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Modulating DNA Methylation in Activated CD8\(^+\) T Cells Inhibits Regulatory T Cell–Induced Binding of Foxp3 to the CD8\(^+\) T Cell IL-2 Promoter

Michelle M. Miller,\(^ {1}\) Nnenna Akaronu,\(^ {2}\) Elizabeth M. Thompson,\(^ {3}\) Sylvia F. Hood, and Jonathan E. Fogle

We have previously demonstrated that CD4\(^+\)CD25\(^+\) regulatory T cells (Tregs) activated during the course of feline immunodeficiency virus (FIV) infection suppress CD8\(^+\) CTL function in a TGF-\(\beta\)–dependent fashion, inhibiting IFN-\(\gamma\) and IL-2 production and inducing G\(_1\) cell-cycle arrest. In this article, we describe the molecular events occurring at the IL-2 promoter leading to suppression of IL-2 production. These experiments demonstrate that Foxp3 induced by lentivirus-activated Tregs in the CD8\(^+\) target cells binds to the IL-2 promoter, actively repressing IL-2 transcription. We further demonstrate that the chronic activation of CD8\(^+\) T cells during FIV infection results in chromatin remodeling at the IL-2 promoter, specifically, demethylation of CpG residues. These DNA modifications occur during active transcription and translation of IL-2; however, these changes render the IL-2 promoter permissive to Foxp3-induced transcriptional repression. These data help explain, in part, the seemingly paradoxical observations that CD8\(^+\) T cells displaying an activation phenotype exhibit altered antiviral function. Further, we demonstrate that blocking demethylation of CpG residues at the IL-2 promoter inhibits Foxp3 binding, suggesting a potential mechanism for rescue and/or reactivation of CD8\(^+\) T cells. Using the FIV model for lentiviral persistence, these studies provide a framework for understanding how immune activation combined with Treg-mediated suppression may affect CD8\(^+\) T cell IL-2 transcription, maturation, and antiviral function. The Journal of Immunology, 2015, 194: 990–998.

Lentiviruses such as HIV and feline immunodeficiency virus (FIV) are able to evade an early, vigorous immune response and establish a persistent infection. Despite an initial, robust expansion in HIV-specific CD8\(^+\) T cells, virus is only partially cleared and CD8\(^+\) cells display signs of dysfunction including a lack of inflammatory cytokine production in response to activation by specific ligand(s) or in response to mitogenic stimulation (1–3). A specific group of HIV-infected individuals referred to as elite controllers are able to control virus in the absence of therapeutic treatment, and researchers have demonstrated that these individuals maintain a highly active population of HIV-specific CD8\(^+\) T cells into the chronic infection stage (4, 5). By comparison, HIV-infected individuals who do not effectively control virus harbor virus-specific CD8\(^+\) T cells with altered functionality leading to disruptions in overall immune homeostasis (1, 4). During chronic HIV/FIV, the virus replicates at low levels, contributing to a state of chronic immune activation followed by immune exhaustion (6–9). These findings illustrate the need for a more detailed understanding of CD8\(^+\) T cell–mediated response to HIV/FIV infection and to define the direct cause for CD8\(^+\) dysfunction.

Using the FIV model for HIV/AIDS, our group and others have demonstrated the progressive activation of regulatory CD4\(^+\)CD25\(^+\) regulatory T cells (Tregs) during the course of infection, consistent with reports of activated regulatory cells being isolated from HIV patients (10–16). Several groups have reported that depletion of Tregs during HIV infection results in boosted antiviral responses and CD8\(^+\) T cell activity (15, 17). Similar to our findings using the FIV model, Kinter et al. (18) reported that CD4\(^+\)CD25\(^+\) T cells in HIV\(^+\) patients significantly suppressed cellular proliferation and cytokine production by CD4\(^+\) and CD8\(^+\) T cells stimulated with HIV peptides in vitro. Although it is evident that Tregs are able to suppress CD4\(^+\) and CD8\(^+\) effector T cells during the course of lentiviral infections, it is not clear whether suppression is always harmful. Several investigations have indicated that CD4\(^+\)CD25\(^+\) T cell activation may play a protective role during the course of lentiviral infections, and it has been reported that there is a significant expansion of CD4\(^+\)CD25\(^+\) T cells in the blood of HIV\(^+\) patients on antiretroviral therapy (19). Taken together, this evidence suggests that timing may be a critical factor, with Treg activation being detrimental during acute infection by inhibiting early T cell responses, and thus aiding in the establishment of persistent infection but performing a beneficial role during chronic infection.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; Ct, cycle threshold; DNMT, DNA methyltransferase; FIV, feline immunodeficiency virus; IP, immunoprecipitation; PLN, peripheral lymph node; qPCR, quantitative PCR; Treg, regulatory T cell.

