IL-10R Polymorphisms Are Associated with Very-early-onset Ulcerative Colitis

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Background: Interleukin-10 (IL-10) signaling genes are attractive inflammatory bowel disease (IBD) candidate genes as IL-10 restricts intestinal inflammation, IL-10 polymorphisms have been associated with IBD in genome-wide association studies, and mutations in IL-10 and IL-10 receptor (IL-10R) genes have been reported in immunodeficient children with severe infantile-onset IBD. Our objective was to determine if IL-10R polymorphisms were associated with early-onset IBD (EO-IBD) and very-early-onset IBD (VEO-IBD).

Methods: Candidate-gene analysis of *IL10RA* and *IL10RB* was performed after initial sequencing of an infantile onset-IBD patient identified a novel homozygous mutation. The discovery cohort included 188 EO-IBD subjects and 188 healthy subjects. Polymorphisms associated with IBD in the discovery cohort were genotyped in an independent validation cohort of 422 EO-IBD subjects and 480 healthy subjects.

Results: We identified a homozygous, splice-site point mutation in *IL10RA* in an infantile-onset IBD patient causing a premature stop codon (P206X) and IL-10 insensitivity. *IL10RA* and *IL10RB* sequencing in the discovery cohort identified five *IL10RA* polymorphisms associated with ulcerative colitis (UC) and two *IL10RB* polymorphisms associated with Crohn's disease (CD). Of these polymorphisms, two *IL10RA* single nucleotide polymorphisms, rs2228054 and rs2228055, were associated with VEO-UC in the discovery cohort and replicated in an independent validation cohort (odds ratio [OR] 3.08, combined $P = 2 \times 10^{-4}$; and OR 2.93, $P = 6 \times 10^{-4}$, respectively).

Conclusions: We identified *IL10RA* polymorphisms that confer risk for developing VEO-UC. Additionally, we identified the first splice site mutation in *IL10RA* resulting in infantile-onset IBD. This study expands the phenotype of *IL10RA* polymorphisms to include both severe arthritis and VEO-UC.

(Inflamm Bowel Dis 2012;000:000–000)

Key Words: IL-10, IL-10 receptor, inflammatory bowel disease, immunodeficiency

Additional Supporting Information may be found in the online version of this article.

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Received for publication February 25, 2012; Accepted March 14, 2012.

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C.J.M. is supported by an award from the Crohn's and Colitis Foundation of America (CCFA). A.M.M. is supported by a transition award from the Crohn's and Colitis Foundation of Canada (CCFC) / Canadian Association of Gastroenterology (CAG) / Canadian Institute for Health Research (CIHR), a Canadian Child Health Clinician Scientist Program (Strategic Training Initiatives in Health Research Program – CIHR) award, and an Early Researcher Award from the Ontario Ministry of Research and Innovation and a CDHNF/NASPGHAN George Ferry Young Investigator Development Award and funded by a Canadian Institute of Health Research – Operating Grant (MOP119457). M.S.S. is supported in part by the Gale and Graham Wright Research Chair in Digestive Diseases. Partial funding to recruit subjects and collect samples was provided by M.S.S. through NIDDK grant DK. This work was funded in part by funding to Dr. Snapper through the Wolpow Chair in IBD Research and Treatment as well as through funding made possible from the Harvard Digestive Disease Center P30 DK034854.

*NEOPICS: interNational Early Onset Pediatric IBD Cohort Study www.NEOPICS.org; see Supporting Material for details.

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DOI 10.1002/ibd.22974

Published online in Wiley Online Library (wileyonlinelibrary.com).

Interleukin-10 (IL-10) is an antiinflammatory cytokine secreted by a variety of cell types and is critical for maintaining immune homeostasis in the gastrointestinal (GI) tract.[1] These immunosuppressive effects include restricting T-cell proliferation, downregulating costimulatory protein expression on antigen-presenting cells, and limiting proinflammatory cytokine production. IL-10 activates downstream signaling by binding to the IL-10 receptor (IL-10R), comprised of two α subunits (encoded by *IL10RA*) and two β subunits (encoded by *IL10RB*). This activates JAK1 and TYK2, leading to phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) and gene transcription. Intact IL-10 signaling is required for restricting inappropriate Th17 cell expansion—an effector cell type that has been associated with Crohn's disease (CD).[2,3] Consistent with the antiinflammatory role of IL-10, mice deficient in either *IL-10* or *IL-10R* develop enterocolitis.[3–5]

Recent studies have shown that loss-of-function mutations in *IL10RA* and *IL10RB*[6,7] and *IL10*[8] in immunodeficient patients are associated with severe, infantile-onset inflammatory bowel disease (IBD). This has stimulated renewed interest in studying IL-10 pathway genes in the pathogenesis of IBD. Genome-wide association studies (GWAS) in IBD populations (including exclusively pediatric-onset disease) have associated single nucleotide polymorphisms (SNPs) in *IL10* and *STAT3* loci with ulcerative colitis (UC) and CD.[9–12]

