Published in final edited form as:

J Acquir Immune Defic Syndr. 2014 January 1; 65(1): 19–26. doi:10.1097/QAI.0b013e3182a1bca4.

Epigenetic modification of *FOXP3* in patients with chronic HIV infection

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Abstract

Objectives—Human immunodeficiency virus type 1 (HIV-1) modulates host cell epigenetic machinery to control its own replication and induce immune suppression. HIV-1 infection leads to activation of T regulatory cell (T_{reg}), but the mechanism underlying this immune modulation is unclear. T_{reg} plays a prominent role in gut-mucosal immune tolerance by restraining excessive effector T-cell responses, a mechanism that is known to be disturbed in chronic HIV-1 infection. DNA methylation plays a major role in T_{reg} lineage commitment and immune homeostasis, which may be regulated by HIV. To investigate the mechanisms of aberrant methylation of the T_{reg} marker *FOXP3* in HIV-1 infection, we evaluated the expression pattern of methylation related enzymes and its correlation to *FOXP3* methylation.

Methods—*FOXP3* promoter methylation in the colon mucosa and peripheral blood from HIV-infected patients and control subjects was measured using Pyrosequencing. Gene expression pattern of DNA methylation enzymes in the colon mucosa was investigated by Microarray and quantitative rt-PCR analysis in the same subjects.

Results—*FOXP3* promoter was significantly ($p < 0.0001$) demethylated in HIV-infected patients compared to control subjects in both tissues. Expression of DNA methyltransferase 1 (*DNMT1*), DNA methyltransferase 1-associated protein 1 (*DMAPI*), methyltransferase like 7B (*METTL7B*), and methyltransferase like 10 (*METTL10*), were significantly down regulated in

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This work was partially presented at HIV & Liver diseases Sept 6-8, 2012 in Jackson Hole, WY, USA and 14th International meeting of Institution of Human Virology, Baltimore, MD Oct 14-17, 2012.

Financial Support. This investigation was supported by Merck Investigator Initiated Studies [IISP #: 38879 (Shata)]; National Institute of Health Grant [K24DK070528 (Sherman)]; and was supported in part by Public Health Service Grant [P30 DK078392 (The Gene Expression Microarray Core Cincinnati Children's Hospital Medical Center)]; National Institute of Health Grant [NIH P30 DK078392 (Bioinformatics Core of the Digestive Disease Research Core Center in Cincinnati)].

Potential conflicts of interest. The authors do not have a commercial or other association that might pose a conflict of interest.

HIV-infected patients compared to controls and had a significant positive correlation to *FOXP3* promoter methylation.

Conclusion—We present evidence suggesting that altered methylation pattern of *FOXP3* and accordingly higher T_{reg} frequency in gut mucosa of HIV infected patients may be due to aberrant methylation processing in HIV.

Keywords

HIV; FOXP3; Methylation; Epigenetics; Methylation enzymes

Introduction

Epigenetic modification of DNA leads to heritable changes in gene expression that are not coded by DNA sequence. The most commonly studied epigenetic changes include DNA methylation, and chromatin modification. The importance of epigenetic changes such as the methylation of 5'-cytosine located in the CG-rich regions (islands) of DNA has emerged in cancer, autoimmune and inflammatory diseases.^{1, 2} The CpG islands are defined as short (0.2-2 kb) DNA regions with highly enriched cytosine/ guanine dinucleotide.³ These CG-rich regions are usually located with higher frequency at the 5' end of genes and are associated with transcriptional promoters.⁴ Most promoter CpG islands are non-methylated or methylated at low levels regardless of transcription status, while high methylation of promoter CpG islands is commonly associated with repression of their transcription.¹

Epigenetic modifications can modulate HIV-1 integration, transcription and latency of infection. Integration of HIV-1 into the host genome is not likely to occur in transcriptionally inactive, “methylated” regions of the genome.⁵ In latently infected CD4⁺ T cells, the transcription of integrated HIV-1 was suppressed by methylation of its promoter.⁶ Epigenetic modification of host genes can presumably affect HIV-1 transcription and replication. Furthermore, HIV-1 can induce *de novo* methylation of the host T cell-specific genes through the induction of DNA methyl transferase 1 (*DNMT1*) *in vitro*.⁷ Recent data suggested that methylation of 5'- LTR of HIV is associated with the control of viral replication in a subset of patients characterized as long-term nonprogressor and elite controllers.⁸