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by dampening immune activation and associated pathologic inflammation during the course of chronic infection. These observations underscore the need to further understand the molecular mechanisms that occur in activated CD8+ T cells after interaction with lentivirus-activated Tregs.

The intranuclear transcription factor Foxp3 serves as a “master molecule” for Treg function. Foxp3 alters gene expression profiles by binding to specific promoters, including the IL-2 promoter, to regulate transcription through control of histone modifications and blocking the assembly of transcriptional machinery (20, 21). For example, Foxp3 and the linker histone H1.5 cooperatively bind the IL-2 promoter and repress IL-2 expression (22). Although Foxp3 has been broadly considered a regulatory cell–specific marker, we and others have demonstrated increased Foxp3 expression in activated CD8+ lymphocytes after interaction with lentivirus-activated Tregs (23, 24). Although some investigations have demonstrated that CD8+Foxp3+ T cells are suppressor cells, we and others were unable to document that CD8+Foxp3+ T cells exhibit suppressor function (23, 25). As part of the same series of experiments, we were able to demonstrate that Tregs inhibited IL-2 mRNA expression and induced G2 cell-culture arrest and anergy in CD8+ lymphocyte targets during both acute and chronic FIV (23, 26, 27). Findings from murine studies have offered clues as to what may be occurring to these cells during the course of lentiviral infection. OVA and LCMV studies have demonstrated that the acquisition of CD8+ effector function and differentiation into mature, virus-specific CTLs is clearly linked to specific epigenetic modifications at the IL-2 promoter (28, 29). These studies have also shown that disruption of differentiation leads to the induction of CD8+ T cell dysfunction and eventual immune exhaustion, which is consistent with what occurs in lentivirus-induced CD8+ T cell immune dysfunction (30).

Based upon our previous findings that Tregs induce anergy and suppression of IL-2 production in CD8+ T cells after coculture, we hypothesized that Foxp3 expressed in CD8+ target cells functions in a similar manner as Foxp3 in other T cell subsets, occupying the IL-2 promoter and directly inhibiting gene expression. Further, we propose a mechanism by which early CD8+ T cell epigenetic changes (DNA demethylation), although essential to antiviral function, render activated CD8+ T cells highly permissive to Treg-suppression thus leads to a loss of IL-2 and effector cell function in the CD8+ target cells, and helps to reconcile the seemingly paradoxical observations that activated CD8+ T cells exhibit characteristic features associated with both chronic activation and altered immunologic function. These results define a key element involved in subverting the early CD8+ T cell response and identify potential avenues for rescuing and augmenting T cell function.

Materials and Methods

**Cats and FIV infection**

Specific pathogen-free cats were obtained from Liberty Labs (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA) and housed at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Cats were inoculated with the NCSCU isolate of FIV, a pathogenic clade A virus, as described by Bucci et al. (31). FIV infection was confirmed by ELISA (SNAP FIV/FelV; Idexx Laboratories), and provirus was detected by PCR using primers specific for the FIV-p24 GAG sequence. At the time samples were taken, cats had been infected with FIV for at least 5 y and were clinically asymptomatic. Noninfected control cats ranged in age from 3 to 6 y and were housed separately from FIV-infected cats. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

**Sample collection and preparation**

Whole blood (28 mL/cat) was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). PBMCs were isolated by Percoll density gradient centrifugation (Sigma-Aldrich, St. Louis, MO) as previously described (32) or by ficoll-Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s guidelines. Single-cell suspensions were prepared from popliteal or submandibular peripheral lymph nodes (PLNs) obtained through surgical biopsies by gently and repeatedly injecting sterile PBS into the tissue using an 18G needle until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion, and viability was always >95%.

**Reagents and Abs**

Recombinant human IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health from Dr. Maurice Gatley (Hoffmann-La Roche). Con A was purchased from Sigma-Aldrich (St. Louis, MO). Streptavidin-PerCP was purchased from BD Biosciences Pharmingen (San Diego, CA). Mouse anti-feline CD25 (mAb 9F23) was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan). Mouse anti-feline CD4 (mAb 30A) and CD8+ (mAb 3.357) were purchased from our laboratory (33). PE-conjugated rat anti-mouse Foxp3 (FJK-16s) used for flow cytometry analysis was purchased from eBioscience (San Diego, CA). Chromatin immunoprecipitation (ChIP) grade anti-Foxp3 Ab (ab2481) was purchased from Abcam (Cambridge, MA). Curcumin (product C22727) and gencitabine (product G6423) were purchased from Sigma-Aldrich.

