ORIGINAL RESEARCH—CLINICAL

TNF Promoter Hypomethylation Is Associated With Mucosal Inflammation in IBD and Anti-TNF Response

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BACKGROUND AND AIMS: Inflammatory bowel diseases (IBDs) are chronic inflammatory conditions influenced heavily by environmental factors. DNA methylation is a form of epigenetic regulation linking environmental stimuli to gene expression changes and inflammation. Here, we investigated how DNA methylation of the tumor necrosis factor (TNF) promoter differs between inflamed and uninflamed mucosa of IBD patients, including anti-TNF responders and nonresponders. **METHODS:** We obtained mucosal biopsies from 200 participants (133 IBDs and 67 controls) and analyzed TNF promoter methylation using bisulfite sequencing, comparing inflamed with uninflamed segments, in addition to paired inflamed/uninflamed samples from individual patients. We conducted similar analyses on purified intestinal epithelial cells from bowel resections. We also compared TNF methylation levels of inflamed and uninflamed mucosa from a separate cohort of 15 anti-TNF responders and 17 nonresponders. Finally, we sequenced DNA methyltransferase genes to identify rare variants in IBD patients and functionally tested them using rescue experiments in a zebrafish genetic model of DNA methylation deficiency. **RESULTS:** TNF promoter methylation levels were decreased in inflamed mucosa of IBD patients and correlated with disease severity. Isolated intestinal epithelial cells from inflamed tissue showed proportional decreases in TNF methylation. Anti-TNF nonresponders showed lower levels of TNF methylation than responders in uninflamed mucosa. Our sequencing analysis revealed 2 missense variants in DNA methyltransferase 1, 1 of which had reduced function in vivo. **CONCLUSION:** Our study reveals an association of TNF promoter hypomethylation with mucosal inflammation, suggesting that IBD patients may be particularly sensitive to inflammatory environmental insults affecting DNA methylation. Together, our analyses indicate that TNF promoter methylation analysis may aid in the characterization of IBD status and evaluation of anti-TNF therapy response.

Keywords: Epigenetics; Methylation; Inflammatory Bowel Disease; Anit-TNF Response

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are complex and heterogeneous chronic inflammatory bowel diseases (IBDs) characterized by cycles of severe, relapsing intestinal inflammation. IBDs are complex disorders of poorly understood origin and are generally thought to result from environmental triggers, such as diet and the microbiome, in genetically predisposed individuals. Large-scale association studies have revealed remarkable genetic

complexity and identified more than 240 risk loci for IBD,[1–3] although estimates indicate that genetic contributions account for less than 15% of IBD cases.[1,2] Nevertheless, while there may be many distinct causes of IBD, clinical manifestation (ie, inflammation) is largely mediated by increased expression of a few key cytokines.[4] Among these, tumor necrosis factor (TNF) is strongly linked to pathogenesis of IBD.[5] Monoclonal antibodies targeting TNF (anti-TNFs) were the first biologic therapeutic agent approved to treat IBD and remain the most effective therapy for achieving endoscopic healing in IBD patients.[6] Additionally, mucosal TNF expression levels closely correlate with disease activity in IBD patients.[7] Therefore, it stands to reason that identifying factors that control TNF levels is critical for understanding the pathophysiology of IBD.

Epigenetics has emerged as a link among environmental triggers, gene expression changes, and aberrant inflammation.[8] Epigenetic modifications, such as DNA and histone methylation, are strongly influenced by environmental stimuli and can regulate gene expression levels.[9] Epigenome-wide association studies have identified many differentially methylated loci associated with IBD.[10–19] Additionally, changes in DNA methylation of specific genes have been proposed to mediate some of the known genetic risks for IBD.[15] Stable epigenetic changes specific to CD or UC patients also show promise as potential biomarkers to differentiate IBD subtypes.[18,20]

We previously found that loss of the DNA methyltransferase gene, DNA methyltransferase 1 (DNMT1), triggered spontaneous intestinal inflammation and an IBD-like phenotype in zebrafish. This phenotype was mediated by impaired *tnf* promoter methylation and derepression of *tnf* in intestinal epithelial cells (IECs). Moreover, hypomethylation and overexpression of *tnf* in IECs occurred prior to other inflammatory phenotypes, such as immune cell infiltration.[21] Similar intestinal phenotypes were later observed in mice lacking DNMT1 and DNMT3b in IECs.[22] Furthermore, both polymorphisms and decreased expression levels of DNMT3A are risk factors for IBD.[23] Together, these studies indicate a critical role for DNA methylation in IECs to suppress inflammation and protect against IBD.

Given the central role of TNF in IBD pathophysiology and its importance as a therapeutic target, we investigated whether DNA methylation of its promoter sequence is altered in intestinal mucosa of CD and UC patients using a case-control study of individuals undergoing colonoscopy for IBD disease activity assessment or controls undergoing screening or diagnostic colonoscopy without colitis (n = 200 total). TNF methylation levels were decreased in both CD and UC patients compared to healthy controls. Importantly, these changes were present only within inflamed mucosa, and TNF methylation levels correlated with the severity of local inflammation. Using an independent cohort, we found that TNF promoter methylation levels differed according to anti-TNF therapy response, with nonresponders also exhibiting hypomethylation in uninflamed mucosa. Finally, we also evaluated whether variants in

DNMT genes could account for any of the observed methylation changes in our cohort. While our data show that causal variants in these genes are likely very rare among IBD patients, we identified a missense variant affecting the catalytic domain of DNMT1 that showed reduced activity using in vivo assays in zebrafish. Overall, our study indicates that intestinal mucosal inflammation is associated with TNF promoter hypomethylation in IBD patients, especially in anti-TNF nonresponders. TNF methylation may represent a safeguard to dampen the inflammatory response in the intestine.

Methods

All authors had access to the study data and had reviewed and approved the final manuscript.

