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Epigenetic repolarization of T lymphocytes from chronic lymphocytic leukemia patients using 5-aza-2'-deoxycytidine

Jason A. Dubovsky^{a,b}, John J. Powers^{a,b}, Yang Gao^{a,b}, Luis F. Mariusso^{a,b}, Eduardo M. Sotomayor^{a,b}, and Javier A. Pinilla-Ibarz^{a,b,*}

^aDepartment of Experimental Therapeutics at H. Lee Moffitt Cancer Center, Tampa, FL, USA

^bDepartment of Malignant Hematology at H. Lee Moffitt Cancer Center, Tampa, FL, USA

Abstract

T cell immune dysfunction has an important role in the profound immunosuppression that characterizes chronic lymphocytic leukemia (CLL). Improper polarization of T cells has been proposed as one of the mechanism involved. Mounting data implicates chromatin regulation, namely promoter methylation, in the plasticity of naïve human T cells. Recent in vitro evidence indicates that this plasticity may be phenotypically altered by using methylation inhibitors which are approved for clinical use in certain types of cancer. These results beg the question: can the ineffective polarization of T lymphocytes in the context of CLL be effectively modulated using methylation inhibitors in a sustainable therapeutic fashion? To answer this question our laboratory has studied the effects of 5-aza-2'-deoxycytidine (5A2) in helper and cytotoxic T lymphocytes from healthy donors and CLL patients in well characterized molecular and epigenetic signaling pathways involved in effective polarization. Moreover, we sought to investigate the consequences of methylation inhibitor treatment on lymphocyte survival, activation intensity, and naïve cell polarization. Our data indicates that 5A2 treatment can repolarize Th2 cells to effectively secrete interferon gamma, signal via T-bet, and achieve demethylation of critical Th1 specific promoters. Moreover, we demonstrate that 5A2 can force Th1 polarization of naïve T cells despite a strong IL-4 stimuli and a lack of IL-12. In conclusion our data seeks to define a modality in which improper or ineffective T cell polarization can be altered by 5AZA and could be incorporated in future therapeutic interventions.

Keywords

5-Aza-2'-deoxycytidine; T helper cell; Epigenetic; Interferon gamma; T-bet

*Corresponding author at: MRC 3E/3056H, 1290 Magnolia Dr, Tampa, FL 33612-9416, USA. Tel.: +1 813 745 6335; fax: +1 813 745 3727. Javier.Pinilla@moffitt.org (J.A. Pinilla-Ibarz).

Conflict of interest

The authors of this manuscript have no relevant conflicts of interest to disclose.

Contributions. J.P.-I. led the project, acquired funding, edited writing, and directed research; J.D. conducted all experiments, designed figures, and wrote the manuscript; E.S. provided guidance, advice, and expertise and helped to design experiments; J.P. processed CLL patient and healthy donor blood samples and purified cells; L.M. conducted RT-PCR experiments on CLL patient samples; and Y.G. conducted DNA methylation assays.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common B cell leukemia in the western world and is characterized by the progressive accumulation of long-lived, slowly proliferating mature B lymphocytes [1–4]. In healthy individuals B-lymphocytes are critically necessary for the polarization of effective T cell responses [5]. However, in CLL an increasingly defective immune synapse enables the malignant B-cell to evade immune detection by inducing T cell anergy as well as improper Th2 polarization [2,6–8]. The end result of this immunosuppression is a high incidence of severe infections that in the setting of therapeutic interventions, often lead to patient morbidity [3,9–11].

Original identification of the Th1 and Th2 T cell subsets established disparate patterns of stimulation underpinned by static and heritable epigenetic changes [12]. More recently, however, the widely observed plasticity in Th cell differentiation was nailed down to bivalent epigenetic marks that maintained heritability yet provided the flexibility to tailor activation status based upon changing external signals [13]. Surprisingly, these bivalent marks were identified on transcription factors previously considered “master regulators” of Th cell differentiation, opening the possibility for diametrically opposed states of activation to alternately persist in a single clonal cell population [14,15].

Azanucleoside hypomethylating agents have become part of the armamentarium for the treatment of myelodysplastic syndrome and are currently under investigation in various hematologic and solid malignancies [16]. Although the mechanism of action relatively well defined, clinical and laboratory evidence has shed light on a variety of sometimes divergent immunologic effects [17]. Many of these immunomodulatory effects have been identified at concentrations well below the chemotherapeutic threshold.

Based upon a new understanding of CLL immune dysfunction and T cell polarization we postulated that sub-cytotoxic levels of a DNA methyltransferase inhibitor may be able to alter T cell polarization and anergy found in CLL patients. Our studies sought to investigate the consequences of methylation inhibitor treatment on lymphocyte survival, activation intensity, and naïve cell polarization. Our results demonstrate the ability to force Th1 cell polarization despite strong Th2 polarizing conditions, increase T cell proliferative capacity, and initiate the conversion of Th2 into Th1 lymphocytes.