**CD8+ coculture assays**

Whole blood from FIV-infected or FIV− control cats was isolated as described and purified by ficoll density gradient. PBMCs were then labeled with specific Ab and purified into CD4+CD25+ and CD8+ groups using a high-speed, high-purity fluorescence activated cell sorter (MoFlo; DakoCytomation). A total of 1 × 10^6 CD8+ cells was left untreated or stimulated with Con A (5 μg/ml) and IL-2 (1000/ml) for 1 h, washed, and then cultured with or without CD4+CD25+ Tregs at a 1:1 ratio. After 24 h, cells were washed and then used directly for flow cytometry or resorted into pure CD8+ populations for analysis by PCR. The purity of FACS sorted cell populations was always >95%.

**Flow cytometric analysis**

For comparison of intracellular staining of cultured PBMCs, at least 5 × 10^6 cells were stained for surface expression of CD8, CD4, and CD25 using specific Abs. For intracellular staining of Foxp3, cells were then washed in PBS, incubated with 4% PFA for 10 min, incubated in 0.1% Triton X-100 for 20 s, annealed at 58˚C for 20 s, and elongated at 72˚C for 30 s with 40 cycles. Reverse transcription and real-time PCR

Reverse transcription was carried out using a reverse transcription system kit from Promega as per the manufacturer’s protocol followed by real-time PCR using Quantitect SYBR green PCR kit (Qiagen, Valencia, CA). Reactions were run in triplicate in 96-well plates, and all reactions were carried out using identical cycling conditions as follows: denatured at 95˚C for 20 s, annealed at 58˚C for 20 s, and elongated at 72˚C for 30 s with 40 cycles.

**DNA methylation analysis and cloning**

Because the feline IL-2 promoter has not been previously characterized, we first investigated the feline IL-2 gene using the National Center for Biotechnology Information database for the felis catus whole genome. We then designed primers to amplify the region up to 1 kb upstream of the transcriptional start site by first amplifying and sequencing smaller, overlapping regions and then piecing together a robust promoter map using sequences of genomic DNA isolated from two FIV− and two FIV+ cat CD8+ samples (data not shown). The resultant sequence was then analyzed for CpG residues and used to design quantitative RT-PCR primers for later ChIP analyses (see Table I for primers). Three CpG residues were identified in this IL-2 promoter region. For analysis of promoter methylation, DNA was isolated from at least 5 × 10^6 lymphocytes using the QIAamp DNA Mini Kit.
kit (Qiagen, Valencia CA) and then subjected to bisulfite modification using an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA). Reduced DNA was used as a template for qPCR amplification using the primers listed in Table I and the following cycling conditions: denatured at 95°C for 30 s, annealed at 60°C for 30 s, and elongated at 72°C for 30 s with 40 cycles. Product was gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and then used as template for TA cloning. In brief, product was ligated into the pGEM T Easy Vector System (Promega, Madison, WI) according to manufacturer’s protocol, and vectors were used to transform JM109 High Efficiency Competent Cells (Promega). Colonies were selected based on Blue/White screening and processed using the ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA). Individual clones were sent for sequencing to Eurofins MWG Operon (Huntsville, AL). Sequence data were analyzed using the Geneious analysis software program (35).

Global DNA methylation

For global methylation analysis, DNA was isolated from 1e6 FACs-purified lymphocytes and analyzed using the MethyLexp Global DNA Methylation kit (Epigentek, Farmingdale, NY) following manufacturer’s instructions.

ChiP assay

After culture, cells were resorted (97–99% purity) and cross-linked with 1% formaldehyde for use in ChiP experiments. Anti-Foxp3 (ab2481; Abcam) ChiPs were performed with an A/G agarose ChiP kit (Thermo Scientific), following the manufacturer’s protocol. After enzymatic digestion, 10% of the chromatin was saved as input controls. The remaining chromatin was immunoprecipitated with 1 μg Ab during an overnight incubation at 4°C. After immunoprecipitation (IP)elution, cross-linking was performed on the purified product, and DNA fragments were collected for use in real-time quantitative PCR (qPCR) following the described cycling conditions and with primers listed in Table I. The relative enrichment was calculated using input controls and a ΔΔ Ct cycle threshold equation as follows:

\[
\frac{(2^{-\Delta\Delta Ct_{IP}}} - 2^{-\Delta\Delta Ct_{input}})}{(2^{-\Delta\Delta Ct_{IP}}} - 2^{-\Delta\Delta Ct_{input}}}
\]

Statistical analysis

The Mann–Whitney U test (t test for nonparametric data) was used for pairwise comparison of parameters. Differences were considered to be significant at p < 0.05.

