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Distinct *IFNG* Methylation in a Subset of Ulcerative Colitis Patients Based upon Reactivity to Microbial Antigens

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Abstract

Background—High antibody reactivity toward microbial antigens in Crohn’s disease patients is predictive of a more aggressive disease course. However, few ulcerative colitis patients exhibit serologic reactivity towards microbial antigens. Mucosal expression of IFN- γ plays a pivotal role in IBD pathogenesis. Recent GWAS surprisingly link UC, but not CD, risk loci to *IFNG*. We recently demonstrated that mucosal T cells from IBD patients exhibit distinct patterns of *IFNG* methylation compared to controls. This study evaluated the relationship between *IFNG* methylation and serologic and clinical profiles in peripheral T cells from IBD patients.

Methods—DNA from peripheral T cells of 163 IBD patients (94 CD and 64 UC) and 43 controls was analyzed for methylation of eight *IFNG* sites. Serum markers ASCA, OmpC, I2, CBir and pANCA were measured by ELISA. IFN- γ secretion was measured by ELISA.

Results—IBD patients requiring surgery exhibited reduced *IFNG* methylation compared to non-surgical patients ($p<0.02$). Enhancement of IFN- γ secretion ($p<0.003$), along with high antibody responses toward multiple microbial antigens ($p<0.017$) in UC, but not CD, patients was correlated with decreased *IFNG* methylation. pANCA levels were not correlated with *IFNG* methylation.

Conclusions—Levels of *IFNG* methylation were correlated with immune response to microbial components, and expression of IFN- γ in UC patients. Serological and epigenetic markers identify a subset of UC patients with an expression profile of a key TH1 pathogenic cytokine. These data may provide a useful tool to classify a more homogeneous subset of UC patients allowing for improved diagnostics and targeted therapeutics.

Keywords

Human; T Lymphocytes; methylation; interferon; IBD

INTRODUCTION

Dysregulated mucosal immune response to enteric bacterial flora in genetically susceptible individuals is believed to trigger the inflammatory bowel diseases (IBD) (1). Although UC and CD each have unique features, they may also share a number of characteristics that can, on occasion, lead to difficulty differentiating the two conditions. Moreover, there is

considerable heterogeneity in disease classification, with patients exhibiting a broad array of symptoms suggesting that CD and UC may represent a series of overlapping disorders. The etiology of disease remains unclear. Genetic factors play a role in IBD pathogenesis with evidence suggesting that CD and UC are related polygenic diseases. In the past, genome wide association studies (GWAS) have largely focused on genes linked to susceptibility for CD although more recently a number of UC GWASs have been performed (2–9). It is now increasingly clear that some susceptibility genes are common for both UC and CD while others are peculiar to each condition. Genes related to innate immune handling of intracellular bacteria, such as *NOD2* (10, 11), and *ATG16L1* (12–14) appear to be CD-specific, whereas genes encoding members of the IL23 and IL17 pathway (*IL23R*, *IL12B*, *JAK2* and *STAT3*) (15–18) are associated with both CD and UC. More recently, novel UC-specific loci including *ECM1*, FCGR2A, chromosomes 1p36 (*OTUD3* and *PLAG2E*) and 12q15 (*IFNG*, *IL26* and *IL22*) (3).

A loss of tolerance to commensal microbiota also contributes to IBD pathogenesis as evidenced by the presence of circulating antibodies against various mucosal micro-organisms. Initial investigations by our group and others described antibodies against the mannose epitopes of *Saccharomyces cerevisiae* (ASCA) and autoantibodies against an unidentified nuclear protein in neutrophils (pANCA) (19–21) of patients with IBD. Subsequently, the list of antibodies has been expanded to include, among others, antibodies to the outer-membrane porin C of *Escherichia coli* (OmpC), a *Pseudomonas fluorescens*-associated sequence (I2) and antibodies against the flagellin CBir1 (anti-CBir1) (22–24). Although there is ambiguity regarding the functional and molecular consequence of antibody reactivity, there is an increasing body of evidence supporting the utility of these markers as a tool for disease stratification (25). High antibody response toward multiple microbial antigens is associated with more aggressive disease and risk for surgery in CD (26–28). In contrast, pANCA is detected in the majority of UC patients and is associated with more aggressive disease whereas only a small subset of UC patients exhibit reactivity towards multiple other microbial antigens (29, 30).