The gastrointestinal (GI) tract houses most of the body's lymphocytes and the GI mucosa represents a key compartment for HIV replication.⁹ DNA methylation regulates lineage commitment of lymphocyte subset T regulatory cell (T_{reg}),¹⁰ which has an essential role in gut mucosal immune tolerance.¹¹ T_{reg} are CD4-positive (CD4⁺) T cells that inhibit immunopathology or autoimmune disease *in vivo*. The function of T_{reg} depends on the expression of the transcription factor *FOXP3* (forkhead box P3), which is considered the master switch for T_{reg}. There are 3 conserved regions for methylation of *FOXP3* in T_{reg}: *FOXP3* promoter, TGFβ-sensor, and TSDR-enhancer regions, which are differentially methylated in different subsets of T cells.^{10, 12} *FOXP3* promoter and TGFβ-sensor are mainly demethylated in stable and induced T_{reg} respectively. T_{reg} cells inhibit immune responses by restraining excessive effector T-cell responses. Accumulated data suggested that there is increased frequency of T_{reg} among CD4⁺ T cells in gastrointestinal mucosal tissue in SIV and HIV-1 infection.¹³ This increase in frequency of mucosal T_{reg} was specifically found in HIV-1 infection but not in other viral infection such as Norovirus.¹⁴ However, the role of T_{reg} in HIV infection is still controversial. Increased T_{reg} frequency is associated with limited immune activation in HIV exposed- uninfected neonates and adults,^{15, 16} and in ART treated patients¹⁷, which has a beneficial effect to the host. On the other hand, T_{reg} might exacerbate HIV infection by down regulation of specific immune responses toward the virus.¹⁸

The present study was designed to examine how HIV-1 infection modifies methylation of the genome, particularly in immune-related genes by which the virus can evade the host immune system, its association with clinical outcomes and possible underlying mechanisms. Specifically, we measured the levels of DNA methylation within *FOXP3* promoter (as a biomarker for T_{reg}) in peripheral blood mononuclear cells (PBMCs) and colon mucosa and studied how HIV-1 infection alters epigenetic modification of *FOXP3*. We also investigated the effect of aberrant DNA methylation level on *FOXP3* gene and protein expression. In addition, we examined the relationship between *FOXP3* methylation and clinical profile of HIV-1 infected patients and its correlation with immunological and virological status. Furthermore, we evaluated the expression pattern of methylation related enzymes in the colon mucosa and its correlation to *FOXP3* methylation.

Methods

Patients and Methods

All participants were recruited from University of Cincinnati clinics. Thirty ml of blood and 3 colonic mucosal biopsies 1-3 mm in size from the distal colon (30-45 cm from the anal verge) were obtained from patients and controls using flexible sigmoidoscopy according to the standard procedure. Consent forms were obtained from participating subjects according to a protocol approved by the University of Cincinnati School of Medicine Human Studies Committee, and Institutional Review Board. To study the effect of HIV-1 infection on *FOXP3* promoter methylation, 10 non-infected controls and 10 HIV-1 infected subjects were enrolled in the study. All HIV-infected patients were receiving anti-retroviral treatment for a median of 11 years. Half of the HIV-1 infected patients were coinfecting with HCV. Only 2 subjects were receiving anti-HCV treatment at the time of sample collection. Demographic data and characterization of the enrolled subjects are summarized in (Table 1). Viral loads were determined in patients' plasma using COBAS AmpliPrep/COBAS *taqMan* HIV-1 Test v2.0 (Roche Diagnostics, Indianapolis, IN) with a threshold of 40 copies/ml. PBMCs were isolated from the blood by density gradient using Ficoll-Paque Plus, Histopaque (Sigma, St. Louis, MO) according to manufacturer's instruction. PBMCs were washed twice in RPMI-1640 (Gibco, Carlsbad, CA), counted and used immediately or stored in 10% DMSO and 90% fetal bovine sera (FBS) (Gibco) at -80°C for further analysis.

DNA Isolation and Na Bisulfite Modification

Tissue biopsies saved in RNA later was disrupted and the lysate was homogenized in the appropriate volume of Buffer RLT Plus (Qiagen, Valencia, CA). The lysate was then centrifuged at maximum speed and the supernatant was collected and used for isolation of DNA using All Prep DNA/RNA Mini Kit (Qiagen) and treated with sodium bisulfite EZ DNA methylation direct kit (Zymo research, Irvine, CA) to convert unmethylated cytosine into uracil while methylated cytosine remained unchanged.¹⁹ Bisulfite modified DNA was amplified using PCR primers for a specific CpG islands in *FOXP3* promoter area (Qiagen). *SssI* treated human genomic DNA was used as 100% methylation control and human genomic DNA amplified by GenomePlex-Complete Whole Genome Amplification Kit (Sigma St. Louis, MO) was used as the non-methylated DNA control.