2. Materials and methods

2.1. Subject populations

Sera and peripheral blood mononuclear cells (PBMCs) were obtained from patients with CLL (Supplementary Table 1). All subjects gave written institutional review board (IRB)-approved informed consent for their blood products to be used for research. Blood was collected at the H. Lee Moffitt Cancer Center (Tampa, FL). PBMCs were stored in 1 ml aliquots at –140 °C and sera were stored in aliquots at –80 °C until used.

2.2. Cell culture, drug treatments, and T cell polarization

Unless otherwise stated cells were cultured in vitro at 37 °C and 5%CO₂ using RPMI1640 medium supplemented with 10% fetal calf serum and antibiotics. Drug treatments were carried out on plate-bound anti-CD3, soluble anti-CD28, and IL-2 (20 U/ml) stimulated magnetically isolated T cells (>95% purity) for 72 h in complete medium using the indicated concentration of 5-aza-2'-deoxycytidine (5A2) (dissolved at 10 mM in DMSO). At the conclusion of treatment cells were washed twice using pre-warmed serum-free RPMI1640 and subjected to the various assays (Supplementary Fig. 1). All CD4 T cells were negatively isolated using magnetic separation (EasySep CD4+ and Naïve CD4+ T-cell enrichment kits, STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer protocol. Th1 and Th2 polarized T cells were obtained from magnetically purified naïve human CD4 T cells by weekly stimulation with plate-bound anti-CD3, soluble anti-CD28, and IL-2 (20 U/ml) in the presence of IL-12 (10 ng/ml) and anti-IL-4 (1:100) for Th1 or IL-4 (5 ng/ml) and anti-IL-12 (1:100). Medium was replaced twice weekly and stimulation was repeated for four weeks upon which a sample was collected and tested via CBA array and qRT-PCR for polarity (Supplementary Fig. 2).

2.3. Reverse transcriptase-PCR (RT-PCR)

Total RNA was prepared from pelleted cells (RNeasy mini columns and RNase free DNase, Qiagen, Valencia, CA). RT-PCR and qRT-PCR reactions were conducted using the Qiagen onestep RT-PCR kit (Qiagen) or the iScript SYBR green RT-PCR kit (BioRad, Hercules, CA) with transcript-specific primers (T-bet: 5'TGACCCAGATGATTGTGCTC, 3'ATCTCCCCCAAGGAATTGAC) (GATA3: 5'AAGGCAGGGAGTGTGTGAAC, 3'TGGATGCCTTCCTTCTTCAT) (IFNG: TTCAGATGTAGCGGATAATGGA, 3'TCAGCCATCACTTGGATGAG) and 200 ng of total RNA. RT-PCR amplification reactions were resolved on 2% agarose gels and the size of the amplified transcript confirmed by comparison with a standard DNA ladder (GelPilot 1 Kb Plus Ladder, Qiagen). qRT-PCR experiments were analyzed using the MyiQ software package. After confirming a single melt curve peak CT values for Actin were compared to CT values for the transcript of interest using the 2^{-CT} method.

2.4. Flow cytometry and cytokine bead array

Flow cytometric analysis was performed using fluorochrome-labeled monoclonal antibodies (mAbs; anti-CD3, -CD4, -CD8, -IL-4, -IFN γ , and anti-pSTAT6, Becton Dickinson, San Jose, CA and eBiosciences, San Diego, CA) and the viability dye 4',6-diamidino-2-phenylindole (DAPI, Sigma). Intracellular staining of IL-4, IFN γ , and pSTAT6 was conducted according the appropriate manufacturer protocol (Becton Dickinson). For intracellular staining of IL-4 and IFN γ PMA and Ionomycin stimulation was utilized. Cytokine bead array (CBA) (Becton Dickinson) was conducted according to the manufacturers published protocol using cellular supernatant from three replicate experiments. Flow cytometric data was analyzed with FlowJo software (Tree Star, Ashland, OR) on a minimum of 30,000 collected events.

2.5. Methylation analysis

For both Methylation specific PCR (MSP) and bisulfite sequencing purified DNA (Qiagen DNA miniprep kit, Qiagen) was bisulfite treated according to the manufacturer protocol using the Epitect Bisulfite treatment kit (Qiagen). MSP was conducted using transcript specific primers designed to specifically recognize either methylated or unmethylated CpG motifs (IFNG-methyl 5'AAGAGTTAATATTTTATTAGGGCGA, 3'TAAACTCCTTAAATCCTTTAACGAT) (IFNG-unmethyl 5'TGAAGAGTTAATATTTTATTAGGGTGA, 3'TAAACTCCTTAAATCCTTTAACAAT). Touchdown PCR was utilized to generate PCR products which were subjected to gel electrophoresis, stained with ethidium bromide and densitometrically analyzed.