Given the severe phenotype of infantile-onset IBD observed with IL-10R mutations as well as that of an infant described in this article who was found to have a homozygous, splice site mutation in *IL-10RA*,[6,7] we hypothesized that *IL10RA* and *IL10RB* polymorphisms contribute to the risk of IBD developing in the very young. To date, SNPs within *IL10RA* and *IL10RB* loci have not been identified by GWAS of IBD. However, the contribution of IL-10R polymorphisms to IBD susceptibility in very young patients may be underestimated in GWAS, as this age group represents a very select population that is poorly represented even in GWAS focusing on pediatric IBD.[13] Rather than studying only the overall population of patients that constitute "pediatric IBD," we subdivided patients so that we could concentrate our analyses on extremely young patients (that may be most likely to have the strongest genetic component to IBD risk). Although the Paris Classification for IBD stratifies pediatric IBD into those diagnosed younger than 10 years of age and those diagnosed between 10 and 18 years of age,[14] we focused on patients diagnosed prior to their 6th birthday—a cohort that comprises ≈15% of pediatric IBD and represents a greater extreme of the disease—since the average age at diagnosis of "pediatric IBD patients" is ≈10 years old.[15] Therefore, we undertook a candidate gene study by deep sequencing of *IL10RA* and *IL10RB* in children with early-onset IBD (EO-IBD, defined as age of onset <18 years old) and subsequently focused on very-early-onset IBD (VEO-IBD, defined as age of onset <6 years old).

MATERIALS AND METHODS

Identification of Novel IL-10RA Mutation in Patients with Infantile Colitis

We identified an IL-10RA mutation in a Caucasian female presenting with infantile colitis who was not recognized a priori to be the product of a consanguineous marriage, but whose parents came from the same geographically isolated region of Canada. Her first year of life was complicated by recurrent fevers (initially at 6 days of life). At 3 months old, the patient developed cellulitis and scalp eczema and generalized erythroderma (Fig. 1A). Bloody diarrhea and a perianal skin tag developed at 5 months of age (Fig. 1B) that progressed to perianal and rectovaginal fistulae. Further complications included urinary tract infections, scalp pustules, and severe, effusive, large joint polyarthritis (Fig. 1C). Joint aspirations demonstrated high neutrophil counts (>100,000/mm^3) despite sterile cultures. The patient did not manifest thyroid involvement. Endoscopic evaluation revealed patchy colonic ulcerations (Fig. 1D) and biopsies showed chronic colitis (Fig. 1E) with the absence of small bowel inflammation. GI symptoms were refractory to antibiotics, intravenous immunoglobulins, corticosteroids, anakinra, sulfasalazine, and azathioprine, and the patient underwent a loop ileostomy at 23 months old.

An extensive immunological evaluation included normal immunoglobulin levels and lymphocyte subsets, normal NADPH oxidase function, a normal thymic biopsy, normal karyotyping, and negative fluorescence in situ hybridization (FISH) for 22q11. Screening for *JAK3, IPEX, RMRP, IL2RA, IRAK4*, and *TRAPS* mutations and mutations associated with chronic granulomatous disease were negative.

Genomic DNA was purified from whole blood using the Puregene Blood Kit (Qiagen, Chatsworth, CA). *IL10RA* and *IL10RB* were amplified using intronic primers flanking each exon and sequenced with the ABI3730 DNA analyzer (Applied Biosystems, Melbourne, Australia). *IL10RA* and *IL10RB* variants are numbered according to GenBank accession numbers NM_001588 and NM_00628, respectively. Amino acid numbering refers to position in the immature protein (including signal peptide).

RNA was isolated from whole blood with the PAXgene Blood RNA kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA). Primers for full-length *IL10RA,* full-length *IL10RB,* and for exon 4 to exon 7 of *IL10RA* were designed and synthesized at the Centre for Applied Genomics (Toronto, ON, Canada). Polymerase chain reaction (PCR) was performed according to standard protocol, and the purified PCR product was cloned