Patient Selection

Patients undergoing colonoscopy and giving written informed consent for research were enrolled in the Duke Gastrointestinal (GI) Tissue Repository under an existing Institutional Review Board protocol (Pro00001662). Enrollment in the GI Tissue Repository has been ongoing since 2009, and this is an open protocol for a longitudinal observational study. Patients presenting for screening or diagnostic colonoscopy without any known history of IBD or colon cancer and giving written informed consent for research were selected as controls. Patients with IBD were enrolled at the time of either surveillance or diagnostic colonoscopy. Both UC and CD patients were included. IBD patients represented the spectrum of disease activity. A second cohort of patient samples was identified from the Pathology Archives of the University of Utah (IRB 00091019). No participants were recruited for these samples.

Clinical Data Collection

For the first cohort, clinical data related to IBD were collected using a detailed chart abstraction process from the electronic medical record. The chart review conducted was performed as part of the existing Duke GI Tissue Repository study. Both cases and controls had basic demographic data collected including age at time of sample collection, sex, and race/ethnicity. IBD patients also had data collected including length of time since diagnosis with IBD, type of IBD (CD, UC, indeterminate), and type of disease (ileal, colonic, upper GI tract, fistulizing, perianal, proctitis only). Disease activity at the time of sample collection was determined by presence of symptoms, endoscopic disease activity determined by review of the endoscopy report, and histologic evidence of disease determined by pathology report generated by a GI clinical pathologist. Family history of IBD and surgical history were noted. Treatment history was classified based on any prior anti-TNF therapy and, if treated, response to that therapy. Other medication use was recorded. Laboratory data closest to the time of index colonoscopy were recorded including erythrocyte sedimentation rate and C-reactive protein (CRP). Exposures such as tobacco and alcohol use were recorded. Clinical recurrence data were also abstracted from the medical record. For the second cohort, archived samples and medical records

were accessed on February 26, 2019; September 30, 2019; December 10, 2019; December 1, 2020; and November 21, 2022. Some authors had access to both clinical data and protected health information. The laboratory research investigators did not have access to the full clinical dataset with protected health information.

Sample Collection

In addition to clinical biopsies, up to 6 research biopsies were collected at the time of colonoscopy by research staff present in the endoscopy room. In patients with IBD undergoing clinical biopsies, the research biopsies were paired with clinical biopsies. For each area where research biopsies were obtained, both endoscopic and microscopic disease activity at that specific location were recorded as part of the research record. In screening colonoscopy patients, clinical biopsies were not typically obtained from normal colonic mucosa and research biopsies were obtained from different parts of the colon to allow comparison with IBD samples from different regions of the colon. In addition to colonic biopsies, in some cases and controls, research samples were also collected from the terminal ileum.

Sample Processing

For the first cohort, research samples obtained during the index colonoscopy were placed in optimal cutting temperature compound and snap frozen over either liquid nitrogen or dry ice and stored at −80 °C. Frozen samples were then processed in Duke's BioRepository & Precision Pathology Core to obtain DNA for the methylation assays.

For the second cohort, scrolls (mucosal biopsies) or unstained slides (resections) were obtained from formalin-fixed paraffin-embedded tissue blocks. Mucosal areas were microdissected (resections). DNA was prepared by the University of Utah Biorepository and Molecular Pathology Shared Resource.

Deidentified DNA samples were processed for bisulfite conversion using the EZ DNA Methylation-Direct kit (Zymo Research), and a TNF promoter fragment was amplified using nested polymerase chain reaction (PCR) with the primers TNF_outer_f   5′-CTAACTAAATATACCAACAACTA-3′   and TNF_outer_r 5′-AGAAATGGAGGTAATAGGTTTT-3′ followed by TNF_inner_f 5′-CCAACAACTACCTTTATATATC-3′ and TNF_inner_r 5′-AGGTTTTGAGGGGTATGGG-3′. PCR amplicons were purified and cloned using the pGEM-T Easy Vector System (Promega). Sanger sequencing of bacterial colonies was performed by Genewiz (Azenta Life Sciences), and sequencing data were analyzed using QUantification tool for Methylation Analysis (http://quma.cdb.riken.jp/).[34]

Statistical Methods

General patient characteristics were described and compared between cases and controls. Age was compared using the Kruskal-Wallis test and sex and race were compared using the Fisher's exact test. Descriptive statistics were compiled for methylation markers overall. These assessments included multiple observations per subject. The distribution of methylation values did not meet the assumption of normality. Thus, the nonparametric Wilcoxon 2-sample test was used to compare methylation values between cases and controls,

between inflamed and uninflamed samples, and between anti-TNF responders and nonresponders assuming independence among observations. TNF methylation levels by inflammation status (paired) within subject were compared using the Wilcoxon signed rank test. Spearman correlation estimates were computed to assess relationships among erythrocyte sedimentation rate (ESR), CRP, and methylation status (results not shown). Analyses were performed in SAS 9.4 and GraphPad Prism 10.0.2.

Isolation of Intestinal Epithelial Cells (IECs)

Colonic epithelial cells were isolated as previously described.[35,36] In brief, dissected colonic mucosa was cut into small pieces and incubated in magnesium-free Hank's balanced salt solution (HBSS) containing 2 mmol/L ethylenediaminetetraacetic acid and 2.5% heat-inactivated fetal bovine serum for 30 minutes with shaking at 37 °C. Collected supernatants were centrifuged, resuspended in HBSS containing 1 mg/mL collagenase type 4 (17104019; Thermo Fisher Scientific), and incubated for 10 minutes at 37 °C to further remove the mucus. The fraction was pelleted, resuspended in HBSS, passed through a 40-μm filter, and overlayered on 50% Percoll. Cells were centrifuged at 2000 rpm for 20 minutes at room temperature and viable colonic IECs were recovered from the interface layer.

Mutational Analysis

Coding exons and intron-exon junctions of DNMT1, DNMT3A, and ubiquitin like with PHD and ring finger domains 1 (UHRF1) (79 exons total) were PCR-amplified and sequenced bidirectionally using BigDye Terminator 3.1 chemistry on an ABI 3730xl automated capillary sequencer (Applied Biosystems). Sequencer (Gene Codes) was used for sequence alignment to reference. Primer sequences are available upon request. Variant frequency information was obtained from gnomAD (gnomAD.broadinstitute.org); in silico prediction information was queried on PolyPhen-2 (genetics.bwh.harvard.edu/pph2/) and Mutation Taster (mutationtaster.org).