Results

Lentivirus-activated Tregs induce Foxp3 expression in CD8* cells during coculture

Our laboratory and others have previously documented a population of CD8*Foxp3* cells exhibiting altered function, including decreased IL-2 production, that are not suppressor cells. Chronic FIV infection is associated with progressive Treg activation; therefore, we asked whether Tregs from FIV+ cats induce Foxp3 expression in autologous, Con A-activated CD8* targets. Because Foxp3 is a transcriptional regulator and is known to modulate IL-2 expression, we hypothesized that activated Tregs induce Foxp3 expression in CD8* T cell targets, leading to IL-2 suppression. To address this hypothesis, we first demonstrated Foxp3 induction in CD8* target cells. Lymphocytes from eight FIV-infected or four control cats were isolated from peripheral blood and sorted into CD8+ target cells. Lymphocytes from eight FIV-infected or four control cats were isolated from peripheral blood and sorted into CD8+ target cells. Lymphocytes from eight FIV-infected or four control cats were isolated from peripheral blood and sorted into CD8+ target cells.

Table I. Real-time qPCR primers and primers for PCR product cloning

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Foxp3 (mRNA)</td>
<td>5'-TTTTTCAACGACATCCCACTTCCTCCTACACG-3'</td>
<td>5'-CTGTGACCTCTGCTTCAAGTCTC-3'</td>
</tr>
<tr>
<td>IL-2 (mRNA)</td>
<td>5'-AACACCTCTCCTTTGAGTGAATTGGCC-3'</td>
<td>5'-CTCTGAGATTTGCTGTTCTCAGG-3'</td>
</tr>
<tr>
<td>GapDH (mRNA and ChIP)</td>
<td>5'-CTTCTTACGACTCTCACTACAT-3'</td>
<td>5'-CTGATGACTTTGCCTTACAGC-3'</td>
</tr>
<tr>
<td>IL-2 (bisulfite trt promoter)</td>
<td>5'-GGTTAATTTATATTTATTTATTTGAGG-3'</td>
<td>5'-CTTATTGATTTGTTTCTCAGG-3'</td>
</tr>
<tr>
<td>IL-2 (ChIP promoter)</td>
<td>5'-GGTTAATTTATATTTATTTATTTGAGG-3'</td>
<td>5'-CTTATTGATTTGTTTCTCAGG-3'</td>
</tr>
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Kaplan-Meier survival analysis, 10% of the chromatin was saved as input controls. The remaining chromatin was immunoprecipitated with 1 μg Ab during an overnight incubation at 4°C. After immunoprecipitation (IP)elution, cross-linking was performed on the purified product, and DNA fragments were collected for use in real-time quantitative PCR (qPCR) following the described cycling conditions and with primers listed in Table I. The relative enrichment was calculated using input controls and a ΔΔ Ct cycle threshold equation as follows:

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suggest that lentivirus-activated Tregs induce Foxp3 expression and Foxp3 binding to the IL-2 promoter, suppressing IL-2 transcription in activated CD8+ T cell targets.

CD8+ T cells from FIV+ cats exhibit epigenetic remodeling at the IL-2 promoter

Activation of CD8+ CTLs during lentiviral infection has been extensively documented (2, 4, 37–39), but epigenetic changes induced by chronic infection at promoters for genes important for antiviral function remain largely uncharacterized. Specifically, alteration in methylation status at these regions can correlate with chromatin structure and impact accessibility of promoters for transcription factor binding. Therefore, we asked whether the chronic immune stimulation during FIV infection led to epigenetic changes at the CD8+ T cell IL-2 promoter, specifically, changes in promoter enrichment in CD8+ cells stimulated with Con A alone (5 μg/ml, 1 h), or Con A–stimulated (5 μg/ml, or Con A–stimulated for 2 h before autologous CD4+CD25+ Treg coculture. After 24 h, RNA was isolated and RT-qPCR was performed on all sample groups. Specific pathogen-free control cats with little antigenic exposure exhibit only a modest response to mitogenic stimulation. IL-2 was decreased by ∼4-fold in Con A–stimulated CD8+ lymphocytes from FIV− cats after Con A+CD25+ coculture (p < 0.05). For both (A) and (B), CD8+ lymphocytes and Tregs from FIV− cats were separated by cell sorting after coculture (∼99% purity).