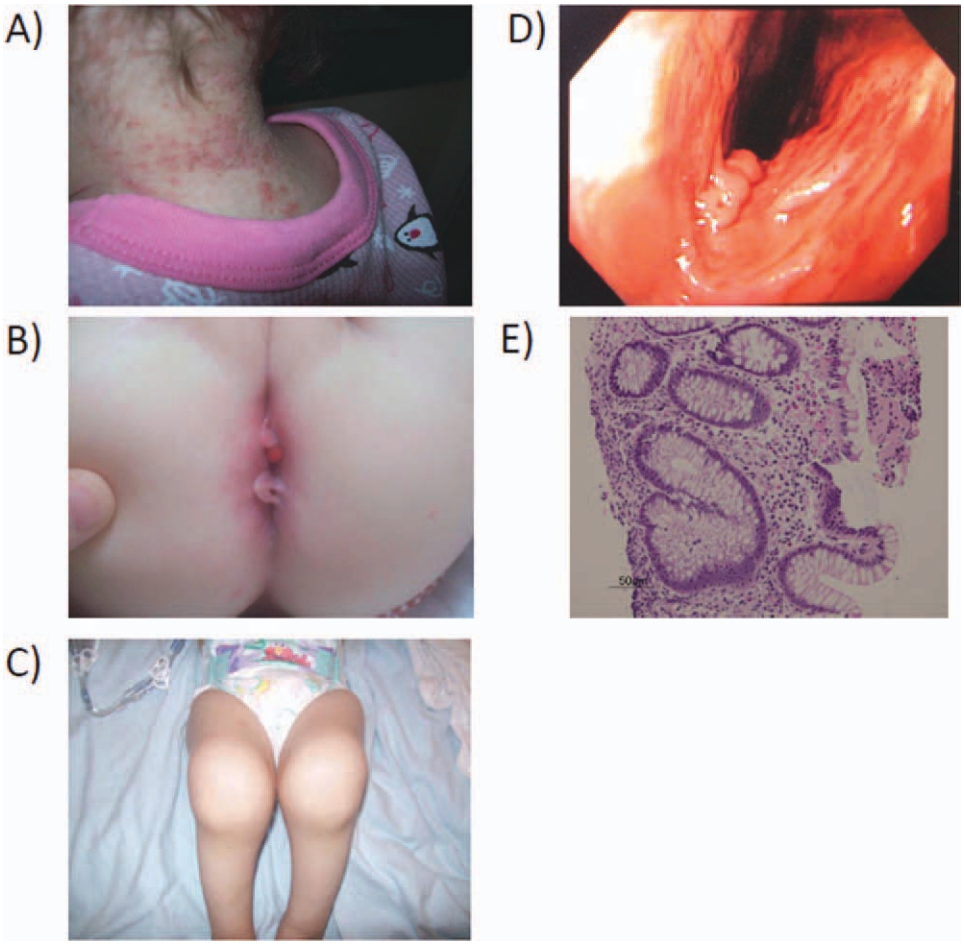

FIGURE 1. Clinical features of patient with severe, infantile-onset IBD. The patient had skin folliculitis (A), perianal disease with fistulae (B), joint effusions (C), and colonic erosions (D). Histology showed colitis with gland branching and mixed lamina propria cell infiltrate with fibropurulent exudate (arrow) (E).

into pJET cloning vector (Fermentas, Burlington, ON, Canada) and sequenced by ABI 3730 DNA analyzer (Applied Biosystems).

Functional IL-10 Testing in IL-10R-Deficient Patient

Peripheral blood mononuclear cells (PBMCs) from the patient and a healthy control were isolated using Polymorphoprep (Nycomed Pharma, Norway). The PBMCs were stimulated with IL-10 (40 ng/mL) or IL-6 (20 ng/mL) for 10 and 30 minutes. Cellular extracts were prepared following standard protocol. The protein concentration was measured by protein assay kit (Bio-Rad, Hercules, CA) and 50 μg protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. The membrane was incubated with antibodies to STAT3 and phosphorylated (Tyr705) STAT3 (p-STAT3) (Cell Signaling, Beverly, MA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The membrane was read using a chemiluminescence system (GE Healthcare, UK). β-Actin was used as a loading control.

Flow Cytometry

Thawed PBMC were stained in 2% fetal bovine serum (FBS) with CD4-PerCP (eBioscience, San Diego, CA) and CD25-PE (Miltenyi Biotech, Auburn, CA) followed by intracellular staining with FoxP3-FITC (eBioscience). Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA). Coexpression of CD25 and intracellular FoxP3 was assessed in cells within $CD4^+$ lymphocyte gate.

IL-10R Sequencing in the Discovery Cohort

The discovery cohort included DNA samples from 188 EO-IBD patients from the Hospital for Sick Children (Toronto, Canada) (49 patients) including some subjects as part of the NEOPICS consortium (Supporting Table 1) and Children's Hospital of Wisconsin (Milwaukee, WI) (139 patients). Control DNA samples were obtained from the Centre for Applied Genomics (Ontario Population Genomics Platform, plates used: 1–4); 95% of this cohort are Caucasians of European ancestry. A complete description can be found at http://www.tcag.ca/facilities/cyto_population_control_DNA.html.