Zebrafish Experiments

Zebrafish (Danio rerio) were used in accordance with Duke University Institutional Animal Care and Use Committee guidelines under the approval from protocol number 170105-02. Zebrafish stocks were maintained and bred as previously described.[37] Genotypes were determined by PCR and DNA sequencing or phenotypic analysis. Male and female breeders from 3 to 18 months of age were used to generate fish for all experiments. Zebrafish larvae (5–6 days postfertilization) from the Ekkwill background were used in this study. Strains used in this study were dnmt1^{s872}[38] and TgBAC(tnfa:green fluorescent protein [GFP])^{pd1028}.[21] Larvae were anesthetized with 0.4 mg/mL MS-222 (Sigma, A5040) dissolved in embryo media for handling when necessary.

For mosaic transgene expression, the coding sequence of DNMT1 (NM_001130823) was subcloned to pDONR221 using gateway cloning (Thermo Fisher Scientific). pDEST-Tol2-QUAS:DNMT1-p2A-mCherry and pDEST-Tol2-cldn15la:QF2 were constructed as previously described[39] and coinjected with transposase copy RNA (cRNA) into 1-cell stage embryos

generated from crosses of _dnmt1^s872_; _TgBAC(tnfa:GFP)^pd1028_ double heterozygotes. Transverse sections of the midintestine of mutant and wild-type (WT) sibling larvae were then collected using a Leica VT1000S vibratome as previously described,[40] and confocal imaging was performed with an Olympus Fluoview FV3000 with a 60×/1.4 N.A. oil objective. Confocal data were analyzed using ImageJ/FIJI (National Institutes of Health) and Graphpad Prism 10.0.2.

For rescue experiments, the coding sequence of DNMT1 (NM_001130823) was subcloned to pCSDest[41] using gateway cloning (Thermo Fisher Scientific). Variant substitutions were introduced using Q5 site-directed mutagenesis (New England Biolabs). DNMT1 WT or variant cRNA was injected into 1-cell stage embryos generated from crosses of _dnmt1^s872_; _TgBAC(tnfa:GFP)^pd1028_ double heterozygotes. Live confocal imaging was performed with an Olympus Fluoview FV3000 with a 10×/0.4 N.A. objective. Confocal data were analyzed using ImageJ/FIJI (National Institutes of Health) and then larvae were genotyped before statistical analysis (Graphpad Prism).

Results

Cohorts

A total of 200 participants were included in the initial phase of this study. Baseline characteristics of the cohort are shown in Table. Among the 133 patients with IBD, 86 cases had a diagnosis of CD (64.7%), 46 cases had a diagnosis of UC (34.6%), and 1 case was of indeterminate type. Duration of disease was estimated for all cases with a median of 16 years since initial diagnosis and range of 0–46 years of disease duration. A family history of IBD was present in 38/128 patients (29.7%) with available data.

Among cases, 72/133 (54.1%) had endoscopic evidence of disease activity at the time of colonoscopy and 91/133 (68.4%) had histologic evidence of disease. Among 132 cases with available exposure data, 49 cases (37.1%) had any tobacco use, 7 (5.3%) were actively using tobacco at the time of index procedure, 94 (71.2%) had any alcohol use, and 72 (54.5%) had current alcohol use.

A history of anti-TNF-alpha therapy occurred in 80/132 cases (60.6%) with available data. Of the 80 participants ever treated with anti-TNF-alpha therapy, 59 (73.8%) received infliximab as their initial therapy. Among 41 patients with data on treatment agents at the time of index procedure, 21 (51.2%) were actively being treated with infliximab, 18 (43.9%) with adalimumab, and 1 with certolizumab (2.4%). Alternatively, some participants were being treated with other biologic agents at the time of index procedure, including 5/131 (3.8%) treated with ustekinumab and 11/131 (8.4%) treated with vedolizumab. Overall, 14/131 (10.7%) participants were taking steroids at the time of index procedure, with 3 of those also taking them in conjunction with an anti-TNF agent and 4 in conjunction with either ustekinumab or vedolizumab.

TNF Hypomethylation Is Associated With Inflammation in CD and UC

To investigate whether TNF methylation is altered in IBD patients, we focused on a cluster of Cytosine-phosphate-Guanine (CpG) sites in the distal promoter (Figure 1A) that was previously shown to be highly and stably methylated in macrophages from healthy human donors.[24] We first obtained colonic mucosal biopsies from 6 healthy donors and found that 3 distal CpG sites (−304, −245, and −239 bp upstream from TNF exon 1) were highly methylated, whereas the more proximal 8 CpG sites had much lower methylation levels, similar to previous reports[24] (Figure 1B and C).

We next compared mucosal TNF methylation levels of IBD cases (n = 199 biopsies from 133 patients) and healthy controls (n = 69 biopsies from 67 donors). Among IBD cases, 87 samples were inflamed and 112 were uninflamed; 58 of the uninflamed samples were paired biopsies collected along with inflamed samples. Without accounting for disease activity, no differences were observed between cases and controls (nonparametric, Wilcoxon 2-sample test; P = .4063) (Figure 2A). By contrast, when cases were compared according to inflammation status, significant differences were found. TNF methylation was higher in uninflamed samples from IBD cases vs inflamed samples (nonparametric, Wilcoxon 2-sample test; P < .0001) (Figure 2B), and

Descriptor	IBD cases (n = 133)	Controls (n = 67)	All (n = 200)	_P_ value
Table. Demographics of Inflammatory Bowel Disease Cases and Screening Colonoscopy Controls				
Age (y)				
Median	48.3	56.1	52.0	<.001
Range	21.2–more than 80	31.8–75.5	21.2–more than 80	
Sex				
Female	78 (58.6%)	36 (53.7%)	114 (57.04%)	.51
Race/Ethnicity				
Black	13 (9.8%)	13 (19.4%)	26 (13.1%)	.12
White	112 (84.8%)	49 (73.1%)	161 (80.9%)	
Other	7 (5.3%)	5 (7.5%)	12 (6.04%)	
Unknown	1	0	1	
Hispanic[a]	1	1	2	