Thus, GWAS studies have implicated components of the innate immune pathway whereas serological responses to commensal bacteria have implicated adaptive immune components. It is thought that the interplay between innate and adaptive immunity ultimately determines host response. While the precise mechanisms involved remains unknown, a recent study (31), examining Toll-like receptor signaling-deficient mice, noted that a defect in the innate immune response (i.e., toll-like signaling) resulted in compensation via enhanced adaptive response (i.e., high-titer of anti-commensal bacteria antibodies). These data suggest a “cooperative flexibility” between innate and adaptive immune pathways (31).

Enhanced Th1 cytokine expression, and in particular IFN- γ expression, has clearly been associated with CD (32–34). A reduced Th1 profile has been proposed but the definitive cytokine profile for UC is more obscure (34). Studies have suggested that lamina propria (LP) T cells from CD patients can be stimulated *in vitro* to produce increased amounts of IFN- γ compared to normal controls, whereas secretion of IFN- γ from UC patients remains unchanged (33, 34). However, a recent study examining the mechanism of action of infliximab, an anti-TNF- α antibody, in UC patients noted an increase of *IFNG* mRNA levels compared to control, which was subsequently down-regulated following treatment (35).

T cell production of IFN- γ has largely been studied at the level of transcriptional regulation. Increasing evidence suggests that epigenetic mechanisms, via chromatin modification such as DNA methylation, histone acetylation, and RNA interference, are important modulators of gene expression are also important. DNA methylation is considered an inherited epigenetic marker for expression competency with reduced DNA methylation generally

correlated with enhanced gene expression. We have previously reported that, *in vivo*, freshly-isolated lamina propria (LP) T cells are inherently activated and poised to secrete IFN- γ , displaying distinct epigenetic *IFNG* DNA methylation patterns in patients with IBD compared to normal controls (36). In this study, we evaluated whether methylation of *IFNG* in peripheral blood (PB) T cells of IBD patients is associated with specific clinical characteristics. We demonstrate that epigenetic modification of *IFNG* may identify a subset of UC patients with an altered response to commensal microbes possibly defining a sub-clinical phenotype.

MATERIALS AND METHODS

Isolation of T cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers or IBD patients by separation on Ficoll-Hypaque gradients. Informed consent (approved by the Institutional Review Board at Cedars-Sinai Medical Center) was obtained from all participating subjects. CD3⁺ T cells were isolated using CD3-immunomagnetic beads (Miltenyi Biotech, Auburn, CA) and were at least 95% pure.

Study Subjects

The IBD patients were recruited through the IBD Center at Cedars-Sinai Medical Center. The diagnoses of Crohn's disease and ulcerative colitis were confirmed using standard clinical, radiological, endoscopic and pathological criteria. The demographics, medications and disease activity of the patients included in this study are outlined in Table 1. The controls are all healthy individuals, free of medication and with no known personal or family history of autoimmune disease or IBD.

IFN- γ assay

IFN- γ was measured by an amplified ELISA (37). Greiner Bio-One (Longwood, FL) ELISA plates were coated overnight with 100 μ l of 5 μ g/ml monoclonal anti-IFN- γ (BD Biosciences, Woburn, MA). Samples and standards were added for 24 h followed by addition of 100 μ l of 2.5 μ g/ml polyclonal biotinylated rabbit anti-IFN- γ (BD Biosciences) for 2 h. This was followed by addition of 100 μ l of 1/1000 diluted alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h. Substrate, 0.2 mM NADP (Sigma-Aldrich, St. Louis, MO) was added for 30 min followed by addition of amplifier (3% 2-propanol, 1 mM iodonitrotetrazolium violet, 75 μ g/ml alcohol dehydrogenase, and 50 μ g/ml diaphorase; Sigma-Aldrich) for 30 min. Plates were read at 490 nm using an E max plate reader (Molecular Devices, Sunnyvale, CA).