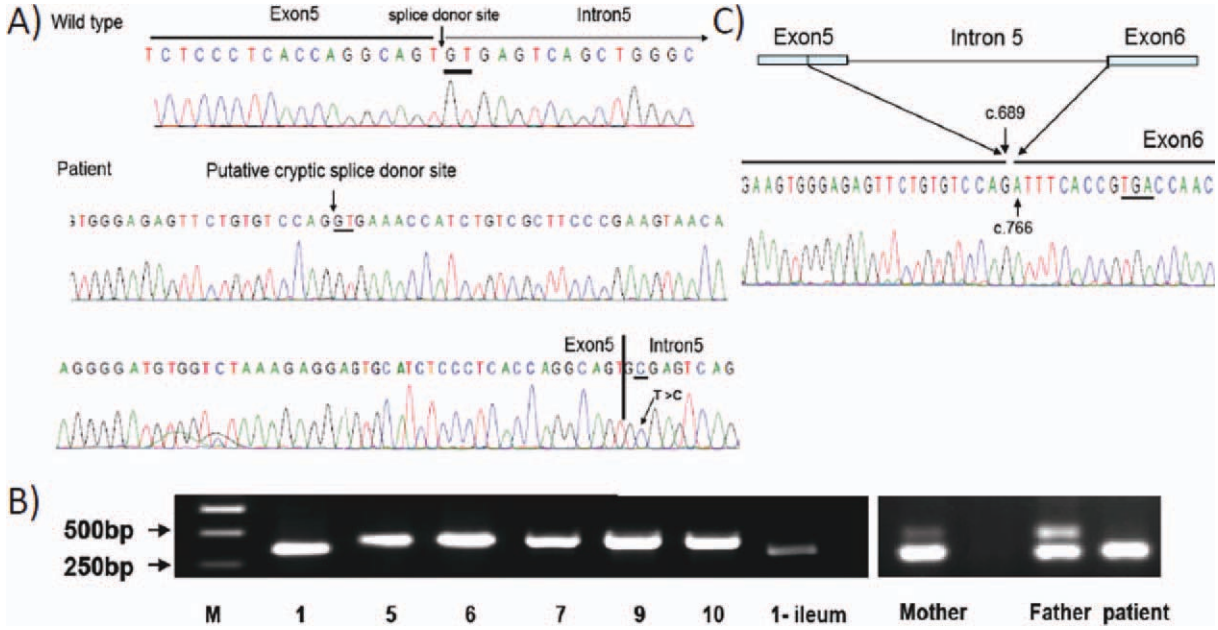

FIGURE 2. Identification of *IL10RA* g.IVS5+2T>C mutation in genomic DNA. (A) Sequencing of exon 5 of *IL10RA* revealed a homozygous g.IVS5+2T>C mutation (bottom), compared to wildtype (top). The splice donor site, putative cryptic splice donor site, and T>C mutation are underlined. (B) RT-PCR of *IL10RA* (exon 4–7) from RNA isolated from blood and ileum. Lane 1 and Lane 1-ileum is from the patient. Lanes 2–6 are controls. Right: RT-PCR of exon 4–7 of *IL10RA* from RNA from the patient and her parents. (C) Sequence of abnormal PCR product shows 76 bp deletion (c.690_765del) leading to a premature stop codon (underlined).

The coding sequences (and intervening sequences and 3′-untranslated regions) of *IL10RA* and *IL10RB* were amplified. PCR products were sequenced by the Sanger method (Beckman-Coulter Genomics, Danvers, MA). SNPs were detected using Polyphred software and all chromatograms with reliability score <99 were manually reviewed.

Allelic frequencies were determined for SNPs identified in the discovery cohort. Testing for Hardy–Weinberg equilibrium was done by χ^2 analysis. Odds ratios (ORs) were calculated by comparing the ratio of individuals with two copies of the major allele to those with one or two copies of a variant allele (DD vs. Dd/dd model) between EO-IBD and control groups. ORs were determined for UC and CD and specific age groups: children diagnosed before their 18th birthday (<18 years old) and children diagnosed before their 6th birthday (<6 years old). *P*-values were calculated (but not adjusted for multiple testing in discovery cohort). SNPs with $P < 0.05$ were considered associated with IBD risk in the discovery cohort. Linkage between SNPs was assessed by χ^2 analysis using a 3×3 table of genotypes. Linkage disequilibrium (LD) plots were created with Golden Helix SVS 7.0 (Bozeman, MT) using expectation-maximization (EM) algorithm to calculate r^2 and D′ values.

Genotyping of IL10RA and IL10RB of Replication Cohort

DNA samples were collected from 422 pediatric IBD patients at the Hospital for Sick Children and genotyped using Taqman probes for SNPs associated with IBD from the discovery cohort. Control DNA samples (distinct from discovery cohort controls) were obtained from the Centre for Applied Genomics. Allelic frequencies were calculated and Hardy–Weinberg equilibrium was assessed by χ^2 analysis. ORs were calculated (DD vs. Dd/dd model) and analyzed by χ^2 analysis. *P*-values were adjusted for multiple testing by Bonferroni method (based on the number of SNPs associated with IBD in discovery cohort), and an adjusted $P < 0.05$ was considered statistically significant. As quality control, allele frequencies in the discovery and validation cohorts were compared by χ^2 analysis. LD analysis and haplotype testing was performed as above.

All probands had a confirmed diagnosis of IBD fulfilling standard diagnostic criteria. Phenotypic characterization was based on the Montreal Classification.[16] Age categories reflect age at diagnosis: EO-IBD, defined as diagnosis before the 18th birthday; VEO-IBD, defined diagnosis before the 6th birthday; and infantile-onset IBD, defined as diagnosis before the 1st birthday.[14] Institutional Review Boards at the Hospital of Sick Children, Children's Hospital of Wisconsin, Mount Sinai Hospital (Toronto, Canada), and Massachusetts General Hospital approved these studies.

RESULTS

Novel, Aberrant IL10RA Splice Site Mutation Identified in Patients with Infantile Colitis

We identified a patient with severe, infantile-onset IBD with significant arthritis and folliculitis (Fig. 1). Sequencing of *IL10RA* and *IL10RB* in this patient identified