FIGURE 2. Treg-induced Foxp3 binds the IL-2 promoter in activated CD8+ T cells, inhibiting IL-2 transcription. (A) CD8+ and CD4+CD25+ Tregs were purified from FIV+ PLNs and used for coculture experiments. Foxp3 ChIP followed by PCR for the IL-2 promoter resulted in IL-2 promoter enrichment in Con A–stimulated (5 μg/ml, 1 h) CD8+ cells cocultured with Tregs (lanes 3, 5, 7, arrows), whereas there was little IL-2 promoter enrichment in CD8+ cells stimulated with Con A alone (5 μg/ml, 1 h, lanes 2, 4, 6). (B) CD8+ lymphocytes from FIV− or FIV+ PLNs were either untreated, Con A–stimulated (5 μg/ml), or Con A–stimulated for 2 h before autologous CD4+CD25+ Treg coculture. After 24 h, RNA was isolated and RT-qPCR was performed on all sample groups. Specific pathogen-free control cats with little antigenic exposure exhibit only a modest response to mitogenic stimulation. IL-2 was decreased by ∼4-fold in Con A–stimulated CD8+ lymphocytes from FIV− cats after Con A+CD25+ coculture (p < 0.05). For both (A) and (B), CD8+ lymphocytes and Tregs from FIV− cats were separated by cell sorting after coculture (∼99% purity).

FIGURE 1. Foxp3 protein and mRNA levels are increased in CD8+ lymphocytes after coculture with lentivirus-activated Tregs. Intracellular expression of Foxp3 was measured by flow cytometry in untreated CD8+ T cells, CD8+ T cells stimulated with Con A (5 μg/ml, 1 h), or in Con A–stimulated CD8+ T cells cocultured with Tregs for 24 h. (A) The percentage of Foxp3 expression is increased ∼2-fold in stimulated CD8+ T cell targets after coculture with Tregs from FIV− cats. Bars represent the mean + SEM for FIV− or FIV+ cats (p < 0.05, four and eight independent experiments, respectively). (B) Representative flow cytometry dot plots from FIV− (upper row) and FIV+ cats (lower row) show the percentage of Foxp3+ cells (upper right corner) for untreated CD8+ T cells (left column, FIV− 1%, FIV+ 4%), CD8+ T cells stimulated with Con A (middle column, FIV− 6%, FIV+ 8%), or in Con A–stimulated CD8+ T cells cocultured with Tregs for 24 h (right column, FIV− 7%, FIV+ 19%). CD8+ T cells were gated and analyzed by forward scatter (FSC, x-axis) versus Foxp3 fluorescence (y-axis). Dot plots demonstrate the change in both the percentage of positive cells and the fluorescence intensity for Foxp3. (C) Foxp3 mRNA induction (fold change) was higher in all treatment groups from FIV− cats when compared with FIV+ cats and in Con A–stimulated CD8+ lymphocytes after Treg coculture. Each bar represents the mean + SEM for five or six experiments (p < 0.05, arrows).
distal sites, CG2 (-1333) and CG3 (-1236), when compared with FIV− cats. The percent demethylation of 30 CpG residues was calculated for each cat (10 unique clones × 3 sites). FIV+ cats displayed a significantly higher percentage of IL-2 promoter demethylation when compared with FIV− cats (p < 0.05; Fig. 3C).

Modulating DNA methylation prevents Foxp3 from binding to the IL-2 promoter in activated CD8+ T cells after Treg coculture

