Distinct Methylation of *IFNG* in the Gut

Rivkah Gonsky, Richard L. Deem, and Stephan R. Targan

Mucosal expression of proinflammatory cytokines plays a pivotal role in inflammatory bowel disease (IBD) pathogenesis. Epigenetic remodeling of chromatin via DNA methylation regulates gene expression. In this study, *IFNG* DNA methylation was analyzed within the mucosal compartment in both normal and IBD populations and compared to its peripheral counterparts. Overall *IFNG* methylation (across eight CpG sites) was significantly lower in lamina propria (LP) T cells compared to peripheral blood (PB) T cells. No methylation differences were detected when comparing PB T derived from normal to IBD patients. However, LP T-cell DNA derived from IBD patients displayed different levels of *IFNG* methylation of the upstream regulatory regions compared to DNA from normal controls. In fact, *IFNG* DNA promoter methylation levels functionally correlate with *IFNG* mRNA expression in unstimulated T cells, using quantitative real-time PCR. A 5% decrease in promoter methylation status is associated with nearly a 3-fold increase in *IFNG* expression. Likewise, methylation of the single -54 bp *IFNG* *Sna*B1 site strongly inhibited *IFNG* promoter expression. These results suggest that the epigenetic methylation status of *IFNG* may play a mechanistic role in the modulation of cytokine secretion in the mucosa.

Introduction

INFLAMMATORY BOWEL DISEASE (IBD) is characterized by overexpression of proinflammatory cytokines. Immune dysregulation of activated mucosal T cells is implicated in the pathogenesis of both Crohn's disease (CD) and ulcerative colitis (UC). Cytokines indicative of Th1 polarization (IL-12, IL-18, TNF ligand-related molecule-TL1A, IFN- γ) are elevated in inflamed mucosa of animal models as well as human CD (Sartor 1994; Fuss and others 1996; Strober and Fuss 2006). IFN- γ plays an important role in the generation and perpetuation of mucosal inflammation in many animal models of IBD and is central to the induction and perpetuation of CD. UC cytokine expression is less defined and thought to result from decreased Th1 cytokine responses possibly mediated by Th17 production (Kobayashi and others 2008). Not only mucosal expression of IFN- γ and other proinflammatory cytokines is critical to the development and maintenance of inflammation but also the absolute amount of IFN- γ appears to modulate the severity of colitis (Blumberg and others 1999). In addition, recent clinical studies suggest that humanized anti-IFN- γ antibody is effective in achieving response and remission in CD patients (Hommes and others 2006; Reinisch and others 2006).

T-cell production of IFN- γ is determined primarily at the transcriptional level. Cis- and trans-regulatory regions play

a key role in this process. Previously, we identified mucosa-specific cis- and trans-regulatory mechanisms in LP T cells that are distinct from those in PB T cells or T-cell lines. In fact, the activation pathways of LP T cells are distinct from those of PB T cells. LP T cells do not respond well to activation via the TCR/CD3 receptor, yet they do exhibit increased proliferation and cytokine production when activated via the CD2 pathway. LP T cells are generally thought to manifest a heightened activation state compared to PB T cells. This activated state can be further amplified in conditions of dysregulated inflammation, such as CD and UC.

An additional level of transcriptional regulation occurs through changes in the epigenetic chromatin structure, allowing for accessibility of these trans-acting factors to bind the DNA of the cytokine locus. These changes come about largely through DNA methylation on the cytosine residues at CpG dinucleotides, and through changes in the chromatin structure via modifications of the histone structure. Published reports suggest that the DNA methylation status of cytokine promoters correlates with transcriptional activation (Lee and others 2001, 2002; Young and others 1994). Results from studies of T-cell lines and primary PBL and NK cells have suggested that *IFNG* is methylated in transcriptionally silent cells and hypomethylated in cells poised to secrete IFN- γ (Pang and others 1992; Young and others 1994; Fitzpatrick and

others 1998, 1999; Tato and others 2004). Early studies defined the -54 bp CpG dinucleotide within the proximal regulatory element as a site-specific hypomethylation region associated with transcriptional competence in CD4⁺ T clones (Young and others 1994). It was further demonstrated that this region is methylated differently during Th2 differentiation and following HIV infection, which is accompanied by down-regulation of *IFNG* expression (Mikovits and others 1998). Results from a study comparing the methylation profiles of DNA from adult T cells to that of neonatal T cells outside this region suggested that regions further upstream and downstream may be associated with transcriptional competence as well (White and others 2002). Two recent studies have corroborated and expanded upon these findings by suggesting that appropriate regulation of *IFNG* expression in murine Th1 clones involves coordinated epigenetic regulation of histone and CpG methylation patterns in regions extending over 100 kb (Chang and Aune 2007; Schoenborn and others 2007).

