19	7.1	M	4.3	22.5	L2 B1	5-ASA, 6-MP, Infliximab
20	13.4	M	2.7	30	L1 B1 P	6-MP, Infliximab
21	14.8	F	3.4	25	L2 B1	5-ASA, 6-MP, Infliximab
22	12.7	M	1.8	25	L3 B1 P	Budesonide, 6-MP
23	10.4	M	4.4	55	L3 B2	Pred, Mtx
24	15.6	F	2.7	40	L1 B2	Budesonide, 6-MP, Infliximab
25	6.4	F	7.7	52.5	L3 B1 P	Mtx, Infliximab

PCDAI, Pediatric Crohn's Disease Activity Index.

Medications: Pred, prednisone; 6-MP, 6-mercaptopurine; 5-ASA, 5-aminosalicylate; Mtx, methotrexate.

* AI age ≤16 years old; L1 ileal location, L2 colonic, L3 ileocolonic; B1 nonstricturing, nonpenetrating, B2 stricturing, nonpenetrating, B3 penetrating, P perianal disease modifier.

TABLE 4
IL-6/STAT3 Dependent Transcriptome Upregulated in Pediatric Crohn's Disease

Gene Symbol	Gene Name	Cytoband	Fold Change		
			CD	CD TR	UC
Immune and Inflammatory Mediators					
C4A	Complement Component 4A	6p21.3	3.6	4.7	7.9
C4B	Complement Component 4B	6p21.3	3.6	4.7	7.9
C4BPA	Complement Component 4-Binding Protein Alpha	1q32	2.1	4.2	13.6
C4BPB	Complement Component 4-Binding Protein Beta	1q32	1.8	3.8	12.1
CD38	CD38 Antigen	4p15	2.6	2.7	4.8
CD74	CD74 Antigen	5q32	2.3	3.2	4.3
FCER1G	Fc Fragment IgE Receptor 1 Gamma	1q23	4.1	6.1	7.2
GALNAC4S-6ST	B-cell Rag Associated Gene	10q26	2.4	3.8	5.3
HCK	Hemopoietic Cell Kinase	20q11-q12	2.7	3.5	3.5
HLA-DQB1	Major Histocompatibility Complex	6p21.3	1.9	2.9	3.8
HLA-DRA	Major Histocompatibility Complex	6p21.3	2.5	3.7	4.8
HLA-DRB1	Major Histocompatibility Complex	6p21.3	2.0	2.9	3.4
HLA-DRB3	Major Histocompatibility Complex	6p21.3	2.0	2.9	3.4
INDO	Indoleamine 2,3 Dioxygenase	8p12-p11	6.2	9.4	6.5
LCP2	Lymphocyte Cytosolic Protein-2	5q33.1-qter	2.9	4.5	5.5
MS4A2	Membrane-Spanning 4 Domain Fc IgE Fragment	11q13	2.3	2.7	3.1
NCF2	Neutrophil Cytosolic Factor 2	1q25	3.8	4.7	5.8
SEMA4D	Semaphorin 4D	—	1.8	1.9	3.0
Chemokines, Cytokines and Growth Factors					
CCL11	Chemokine Ligand 2 (CC Motif)	17q21.1-q21.2	4.2	11.0	7.9
CSF1R	Colony Stimulating Factor 1 Receptor	5q33.2-q33.3	2.3	3.6	4.2
CSF2RA	Colony Stimulating Factor 2 Receptor Alpha	Xp22.32	1.7	1.7	2.1
CXC3R1	Chemokine Receptor 3	Xq13	3.1	4.4	6.2
CXCL1	Chemokine Ligand 1	4q12-q13	8.9	13.3	22.1
CXCL2	Chemokine Ligand 2	4q12-q13	3.0	4.0	5.2
CXCL3	Chemokine Ligand 3	4q12-q13	3.6	4.1	7.1
CXCL5	Chemokine Ligand 5	4q12-q13	15.0	6.6	26.3
CXCL6	Chemokine Ligand 6	4q12-q13	10.3	13.5	31.6
CXCL9	Chemokine Ligand 9	4q21	7.0	10.1	7.4
CXCL10	Chemokine Ligand 10	4q21	3.6	5.8	3.8
CXCL11	Chemokine Ligand 11	4q21.2	7.4	12.7	8.3
CXCL12	Chemokine Ligand 12	10q11.1	1.7	3.3	2.5
IL-6	Interleukin 6	7p21	7.3	3.5	6.2
IL-8	Interleukin 8	4q12-q13	5.7	4.3	7.6
IL-8RB	Interleukin 8 Receptor Beta	2q35	5.4	2.6	6.3
IL-12RB1	Interleukin 12 Receptor Beta 1	19p13.1	1.7	2.0	2.7
IL-2RB	Interleukin 2 Receptor Beta	22q11.1-q13	2.2	2.5	3.9
OSMR	Oncostatin M Receptor	—	1.9	2.7	5.4
Cancer and Cell Proliferation/STAT1 and 3					
FGR	Gardner-Rasheed Feline Sarcoma Viral Oncogene	1p36.1-p36.2	3.1	3.6	4.3
JAK2	Janus Kinase 2	9p24	1.8	2.3	2.9
V-kit Hardy-Zuckerman Feline Sarcoma Viral					
KIT	Oncogene	4q12	2.7	5.2	6.0
PIM2	Oncogene PIM2	X Chrom.	2.6	3.1	6.7
PRAP1	Proline Rich Acidic Protein 1	10q26	0.31	0.32	0.15
PTPRG	Protein-Tyrosine Phosphatase Receptor Gamma	3p14.2	1.6	2.1	3.1
VAV1	Vav1Oncogene	19p13.3-p13.2	1.7	1.9	2.3
STAT1	Signal Transducer and Activator of Transcription 1	2q32.2-q32.3	2.0	1.8	2.2
STAT3	Signal Transducer and Activator of Transcription 3	17q21	1.2	1.3	1.5
ECM/Tissue Remodeling/Cell Adhesion					
ANPEP	Alanyl aminopeptidase	15q25-q26	0.35	0.19	0.09