Pyrosequencing

The PCR product was measured by quantitative Pyrosequencing using PyroMark Q96 MD (Qiagen) in the Pyrosequencing core Lab for Genomic and Epigenomic research (Division of Asthma Research, CCHMC). Data was determined using the pyro Q-CpG methylation software (Qiagen) and was presented as percent methylation of each CpG dinucleotide tested.

Microarray Analysis

Gene expression pattern were investigated by Chip Selection Human Gene 1.0 ST microarray according to manufacturer's instructions. For detailed description please see SDC 1.

Quantitative RT-PCR and Gene Expression Analysis

RNA was converted to cDNA using QuantiTect reverse transcription kit (Qiagen). The prepared cDNA was amplified using QuantiTect primer assay for *FOXP3* (NM_014009), *METTL10* (NM_212554), *METTL7* (NM_152637), *DMAP1* (NM_019100), *DNMT1* (NM_001379), and *GAPDH* (NM_002046) and the QuantiTect SYBER Green PCR kit (Qiagen) according to manufacturer's instructions.

Immunohistochemical (IHC) Staining

FOXP3 protein expression was evaluated by Immunohistochemistry. For detailed description of procedure and analysis please see SDC 1.

Statistical Analysis

The Student t test was used to examine differences between groups with a significance value at $p = 0.05$. Correlations between parameters measured were calculated using Spearman's correlation coefficient for patients and controls.

Results

For promoter methylation, the average percent methylation of the selected CpG sites was compared between the healthy controls and the HIV-1 infected subjects. The initial screening experiment revealed that CpG sites tested in the *FOXP3* promoter area have a significant lower average percent methylation in HIV-1 infected patients compared to the controls. The mean percent methylation and standard deviation in each group was as follows: control Lymphocytes (39.6 ± 1.25), control colon tissue (35.4 ± 2.8), HIV lymphocytes (1.9 ± 0.1) and HIV colon tissue (1.8 ± 0.4). No significant difference was seen between HIV and HIV/HCV coinfecting patients; therefore, in later analysis, we grouped them together. The difference in methylation was statistically significant between patients and controls in both lymphocytes and colon tissue respectively ($p < 0.0001$ and $p < 0.0001$ adjusted for age, gender and race) (Fig. 1A).

The methylation status of *FOXP3* was compared in PBMCs and colon tissue of five HIV-1 infected patients and six control subjects to determine if this altered methylation status is different between the two types of tissues within the same control and HIV infected subjects. The level of *FOXP3* methylation was comparable in PBMCs and colon tissue from the same subject as shown in the heat map methylation profile (Fig. 1B).

To study the effect of *FOXP3* promoter methylation on its gene expression in colon tissue, *FOXP3* gene expression was quantified by RT-PCR. As shown in (Fig. 2), There was significantly ($p=0.009$) higher expression level of the *FOXP3* gene in HIV-infected patient samples compared to controls. The methylation of *FOXP3* promoter was significantly negatively correlated with the relative expression of the gene in colon tissue (Spearman $r = -0.6606$, $p=0.0438$). Immunohistochemistry of colon tissues revealed a higher FOXP3 protein expression level and a higher frequency of infiltrating FOXP3⁺ T_{reg} in colon tissue from HIV-1 infected patients compared to the control, (Mean score $\pm$ SD were 20 ± 1.7 cells/HPF and 2 ± 0.85 cells/HPF, respectively, Fig. 3, SDC 2).

All HIV-infected patients included in this study were receiving anti-retroviral treatment with successful control of viral load except for one patient (1074 copies/ml) (Table1).

The CD4⁺ count in HIV-1 infected patients ranged from 320-581 cells/ μ l with an average $\pm$ SD: 472 $\pm$ 99. In contrast, in the non-infected controls the range was 420-890 cells/ μ l and the average $\pm$ SD was 699 $\pm$ 138. The overall difference in CD4⁺ count was significant between the control and patient groups ($p < 0.000$) (Table 1). The level of methylation of *FOXP3* promoter is significantly positively correlated with CD4⁺ count (Spearman $r = 0.5963$, $p = 0.0055$)