Despite the pivotal role of cytokine dysregulation in the pathogenesis of IBD, the epigenetic mechanisms regulating cytokine expression are unknown. No studies have evaluated the methylation status of *IFNG* within the mucosal immune compartment of the gut in IBD and most other methylation studies have used an *in vitro* Th1-skewed cell population or clonal analysis. The investigations reported herein were based on the hypothesis that freshly isolated LP T cells are inherently activated and poised *in vivo* to secrete IFN- γ , and, therefore, would display a less methylated profile than their PBL counterparts. This study demonstrates that *IFNG* DNA from mucosal LP T cells is less methylated compared to PB T cells. Furthermore, mucosal, but not peripheral, T cells from patients with IBD display distinct DNA methylation patterns

of the *IFNG* locus compared to LP T cells from normal controls. The data suggest that epigenetic control of DNA methylation of the *IFNG* promoter may be functionally important in regulation of the *IFNG* transcription within the mucosal immune compartment of the gut.

Materials and Methods

Isolation of T cells

PBMC were isolated from healthy volunteers or IBD patients by separation on Ficoll-Hypaque gradients. Intestinal specimens were obtained from patients undergoing surgical resection of the colon at Cedars-Sinai Medical Center, Los Angeles. Approval for the use of human tissue was granted by the Institutional Review Board at Cedars-Sinai Medical Center. An informed consent was obtained from all participating subjects. In this study, all tissue specimens were taken from an uninvolved area of colon resected from non-IBD patients with colonic carcinoma (control), involved and uninvolved areas from patients with UC and CD, as defined in Table 1. Lamina propria mononuclear cells (LPMC) were isolated from the resection samples using a technique modified from that described previously (Shanahan and others 1987). CD3⁺ T cells were isolated using CD3-immunomagnetic beads (Miltenyi Biotec, Auburn, CA) and were at least 95% pure. The diagnoses for UC and CD were based on the presence of characteristic endoscopic findings and the results of pathological examination. The medications and demographics of the patients from which the lamina propria (LP) and peripheral blood (PB) samples were obtained are outlined in Table 1.

TABLE 1. PATIENT DEMOGRAPHICS

Demographic	Source					
	Lamina propria			Peripheral		
	CD	UC	Control	CD	UC	Control
Number	23	20	25	90	73	41
Sex						
Male	12	12	17	54	43	15
Female	11	8	8	36	30	26
Age						
Mean	37	38	66	39	46	40
Range	22–73	10–76	37–91	20–78	19–73	24–61
Location						
Colon	16	19	23	—	—	—
Ileum	8	1	3	—	—	—
Gross pathology						
Inflamed	20	20	0	—	—	—
Normal	11	8	25	—	—	—
IBD medication						
None	5	6		10	18	—
Salicylates	5	2		46	38	—
6MP	1	2		47	42	—
Steroids	7	5		45	38	—
Anti-TNF- α	2	2		46	49	—
Other	4	1		4	2	—

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

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 µg 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 2. 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.

Site-specific methylation of *IFNG* promoter-reporter constructs and cell transfection

IFNG promoter-reporter constructs -2.7 kb, -538 bp, and -204 bp were as previously described (Gonsky and others 2000). *IFNG* promoter-reporter constructs were methylated at the single -54 bp *SnaBI/BsaA1* site with the *BsaA1* methylase expression vector pIH919/*BsaA1* (gift from New England Biolabs, Ipswich, MA) following cotransformation. Non-methylated *IFNG* promoter-reporter constructs were cotransformed with empty pIH919 vector. Resistance to *SnaBI* digestion confirmed vector methylation. PBMC or LPMC were transfected as previously described (Gonsky and others 2000). A control plasmid containing the β -actin promoter driving a Renilla luciferase (provided by Dr. Christopher Wilson, University of Washington) was cotransfected as an internal standard. Cells were activated

with anti-CD2 antibodies (clones CB6 and GD10, a gift from Chris Benjamin, Biogen, Cambridge, MA) at 0.1 µg/10⁶ cells or PMA/ionomycin (PMA 40 ng/mL and ionomycin 1 µg/mL, Sigma Chemical Company, St. Louis, MO) for 4 h and luminescence was determined.