Gene Symbol	Gene Name	Cytoband	Fold Change		
			CD	CD TR	UC
CTSK	Cathespin K	1q21	2.3	3.8	6.0
HAS2	Hyaluronon Synthase 2	8q24.12	2.2	5.5	6.0
ICAM1	Intercellular Adhesion Molecule 1	19p13.3-p13.2	2.2	2.4	2.9
ITGB2	Integrin B2	21q22.3	2.6	3.6	4.5
MMP10	Matrix Metalloproteinase 10	11q22.3-q23	8.3	7.9	95.0
PECAM1	Platelet-Endothelial Cell Adhesion Molecule 1	17q23	1.9	2.6	3.8
Metabolic Pathways and Transcription Factors					
ACOT8	Acyl-CoA Thioesterase 8	20q13.1	0.63	0.45	0.36
CYP2C9	Cytochrome P450 Subfamily IIC	10q24	0.32	0.26	0.13
CYP2C18	Cytochrome P450 Subfamily IIC	10q24	0.51	0.48	0.44
MITF	Microphthalmia-Associated Transcription Factor	3p14.1-p12.3	1.8	2.5	3.0
NMU	Neuromedin U	Chrom. 4	1.6	2.2	2.8
NOX1	NADPH Oxidase	Xq22	1.7	1.9	1.7
RGS18	Regulator of G Protein Signaling 18	1q31.2	2.8	4.8	5.5
SERPINA1	Proteinase Inhibitor I	14q32.1	1.9	3.1	3.5
STAP2	Signal Transducing Adapter Protein 2	Chrom. 19	0.55	0.41	0.27
WARS	Tryptophanyl-tRNA Synthetase	14q32.31	2.8	2.8	3.6
XBPI	X-Box Binding Protein 1	22q12	1.9	2.4	3.9