Bisulfite sequencing was conducted using primers indifferent to methylation status (IFNG-Promoter 5'TAGAATGGTATAGGTGGGTATAATGG, 3'ATAACAACCAAAAAACCCAAAAC) (CNS-6kb 5'TGAGTAAAGGTTTAGGGTATTTTTT, 3'ACTCACTACAACTCTACCTCCC). PCR amplicons were cloned into plasmid vectors using a T-A cloning kit (Qiagen) and transformed bacterial colonies were directly sequenced using vector primers. A minimum of 12 bacterial colonies were sequenced for each sample. Sequencing results were used to calculate methylation status of CpG motifs and average methylation stats was depicted in graphical form.

2.6. Western blot analysis

Western blotting experiments were conducted using conventional methodology previously described [18]. Blotting was conducted using pSTAT1 Y701 (Cell Signaling Technologies, Danvers, MA), T-bet (eBiosciences), STAT1, and β -Actin (Santa Cruz Biotechnology, Santa Cruz, CA) specific antibodies.

3. Results

3.1. T lymphocytes display phenotypic Th1 repolarization after treatment with 5A2

To understand the activity of DNA methyltransferase inhibitors (DNMTi) on T helper cell polarization we examined the effects of a single dose of 5A2 (0.3 μ M, 1 μ M, 3 μ M, 10 μ M, and 30 μ M) on fresh CD4 T lymphocytes obtained from healthy blood donors. Intracellular staining revealed an increase in IFN γ (Th1 cytokine) positive cells, ranging from 10–13% at baseline to 39% in the 30 μ M dosage (Fig. 1A). To identify any potential detriment to CD8 T lymphocyte responses we examined IFN γ production in CD8 T cells from healthy donors and observed a similar increase which directly correlated with drug treatment (Supplementary Fig. 2).

To more specifically address alterations to Th2 cells we conducted phosflow analysis of pSTAT6 levels in CD4 T cells from healthy donors. Since pSTAT6 levels in Th2 cells quickly rise following activation via CD3 ζ and CD28 we conducted a timecourse analysis spanning 15–75 min post activation [19]. Interestingly, pSTAT6 was found to decrease with

drug treatment indicating that Th2 signaling patterns were abrogated by the DNMTi (Fig. 1B).

To better understand the effects of 5A2 on Th1 and Th2 cell populations in vitro polarized T cells were subjected to 5A2 treatment and the secreted levels of IFN γ were measured by CBA (Fig. 2A and Supplementary Fig. 3). The results confirmed that IFN γ production in Th2 cells increased in a dose dependent manner with 5A2. It was also noted that Th1 cells produced more IFN γ , however this was less pronounced.

In addition we tested the resulting polarity of 5A2 treated naïve cells after stimulation under strong Th1 or Th2 polarizing conditions using intracellular staining of IFN γ and IL4. As expected, in the control sample Th2 polarizing conditions resulted in exclusively IL4 secreting cells, however 5A2 was capable of eliminating the IL4 response and inducing the secretion of IFN γ in a dose dependent manner (Fig. 2B). Our data also confirmed that Th1 polarizing conditions resulted in exclusive IFN γ production which was further exacerbated by 5A2 treatment.

3.2. T cells from CLL patients alter polarization towards Th1 in response to 5A2

It has been previously demonstrated that T cells from CLL patients generally proliferate poorly after in vitro stimulation with low IL2 (20 U/ml) and secrete high levels of IL4, and relatively low levels of IFN γ [20,21]. Given our previous data we wanted to test the repolarizing effects of 5A2 on these cells. We started by examining the IFN γ secreted by T cells isolated from four CLL patients using CBA. Our results matched the characteristic dose-dependent increase in IFN γ revealed earlier in healthy donor T cells (Fig. 3A). These results were further confirmed using intracellular staining to examine the levels of IFN γ produced in response to stimulation (Fig. 3B). In addition, we found that the percentage of IL4 positive T cells decreased under 5A2 treatment, indicating that a high percentage of Th2 polarized cells were no longer responding to in vitro stimulation via IL4.

As mentioned earlier, the proliferation of T cells from CLL patients after in vitro stimulation is characteristically poor, thus we were curious if 5A2 treatment could alleviate this anergic condition. Using MTX proliferation assays we found that CD4 T cell proliferation directly correlated with 5A2 dose resulting in a 500% increase in proliferation compared to untreated samples (Fig. 3C).