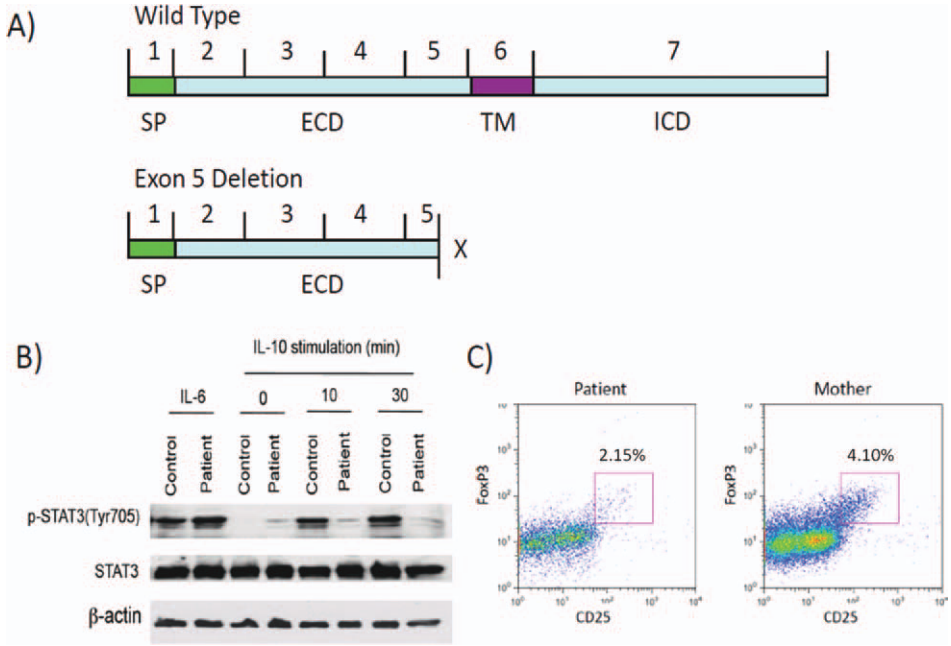

FIGURE 3. Altered IL-10 signaling in a patient with *IL-10RA* mutation. (A) Truncated *IL10RA* protein product found in the patient compared to full-length *IL10RA* in parents/controls. Extracellular (ECD), intracellular (ICD), signaling peptide (SP), and transmembrane (TM) domains are shown. (B) Western blot analysis of IL-10-induced STAT3 phosphorylation in PBMCs from patient and control. (C) Flow cytometric analysis of peripheral blood FoxP3$^+$ Tregs of patient and mother. Cells depicted are CD4+ in small lymphocyte gate and are plotted CD25 versus intracellular FoxP3.

a homozygous mutation in the intervening sequence of intron 5 at the second base of the conserved GT splice donor site (g.IVS5+2T>C) (Fig. 2A). This novel mutation was confirmed by sequencing in an independent laboratory (data not shown) and has not been identified in the 1000 Genomes Project.

This mutation was located in the 5′ splice donor site, a critical position for RNA splicing. Mutation of the invariant dinucleotide GT at the 5′ splice donor site impairs normal splicing and allows activation of cryptic splice site(s).[17] We identified a cryptic splice donor site (CAGgtgaaa) in exon 5 of *IL10RA* using Human Splicing Finder (Fig. 2A).[18] To confirm aberrant *IL10RA* splicing, RNA was isolated from whole blood and *IL10RA* transcripts were analyzed by reverse transcription (RT)-PCR. The expected 433-bp PCR fragment was detected in five healthy donors. However, only a smaller fragment was observed in the patient (Fig. 2B) and sequencing of this fragment revealed a 76-bp deletion (c.690_765del) from the 3′ end of exon 5 (Fig. 2C). The resulting reading frame-shift mutation led to incorporation of three aberrant amino acids of exon 6 (p.204ISP206) and a premature stop codon at P206X (Fig. 2C). The truncated protein contained only the extracellular domain of IL10RA (Fig. 3A). The patient's asymptomatic parents were g.IVS5+2T>C carriers displaying wildtype and altered splicing variants (Fig. 2B).

IL10RA Splice Mutation Prevents IL-10-dependent STAT3 Phosphorylation

In order to determine the functional effects of P206X IL10R1, we assessed in vitro STAT3 phosphorylation, a required step in the IL-10 signaling cascade for downstream immunosuppressive effects.[19] PBMCs were stimulated with IL-10 and STAT3 phosphorylation was measured by western blot analysis. IL-10-induced STAT3 phosphorylation was defective in the patient. IL-6-induced STAT3 phosphorylation was intact (Fig. 3B). Thus, P206X IL-10R1 leads to defective IL-10 downstream signaling.

FoxP3+ Regulatory T Cells Are Present in the Absence of IL-10 Signaling

Regulatory T cells (Tregs) that express the transcription factor FoxP3 possess suppressive ability, and defects in the *FOXP3* gene (in mice and humans) cause systemic autoimmunity.[20,21] As some but not all studies have suggested that IL-10 signaling is critical for the stability of FoxP3 expression,[3,22] we sought to determine whether FoxP3$^+$ Tregs were present in the absence of IL-10 signaling. FoxP3$^+$ Tregs were identified in the peripheral blood of the affected patient (Fig. 3C). This provides evidence in humans that FoxP3$^+$ Tregs can develop in the absence of intact IL-10 signaling.