In humans, DNA methylation is maintained by DNA methyltransferases and modified by other methylation enzymes. It has been previously shown that HIV-1 can induce *de novo* methylation of the host T cell-specific genes through the induction of DNA methyltransferase 1 (*DNMT1*) *in vitro*.⁷ To identify possible mechanisms by which HIV infection modifies DNA methylation of host genes *in vivo*, we examined correlation between *FOXP3* promoter methylation in HIV-1 infected patients and controls with the expression levels of DNA methyltransferase enzymes in colon tissue first by microarrays and further validated by quantitative RT-PCR. DNA methyltransferase 1-associated protein 1 (*DMAP1*), methyltransferase like 7B (*METTL7B*), and methyltransferase like 10 (*METTL10*) was significantly down regulated in HIV-1 infected patients compared to the controls in both microarray analysis and qrt-PCR measurement ($p < 0.05$) (Fig. 4A). In addition, we also found that *DNMT1*, which was not included in our array analysis, was significantly down regulated by quantitative RT-PCR (Fig. 4A). There was a significant positive correlation between the methylation level of *FOXP3* and the expression levels of *DNMT1*, *DMAP1*, *METTL7B*, and *METTL10* (Spearman $r = 0.7112$, $p = 0.0254$, $r = 0.8909$, $p = 0.0022$, $r = 0.8667$, $p = 0.0011$; and $r = 0.7455$, $p = 0.0174$ respectively) (Fig. 4B).

Discussion

HIV infection is accompanied by a perturbed gastrointestinal mucosal immune response with subsequent persistent inflammation and viral propagation.⁹ Viruses have developed several mechanisms by which they can escape the host immune response. Aberrant methylation of the HIV-1 and its surrounding environment is one mechanism that might help the virus escape the host immune response and maintain its existence.²² Decreased methylation of the *FOXP3* promoter can lead to the induction and stabilization of T_{reg}, a key player in maintaining immune-homeostasis of the gut mucosa.²³ However, the effect of HIV-1 infection on the epigenetic modification of the *FOXP3* promoter, a marker of T_{reg} cells, is unknown.

The present study was designed to examine the effect of HIV-1 infection on T_{reg} by testing DNA methylation level of *FOXP3* and its expression in colon mucosa and PBMCs from HIV-1 infected subjects. Our results indicate that the tested *FOXP3* promoter area had a significantly lower level of methylation in colon mucosa and PBMCs from chronic HIV-1 infected patients as compared to control subjects (Fig. 1). This lower level of methylation was significantly associated with higher number of T_{reg} infiltrating the colon mucosa of chronic HIV infected patients and is in agreement with earlier reports that showed an increased number of infiltrating T_{reg} in the gut mucosa of HIV-1 infected patients despite lower CD4⁺ counts²⁴ (Fig. 3 and Table1). However, the underlying mechanism of T_{reg} induction in the colon mucosa of HIV infected subjects was not clear. There is strong evidence suggesting that *FOXP3* expression is regulated by DNA methylation of conserved elements in its promoter.¹² Others have also suggested that decreased methylation of *FOXP3* promoter might be considered a marker of higher percentage of natural T_{reg}.²⁵ At the cellular level, CD4⁺CD25^{hi}*FOXP3*⁺ T cells (T_{reg}) were not methylated, while

CD4⁺CD25^{lo} (activated T cells) displayed intermediate *FOXP3* methylation.²⁶ Additionally, *FOXP3* expressing cells exhibited suppressive abilities that correlate to the methylation status of the *FOXP3* promoter.²⁶ Moreover, the use of demethylation agents such as 5 aza-2' deoxycytidine leads to increased expression of *FOXP3* in NK cells.²⁷ Collectively, our data suggested that decreased methylation of *FOXP3* promoter of mucosal T cell populations is the cause of increased *FOXP3* expression and consequently increased T_{reg} frequency in chronic HIV infection.

We next tested the difference in *FOXP3* methylation in two tissues (PBMCs and colon tissue) obtained from the same individual assuming that there will be an intra-tissue methylation difference as reported earlier³. No difference in the methylation level of *FOXP3* promoter region in colon tissue or PBMCs either in control or HIV-infected subjects were observed. This suggests that the lower level of methylation in *FOXP3* promoter is a systemic effect of HIV infection. It also indicates that the region we investigated is not a tissue specific differentially methylated region; instead, methylation level in this region may serve as a cross-tissue, uniform biomarker for HIV infection. Several mechanisms have been suggested to explain the increased frequency of T_{reg} during the course of HIV infection.²⁸ Increased proliferation and expansion of T_{reg} population in the gut was documented earlier. However, this proportional T_{reg} expansion was controlled by anti-retroviral treatment.²⁹ In this study all patients were receiving antiretroviral treatment and their viral load was controlled, except one, which suggests that T_{reg} expansion does not play a role in the methylation pattern observed in the HIV- patients examined. Another possibility is the increased homing of *FOXP3*⁺ T_{regs} from blood to the lymphoid tissue in the gut,³⁰ which is associated with slower restoration of CD4⁺ T in the gut compared to the blood in treated HIV-infected patients.³¹ This dynamic change in T cell homing might lead to apparent increase in gut T_{reg}. However, we noticed the same level of *FOXP3* demethylation in both the PBMCs and colon tissue in the treated HIV-infected patients examined in our study.