IBD, inflammatory bowel disease.
[a]Counted in Other Race/Ethnicity category.
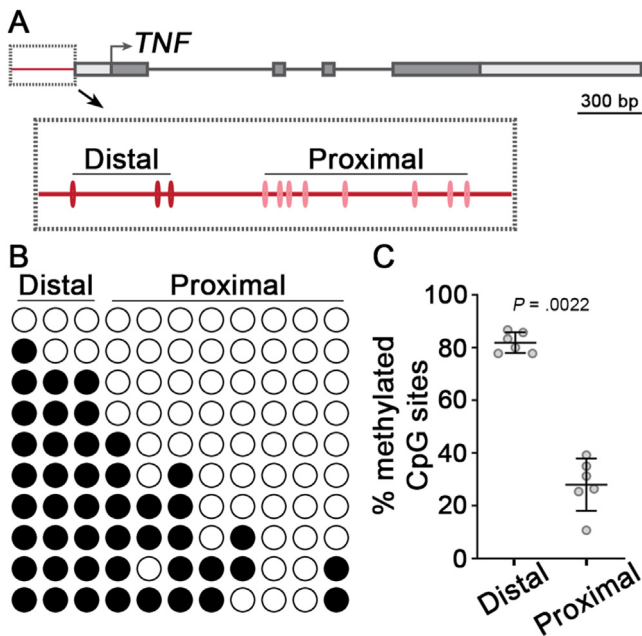
Figure 1. Methylation analysis of the TNF promoter from healthy human colon mucosa. (A) Schematic of the human TNF gene. CpG sites of the promoter region (red line) are shown in the boxed inset. (B) Methylation analysis of the TNF promoter from healthy human colon using targeted bisulfite sequencing. Columns represent individual CpG sites, and rows are 10 sequencing replicates for a single donor. Filled circles are methylated CpG sites, while open circles are nonmethylated. (C) Quantification of methylation analysis from healthy human colon. Individual data points are average methylation values (n = 6 samples each). Mean ± SD are plotted. P value was calculated using a nonparametric Wilcoxon 2-sample test. CpG, Cytosine-phosphate-Guanine; SD, standard deviation; TNF, tumor necrosis factor.

methylation levels of uninflamed samples from both CD and UC cases were more similar to healthy controls than inflamed samples (Figure 2C). We validated these findings using an independent cohort of uninflamed and inflamed mucosal samples from CD cases (n = 26 biopsies from 15 patients) obtained from a separate study site (nonparametric, Wilcoxon 2-sample test, P = .0406) (Figure 2D).

Because inflamed biopsies from cases exhibited TNF hypomethylation, we hypothesized that the severity of disease activity might influence methylation levels. To explore this possibility, we analyzed TNF methylation levels in uninflamed samples from cases with or without active disease compared to inflamed samples of varying severity. Median TNF methylation levels were negatively associated with the degree of disease activity ($P < .0001$, Kruskal-Wallis test) (Figure 3A). To further investigate the relationship between inflammation and TNF methylation, we analyzed paired inflamed and uninflamed biopsies from individual cases with active disease (n = 58 cases). Inflamed samples were significantly hypomethylated relative to their paired uninflamed samples (nonparametric, Wilcoxon matched-pairs signed rank test, $P = .0001$) (Figure 3B). Although inflamed samples of both groups

showed decreases in TNF methylation, the differences were significantly more pronounced in UC than in CD cases (nonparametric, Wilcoxon matched-pairs signed rank test; UC, $\Delta = -22.03\%$, $P = .0002$; CD, $\Delta = -7.89\%$, $P = .0832$).

Analysis of TNF Methylation Association With Covariates Reveals Link to Anti-TNF Therapy Response

We next investigated if systemic inflammatory markers were associated with mucosal TNF methylation levels. The clinical CRP and ESR values closest to the index colonoscopy when tissue samples were collected demonstrated that CRP and ESR correlated (test of Spearman correlation; CRP vs TNF, $P = .93$; ESR vs TNF, $P = .68$; ESR vs CRP, $P < .0001$). Among all observations (cases and controls), neither CRP nor ESR significantly correlated with TNF methylation, and within cases alone, CRP and ESR also were not correlated with mucosal TNF methylation. Some IBD risk exposures, such as tobacco smoke, can have modifying effects on DNA methylation of certain loci.[15,25] However, neither tobacco nor alcohol use correlated with mucosal TNF methylation levels.

We next explored whether mucosal TNF methylation levels differed among anti-TNF responders and nonresponders. In the initial cohort, the number of nonresponders was small with only 5 cases. Therefore, we analyzed our second cohort, which was comprised of a comparable number of responders (15 cases) and nonresponders (17 cases, 14 of which were primary nonresponders). Strikingly, uninflamed mucosa of nonresponders had reduced levels of TNF methylation compared to responders ($\Delta = -9.31$ [-12.9%]; parametric, unpaired t-test, $P = .0359$) (Figure 4A). Nonresponders had similar levels of TNF methylation in uninflamed and inflamed mucosa (Figure 4A and B). These results contrast with those of anti-TNF responders, in which TNF methylation values strongly correlated with inflammation status (Figures 2 and 3).