Microbial Antibody Responses

All blood samples were taken at the time of consent and enrolment. Sera were analyzed for expression of ASCA, anti-OmpC, anti-I2 anti-CBir1 antibodies in a blinded fashion by ELISA as previously described (23, 26). Antibody levels were determined and results expressed as ELISA units (EU/ml), which are relative to a Cedars-Sinai Laboratory standard, which is derived from a pool of patient sera with well-characterized disease found to have reactivity to this antigen.

Pyrosequencing

DNA was extracted from T cells using a QIAmp DNA isolation kit (Qiagen Inc., Valencia, CA). All samples were analyzed in a blinded fashion using the Biotage custom pyrosequencing service (Biotage, Inc, Foxboro, MA). Briefly, bisulfite treatment of 2 μ g of DNA was carried out using the EZ DNA methylation kit (Zymo Research, Orange, CA)

according to manufacturer's instructions. Hot-start PCR was carried out with HotStart Taq (Qiagen Inc) using 100 ng of bisulfite treated DNA. PCR and pyrosequencing primers are shown in Table 3. Direct quantification of the ratio of unmethylated to methylated cytosines was determined for each site using Pyro Q-CpG software. The *IFNG* non-CpG cytosine at site -181 bp served as an internal control and revealed that bisulfite conversion of DNA was greater than 95%. Likewise, only slight variability was detected in DNA samples treated with bisulfite on different days. The naïve NK92 cell line demonstrated complete conversion following bisulfite treatment and served as a demethylation control.

Statistical Analysis

Quartile sum score: To evaluate the association between disease phenotype characteristics and the combination of the level of immune response toward microbial antigens, the sums of quartile scores for ASCA (IgA and IgG), anti-OmpC, anti-I2, and anti-CBir1, were calculated. For each antigen, patients whose antibody levels were in the 1st, 2nd, 3rd, and 4th quartile of the distribution were assigned a quartile score of 1, 2, 3, and 4, respectively. Because ASCA can be expressed in both an IgA and an IgG class, positivity to ASCA was determined if either class of antibody was above the reference range. In determining a quantitative measure of ASCA, the reactivity was first log-transformed and standardized. Among the two classes, the higher one was then used to determine the quartile of reactivity. The magnitude of reactivity to the other 3 antigens was not standardized because each is represented by a single class of antibody. By adding individual quartile scores for each microbial antigen, a quartile sum score (range, 4–16) was obtained to represent the cumulative quantitative immune response toward all 4 antigens for each patient. The quartile sum score was calculated for patients with CD, and UC, and evaluated as two separate cohorts. The quartile ranking reflects each group independently and is not directly comparable between groups.

Correlation between methylation and serologic markers: Correlation between methylation and serologic markers was calculated using the non-parametric Spearman's rank correlation test. Serology sum was calculated for both quartile sum score, and for sum of normalized, log-transformed continuous values.

Multivariable analysis: For the association between methylation levels, serological sums and additional covariates (ESR, CD3-activated IFN- γ , etc.), multivariable logistic regression was used.

ETHICAL CONSIDERATIONS

All studies involving human subjects were approved by the Institutional Review Board at Cedars-Sinai Medical Center.

RESULTS

Prior studies that have examined expression of IFN- γ secretion in IBD patients have, for the most part, focused on the competency of mucosal LP T lymphocytes. Little is known regarding IFN- γ secretion anomalies in IBD patients within the peripheral immune compartment. In this study peripheral circulating T lymphocytes were isolated from patients with IBD. We evaluated IFN- γ secretion following 24 hour T cell activation via the TCR/CD3 pathway. As seen in figure 1A, PB T cells isolated from IBD patients displayed reduced IFN- γ secretion compared to PB T cells isolated from healthy controls. No differences in IFN- γ secretion was detected between UC and CD patients (figure 1B). Likewise, no relationship between the level of IFN- γ secretion and concomitant drug regimen or age of patient was seen (data not shown).