Although the presence of HIV/HCV co-infection further impairs liver function and augment the severity of microbial translocation and immunopathology in co-infected patients,³² in our study HCV co-infection did not further modify the level of methylation or expression of *FOXP3* in the colon tissue, which suggests that the effect on T_{reg} is mainly due to HIV. Because HIV infection alone already decreased the level of *FOXP3* methylation to nearly zero, further HCV infection may use mechanisms other than *FOXP3* promoter methylation to exert its negative impact in patients. We noticed a higher methylation level for *IL-17* promoter in the same HIV-infected patient samples (SDC 3) compared to *FOXP3* promoter (manuscript in preparation). In addition, in contrast to *FOXP3* promoter, there is no significant difference for *IL-17* promoter methylation between patients and controls in PBMCs, while a significant ($p=0.01$) higher methylation of *IL-17* promoter was noticed in HIV colonic tissue. This differential methylation of genes in T cells indicates that the lower methylation in *FOXP3* promoter is a unique effect of HIV infection on T_{reg} cells master-regulator gene.

It is not clear if *FOXP3* expression affects HIV replication in chronic HIV-infected patients. However, in an *in vitro* model of HIV infection, it has been shown that *FOXP3* expression increases HIV -1 gene expression through histone acetylation of HIV LTR. The opposite effect was found on the IL-2 promoter.³² Lower methylation of the HIV LTR was also reported in latently infected resting CD4⁺ T cells isolated from HIV-1 infected individuals receiving antiretroviral therapy.³³ However, in that study, the methylation pattern of subsets of T cells promoters were not examined.

Whether HIV infection has a direct or indirect effect on the methylation of *FOXP3* is a matter of a debate and needs further investigation. Although the development of *in vitro*

model with T_{reg} cells transduced with HIV infectious clones might appear as a reasonable approach to investigate this further, this model has some limitations; including the lack of sufficient numbers of T_{reg} which would be resistant to apoptosis by replicating HIV virus,³⁴ and the inability to create a microenvironment that represents T_{reg} cells *in vivo* during chronic HIV infection.

In order to investigate whether the level of *FOXP3* promoter methylation is influenced by immunological status in HIV-infected patients, the association between *FOXP3* promoter methylation level and CD4⁺ counts was analyzed. CD4⁺ T-lymphocytes are the primary target for HIV infection and CD4⁺ count is a useful biomarker to monitor disease progression and responsiveness to anti-retroviral therapy. In this study, all the HIV-infected patients were receiving antiretroviral treatment for a minimum of 3 years and improved their CD4⁺ counts but not to the normal level. Interestingly, we found a significant positive correlation between the level of *FOXP3* methylation and blood CD4⁺ counts. Decreased levels of *FOXP3* methylation is associated with an increase in FOXP3⁺T_{reg} number in the colon mucosa and correlated with lower CD4⁺ count in patients. This can be explained by previous observations that activated T_{reg} can non-specifically suppress HIV-specific inflammation and recruitment of CD4⁺ to the colon mucosa.³⁵ Almost all HIV infected patients included in this study had a controlled viral replication by a successful antiretroviral therapy, which limits our ability to study the correlation between *FOXP3* methylation and viral loads.

To investigate possible mechanisms of aberrant methylation of *FOXP3* by HIV-1 infection, gene expression pattern of DNA methylation enzymes in the colon mucosa from chronic HIV-infected patients and control subjects was tested by Microarray analysis and quantitative rt-PCR. Expression of *DNMT1*, *DMAP1*, *METTL7B*, and *METTL10*, were significantly down regulated in HIV-infected patients compared to controls and had a significant positive correlation to *FOXP3* promoter methylation (Fig. 4). DNA Methyltransferases are a group of enzymes that catalyze the transfer of the methyl group from the ubiquitous methyl donor S-adenosyl-L-methionine (AdoMet) to different accepting molecules including proteins, small molecules, lipids, and nucleic acids.³⁶ Both *METTL10* and *METTL7* protein has been annotated in UniProt database as having a seven beta strand AdoMet binding domain.³⁷ To our knowledge, the association between the expression pattern of these two enzymes and *FOXP3* methylation was reported for the first time by our study and has not been reported previously in HIV infection. The specific methylation reaction catalyzed by *METTL10* and *METTL7B* remains to be characterized.