Contribution of the Epithelium to TNF Methylation and Mucosal Inflammation

To investigate the possibility that the methylation differences we observed in inflamed mucosa could derive from changes in cell composition, we tested whether methylation changes within IECs can account for any of the changes we observe in mucosal biopsies. To this end, we isolated IECs using fluorescence-activated cell sorting from sigmoid or ascending colon bowel resections of non-IBD controls or inflamed CD cases. IECs isolated from inflamed CD tissue were hypomethylated compared to control IECs (nonparametric, Wilcoxon rank sum test, $P = .0368$) (Figure 5A). Furthermore, IECs isolated from inflamed tissue showed greater reduction in TNF methylation compared to control IECs than did inflamed mucosa compared to their respective controls (Figure 5A, IECs, $\Delta = -13.87$ [-15.2%]; Figure 2A and B, mucosa, $\Delta = -8.58$ [-11.7%]). These findings indicate that at least some of the inflammatory changes in mucosal TNF methylation are due to changes in IECs
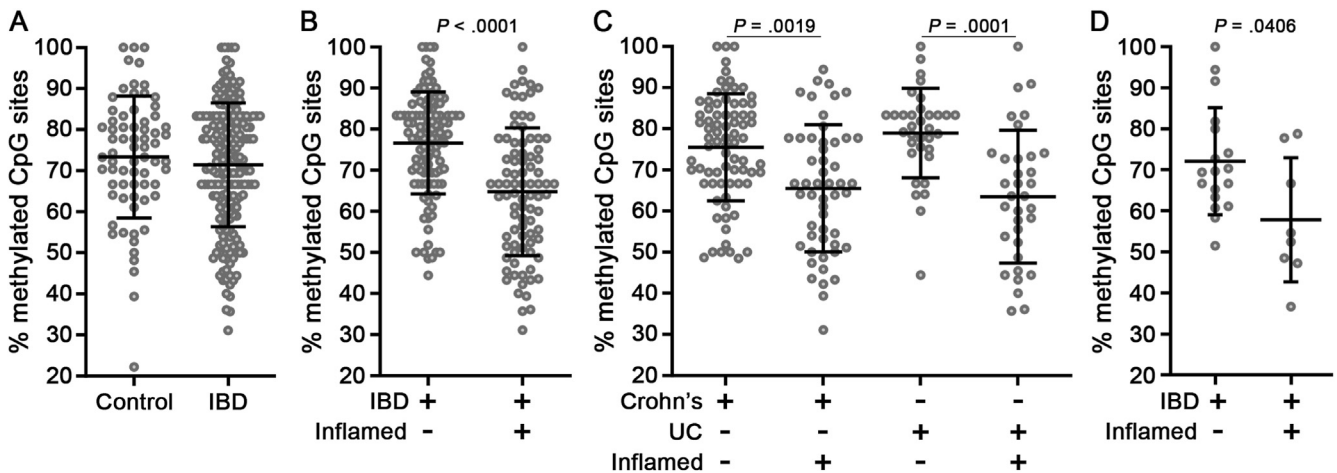
Figure 2. Inflamed mucosa of IBD cases exhibit TNF hypomethylation. (A) Methylation analysis of the TNF promoter from mucosa of controls (n = 69 samples from 67 donors) and IBD cases (n = 199 samples from 133 donors). (B) IBD cases were stratified according to biopsy inflammation status (uninflamed, n = 112 samples; inflamed, n = 87 samples). (C) IBD cases were stratified according to disease subtype and biopsy inflammation status (CD uninflamed, n = 77 samples; CD inflamed, n = 52 samples; UC uninflamed, n = 34 samples; UC inflamed, n = 34 samples). (D) Methylation analysis of an independent cohort of IBD cases (n = 26 samples from 15 donors) obtained from a separate study site. Data points are average methylation values for individual biopsies. Mean ± SD are plotted. *P* values were calculated using nonparametric Wilcoxon 2-sample tests (A, B, and D) or a nonparametric Kruskal-Wallis H test (C). CD, Crohn's disease; IBD, inflammatory bowel disease; SD, standard deviation; TNF, tumor necrosis factor; UC, ulcerative colitis.

themselves rather than to recruitment of inflammatory cell types with diverse methylation levels.

To further explore the role of the epithelium in this process, we turned to a genetic model of spontaneous intestinal inflammation. Zebrafish mutants for the DNA methyltransferase gene, DNMT1, develop hallmarks of inflammation in the gut (ie, cytokine induction, immune cell infiltration, loss of barrier function) in early larval stages.[21]
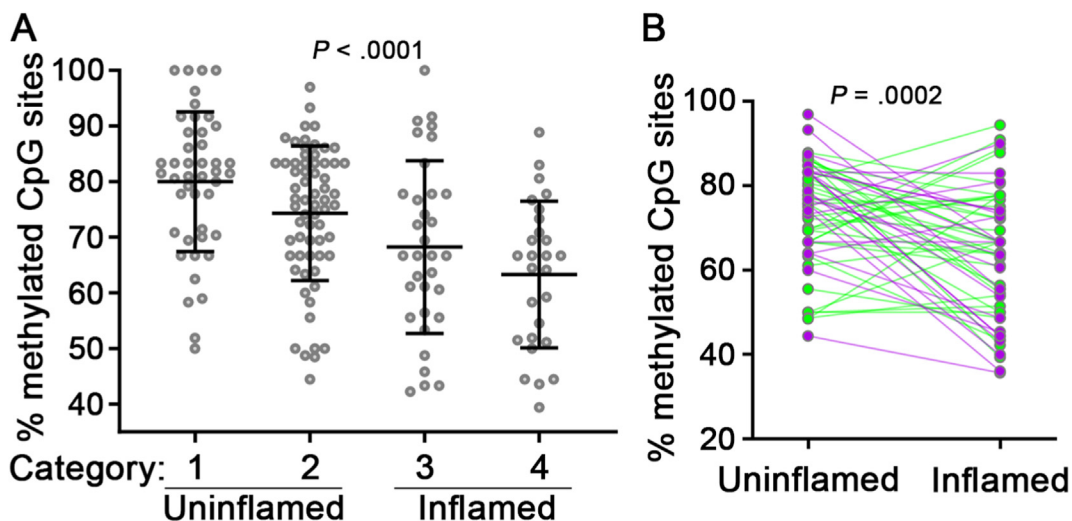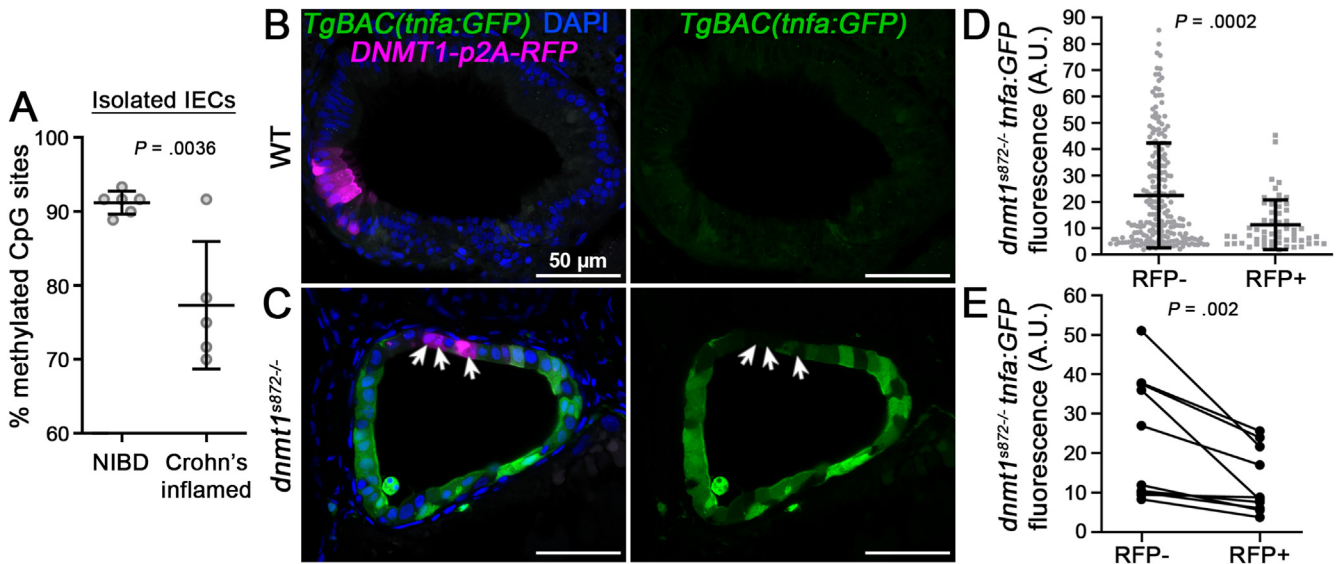