We have previously reported that T cells isolated from the mucosa of IBD patients display distinct *IFNG* DNA methylation patterns compared to normal controls (36). DNA methylation patterns of PB T cells isolated from patients with either Crohn's disease or ulcerative colitis were compared to methylation patterns of PB T cells isolated from healthy volunteers. *IFNG* DNA methylation patterns were measured across eight CpG islands, as previously described (36). The overall methylation index was calculated for each sample as the mean methylation value for all sites examined (36). Table 1 shows the demographics of the UC and Crohn's disease patients and healthy volunteers. Statistical analysis revealed no correlation between methylation index and anatomic location (e.g., colon vs. ileum) of disease or patient gender. A small but statistically significant increase in the overall methylation index of *IFNG* was detected in PB T cell DNA from IBD patients 48% ($p < 0.02$) compared to normal controls (44%) (Figure 2A). There was no statistically significant difference in methylation between UC and CD patients (Figure 2B).

The functional significance of altered epigenetic methylation patterns was examined within the IBD patient population based on the premise that epigenetic regulation reflects a different potential for gene expression, which leads to distinct subclinical or disease attributes. Patients were assessed at time of clinical intake and characterized as having active or inactive disease or being within two months of surgery performed for medically refractive disease. As seen in figure 3A, methylation of *IFNG* was independent of clinical disease assessment. However, a significant decrease in *IFNG* DNA methylation was detected in IBD patients who were medically refractive requiring surgical intervention (57%, $p < 0.04$) compared to patients who were medically responsive (62%) (Figure 3B).

We have previously reported that *IFNG* DNA methylation functionally correlates with *IFNG* mRNA expression in untreated LP T cells. A similar analysis was performed in order to determine whether methylation of *IFNG* functionally correlates with IFN- γ secretion in activated PB T cells from IBD patients. As seen in figure 4A, a significant inverse correlation ($p < 0.003$) was observed between IFN- γ secretion and the corresponding methylation index of *IFNG* in PB T cells from UC patients. In contrast, no correlation was detected for patients with CD (figure 4B).

There is considerable evidence for the existence of Crohn's disease-specific response to microbial antigens that are associated with a more complicated disease phenotype (38). Severity of disease is associated not only with the magnitude of response, but with the number of microbial antigens to which the patient is responsive. Response to four microbial antigens was evaluated in association with methylation levels in IBD patients. The level of immune response toward ASCA, I2, CBir and OmpC was measured as a quartile score (Figure 5A) or as a continuous variable (Figure 5B). Quartile scores for each serological marker was calculated, as previously described (39). For each antigen, a score of 1–4 was assigned for individual patients based on the individual antibody response levels and then the cumulative response toward all four antigens was calculated by adding the individual quartile scores for each microbial antigen. Since the quartile scores were derived separately from CD and UC patient populations and were based on the distribution of sero-reactivity within each individual cohort, the absolute magnitude of response should not be compared between groups. As expected, the absolute magnitude of serologic response for the CD group was much higher than that of the UC group, which, for the most part, fell within the "normal" range. Nevertheless, as seen in figure 5A and 5B, a decrease in the *IFNG* methylation level was associated with increased antibody reactivity to microbial antigens in UC patients (regression for continuous $p < 0.001$, quartile $p < 0.017$). Surprisingly, no correlation between methylation and antibodies against microbial antigens was detected in the CD patient population. No correlation was found between methylation and antibodies against pANCA or CRP levels within UC or CD patients.