In contrast, *DMAP1* protein is known to be a co-repressor of transcription that stimulates universal and local DNA methylation.³⁸ Knocking down *DMAP1* in human cell lines caused hypo-methylation of the tumor suppressor gene *p16* that leads to cell growth arrest. In a similar manner, the knocked down *DMAP1* triggered hypo-methylation of DNA repair products with resulting elevated genomic instability.³⁹ The lower expression level of *DMAP1* we observed in the colon tissue from HIV infected patients may indicate a low level of *DMAP1* activity.

DNMT1 has been described as the main enzyme responsible for *de novo* methylation and propagation of DNA methylation pattern in vertebrates.⁴⁰ In addition, *DNMT1* was shown to maintain a repressive chromatin structure (deacetylated) through its binding to *DMAP1* and recruitment of the enzyme histone deacetylase 2 (HDAC2) to the replication foci of late S-phase *in vivo*.⁴¹ In agreement with previous report of lower methylation of HIV virus promoter in treated chronic HIV-infected patients,³⁵ our data shows a lower expression of *DNMT1* in chronic HIV-1 infected patients under treatment compared to controls. The lower expression of *DNMT1* correlated positively with methylation status of *FOXP3* promoter in

patients and controls. Interestingly, although there is evidence that HIV-1 early proteins (Nef and Tat) induced DNMT1 expression from a reporter construct transfected Hela cells,⁷ FOXP3 suppresses the transcriptional activity and the promoter DNA binding of AP-1,⁴² which was shown in the previous study to inhibit the induction of DNMT1 expression by HIV-1.⁷ Our data highlight for the first time the possible mechanisms by which HIV-1 infection may alter methylation pattern of *FOXP3* in T_{reg} and immune homeostasis. Limitations of our study including that it is cross-sectional and did not have samples from HIV-infected untreated patients to investigate the effect of treatment.

In summary, we found lower level of *FOXP3* promoter methylation in the gut mucosa and blood of chronic HIV-1 infected patients who were receiving prolonged antiretroviral therapy. This decreased methylation level of *FOXP3* promoter was significantly associated with changes in the gene and protein expression of FOXP3 and a higher frequency of infiltrating T_{reg} in the gut mucosa of infected patients. Collectively our data support that higher T_{reg} frequency in gut mucosa of HIV infected patients may be caused by aberrant methylation process associated with HIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the entire participant in this study particularly the patients. We thank the Pyrosequencing Lab for Genomic and Epigenomic Research, Digestive Health Center, The Gene Expression Microarray Core, and Bioinformatics Core in Cincinnati Children's Hospital Medical Center.

References

1. Bird A. The essentials of DNA methylation. *Cell*. 1992; 70:5–8. [PubMed: 1377983]
2. Martino DJ, Prescott SL. Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease. *Allergy*. 2010; 65:7–15. [PubMed: 19796189]
3. De Bustos C, Ramos E, Young JM, et al. Tissue-specific variation in DNA methylation levels along human chromosome 1. *Epigenetics Chromatin*. 2009; 2:7. [PubMed: 19505295]
4. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. *J Mol Biol*. 1987; 196:261–282. [PubMed: 3656447]
5. Bushman F, Lewinski M, Ciuffi A, et al. Genome-wide analysis of retroviral DNA integration. *Nat Rev Microbiol*. 2005; 3:848–858. [PubMed: 16175173]
6. Kauder SE, Bosque A, Lindqvist A, et al. Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS Pathog*. 2009; 5:e1000495. [PubMed: 19557157]
7. Youngblood B, Reich NO. The early expressed HIV-1 genes regulate DNMT1 expression. *Epigenetics*. 2008; 3:149–156. [PubMed: 18567946]
8. Palacios JA, Perez-Pinar T, Toro C, et al. Long-Term Nonprogressor and Elite Controller Patients Who Control Viremia Have a Higher Percentage of Methylation in Their HIV-1 Proviral Promoters than Aviremic Patients Receiving Highly Active Antiretroviral Therapy. *J Virol*. 2012; 86:13081–13084. [PubMed: 22973038]
9. Shacklett BL, Anton PA. HIV Infection and Gut Mucosal Immune Function: Updates on Pathogenesis with Implications for Management and Intervention. *Curr Infect Dis Rep*. 2010; 12:19–27. [PubMed: 20174448]
10. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*. 2009; 9:83–89. [PubMed: 19114986]
11. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity*. 2009; 30:626–635. [PubMed: 19464985]

12. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood*. 2009; 114:3727–3735. [PubMed: 19641188]
13. Allers K, Loddenkemper C, Hofmann J, et al. Gut mucosal FOXP3+ regulatory CD4+ T cells and Nonregulatory CD4+ T cells are differentially affected by simian immunodeficiency virus infection in rhesus macaques. *J Virol*. 2010; 84:3259–3269. [PubMed: 20071575]
14. Epple HJ, Loddenkemper C, Kunkel D, et al. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood*. 2006; 108:3072–3078. [PubMed: 16728694]
15. Legrand FA, Nixon DF, Loo CP, et al. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. *PLoS One*. 2006 Dec 20; 1:e102. [PubMed: 17183635]
16. Card CM, McLaren PJ, Wachhihi C, et al. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. *J Infect Dis*. 2009; 199:1318–1322. [PubMed: 19301980]
17. Weiss L, Piketty C, Assoumou L, et al. Relationship between regulatory T cells and immune activation in human immunodeficiency virus-infected patients interrupting antiretroviral therapy. *PLoS One*. 2010; 5:e11659. [PubMed: 20657770]
18. Keynan Y, Card CM, McLaren PJ, et al. The role of regulatory T cells in chronic and acute viral infections. *Clin Infect Dis*. 2008; 46:1046–1052. [PubMed: 18444822]
19. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A*. 1992; 89:1827–1831. [PubMed: 1542678]
20. Roncador G, Brown PJ, Maestre L, et al. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol*. 2005; 35:1681–1691. [PubMed: 15902688]
21. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. *Anal Quant Cytol Histol*. 2001; 23:291–299. [PubMed: 11531144]
22. Rodriguez-Cortez VC, Hernando H, de la Rica L, et al. Epigenomic deregulation in the immune system. *Epigenomics*. 2011; 3:697–713. [PubMed: 22126290]
23. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol*. 2003; 3:331–341. [PubMed: 12669023]
24. Shaw JM, Hunt PW, Critchfield JW, et al. Increased Frequency of Regulatory T Cells Accompanies Increased Immune Activation in Rectal Mucosae of HIV-Positive Noncontrollers. *J Virol*. 2011; 85:11422–11434. [PubMed: 21880771]
25. Toker A, Huehn J. To be or not to be a Treg cell: lineage decisions controlled by epigenetic mechanisms. *Sci Signal*. 2011; 4:e4.
26. Janson PC, Winerdal ME, Marits P, et al. FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS One*. 2008; 3:e1612. [PubMed: 18286169]
27. Zorn E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood*. 2006; 108:1571–1579. [PubMed: 16645171]
28. Moreno-Fernandez ME, Presicce P, Chougnet CA. Homeostasis and function of regulatory T cells in HIV/SIV infection. *J Virol*. 2012; 86:10262–10269. [PubMed: 22811537]
29. Presicce P, Orsborn K, King E, et al. Frequency of circulating regulatory T cells increases during chronic HIV infection and is largely controlled by highly active antiretroviral therapy. *PLoS One*. 2011; 6:e28118. [PubMed: 22162758]
30. Ji J, Cloyd MW. HIV-1 binding to CD4 on CD4+CD25+ regulatory T cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: an additional mechanism of immunosuppression. *Int Immunol*. 2009; 21:283–294. [PubMed: 19208751]
31. Mavigner M, Cazabat M, Dubois M, et al. Altered CD4+ T cell homing to the gut impairs mucosal immune reconstitution in treated HIV-infected individuals. *J Clin Invest*. 2012; 122:62–69. [PubMed: 22156200]

32. Marchetti G, Nasta P, Bai F, et al. Circulating sCD14 is associated with virological response to pegylated-interferon-alpha/ribavirin treatment in HIV/HCV co-infected patients. *PLoS One*. 2012; 7:e32028. [PubMed: 22363790]
33. Holmes D, Knudsen G, Mackey-Cushman S, et al. FoxP3 enhances HIV-1 gene expression by modulating NFkappaB occupancy at the long terminal repeat in human T cells. *J Biol Chem*. 2007; 282:15973–15980. [PubMed: 17416586]
34. Blazkova J, Murray D, Justement JS, et al. Paucity of HIV DNA methylation in latently infected, resting CD4+ T cells from infected individuals receiving antiretroviral therapy. *J Virol*. 2012; 86:5390–5392. [PubMed: 22345448]
35. Oswald-Richter K, Grill SM, Shariat N, et al. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol*. 2004; 2:E198. [PubMed: 15252446]
36. Larkin J 3rd, Picca CC, Caton AJ. Activation of CD4+ CD25+ regulatory T cell suppressor function by analogs of the selecting peptide. *Eur J Immunol*. 2007; 37:139–146. [PubMed: 17154263]
37. Martin JL, McMillan FM. SAM (dependent) I AM: the S-adenosylmethionine-dependent methyltransferase fold. *Curr Opin Struct Biol*. 2002; 12:783–793. [PubMed: 12504684]
38. Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic Acids Res*. 2012; 40:D71–75. [PubMed: 22102590]
39. Lee GE, Kim JH, Taylor M, et al. DNA methyltransferase 1-associated protein (DMAP1) is a co-repressor that stimulates DNA methylation globally and locally at sites of double strand break repair. *J Biol Chem*. 2010; 285:37630–37640. [PubMed: 20864525]
40. Aubol BE, Reich NO. Murine DNA cytosine C(5)-methyltransferase: in vitro studies of de novo methylation spreading. *Biochem Biophys Res Commun*. 2003; 310:209–214. [PubMed: 14511672]
41. Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat Genet*. 2000; 25:269–277. [PubMed: 10888872]
42. Lee SM, Gao B, Fang D. FoxP3 maintains Treg unresponsiveness by selectively inhibiting the promoter DNA-binding activity of AP-1. *Blood*. 2008; 111:3599–3606. [PubMed: 18223166]