TABLE 1. IL-10R Polymorphisms Associated with EO-IBD

		Discovery Cohort		Validation Cohort		Combined Cohorts	
IL10RA SNP		OR	*P*-value	OR	*P*-value	OR	*P*-value
rs10892202	EO-IBD	0.6	0.067	0.88	0.405	0.79	0.057
	EO-UC	0.3	0.011	0.82	0.437	0.62	0.034
	EO-CD	0.79	0.469	0.9	0.562	0.87	0.358
rs2228054	EO-IBD	1.87	0.056	0.93	0.711	1.15	0.36
	EO-UC	3.06	0.003	1.25	0.452	1.71	0.019
	EO-CD	1.24	0.691	0.83	0.432	0.92	0.686
rs2228055	EO-IBD	1.6	0.158	1.03	0.885	1.17	0.295
	EO-UC	2.52	0.016	1.36	0.302	1.71	0.021
	EO-CD	1.1	0.848	0.9	0.668	0.95	0.82
rs4252249	EO-IBD	0.68	0.143	0.86	0.36	0.82	0.104
	EO-UC	0.3	0.012	0.78	0.329	0.6	0.024
	EO-CD	0.94	0.774	0.92	0.64	0.92	0.605
rs4252270	EO-IBD	0.63	0.107	0.88	0.409	0.79	0.06
	EO-UC	0.3	0.012	0.77	0.306	0.59	0.021
	EO-CD	0.84	0.665	0.91	0.592	0.89	0.438
rs1058867	EO-IBD	1.78	0.011	0.91	0.492	1.18	0.109
	EO-UC	1.35	0.373	0.96	0.863	1.06	0.74
	EO-CD	2.12	0.004	0.89	0.46	1.12	0.369
rs8178561	EO-IBD	0.34	0.004	0.92	0.68	0.75	0.06
	EO-UC	0.85	0.834	1.07	0.814	1	1
	EO-CD	0.1	<0.001	0.9	0.646	0.64	0.030

Odds ratios using DD vs. Dd/dd model. Discovery cohort: EO-IBD (n=188), UC (n=65), CD (n=121), and controls (n=188). Validation cohort: EO-IBD (n=422), UC (n=122), CD (n=300), and controls (n=480). Threshold for significance in validation cohort was 0.007 to correct for multiple testing.

IL10RA and IL10RB Polymorphisms Are Associated with IBD

In order to determine whether *IL10RA* and *IL10RB* mutations are associated more generally with IBD and extend beyond the rare patients with infantile-onset IBD that have homozygous, loss-of-function mutations, we performed deep sequencing of coding and flanking intronic regions of *IL10RA* and *IL10RB* in a cohort of 188 EO-IBD patients and 188 controls (Supporting Table 2). The EO-IBD group included 121 children with CD, two with IBD-unclassified, and 65 with UC. The ages of children ranged from 6.5 months to 11 years old, with 68 VEO-IBD subjects (including two infants).

In this discovery cohort, 59 SNPs (28 novel) were found in *IL10RA* and 39 SNPs (15 novel) in *IL10RB* (Supporting Table 3). There were 14 (six novel) nonsynonymous SNPs (12 in *IL10RA* and two in *IL10RB*). Each novel, nonsynonymous SNP was identified only in the heterozygous state, and none of the resulting amino acid changes were predicted to be deleterious by SIFT[23] or Polyphen.[24] Additionally, we found five SNPs in *IL10RA* were associated with EO-UC and two SNPs in *IL-10RB* were associated with EO-CD (Table 1; Supporting Table 3). Three *IL10RA* SNPs (rs10892202, rs4252249, and rs4252270) were protective for EO-UC, while the remaining two *IL10RA* SNPs (rs2228054 and rs2228055) were associated with increased risk for EO-UC and VEO-IBD. In subgroup analysis of these five *IL10RA* SNPs, four of the SNPs were found to be associated with VEO-UC, while there was a positive trend for VEO-UC in rs2228055 (Table 2). Of the *IL10RB* SNPs that were associated with EO-CD, rs1058867 was associated with increased CD risk and rs8178561 was protective for EO-CD. The minor allelic frequencies of the SNPs described above did not differ between the IBD cohorts from Wisconsin and Toronto (data not shown).

Linkage analysis of these SNPs showed that rs2228054 and rs2228055 were in LD ($P < 0.0001$) (Haplotype Block 1 AG) as were rs10892202, rs4252249, and rs4252270 (Haplotype Block 2 CAT) ($P < 0.0001$) (LD plot in Supporting Fig. 1). Haplotype Block 1 AG was associated with VEO-IBD (OR 2.35, $P = 0.020$), UC (OR 2.41, $P = 0.016$) and VEO-UC (OR 2.48, $P = 0.025$) but not for EO-IBD or EO-CD (Supporting Table 4).

TABLE 2. IL-10R Polymorphisms Associated with Very Early Onset IBD

		Discovery Cohort		Validation Cohort		Combined Cohorts	
IL10RA SNP		OR	*P*-value	OR	*P*-value	OR	*P*-value
rs10892202	VEO-IBD	0.41	0.044	1.17	0.701	0.6	0.086
	VEO-UC	0.28	0.03	0.71	0.585	0.4	0.032
	VEO-CD	0.62	0.23	2.35	0.179	0.98	0.951
rs2228054	VEO-IBD	2.69	0.016	2.58	0.024	2.49	$3x10^{-3}$
	VEO-UC	3.29	0.013	3.86	0.005	3.08	$2x10^{-4}$
	VEO-CD	1.85	0.14	1.93	0.404	1.66	0.273
rs2228055	VEO-IBD	2.38	0.032	2.8	0.014	2.56	$2x10^{-3}$
	VEO-UC	2.58	0.058	4.21	0.003	2.93	$6x10^{-4}$
	VEO-CD	2.08	0.057	2.1	0.345	2.01	0.106
rs4252249	VEO-IBD	0.41	0.045	1.18	0.701	0.61	0.882
	VEO-UC	0.28	0.046	0.71	0.585	0.4	0.033
	VEO-CD	0.63	0.24	2.35	0.179	0.98	0.958
rs4252270	VEO-IBD	0.41	0.045	1.16	0.722	0.6	0.081
	VEO-UC	0.28	0.046	0.7	0.572	0.4	0.031
	VEO-CD	0.62	0.24	2.32	0.186	0.97	0.938
rs1058867	VEO-IBD	1.55	0.186	0.94	0.868	1.16	0.515
	VEO-UC	1.28	0.596	1.13	0.814	1.1	0.748
	VEO-CD	2.11	0.058	0.56	0.364	1.26	0.519
rs8178561	VEO-IBD	0.7	0.528	1.23	0.687	0.92	0.804
	VEO-UC	1.04	1	1.89	0.267	1.32	0.447
	VEO-CD	0.23	0.212	0.74	0.26	0.37	0.160