Taken together, the results of Figs. 2 and 3 suggest that CD8+ T cell activation renders the IL-2 promoter permissive to Treg-induced Foxp3 binding and suppression of IL-2 transcription. Therefore, we asked whether modulating DNA demethylation in activated CD8+ T cells might inhibit Foxp3 binding. Gene promoter regions can be activated by demethylation or silenced by hypermethylation, and enzymes such as DNA methyltransferases (DNMTs) are integral to both these functions (41–44). Several compounds have been reported to modulate DNA methylation activity in vitro, and for our purposes, we selected gemcitabine and curcumin, which are reported to inhibit both DNA methylation and demethylation activity presumably through altering methyl group transfer and repair (41–44). Recent findings suggest that the combination of gemcitabine and curcumin provides maintenance of methylation status ex vivo and prevents hypermethylation after mitogenic stimulation (45–47). We first performed a dose–response curve to determine whether treatment of purified CD8+ lymphocytes from an FIV− cat with various concentrations of curcumin or gemcitabine would result in cell death as measured by FACS analysis of propidium iodide–labeled cells (data not shown). After selecting the highest concentration for each compound that did not result in cell death (greater than controls) after a 12-h incubation, we performed a second set of experiments to determine the combined effects of treating lymphocytes with these doses of compound over increasing lengths of time. As shown in Fig. 4A, curcumin (1 mmol) plus gemcitabine (100 nmol) treatment did not cause significant cell death until after 12 h of incubation time. We next determined whether 2 h of treatment with each compound would be sufficient to modulate DNA methylation in mitogen-activated CD8+ T cells from FIV− cats by performing global methylation analyses on sorted CD8+ cells. Curcumin has a high degree of yellow pigmentation that interfered with OD measurement in the global methylation assay, and we were unable to assess global methylation with curcumin alone or with curcumin plus gemcitabine (data not shown). As shown in Fig. 4B, gemcitabine (100 nmol) treatment for 2 h before Con A stimulation was sufficient to prevent global demethylation and induced a small degree of hypermethylation when compared with untreated controls. CD8+ T cells were then treated with curcumin and gemcitabine before Con A activation and cocultured with autologous CD4+CD25+ Tregs for 24 h to demonstrate that blocking DNA demethylation in activated CD8+ lymphocytes inhibits Foxp3 binding. As shown in Fig. 4C, CD8+ T cells treated with Con A alone displayed little Foxp3 enrichment at the IL-2 promoter (gray bar). However, coculture with CD4+CD25+ Tregs resulted in significant Foxp3 protein binding to the IL-2 promoter in CD8+ cells (Fig. 4C, black bar), consistent with our previous findings from Fig. 2A. Importantly, pretreatment of the CD8+ lymphocytes with curcumin and gemcitabine completely blocked the binding of Foxp3 protein to the IL-2 promoter, suggesting that DNA demethylation of the IL-2 promoter is required for the binding of this repressive transcription factor (Fig. 4C, hatched bar). Collectively, these data indicate that Tregs induce the expression of Foxp3 in activated CD8+ lymphocytes, which, in turn, suppresses IL-2 production by binding at the demethylated IL-2 promoter and interfering with transcription. Based upon these findings, we have developed a model of “active suppression,” where Treg cells target activated CD8+ T cells for suppression (Fig. 5).

Discussion

The transcription factor Foxp3 is upregulated in CD8+ lymphocytes activated by mitogenic stimulation or antigenic stimulation, and after interaction with lentivirus-activated Tregs (23, 24). In this

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consistent with reports that gemcitabine inhibits DNMT activity, feline CD8+ lymphocytes after mitogenic stimulation, antigenic stimulation, and upon interaction with lentivirus-activated Tregs (23, 24). Although others have demonstrated that these CD8+Foxp3+ cells are indeed suppressor cells, our laboratory was unable to document that CD8+ lymphocytes exhibited suppressor function after Treg coculture, despite the induction of CD8+ Foxp3 expression (23, 25). As part of the same series of experiments in our laboratory, we were able to demonstrate that Tregs induce G1 cell-cycle arrest and anergy in CD8+ T cell targets during both acute and chronic FIV infection (23, 26, 27). Figs. 1 and 2 clearly demonstrate that Foxp3 is increased in CD8+ T cells after coculture with lentivirus-activated Tregs and, more importantly, that Foxp3 binds the IL-2 promoter in CD8+ T cells inhibiting transcription of this essential cytokine. Our observations are consistent with those previously reported by Dieckman et al. (50), who reported that Tregs inhibited both CD4+ Th and CD8+ T cell function, but induced suppressor function only in CD4+ T cells.

Activated T cells exhibit varying degrees of demethylation at the IL-2 promoter. For example, Northrop et al. (51) reported CD8+ T cell IL-2 promoter demethylation that followed a trend of progressive distal-to-proximal demethylation. They further demonstrated that IL-2 promoter demethylation was augmented in Ag-specific CD8+ T cells that had received CD4+ T cell help, as compared with CD8+ T cells from Th-deficient mice (51). Consistent with our findings in this study, Nakayama-Hosoya et al. (52) have demonstrated that both CD4+ T cells and CD8+ T cells exhibit a trend toward progressive distal-to-proximal IL-2 promoter demethylation in HIV-infected patients, whereas CD4+ demethylation was also dependent upon CD57 expression and maturation status. Based upon these findings, we asked whether persistent activation of CD8+ T cells during chronic FIV infection leads to demethylation of the IL-2 promoter, which is associated with actively transcribed eukaryotic. To address this hypothesis, we first performed a full sequence analysis on the feline IL-2 promoter. We identified three CpG residues within the 1-kb region upstream of the IL-2 gene and evaluated the methylation status of these cytosine residues in purified CD8+ T cells from FIV+ and FIV– cats. Three separate CpG residues were identified within the IL-2 promoter (Fig. 3A, shaded regions). None of the FIV+ cats exhibited demethylation at CG1 (-1436) or CG3 (-1236) and modest demethylation at CG2 (-1333). Like the FIV+ cats, the FIV– cats exhibited demethylation at CG1 (-1436) or CG3 (-1236) and modest demethylation at CG2 (-1333). The FIV– controls, the FIV+ cats also had no demethylation at CG1 (-1436). More importantly, the FIV+ cats exhibited greater demethylation at both distal sites, CG2 (-1333) and CG3 (-1236), and modest demethylation at CG2 (-1333). Like the FIV– controls, the FIV+ cats also had no demethylation at CG1 (-1436).