Quantitative RT-PCR

Total RNA was isolated from CD3⁺ T cells and gene expression was measured by real-time quantitative RT-PCR. Five hundred nanograms of total RNA were used in each RT reaction, with oligo(dT) as primer, using the Omniscript kit and protocol (Qiagen). *IFNG* primer and probe sequences were as follows: 5'-GTG TGG AGA CCA TCA AGG AAG ACA-3', 5'-CAG CCA TCA CTT GGA TGA GTT CAT GT-3', and 5'/56-FAM/CGG TAA CTG ACT TGA ATG TCC AAC GCA/3BHQ_1/-3'. Levels of gene expression were normalized to β -actin expression.

Statistical analysis

The methylation index (MI) for each sample was calculated as the average values of $^mC/(^mC + C)$ for CpG sites within defined regions of the *IFNG*. Tests for statistical significance: parametric (Student's *t*-test or Tukey-Kramer HSD) or nonparametric (Spearman's rank correlation coefficient) analyses were determined as applicable using JMP Statistical Software (SAS Institute GmbH, Heidelberg, Germany).

Results

Methylation of *IFNG* differs in LP T cells compared to PB T cells regardless of disease status

Decreased DNA methylation is commonly associated with a poised state of transcriptional responsiveness. Therefore, we hypothesized that the heightened activation state of LP T cells compared to PB T cells would be reflected in an altered DNA methylation profile. We further wished to determine

TABLE 2. PCR AND PYROSEQUENCING PRIMERS AND CONDITIONS

Assay CpG	Primers	PCR conditions
-295, -186	Fwd PCR GTGAATGAAGAGTTAATATTTTATTAGG Rev PCR Biotin-TTCCTTTAAACTCCTTAAATCCTT	94°C 15 min 52°C 30 s ×45 cycles 72°C 5 min
-295	Seq GTGAATGAAGAGTTAATATTTTATTAGG	72°C 15 s
-186	Seq GGTGGGTATAATGGGTTTG	
	Fwd PCR GGATTTAAGGAGTTTAAAGGAAA	95°C 15 s
-54	Rev PCR Biotin-AAAACAATATACTACACCTCCTCT Seq TTA AAAAATTTGTGAA	94°C 15 min 52°C 30 s ×45 cycles 72°C 5 min
	Fwd PCR TTTTGGATTGATTAGTTTGATA	72°C 15 s
122, 128, 171	Rev PCR Biotin-AAATCCTAACAATAACAACCA-3	95°C 15 s
122, 128	Seq GATTAGTTTGATATAAGAAT	94°C 15 min 58°C 30 s ×45 cycles 72°C 5 min
171	Seq GGAAAYGATGAAATAT	72°C 15 s
	Fwd PCR GATATTAGTATTAGGTGGGGGAGA	95°C 15 s
-16092	Rev PCR CCCATATCCTTCCTATAAATCA Seq AGGTGGGGGAGAAGA	95°C 15 min 56°C 30 s ×45 cycles 72°C 5 min
	Fwd PCR TGGGAAGTTAGGTTAGAATTGGTT	72°C 15 s
-16241	Rev PCR CAAAAATTTCCCCCAAATACCT Seq CCAAAATACCTTCCATCT	95°C 15 min 56°C 30 s ×45 cycles 72°C 5 min
		72°C 15 s

whether differences in DNA methylation are associated with disease. Bisulfite/pyrosequencing assays were designed to analyze methylation patterns of eight CpG islands, within the regulatory conserved noncoding sequence (CNS-22), proximal promoter, or immediate transcribed *IFNG* regions (Fig. 1A). The overall MI was calculated for each sample as the mean value of the fraction of methylated cytosines divided by the sum of unmethylated and methylated cytosines for all CpGs examined at the *IFNG* locus.