FIGURE 1.

FOXP3 promoter methylation levels in PBMCs and colon tissue. Panel A shows that *FOXP3* has a significant lower average percent methylation in HIV infected patients compared to control subjects. The mean percent methylation and standard deviation in each group was as follows: control Lymphocytes (37.5 ± 4.9), control colon tissue (36.7 ± 1.6), patients' lymphocytes (1.9 ± 0.6) and patients' colon tissue (1.2 ± 0.4). The difference in methylation was statistically significant between patients and control lymphocytes and colon tissue respectively ($p < 0.0001$, $p < 0.0001$ after adjustment of age, gender and race). In panel B the levels of *FOXP3* methylation at different CpG islands were comparable in PBMCs and colon tissue from the same subject but different between controls and HIV-infected patients.

FIGURE 2.

The level of *FOXP3* gene expression in colon tissue examined by quantitative rt-PCR. Significantly ($p=0.009$) higher relative expression levels of the *FOXP3* gene (normalized to the housekeeping gene *GAPDH*) in patient samples compared to controls after adjusting for age, gender and race of patients) is shown.

FIGURE 3.

Immunohistochemical staining of FOXP3 protein in colon tissue. Two representative slides of FOXP3 protein expression in colon tissue using Immunohistochemical staining as described in methods were shown. A and B represent the staining of FOXP3 in control while C and D represent the staining of FOXP3 in HIV patient colon tissue at 10X and 40X respectively. The mean score $\pm$ SD of FOXP3+ T_{reg} cells were 20 ± 1.7 cells/HPF in HIV and 2 ± 0.85 cells/HPF in controls.

FIGURE 4.

Relative expression levels of methylation enzymes using quantitative RT-PCR and correlation with *FOXP3* methylation. In panel A, *DNMT1*; *DMAP1*, *METTL7B*, and *METTL10* relative expression levels (normalized to the housekeeping gene *GAPDH*) were significantly ($p < 0.05$) down regulated in HIV infected patients compared to controls. In panel B, *FOXP3* promoter methylation levels has a significant positive correlation to the relative expression levels of *DNMT1*, *DMAP1*, *METTL7B*, and *METTL10* (Spearman $r=0.7112$, $p=0.0254$, $r=0.8909$, $r=0.8667$, $p=0.0022$, $p=0.0011$; and $r=0.7455$, $p=0.0174$ respectively).

TABLE 1
Demographic and Clinical Data Characteristics of HIV-1 Infected Patients and Non-Infected Control Subjects

Demographic Characters	Control N=10	HIV N=10	p
Sex:			
Male	4	8	-
Female	6	2	-
Age:			-
Range	42-64	24-54	
(Average $\pm$ SD)	(56 $\pm$ 6.8)	(43 $\pm$ 10.7)	0.0045
Race:			
White	7	6	-
AA	3	2	-
Others	0	1	-
CD4 ⁺ counts (cells/ μ l):			
Range	420-890	310-581	-
(Average $\pm$ SD)	(699 $\pm$ 138)	(472 $\pm$ 99)	0.0005
HIV viral load (Copies/ml):			
Range	NA	0-1074	-
(Average $\pm$ SD)		(120.5 $\pm$ 336.8)	-
Anti-Retroviral treatment:	NA	10	-
HCV positive			-
number	0	5	
HCV viral load (10^6 IU/ml):			
Range	NA	0-7.5	-
(Average $\pm$ SD)		(2.5 $\pm$ 4.3)	
HCV treatment:	NA	2	-