Figure 3. Association of TNF hypomethylation with mucosal inflammation. (A) TNF methylation analysis of IBD cases stratified by local mucosal disease activity. Categories: 1, uninflamed biopsies from IBD patients without active disease (n = 44 samples); 2, uninflamed biopsies from IBD patients with active disease (n = 65 samples); 3, inflamed biopsies from IBD patients exhibiting mild or focal inflammation (n = 33 samples); 4, inflamed biopsies from IBD patients exhibiting chronic or acute colitis (n = 27 samples). Data points are average methylation values for individual biopsies. Mean ± SD are plotted. (B) TNF methylation analysis of paired uninflamed and inflamed mucosal samples from individual IBD cases (CD, green lines, n = 36 paired samples; UC, purple lines, n = 22 paired samples). Connecting lines indicate paired samples. *P* values were calculated using nonparametric Kruskal-Wallis (A) or Wilcoxon matched-pairs signed rank tests (B). CD, Crohn's disease; IBD, inflammatory bowel disease; SD, standard deviation; TNF, tumor necrosis factor; UC, ulcerative colitis.
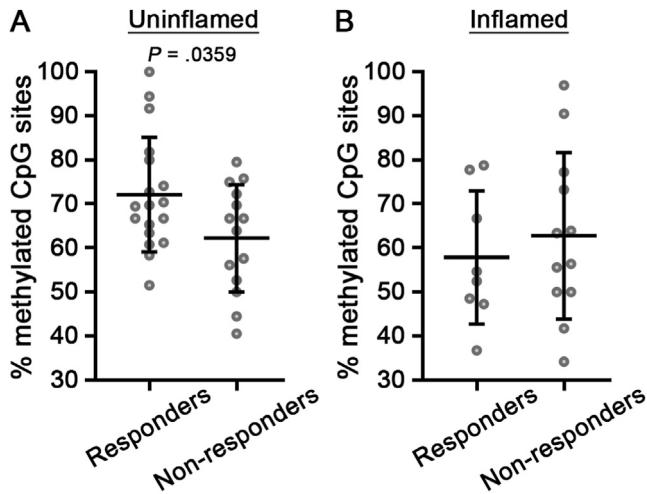
Figure 4. Anti-TNF nonresponders have lower levels of TNF methylation than responders. (A and B) TNF methylation analysis of anti-TNF responders and nonresponders in uninflamed (A) or inflamed (B) mucosa (responders, uninflamed, n = 18 samples; nonresponders, uninflamed, n = 14 samples; responders, inflamed, n = 8 samples; nonresponders, inflamed, n = 12 samples). Data points are average methylation values from individual cases. Mean ± SD are plotted. *P* values were calculated using parametric unpaired *t*-tests. SD, standard deviation; TNF, tumor necrosis factor.

Furthermore, upregulation of TNF within mutant IECs was associated with TNF promoter hypomethylation and was found to immediately precede subsequent inflammatory phenotypes in DNMT1 mutants.[21]

To test whether DNMT1 activity in IECs is sufficient to suppress inflammation, we performed rescue experiments by mosaically expressing human DNMT1 in small clones of IECs in DNMT1 mutants, using p2A-mCherry as a reporter for transgenic DNMT1 expression. In this scenario, mutants are devoid of maintenance DNA methylation except for the sparsely labeled IECs that express human DNMT1. To monitor TNF expression, we used a transgenic bacterial artificial chromosome reporter line we previously generated[21] and quantified *tnfa:GFP* levels in *DNMT1-p2a-mCherry* + IECs, using neighboring mutant IECs as internal controls (Figure 5B and C). We found that mutant IECs expressing DNMT1 showed a 49.7% reduction in *tnfa:GFP* expression relative to DNMT1-negative controls (nonparametric, Wilcoxon rank sum test, *P* = .0002) (Figure 5C and D). Paired DNMT1-positive and DNMT1-negative IECs from individual tissue sections showed similar changes (nonparametric, Wilcoxon matched-pairs signed rank test, *P* = .002) (Figure 5E). These results suggest that DNA methylation within IECs may influence local TNF expression levels.