DISCUSSION

DNA methylation is a well established epigenetic mechanism for regulating gene expression. We have recently shown that, in the mucosal compartment, LP T cells display unique methylation patterns of *IFNG*, distinct from those seen in PB T cells (36). Those studies suggest that epigenetic control of DNA methylation of the *IFNG* may be functionally important in regulating *IFNG* expression within the mucosal immune compartment of the gut. In the present study, we focused on the peripheral immune compartment and investigated whether *IFNG* DNA methylation in IBD patients is associated with disease-specific phenotypes. Distinct *IFNG* methylation patterns were detected when comparing PB T derived from normal subjects to those from IBD patients. In IBD patients, a decrease in *IFNG* methylation levels was associated with the need for surgical intervention for disease management. Surprisingly in UC, but not CD, reduced *IFNG* DNA methylation levels were correlated with a functional enhancement of IFN- γ secretion. Likewise, in the UC, but not CD patients, decreased methylation of the *IFNG* correlated with antibody responses toward multiple microbial antigens commonly associated with CD.

It is a generally accepted that CD is driven, at least in part, by Th1 and Th17 cytokines, whereas the cytokine profile for UC is less defined, but includes both Th1 and Th2 cytokines (34, 40, 41). This premise is based on studies which have utilized either mucosal biopsies or purified LP T cells. All previous studies utilized cells isolated from patients broadly classified as having CD or UC and cytokine secretion was not stratified for subsets of UC or CD populations. Previous studies examining the interrelationship between serological parameter as a predictor of clinical response have demonstrated that these associations are specific for IBD patient/clinical subtypes (26, 42–45). In the present study, we examined whether serologic or clinical markers commonly used to stratify patients with IBD was associated with epigenetic modification and cytokine secretion competency of PB T cells. Cytokine secretion competence of peripheral blood T cells from IBD patients has not been examined in a comprehensive manner. An early study comparing IFN- γ secretion from PB T cells of four CD patients suggested that enhanced secretion of IFN- γ is dependent on the mode of T cell activation (34). More recently a significant decrease in the number of Th1 positive PB T cells was noted in pediatric IBD patients (46). We observed decreased IFN- γ secretion in PB T cells from IBD patients, which was correlated with an increase in *IFNG* methylation. These findings are in contrast to what was observed for the mucosal compartment, in which enhanced IFN- γ expression paralleled a decrease in *IFNG* methylation. Thus, epigenetic methylation patterns are disrupted within both the intestinal and peripheral compartments of patients with IBD, albeit with reverse methylation and expression patterns. A similar inverse correlation of cytokine secretion competency between the mucosa and periphery has previously been reported in CD patients following treatment with anti-TNF- α therapies (47). IFN- γ expression from LPMC is decreased in clinically responsive patients, whereas an increase in IFN- γ production is detected in PBMC. This study suggests that anti-TNF- α treatment may prevent trafficking of lymphocytes from the periphery to the gut (47), with the hypothesis being that during active disease the inflammatory cells remain within the inflamed tissue of the gut. Following treatment with anti-TNF- α agents these cells are no longer “stuck” within the gut, but are markedly increased within the circulating T cell population of IFN- γ competent cells. Similarly, we hypothesize that in active disease, IFN- γ competent cells, marked by reduced *IFNG* DNA methylation, stay within the gut, whereas circulating peripheral T cells, marked by higher *IFNG* methylation levels, exhibit decreased IFN- γ secretion. Indeed, studies have suggested that lymphocyte trafficking of T cell subsets is far from a random process, with selective lymphocyte trafficking between the mucosa and peripheral compartments described in CD. Moreover, expression of the CCR9 chemokine receptor is enhanced in small bowel LP T cells compared with colonic LP or PB T cells, and these cells produce high levels of IFN- γ

(48). However, the precise mechanism that ultimately determines cell migration within the intestine or periphery, as well as the adhesion and chemoattractant molecules involved, are poorly understood. Considering that DNA methylation patterns in T cells are generally believed to be stably inherited, our present study provides additional insight, suggesting that epigenetic changes in *IFNG* methylation patterns may be a hallmark distinguishing sites of inflammation in the gut from the periphery, with limited trafficking between.