Previously, we have demonstrated that lentivirus-activated Tregs induce stable Foxp3 expression and suppressor function in CD4+ Th cells, after coculture (48, 49). We and others have also demonstrated increased Foxp3 expression in activated CD8+ lymphocytes after mitogenic stimulation, antigenic stimulation, and upon interaction with lentivirus-activated Tregs (23, 24). Although others have demonstrated that these CD8+Foxp3+ cells are indeed suppressor cells, our laboratory was unable to document that CD8+ lymphocytes exhibited suppressor function after Treg coculture, despite the induction of CD8+ Foxp3 expression (23, 25). As part of the same series of experiments in our laboratory, we were able to demonstrate that Tregs induce G1 cell-cycle arrest and anergy in CD8+ T cell targets during both acute and chronic FIV infection (23, 26, 27). Figs. 1 and 2 clearly demonstrate that Foxp3 is increased in CD8+ T cells after coculture with lentivirus-activated Tregs and, more importantly, that Foxp3 binds the IL-2 promoter in CD8+ T cells inhibiting transcription of this essential cytokine. Our observations are consistent with those previously reported by Dieckman et al. (50), who reported that Tregs inhibited both CD4+ Th and CD8+ T cell function, but induced suppressor function only in CD4+ T cells.

Activated T cells exhibit varying degrees of demethylation at the IL-2 promoter. For example, Northrop et al. (51) reported CD8+ T cell IL-2 promoter demethylation that followed a trend of progressive distal-to-proximal demethylation. They further demonstrated that IL-2 promoter demethylation was augmented in Ag-specific CD8+ T cells that had received CD4+ T cell help, as compared with CD8+ T cells from Th-deficient mice (51). Consistent with our findings in this study, Nakayama-Hosoya et al. (52) have demonstrated that both CD4+ T cells and CD8+ T cells exhibit a trend toward progressive distal-to-proximal IL-2 promoter demethylation in HIV-infected patients, whereas CD4+ demethylation was also dependent upon CD57 expression and maturation status. Based upon these findings, we asked whether persistent activation of CD8+ T cells during chronic FIV infection leads to demethylation of the IL-2 promoter, which is associated with actively transcribed eukaryotic. To address this hypothesis, we first performed a full sequence analysis on the feline IL-2 promoter. We identified three CpG residues within the 1-kb region upstream of the IL-2 gene and evaluated the methylation status of these cytosine residues in purified CD8+ T cells from FIV+ and FIV– cats. Three separate CpG residues were identified within the IL-2 promoter (Fig. 3A, shaded regions). None of the FIV+ cats exhibited demethylation at CG1 (-1436) or CG3 (-1236) and modest demethylation at CG2 (-1333). Like the FIV– controls, the FIV+ cats also had no demethylation at CG1 (-1436). More importantly, the FIV+ cats exhibited greater demethylation at both distal sites, CG2 (-1333) and CG3 (-1236), when compared with FIV– cats. Why CG1 exhibited no demethylation in either FIV+ or FIV– cats is uncertain. However, our findings suggest a trend toward distal promoter demethylation in CD8+ T cells from FIV+ cats, consistent with the previous reports from Northrop et al. (51) and Nakayama-Hosoya et al. (52) mentioned earlier. When total IL-2 promoter demethylation was examined, as shown in Fig. 3C, there was increased demethylation in the IL-2 promoter in CD8+ T cells from FIV+ cats when compared with FIV– cats. Collectively, these data support our hypothesis that the CD8+ T cell IL-2 promoter is in an “open” conformation, poised for IL-2 transcription.
We then asked whether DNA demethylation resulted in increased susceptibility to Foxp3 suppression and whether blocking demethylation might inhibit Foxp3 binding to the IL-2 promoter. First, we demonstrated that we could inhibit DNA demethylation during CD8^+ T cell activation by treatment with gemcitabine. We also treated cells with curcumin, which is reported to prevent DNA hypermethylation and likely inhibits DNMT activity (44, 53). Although our target was inhibition of DNMT activity in activated CD8^+ T cells, both curcumin and gemcitabine are reported to have other effects upon epigenetic conformation and cellular function. For example, curcumin can modulate histone deacetylase activity, as well as histone acetyltransferase activity (54–56). One of the primary mechanisms of action for gemcitabine is incorporation into the growing DNA strand with chain termination through DNA polymerase inhibition (57). Therefore, it is possible that these compounds may have affected other cellular pathways in CD8^+ target cells and is a limitation of the studies reported in this article. It is also likely that these compounds modulate Treg function. Therefore, to avoid this confounding factor, CD8^+ T cells targets were treated before Treg coculture to assess the effects directly upon CD8^+ T cells. Finally, of primary concern was cellular toxicity; therefore, toxicity and dose–response curves were determined for each compound. As demonstrated in Fig. 4A and 4B, at these drug concentrations, DNA methylation was significantly reduced and no significant cell death was observed. We next demonstrated that Foxp3 directly binds the IL-2 promoter region in activated CD8^+ T cells after Treg coculture by performing Foxp3 ChIP and identifying enrichment of the IL-2 promoter by the Foxp3-bound chromatin (Figs. 2A and 4C). Finally, we treated CD8^+ T cells with curcumin and gemcitabine to block DNMT activity, before activation, and demonstrated that this prevented Foxp3 binding to the IL-2 promoter. These findings are consistent with our hypothesis that DNA demethylation of the IL-2 promoter in activated CD8^+ T cells increases susceptibility to direct suppression of IL-2 by Foxp3.