Figure 5. TNF hypomethylation of IECs is associated with inflammation and increased IEC TNF expression. (A) TNF methylation analysis of FACS isolated IECs obtained from bowel resections of non-IBD (NIBD) controls and inflamed CD cases. Data points are average methylation values from individual cases and controls (NIBD, n = 6 samples; Crohn's inflamed, n = 5 samples). Mean ± SD are plotted. (B and C) 1-cell stage *dnmt1^s872* zebrafish mutants and WT siblings expressing *TgBAC(tnfa:GFP)^pd1028* as an inflammation reporter were injected with transgenic constructs to mosaically express human *DNMT1-p2A-RFP* specifically in IECs. At 5 days postfertilization (dpf), transverse sections of the intestine were collected and imaged by confocal microscopy. Arrows point to sparsely labeled RFP + cells expressing *DNMT1* in *dnmt1^s872* mutants. Scale bars are 50 μm. (D) Quantification of *tnfa:GFP* expression in *dnmt1^s872* mutants (n = 217 [RFP-] and 57 [RFP+] IECs from 10 larvae). Mean ± SD are plotted. (E) Quantification of *tnfa:GFP* expression in *dnmt1^s872* mutants from paired RFP- and RFP+ IECs from individual tissue sections (n = 10 larvae). *P* values were calculated using nonparametric Wilcoxon 2-sample tests (A and D) or a Wilcoxon matched-pairs signed rank test (E). CD, Crohn's disease; FACS, fluorescence-activated cell sorting; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; SD, standard deviation; TNF, tumor necrosis factor.

### Identification of Candidate Mutations in DNMT1 From IBD Patients

To investigate whether variants in methyltransferase genes could account for any changes in TNF methylation we observed in our cohort, we conducted bidirectional Sanger sequencing of the coding exons and intron-exon junctions in DNMT1, DNMT3A, and UHRF1 in genomic DNA extracted from mucosal biopsies of 27 controls and 70 cases. We identified 2 rare missense variants of interest in DNMT1 (NM_001130823.3: c.229G>A; p.G77S, and c.4428T>G; p. H1476Q) that were present in separate cases exhibiting TNF hypomethylation but were not present in controls and were rare in publicly available reference datasets (genome aggregation database; gnomAD v2.1.1). The G77S variant is evolutionarily constrained and predicted to be pathogenic by 2 different in silico prediction algorithms (PolyPhen-2, probably damaging; Mutation Taster, disease causing). On the other hand, the H1476Q variant is not predicted to be pathogenic, although it affects the target recognition domain within the methyltransferase domain of DNMT1. We tested if the function of these variants is impaired using rescue experiments in zebrafish. We injected cRNA encoding WT, G77S, or H1476Q DNMT1 into crosses of DNMT1 heterozygotes and then quantified *tnfa:GFP* expression levels in mutant and WT sibling (controls) IECs. Expression of WT DNMT1 in mutants strongly suppressed *tnfa:GFP* to levels similar to that of WT control siblings (Figure 6A–C, and F). The G77S variant also showed relatively strong activity and partially rescued *tnfa:GFP* expression (Figure 6B, D, and F). By contrast, the H1476Q variant showed significantly lower activity and failed to rescue *tnfa:GFP* levels (Figure 6B, E, and F).

Discussion

IBD is a complex disorder in which environmental triggers lead to an uncontrolled immune response in the intestine. Although hundreds of genes can confer risk to IBD, inflammation is largely propagated by increased expression of a handful of cytokines, most notably TNF.[4,5] Given the central role of TNF and the strong influence of environmental factors on IBD, we investigated how epigenetic marks of the TNF locus are affected in the mucosa of IBD patients. We found that TNF promoter methylation is decreased within inflamed mucosa and IECs isolated from inflamed tissue. Mucosal hypomethylation does not correlate with systemic inflammatory markers and, even among patients with severe active IBD, uninflamed mucosal segments show largely normal levels of TNF methylation. We also found that TNF hypomethylation correlates with the severity of local inflammation. We discovered that anti-TNF nonresponders show distinct patterns of TNF methylation, with inflamed and uninflamed segments alike exhibiting hypomethylation. Altogether, our study identifies an association of TNF promoter methylation with inflammation in IBD and reveals a novel biomarker that may help to distinguish anti-TNF nonresponders from responders.

Our studies using paired inflamed and uninflamed biopsies from individual patients suggest that TNF
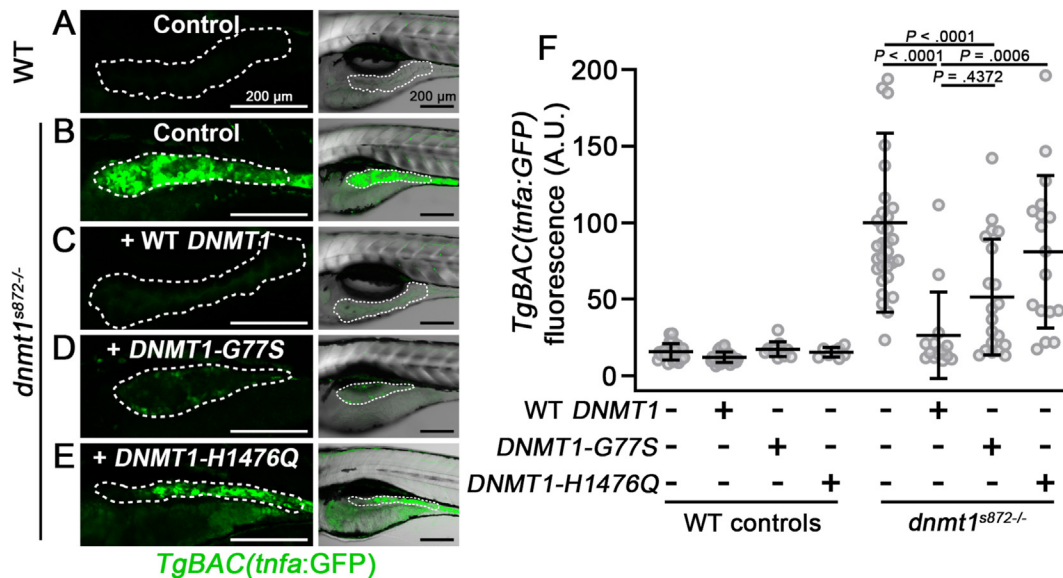
Figure 6. Functional analysis of rare *DNMT1* variants in a zebrafish *dnmt1* mutant model. (A–E) Live confocal imaging of *TgBAC(tnfa:GFP)* in 5 dpf *dnmt1^s872* mutant larvae and WT siblings. 1-cell stage embryos were injected with cRNA encoding WT or variant DNMT1, raised to 5 dpf, imaged, and then genotyped. Dotted line demarcates the anterior intestinal epithelium. Right panels show GFP overlayed with brightfield images. Scale bars are 200 μm. (F) Quantification of *tnfa*:GFP intensity in the anterior intestinal epithelium from experiments in panels A–E. Mean $\pm$ SD are plotted. *P* values were calculated using 2-way ANOVA. Data points are mean pixel intensity values from individual larvae (n $\geq$ 8 larvae per group). ANOVA, analysis of variance; GFP, green fluorescent protein; SD, standard deviation.