Odds ratios using DD vs. Dd/dd model. Discovery cohort: VEO-IBD (n=65), VEO-UC (n=41), VEO-CD (n=24), and controls (n=188). Validation cohort: VEO-IBD (n=28), VEO-UC (n=18), VEO-CD (n=10), and controls (n=480). Threshold for significance in validation cohort was 0.007 to correct for multiple testing.

Haplotype Block 2 had a protective effect for VEO-IBD (OR 0.40, $P = 0.025$), UC (OR 0.29, $P = 0.007$) and VEO-UC (OR 0.28, $P = 0.025$), EO-IBD (OR 0.57, $P = 0.030$) but had no significant effect in EO-CD.

Validation Study Confirms IL10RA SNP Associations with VEO-UC

We determined the frequency of the SNPs (found in the discovery cohort) in an independent cohort of 422 children with EO-IBD and 480 healthy controls (validation cohort) by Taqman genotyping. This EO-IBD cohort was comprised of 122 children with UC and 300 children with CD (including 18 VEO-UC and 10 VEO-CD). χ^2 analysis of discovery and validation controls showed that they were similar with respect to the studied SNPs (data not shown).

Although the association with the seven IL10R SNPs and EO-IBD did not replicate in the validation cohort, combined analysis of the discovery and showed nominal association with all the *IL10RA* SNPs (Table 1) and EO-UC. Furthermore, the association with VEO-UC with both rs2228054 and rs2228055 was replicated in the validation VEO-UC cohort, (rs2228054 OR 3.86 [95% CI 1.39–10.71, $P = 0.005$, adjusted $P = 0.044$] and rs2228055 OR 4.21 [95% CI 1.51-11.69, $P = 0.003$, adjusted $P = 0.024$], Table 2) and remained significant in a combined analysis of the discovery and validation cohorts (OR 3.08, combined $P = 2 \times 10^{-4}$; and OR 2.93, $P = 6 \times 10^{-4}$, respectively).

Linkage analysis of Haplotype Block 1 AG and Haplotype Block 2 CAT showed that these SNPs were also in LD in the validation cohort ($P < 0.0001$). The Haplotype Block 1 AG was also associated with VEO-IBD (OR 2.43, $P = 0.023$), and VEO-UC (OR 3.40, $P = 0.006$) but not for overall IBD or EO-UC (Supporting Table 4). Haplotype Block 2 CAT was not associated with VEO-IBD ($P = 0.89$) or VEO-UC ($P = 0.51$).

DISCUSSION

Homozygous, loss-of-function mutations in *IL10RA* and *IL10RB* have been reported to cause severe, infantile-onset IBD.[6,7] In this report, we identify a novel splice site mutation in *IL-10RA* resulting in defective IL-10 signaling. In addition to the colitis, severe perianal disease, and

recurrent infections that have been previously described in patients with IL-10R mutations, this patient also suffered from severe arthritis.

The contribution of IL10R variants to IBD susceptibility more generally in children was heretofore unclear. Our study is the first to specifically examine by deep sequencing the role of IL-10R SNPs in EO-IBD and VEO-IBD. We identified two *IL10RA* SNPs associated with VEO-UC and these results have been validated in an independent replication cohort with OR of 2.9 and 3.1. These two SNPs, rs2228054 and rs2228055, were frequently found in the heterozygous state among IBD patients and inherited as a haplotype. The conferred risk may be due to one or both SNPs. Alternatively, the increased risk may reside in a regulatory region (e.g., promoter) in LD with these SNPs. We propose that this risk haplotype exerts a mild phenotype in the general population resulting in disease only in the presence of another genetic variant(s) or environmental trigger. In contrast to the complete loss-of-function IL-10R mutations (described here and previously[6,7]) which present with symptoms more typical of CD, these SNPs are associated with UC, which is consistent with the association of SNPs in IL-10 signaling genes and UC.[10,12] Although this study points to an important role of the IL-10 pathway in VEO-UC, due to the limited number of VEO-IBD patients, this association with VEO-UC will require further functional investigations and replication.