DNA methylation of PB T cells isolated from healthy volunteers or IBD patients was compared to LP T cells from control or IBD patients undergoing surgical resection. Table 1 shows the clinical characteristics of control and IBD patients whose PB and LP T cells were examined. Statistical analysis revealed no relationship between methylation level and concomitant drugs or anatomic location (e.g., colon vs. ileum) of the specimen or patient gender. Likewise, although the LP T-cell control group tended to be from an older population, statistical analysis of the methylation level of LP T cell, irrespective of disease status, revealed that there was no correlation between age and MI. The overall MI of the *IFNG* locus in PB T-cell DNA from either normal (44%) or IBD patients' (48%) cells was statistically higher compared

with LP T cells from control (35%, $P < 0.001$) and IBD patients (38%, $P < 0.001$ –0.03) (Fig. 1B). Likewise, differences in the MI were noted when comparing LP T cells from normal controls to LP T cells from IBD patients ($P < 0.05$). No significant difference in MI was seen when comparing CD or UC patients with either PB or LP T-cell populations (data not shown). Matched pairs from uninvolved and involved regions were analyzed for three IBD patients. There was no statistical significant differences in methylation detected between the involved and uninvolved regions (MI was 42.0 for uninvolved and 39.8 for involved). Thus, the overall MI of *IFNG* differs between the peripheral compared to mucosal compartment irrespective of disease status.

IFNG methylation is region-specific and is associated with IBD diagnosis in LP T cells

Recent studies have suggested that multiple conserved nucleotide sequences contribute to regulated expression of *IFNG* (Chang and Aune 2007; Schoenborn and others 2007). Distinct methylation patterns have been observed in these regions in the mouse *IFNG* locus. In particular, in addition to the region immediately upstream and downstream of the transcriptional start site, the conserved noncoding sequence at -22 kb is believed to be essential in regulation of *IFNG* expression, and it has been suggested that it functions as a boundary element marking the end of the *IFNG* regulatory region (Hatton and others 2006). Detailed analysis was carried out to determine whether MI differences in the *IFNG* were regional in nature. The MI of *IFNG* was broken down into the three different regions: CNS-22 (mean of MI at -16092 and -16241 bp), proximal promoter (mean of MI at -295 , -186 , -54 bp), and the transcribed region (mean of MI at $+122$, $+128$, and $+171$ bp). *IFNG* CpG methylation differs in LP T cells compared to PB T cells across all regions examined, regardless of disease status (Fig. 2A and Table 3). However, the nature of these differences varies among regions. The CNS-22 element was found to be the least methylated of all *IFNG* CpG regions examined. This region was almost completely demethylated ($>90\%$) in DNA from PB T cells. Surprisingly, LP T cells were more methylated (10.5) compared to PB T cells (4.0). In contrast, the regions flanking the transcription start site, both upstream (promoter) and downstream (transcribed), displayed significantly decreased CpG methylation in LP T cells compared to PB T cells. Methylation of the *IFNG* promoter inversely correlates with the CNS-22 region whereas a positive correlation was detected for the transcribed region (Fig. 2B). Thus, higher *IFNG* promoter methylation correlates with lower CNS-22 methylation.

The data from Figure 2 was further stratified based on disease status. Methylation profiles differ between LP T-cell populations based upon diagnosis of IBD. LP T-cell DNA from IBD patients differs from normal controls for the CNS-22 and promoter regions, with an inverse relationship between these regions (Fig. 3 and Table 3). LP T cells from IBD patients were less methylated for the CNS-22 region and more methylated for the promoter region compared to normal controls. No significant differences in methylation of *IFNG* were seen for the transcribed region. No regional methylation differences were detected within the peripheral compartment when comparing PB T cells from normal or

FIG. 1. DNA methylation index of *IFNG* from PB T cells compared to LPT cells. (A) Diagram of CpG sites in human *IFNG* locus analyzed (not to scale). Rectangles mark CpG sites and arrow transcriptional start site. (B) Overall methylation index of PB T cells compared to LPT cells categorized by disease status. The overall methylation index is a composite of all *IFNG* sites defined in A. Means $\pm$ SEM are displayed. Number of subjects is as defined in Table 1.