hypomethylation is a consequence of local mucosal inflammation. This conclusion is supported by a recent study showing that inflamed mucosa of IBD patients has reduced expression of DNMT genes,[23] and mouse intestinal organoids treated with TNF strongly downregulate DNMT3a.[23] On the other hand, our rescue experiments using zebrafish DNMT1 mutants indicate that DNA methylation may have cell-autonomous protective effects in an inflammatory microenvironment. Together, these studies suggest that impaired DNA methylation may trigger a feedback loop that exacerbates cytokine expression and inflammation. However, our DNMT1, DNMT3A, and UHRF1 sequencing studies indicate that rare variants in methyltransferase genes are not a predominant contributor-impaired DNA methylation in IBD. It would be informative to explore whether DNA methylation in IBD patients is more sensitive to environmental insults, such as infection or diet-associated inflammation.

A key problem in IBD management is that personalized therapy approaches are not currently part of clinical practice. Therapeutic agents are selected empirically rather than in response to a patient's specific biology. Although anti-TNF therapy remains the most effective treatment for achieving clinical remission and mucosal healing in IBD patients,[6] approximately one-third of IBD patients show poor clinical response to anti-TNFs. Because of the incomplete response rate, adverse side effects, and high costs, there is urgent need for markers that can predict a patient's response to anti-TNF therapy before beginning treatment.[26] We found that uninflamed mucosa of anti-TNF nonresponders show reduced levels of TNF methylation relative to that of responders. These findings suggest that TNF methylation is a quantitative parameter that can be measured pretherapeutically and could therefore inform the potential for anti-TNF response. Larger, prospective studies examining TNF methylation in inflamed and uninflamed tissues of IBD patients before anti-TNF treatment could be helpful to establish TNF methylation status as a biomarker to predict anti-TNF response.

Our finding that TNF methylation status is correlated with anti-TNF response raises the possibility that TNF hypomethylation could influence the response to anti-TNF therapy. Poor response to anti-TNF therapy can, in part, stem from elevated mucosal TNF levels that exceed the concentration of locally available anti-TNF agents.[27] It is conceivable that widespread TNF hypomethylation in both inflamed and uninflamed tissue of nonresponders may sensitize these patients to heightened TNF expression that overwhelms anti-TNF antibody levels. Future studies investigating how mucosal TNF promoter methylation relates to local TNF levels will help to describe its potential role in anti-TNF response. On the other hand, anti-TNF nonresponse can also stem from immunogenicity or an underlying biological mechanism independent of TNF. As a result, several biologics and small molecule inhibitors that target other pathways have been evaluated for treatment of anti-TNF nonresponders.[28] While it is unclear how TNF methylation relates to the efficacy of these alternative IBD therapeutics, such studies may help to devise personalized approaches for IBD management.

Treatment decisions for IBD are made through assessment of disease activity, which is monitored using scoring indices encompassing clinical symptoms, systemic biomarkers, and endoscopic observations.[29] Clinical and endoscopic indices vary widely, and many have been validated for CD and UC.[29] However, histologic assessment may provide a more accurate indicator of disease activity, mucosal healing, and potential relapse.[30,31] Nonetheless, standardized indices for measuring histologic activity of IBD are lacking, particularly for CD.[30,32] Incorporating molecular biomarkers, such as TNF promoter methylation, may aid in the development of an objective scoring index of histologic-level disease activity in CD and UC.

While the differences in TNF methylation we observed were robust, prior studies, including genome-wide analyses,[10–18] did not identify TNF as differentially methylated in IBD. A likely reason that TNF was not identified in genome-wide methylation studies of IBD is that the chip arrays used for such analyses, including the latest EPIC BeadChip DNA methylation array,[20] do not include the CpG sites we selected for our study. While genome-wide methylation studies have been instrumental in identifying potential biomarkers for distinguishing IBD cases, our study highlights the utility of targeted methylation studies of IBD candidate genes that may not be captured by commercial arrays.

One limitation of this study is the modest samples sizes in some subgroups. Although our study included 133 cases and 67 controls, stratifying cases to compare methylation changes with disease activity or location resulted in smaller sample sizes. Similarly, our sample sizes for analyzing methylation changes between anti-TNF responders and nonresponders were also small.

Another limitation of this study is that we did not fully define the cellular source(s) of TNF hypomethylation in inflamed mucosa from IBD patients and in mucosa from anti-TNF nonresponders. IECs represent a major cell type in mucosal biopsies, and our analysis of IECs isolated from inflamed IBD mucosa and healthy donors demonstrated significant methylation differences that were proportional to those of complete mucosal biopsies. This finding supports the hypothesis that IECs are an important source of TNF hypomethylation in inflamed IBD mucosa. However, our studies do not exclude the possibility that some of the methylation differences we observed derive from changes in cellular composition. DNA methylation signatures vary among different cell lineages,[33] and biopsies from acutely inflamed mucosa have increased neutrophils and plasma cells. Thus, it is possible that some of the TNF methylation differences we observed could be related to increased proportions of differentially methylated cell types and not entirely due to hypomethylation within IECs.

In conclusion, our study reveals an association between TNF promoter methylation and mucosal inflammation in CD

and UC patients. IECs represent a major source of these TNF methylation differences: IECs isolated from CD patients show significantly less TNF methylation than IECs from healthy controls, and clinically relevant variants in DNMT1 influence TNF expression in IECs in a vertebrate model system. This study lays the foundation for developing a quantitative biomarker based on TNF methylation to help predict anti-TNF therapy responses.

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