Although GWAS of adult and pediatric IBD have identified novel pathways important in the pathogenesis of IBD, the SNPs detected by GWAS only account for ≈25% of the assumed genetic heritability.[11] The risk for IBD associated with SNPs in *IL10, TYK2*, and *STAT3* loci demonstrate the importance of IL-10 in IBD pathogenesis.[9,12] However, GWAS have not identified an association between SNPs in the *IL10RA* or *IL10RB* loci and IBD risk in the pediatric population.[13,25] Similarly, the recently published meta-analysis of GWAS of UC patients failed to show a statistically significant increase in risk for rs2228054.[26] This is most likely due to the unique subset of patients examined in our study, and highlights the importance of candidate gene studies examining rare presentations of IBD. GWAS evaluating exclusively pediatric IBD focus primarily on EO-IBD patients, which has been shown to be phenotypically more similar to adult-onset IBD than VEO-IBD.[14] Therefore, the contribution of IL-10R SNPs to the susceptibility of IBD in very young children has been underestimated in recent adult and late-onset pediatric GWAS, as VEO-IBD represents a unique population that is poorly represented even in GWAS focusing on pediatric IBD.[13]

Although defective IL-10 signaling causes a strong inflammatory phenotype in the GI tracts of both humans and mice, it is still unclear which is the most critical cell types(s) that require IL-10 signaling. While Murai et al[22] suggested that an IL-10-dependent signal was required for the maintenance of FoxP3$^+$ Tregs, Chaudhry et al[3] demonstrated in mice with a Treg lineage-specific deletion of IL-10R that IL-10-dependent signals were required for the function but not the maintenance of Tregs. Peripheral blood FoxP3$^+$ Tregs were present in the patient studied in this report with defective IL-10 signaling, demonstrating that this signal is not required for the development of FoxP3$^+$ Tregs.

In summary, we have described a novel splice site mutation in *IL10RA* that broadens the existing phenotype of IL-10R deficiency to include severe arthritis. The discovery of this patient in a cohort of VEO-IBD patients reinforces that IL-10R deficiency should be considered in all infantile-onset IBD cases, especially those with severe perianal disease and now arthritis, and that this diagnosis should prompt consideration of stem cell transplantation. Additionally, the association of SNPs in *IL10RA* with VEO-UC implicates a role for IL-10R in the pathogenesis of IBD in a broader population of patients than recently appreciated. Such information may permit stratification of patients for clinical trials that target the IL-10 pathway.

ACKNOWLEDGMENTS

We thank B.P. and her family and all patients and families who participated. We thank Karoline Fielder, Kathleen Kelleher, and Karen Frost for collection of patient samples and Joanne Stempak for sample handling. We thank Dr. Ken Croitoru and members of Dr. Snapper's laboratory for critical reading of the article. We thank the Massachusetts General Hospital Clinical Research Program for assistance with biostatistical calculations. Author contributions: A.M.M. and S.B.S. conceived and designed all experiments. M.S.S., A.M.M., A.M.G., T.D.W., and S.K. provided study samples. C.J.M. and T.D.W. analyzed the data. C.J.M., V.M.W., and C.H.G. performed functional analysis under the supervision of A.M.M. and S.B.S. C.J.M., S.B.S., and A.M.M. wrote the article with contributions from all authors.

REFERENCES

1. Moore KW, de Waal Malefyt R, Coffman RL, et al. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 2001;19:683–765.
2. Huber S, Gagliani N, Esplugues E, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3(+) regulatory CD4(+) T cells in an interleukin-10-dependent manner. *Immunity*. 2011;34:554–565.
3. Chaudhry A, Samstein RM, Treuting P, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of th17 cell-mediated inflammation. *Immunity*. 2011;34:566–578.
4. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993;75:263–274.
5. Spencer SD, Di Marco F, Hooley J, et al. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J Exp Med*. 1998;187:571–578.

6. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med*. 2009;361:2033–2045.
7. Begue B, Verdier J, Rieux-Laucat F, et al. Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. *Am J Gastroenterol*. 2011;106:1544–1555.
8. Glocker EO, Frede N, Perro M, et al. Infant colitis—it's in the genes. *Lancet*. 2010;376:1272.
9. Franke A, McGovern DP, Barrett JC, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet*. 2010;42:1118–1125.
10. Franke A, Balschun T, Karlsen TH, et al. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. *Nat Genet*. 2008;40:713–715.
11. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet*. 2008;40:955–962.
12. Franke A, Balschun T, Karlsen TH, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet*. 2008;40:1319–1323.
13. Imielinski M, Baldassano RN, Griffiths A, et al. Common variants at five new loci associated with early-onset inflammatory bowel disease. *Nat Genet*. 2009;41:1335–1340.
14. Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis*. 2011;17:1314–1321.
15. Heyman MB, Kirschner BS, Gold BD, et al. Children with early-onset inflammatory bowel disease (IBD): analysis of a pediatric IBD consortium registry. *J Pediatr*. 2005;146:35–40.
16. Silverberg MS, Satsangi J, Ahmad T, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol*. 2005;19(Suppl A):5–36.
17. Roca X, Sachidanandam R, Krainer AR. Intrinsic differences between authentic and cryptic 5' splice sites. *Nucleic Acids Res*. 2003;31:6321–6333.
18. Desmet FO, Hamroun D, Lalande M, et al. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res*. 2009;37:e67.
19. Williams L, Bradley L, Smith A, et al. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol*. 2004;172:567–576.
20. Godfrey VL, Wilkinson JE, Russell LB. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol*. 1991;138:1379–1387.
21. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet*. 2002;39:537–545.
22. Murai M, Turovskaya O, Kim G, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol*. 2009;10:1178–1184.
23. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res*. 2001;11:863–874.
24. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7:248–249.
25. Kugathasan S, Baldassano RN, Bradfield JP, et al. Loci on 20q13 and 21q22 are associated with pediatric-onset inflammatory bowel disease. *Nat Genet*. 2008;40:1211–1215.
26. Anderson CA, Boucher G, Lees CW, et al. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet*. 2011;43:246–252.