FIG. 2. Region-specific *IFNG* CpG methylation from PB T cells compared to LP T cells. Analysis includes both normal and IBD patients for each compartment. (A) Region-specific methylation. Means $\pm$ SEM are displayed. (B) Correlation of methylation status of *IFNG* promoter region with CNS-22 and transcribed regions for both PB T and LP T cells. Number of subjects is as defined in Table 1. Spearman's correlation coefficient is -0.28 ($P < 0.001$) for the CNS-22 and 0.64 ($P < 0.001$) for the transcribed regions.

IBD patients. Therefore, while epigenetic differences are evident when comparing PB T to LP T populations, it seems likely these differences involve complex epigenetic regulation of multiple regulatory regions.

Methylation of -54 bp CpG functionally silences *IFNG* promoter activity in both PBMC and LPMC

The -54 bp *IFNG* CpG island lies within the promoter region and represents a conserved *SnaBI* site, which has been the most widely studied of all *IFNG* methylated regions. Recent studies in the mouse have suggested that methylation of the corresponding -53 bp CpG dramatically inhibits *IFNG* promoter expression (Jones and Chen 2006). Considering the strong correlation observed between hypomethylation of this site and *IFNG* expression in T cells,

TABLE 3. DIFFERENTIAL DEMETHYLATION OF *IFNG* IN LP T CELLS

Cell source		<i>P</i> value*		
		CNS-22	Promoter	Transcribed
PB T all	vs. LP T all	<0.001	<0.001	<0.001
PB T NL	PB T IBD	ns (>0.3)	ns (>0.05)	ns (>0.3)
PB T NL	vs. LP T NL	<0.001	<0.001	<0.001
PB T NL	vs. LP T IBD	<0.003	<0.004	<0.004
PB T IBD	vs. LP T NL	<0.001	<0.001	<0.001
PB T IBD	vs. LP T IBD	<0.03	<0.001	<0.001
LP T NL	vs. LP T IBD	<0.005	<0.02	ns (>0.2)

*ns, *P* values not significant.

we wished to study whether artificial methylation of this site could attenuate *IFNG* promoter expression. To determine the effect of methylation of this site on transcriptional silencing of the human *IFNG* promoter, we utilized a *BsaAI* methylase, which selectively methylates the single -54 bp *SnaBI/BsaAI* CpG site, while leaving all other CpG islands intact (Fig. 4A). The effect of methylation of this single site was studied in transfected PBMC and LPMC. As seen in Figure 4B, selective methylation of the -54 bp CpG site inhibited promoter expression by 60–80% in both PBMC and LPMC. Moreover, inhibition was detected in the -204 and -538 bp promoter, as well as the -2.7 kb *IFNG* promoter constructs, suggesting that methylation of this single site alone significantly influenced expression over extended promoter regions.

Hypomethylation is associated with enhanced *IFNG* expression

Since demethylated DNA generally correlates with a predisposed state of gene expression, and since this study

FIG. 3. Region-specific *IFNG* CpG methylation from PB T cells compared to LP T cells categorized by disease status. Means $\pm$ SEM are displayed. Statistical significance of data is displayed in Table 3. Number of subjects is as defined in Table 1.

FIG. 4. Site-specific methylation of -54 bp CpG inhibits *IFNG* promoter activity. **(A)** Diagram of human *IFNG* promoter-reporter constructs used. Arrow marks transcriptional start site. **(B)** Single-site methylation of the -54 bp CpG by *BsaA1* methylase was carried out with the indicated *IFNG* promoter-reporter constructs. Constructs were transfected into PBMC and LPMC and promoter activity of methylated vector compared to unmethylated vector was determined following stimulation with anti-CD2 antibodies or PMA/ionomycin. Representative of three experiments with similar results.

demonstrates that the *IFNG* locus in freshly isolated LP T cells is inherently demethylated *in vivo* compared to PB T cells, quantitative real-time PCR analysis was performed to assess the expression of *IFNG* mRNA in unstimulated LP T compared to PB T cells. As seen in Figure 5A, the levels of

spontaneous *IFNG* mRNA expression are low in unstimulated cells. Nevertheless, LP T cells expressed significantly more *IFNG* mRNA compared to PB T ($P < 0.001$). More importantly, a significant inverse correlation ($P < 0.001$) was observed between the *de novo* *IFNG* expression and the corresponding methylation status of the promoter *IFNG* region (Fig. 5B). In fact, a 5% decrease in methylation is associated with nearly a 3-fold increase in *IFNG* expression. Likewise a significant, though less pronounced, inverse correlation ($P < 0.04$) was observed between the *de novo* *IFNG* expression and the corresponding methylation status of the transcribed region (Fig. 5B). However, no correlation was found for the CNS-22 region (Fig. 5B).