CD and UC are multifactorial diseases that exhibit significant clinical heterogeneity. Clinical features can vary, depending on the site of bowel involvement, adding to the challenge of definitive diagnosis. Moreover, it is widely believed that UC and CD actually include a spectrum of multiple disease subtypes, which are broadly classified as UC and CD based upon similar clinical manifestations. The presence of antibody markers in IBD patients has been proposed as an additional tool to differentiate between UC and CD (49). Indeed, the combination of clinical, radiological and pathological assessment in addition to response to microbial antigens, has allowed for the stratification of subsets of disease phenotypes (50). Equally important is a significant association between the presence of microbial antibodies and disease progression, suggesting that molecular characterization may predict clinical outcome and help stratify patients for targeted therapeutics. Numerous serologic markers (i.e., ASCA, I2, OmpC, and CBir) have been reported to be associated with CD, whereas autoantibodies to pANCA are the single serologic marker commonly associated with UC. Expression of pANCA in UC is generally associated with more aggressive disease and the need for surgical intervention. In fact, a subset of UC patients expressing high levels of pANCA are more likely to develop chronic pouchitis after ileal pouch-anal anastomosis (30). In a recent pediatric study, pANCA reactivity was suggested as a defining marker for non-response to anti-TNF therapy (51). In CD patients, an increase in the number of sero-positive antibodies and a greater level of antibody response is indicative of more complicated disease and the need for surgery (26, 52). Likewise, in pediatric patients, the presence of serological markers is predictive for disease course (53). Thus, even though most studies evaluating the prognostic value of cumulative high level serological markers have focused on CD patients, it is important to keep in mind that a small percentage of UC patients display reactivity to some “CD-associated” antigens. Moreover, these same “CD-associated” antibodies are detected in unaffected relatives, as well as normal controls, albeit at lower levels where expression is within the normal range. Within these cohorts an elevation in the level of combined antibodies is noted in the sub-population of unaffected individuals carrying variants of the *NOD2* (*CARD15*) gene commonly associated with CD (39). The data presented here defines a sub-population of higher-level sero-reactive individuals among UC patients who display reduced *IFNG* methylation levels. Indeed, a recent GWAS identified two independent UC, but not CD, risk regions on chromosome 12 upstream and downstream of *IFNG* (3), suggesting that *IFNG* may play a more central role in the pathogenesis of UC than was originally proposed. A combination of serological and epigenetic markers may define the subset of UC patients related to this *IFNG* genetic variation. Taken together, our study suggest that within a UC-defined patient population, response to “CD-associated” markers may identify a subset of patients with a distinct *IFNG* epigenetic profile in which IFN- γ may play a role in the inflammatory process.

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Figure 1.

IFN- γ secretion in PB T cells from IBD patients compared to normal controls. Cells were activated for 18 hours with OKT3, and supernatants were harvested and analyzed by ELISA. A, Comparison of IFN- γ secretion levels from activated PB T cells from IBD patients compared to normal volunteers. B, Comparison of IFN- γ secretion from PB T cells from IBD patients based on disease diagnosis. Bars represent means $\pm$ SEM.

Figure 2. Methylation index of *IFNG* in PB T cells from IBD patients compared to normal controls. A, Comparison of MI from resting PB T cells from IBD patients vs. normal volunteers. B, Comparison of MI from PB T cells from IBD patients based on disease diagnosis. Bars represent means $\pm$ SEM.

Figure 3.

IFNG DNA methylation index as a function of disease activity and surgery. *IFNG* DNA methylation index in PB T cells from IBD patients categorized by disease activity as (A) active or inactive or (B) medically refractive and requiring surgery. Bars represent means $\pm$ SEM.

Figure 4. *IFNG* DNA methylation inversely correlates with IFN- γ secretion in UC but not CD Patients. Correlation of IFN- γ secretion levels to methylation of *IFNG* for PB T cells from (A) UC or (B) CD patients. Spearman correlation coefficient is -0.66 ($p < 0.003$) for UC patients.

Figure 5. Decreased *IFNG* methylation is associated with increased antibody reactivity to microbial antigen in UC but not CD patients. A, Quartile or B, Continuous regression analysis of the UC or CD cohorts for four serological markers (ASCA, OmpC, I2 and CBir) was correlated with *IFNG* DNA methylation index.