Studies using LCMV have offered some insight in what may be occurring at the IL-2 promoter during the course of lentiviral infections. Northrop et al. (29, 51) have clearly demonstrated that the generation of phenotypically mature, fully functional CD8^+ lymphocytes is dependent upon DNA demethylation at the IL-2 promoter region. Conversely, dysfunctional CD8^+ lymphocytes exhibited increased DNA methylation at the IL-2 promoter and failure to undergo complete maturation. Studies of LCMV have also demonstrated that during protracted viral infection, CD8^+ lymphocytes are persistently activated yet exhibit progressive loss of IL-2 production (58). Collectively, these data suggest epigenetic patterns established early during the course of viral infection contribute to either a robust cellular immune response or to CD8^+ T cell dysfunction, poor maturation, and progressive loss of CD8^+ cytokine function.

Although not the focus of these investigations, lentiviruses may also directly influence DNA methylation profiles. For example, Abdel-Hameed et al. (59) recently reported that chronic HIV infection is associated with alterations in expression of methylation machinery including suppression of DNMT-1 activity, leading to increased Foxp3 promoter demethylation. However, others have demonstrated that HIV may enhance the activity of DNMTs, leading to hypermethylation and inactivation of genes important for antiviral function, such as IFN-γ (60). Collectively, these findings suggest that lentiviruses may directly influence methylation at the IL-2 promoter region, but how this may affect IL-2 transcription is uncertain. Further, Figs. 2 and 4 clearly demonstrate that Foxp3 binds the IL-2 promoter, and this binding is associated with decreased IL-2 mRNA.

We and others have previously established that CD8^+ T cells exhibit an activated phenotype during the course of FIV infection (23, 31, 61). Therefore, the focus of this investigation was to demonstrate how Tregs may use CD8^+ T cell activation as a route for CD8^+ T cell suppression. Using the FIV model for AIDS
lentiviral infections, this study provides a key mechanistic insight into IL-2 suppression in CD8* lymphocytes. These studies clearly demonstrate that Foxp3 binds the IL-2 promoter in mitogen-activated CD8* T cells from FIV+ cats, after coculture with autologous Tregs. Further, we demonstrate that the CD8* T cell IL-2 promoter is demethylated in FIV* cats when compared with FIV* cats. One of the limitations of this study is the potential “off-target” effects of gemcitabine and curcumin on CD8* T cells. Despite this limitation, these findings support a novel mechanism of CD8* T cell dysfunction during the course of lentivirus infection. These data also help to reconcile, at a molecular level, the seemingly paradoxical observation that phenotypically activated CD8* T cells exhibit compromised effector function during the course of lentiviral infection. From these data we propose a mechanism by which Tregs induce the expression of Foxp3 in activated CD8* lymphocytes, which, in turn, suppresses IL-2 production by binding at the demethylated IL-2 promoter and interfering with transcription. Investigations are under way to identify these mechanisms in virus-specific CD8* T cells during the acute stage of infection and follow them through the chronic stage of infection. A schematic representation of this “active suppression” model is shown in Fig. 5.

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