Discussion

In the present study, we used bisulfite/pyrosequencing and functional studies to identify regional epigenetic features of *IFNG* that differ between mucosal and peripheral T cells. Previous *IFNG* epigenetic studies have mostly utilized Th1-skewed T-cell lines and clone models rather than primary T-cell populations. To our knowledge, the study described herein is the first to address whether unstimulated LP T cells inherently display different methylation levels of a key Th1 cytokine distinct from those seen in PB T cells. Moreover, distinct *IFNG* gene methylation can be seen within the mucosal T-cell compartment in IBD. Hypomethylation of *IFNG* is seen in mucosally derived LP T cells compared to their PB counterparts across all regions examined. In IBD, the *IFNG* LP T-cell MI is lower for the far upstream CNS-22 region, whereas regions flanking the transcriptional start site display higher MI compared to those from normal patients. No methylation differences were seen for the peripheral compartment when comparing DNA from normal to IBD patients. Moreover, on a functional level, hypomethylation of regions flanking the *IFNG* transcriptional start site correlates to the level of *IFNG* expression even in unstimulated

FIG. 5. *IFNG* mRNA expression in unstimulated PB compared to LP T cells. **(A)** *IFNG* mRNA as determined by real-time PCR in PB T cells versus LP T cells. **(B)** Correlation of *IFNG* mRNA levels to methylation status of the CNS-22, promoter, and transcribed *IFNG* CpG regions for PB T and LP T cells. Spearman's correlation coefficient is -0.06 ($P < 0.001$) for the promoter and 0.14 ($P < 0.04$) for the transcribed regions.

T cells, with a decrease in methylation levels associated with marked enhancement of *IFNG* expression.

Enhanced *in vitro* induced LP Th1 cytokine expression, and in particular IFN- γ expression, has clearly been associated with CD (Sartor 1994; Fuss and others 1996; Strober and others 1998). In this report, we show that freshly isolated LP T cells from IBD patients are less methylated at the *IFNG* locus compared to PB T cells. It is interesting to note that in LP T cells from control specimens, a further drop in the methylation is detected. These results seem at first glance to be contradictory to the hypothesis that the DNA methylation status correlates with *IFNG* gene expression. However, while all *IFNG* regions displayed decreased DNA methylation of LP compared to PB T cells, decreased DNA methylation of control compared to IBD specimens was attributed only to the reduced methylation of the promoter region. In fact, even though the CNS-22 site is almost completely demethylated in both LP and PB T cells, an enhanced methylation profile was detected when comparing LP T cells from control subjects to IBD patients. The functional significance of subtleties in DNA methylation levels may be related to the fact that it has been proposed that CNS-22 *IFNG* functions as both an enhancer and boundary element protecting *IFNG* expression from interference by flanking regulatory sequences. Although a strong correlation exists between DNA methylation and promoter activity, as noted earlier, methylation does not solely account for differential gene expression. Even within the *IFNG* locus, hypomethylation has been reported in epithelial cell lines incapable of secreting IFN- γ (Fukunaga and others 1986; Kiyomasu and others 1999). CD4⁺ T cells, which normally do not secrete IFN- γ , can be primed with IL-4, in the absence of TCR stimulation, to secrete large amounts of IFN- γ in response to PMA/ionomycin activation (Kiyomasu and others 1999). These cells differ in their DNase I hypersensitivity and do not require promoter hypomethylation for *IFNG* expression. Furthermore, the *IFNG* locus is hypomethylated in resting NK cells prior to activation. Our present data suggest that the regions flanking the *IFNG* transcriptional start site correlates with *IFNG* mRNA expression while the regulatory CNS-22 does not. Thus, discrepancies have been noted between DNA methylation patterns in Th1 and Th2 cytokine loci, suggesting that additional regulatory events might be utilized by these cells to modulate cytokine expression. Regulation of gene expression is probably mediated by multiple epigenetic regions and regulatory events as well as subtle differences in methylation levels.

It is important to keep in mind that other cytokine gene methylation studies have used methylation-sensitive restriction enzymes or methylation-specific PCR reactions. Although the information these studies have provided is useful, it is only semiquantitative. The degree of DNA conversion following bisulfite treatment is hard to ascertain, making the data difficult to interpret. Pyrosequencing technology utilized in this study is highly sensitive and quantitative, allowing precise determination of the ratio of methylated CpGs in DNA, which might otherwise go undetected by other techniques. Additionally, most studies looking at *IFNG* secretion in IBD have compared cells from controls to CD and UC following stimulation. In fact, the level of IFN- γ secretion in unstimulated LP T cells, irrespective of source, is barely, if at all, detectable (Lieberman and others 1988; West and others 1996; Gotteland and others

1999). In this study, we have examined the methylation status in freshly isolated cells prior to stimulation. We believe this study provides an indication of the native status of the *IFNG* gene and serves as measure of the poised epigenetic status of the gene *in vivo*. The enhanced secretion observed in IBD may well result from additional activation modalities aside from an epigenetic poised state.

In conclusion, this is the first study to identify selective epigenetic modifications at the level of DNA methylation of a key Th1 cytokine locus within the mucosal immune compartment. Compared to peripheral T cells, mucosal T cells exhibit lower MI of the *IFNG* locus. Differential *IFNG* methylation is detected within the mucosal, but not peripheral, compartment of patients with IBD. These studies suggest that methylation patterns of cytokine loci may play a role in regulation of mucosal cytokine secretion.

Acknowledgments

We thank Jimmy Nguyen for providing cultured lamina propria mononuclear cells. This work was supported by United States Public Health Service Grants DK043211 and DK046763 and Cedars-Sinai Medical Center Inflammatory Bowel Disease Research Funds.

References

- Blumberg RS, Saubermann LJ, Strober W. 1999. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr Opin Immunol* 11(6):648–656.
- Chang S, Aune TM. 2007. Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells. *Nat Immunol* 8(7):723–731.
- Fitzpatrick DR, Shirley KM, Kelso A. 1999. Cutting edge: stable epigenetic inheritance of regional IFN-gamma promoter demethylation in CD44^{high}CD8⁺ T lymphocytes. *J Immunol* 162(9):5053–5057.
- Fitzpatrick DR, Shirley KM, McDonald LE, Bielefeldt-Ohmann H, Kay GE, Kelso A. 1998. Distinct methylation of the interferon gamma (IFN-gamma) and interleukin 3 (IL-3) genes in newly activated primary CD8⁺ T lymphocytes: regional IFN-gamma promoter demethylation and mRNA expression are heritable in CD44^{high}CD8⁺ T cells. *J Exp Med* 188(1):103–117.
- Fukunaga R, Matsuyama M, Okamura H, Nagata K, Nagata S, Sokawa Y. 1986. Undermethylation of interferon-gamma gene in human T cell lines and normal T lymphocytes. *Nucleic Acids Res* 14(11):4421–4436.
- Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. 1996. Disparate CD4⁺ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 157(3):1261–1270.
- Gonsky R, Deem RL, Bream JH, Lee DH, Young HA, Targan SR. 2000. Mucosa-specific targets for regulation of IFN-gamma expression: lamina propria T cells use different cis-elements than peripheral blood T cells to regulate transactivation of IFN-gamma expression. *J Immunol* 164(3):1399–1407.
- Gotteland M, Lopez M, Munoz C, Saez R, Altshiller H, Llorens P, Brunser O. 1999. Local and systemic liberation of proinflammatory cytokines in ulcerative colitis. *Dig Dis Sci* 44(4):830–835.
- Hatton RD, Harrington LE, Luther RJ, Wakefield T, Janowski KM, Oliver JR, Lallone RL, Murphy KM, Weaver CT. 2006. A distal conserved sequence element controls *Ifng* gene expression by T cells and NK cells. *Immunity* 25(5):717–729.

- Hommes DW, Mikhajlova TL, Stoinov S, Stimac D, Vucelic B, Lonovics J, Zakuciova M, D'Haens G, Van Assche G, Ba S, Lee S, Pearce T. 2006. Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. *Gut* 55(8):1131-1137.
- Jones B, Chen J. 2006. Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development. *EMBO J* 25(11):2443-2452.
- Kiyomasu T, Katamura K, Ueno H, Iio J, Ohmura K, Heike T, Furusho K. 1999. Hypomethylation of the proximal and intronic regulatory regions of the IFN-gamma gene is not essential for its transcription by naive CD4+ T cells cultured with IL-4. *Immunol Lett* 69(2):239-245.
- Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, Chinen H, Saito R, Kitazume MT, Nakazawa A, Sugita A, Koganei K, Isobe K, Hibi T. 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* 57(12):1682-1689.
- Lee DU, Agarwal S, Rao A. 2002. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* 16(5):649-660.
- Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, Cherry SR, Tsai JH, Tucker SM, Weaver WM, Kelso A, Jaenisch R, Wilson CB. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15(5):763-774.
- Lieberman BY, Fiocchi C, Youngman KR, Sapatnekar WK, Proffitt MR. 1988. Interferon gamma production by human intestinal mucosal mononuclear cells. Decreased levels in inflammatory bowel disease. *Dig Dis Sci* 33(10):1297-1304.
- Mikovits JA, Young HA, Vertino P, Issa JP, Pitha PM, Turcoski-Corrales S, Taub DD, Petrow CL, Baylin SB, Ruscetti FW. 1998. Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in de novo methylation of the gamma interferon (IFN-gamma) promoter and subsequent downregulation of IFN-gamma production. *Mol Cell Biol* 18(9):5166-5177.
- Pang Y, Norihisa Y, Benjamin D, Kantor RR, Young HA. 1992. Interferon-gamma gene expression in human B-cell lines: induction by interleukin-2, protein kinase C activators, and possible effect of hypomethylation on gene regulation. *Blood* 80(3):724-732.
- Reinisch W, Hommes DW, Van Assche G, Colombel JF, Gendre JP, Oldenburg B, Teml A, Geboes K, Ding H, Zhang L, Tang M, Cheng M, van Deventer SJ, Rutgeerts P, Pearce T. 2006. A dose escalating, placebo controlled, double blind, single dose and multidose, safety and tolerability study of fontolizumab, a humanised anti-interferon gamma antibody, in patients with moderate to severe Crohn's disease. *Gut* 55(8):1138-1144.
- Sartor RB. 1994. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. *Gastroenterology* 106(2):533-539.
- Schoenborn JR, Dorschner MO, Sekimata M, Santer DM, Shnyreva M, Fitzpatrick DR, Stamatoyannopoulos JA, Wilson CB. 2007. Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. *Nat Immunol* 8(7):732-742.
- Shanahan F, Brogan M, Targan S. 1987. Human mucosal cytotoxic effector cells. *Gastroenterology* 92(6):1951-1957.
- Strober W, Fuss IJ. 2006. Experimental models of mucosal inflammation. *Adv Exp Med Biol* 579:55-97.
- Strober W, Ludviksson BR, Fuss IJ. 1998. The pathogenesis of mucosal inflammation in murine models of inflammatory bowel disease and Crohn disease. *Ann Intern Med* 128(10):848-856.
- Tato CM, Martins GA, High FA, DiCioccio CB, Reiner SL, Hunter CA. 2004. Cutting Edge: Innate production of IFN-gamma by NK cells is independent of epigenetic modification of the IFN-gamma promoter. *J Immunol* 173(3):1514-1517.
- West GA, Matsuura T, Levine AD, Klein JS, Fiocchi C. 1996. Interleukin 4 in inflammatory bowel disease and mucosal immune reactivity. *Gastroenterology* 110(6):1683-1695.
- White GP, Watt PM, Holt BJ, Holt PG. 2002. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO+ T cells. *J Immunol* 168(6):2820-2827.
- Young HA, Ghosh P, Ye J, Lederer J, Lichtman A, Gerard JR, Penix L, Wilson CB, Melvin AJ, McGurn ME. 1994. Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. *J Immunol* 153(8):3603-3610.

Address correspondence to:

Dr. Stephan R. Targan
Inflammatory Bowel Disease Research Center
Cedars-Sinai Medical Center
8700 Beverly Blvd., D4063
Los Angeles, CA 90048

Tel: 310-423-7723

Fax: 310-423-0224

E-mail: targans@cshs.org

Received 23 November 2008/Accepted 15 January 2009