3.3. Th2 cells treated with 5A2 induce constitutive pSTAT1 signaling and express T-bet

The phenotypic alterations we have identified thus far must derive from a specific set of molecular signaling networks. T-bet is considered the master regulator of Th1 phenotype and it is controlled by activated STAT1 signaling. We decided to investigate the protein expression levels of T-bet and pSTAT1 in healthy donor and CLL CD4 T lymphocytes by Western blot. As depicted in Fig. 4A T-bet in healthy donor CD4 T cells is expressed at a basal level, however treatment with 5A2 increases this expression. This correlates with the increased phosphorylation of Tyr 701 on STAT1. Similar results were observed in T cells obtained from a CLL patient; however both T-bet and pSTAT1 were not identified at a basal level (Fig. 4B).

Our prior experiments revealed phenotypic repolarization was restricted to the Th2 cells, thus we wanted to confirm that molecular alterations in purified populations of Th1 and Th2 were restricted to the Th2 population. Using Western blot analysis on polarized T cells we identified an increase in pSTAT1 Tyr 701 in Th2 cells when treated with 5A2. Notably, pSTAT1 levels were maintained at a higher basal level in Th1 cells potentially rendering any alteration of pSTAT1 levels by 5A2 less evident.

3.4. 5A2 treatment of T cells stimulates a well characterized IFN γ autocrine loop

Our data identified a link between T-bet, pSTAT1, and 5A2 treatment of CLL and Th2 T cells. We wanted to know if the demethylating agent was eliciting the expression and secretion of IFN γ thus inducing a well defined STAT1 α mediated autocrine feedback loop. To explore this possibility we decided to abrogate this autocrine loop using anti-IFN γ . Our experiments revealed that soluble IFN γ was directly linked to the activation of STAT1 α in response to 5A2, confirming our hypothesis (Fig. 5A). To confirm that the IFN γ and T-bet were upregulated at the transcriptional level we conducted qRT-PCR. Our examination of mRNA levels indicated that both T-bet and IFN γ mRNA were increased after 5A2 treatment (Fig. 5B and C).

3.5. 5A2 treatment of CLL T cells specifically induces demethylation of the IFNG promoter

In order to link the phenotypic changes with the molecular alterations we observed it was necessary to provide a mechanism for 5A2 induced IFN γ expression. The IFNG locus spans a 110 kb region on human chromosome 12. Using methylation specific PCR and bisulfite sequencing we identified a region comprised of 200 bp proximal to exon I of IFNG which was demethylated by 5A2 (Fig. 6A–C). Our results reveal the methylation percentage of two independent CpG sites was decreased in T cell populations from CLL patients after treatment with 5A2. Although, using Methyl-ChIP, we tested other regions of the IFNG locus, including the Conserved noncoding sequence (CNS) –56 kb, –54 kb, –34 kb, Intron I, +18–20 kb, +24 kb, +46 kb, and +55 kb, we could only identify significant alterations in methylation within the promoter region (data not shown). Notably, the basal methylation level of untreated CLL cells resembled the highly methylated state seen in purified Th2 T cells.

In mouse studies it has been shown that the CNS-6 kb region was divergently methylated in Th1 and Th2 cells [22]. Additionally, it has been postulated that 5A2 treatment demethylates this region resulting in transcription of the IFNG gene in human T cells [23]. Our studies confirmed the notion that Th1 and Th2 cells differ in their respective methylation levels within the CNS-6 kb region using bisulfite sequencing, however after detailed analysis we could not confirm demethylation in T cells after treatment with 5A2 indicating that this region is not responsible for the phenotypic and molecular changes in T cell polarity that we have identified (Supplementary Fig. 4A and B). Fig. 6D shows our proposed schema for the activation of T-bet using 5A2 based on the evidence at hand.

4. Discussion

The heritable epigenetic alterations which underpin T cell polarity can also become chromatin-based patterns which are altered in disease states. Our studies have shed light on a potential mechanism by which therapeutic application of demethylating agents such as 5A2 can induce the signaling patterns of Th1 phenotype in what would generally be considered Th2 T cells. We have also demonstrated this in a disease state, CLL, for which Th2 polarization regularly results in the inability to adequately deal with external pathogens. As of yet it is uncertain how long these molecular alterations can persist in the absence of continued 5A2 treatment. In unpublished studies we have identified phenotypic changes which continue for weeks post treatment and our ongoing studies will work to identify any temporal limitations to these chromatin modifications. It is conceivable, that even temporary alteration of T cell phenotype may have an impact in the T cell immunosuppression seen in patients with CLL. Altogether, our current and published data builds a therapeutic application upon a newly established framework of basic science indicating that methylation induced silencing of the IFNG locus is critical to Th2 cell differentiation [22,24].

With the general goal of chemotherapeutic effect many of the clinical trials to date have used concentrations of 5A2 which are detrimental to the healthy lymphoid compartment. We too have occasionally witnessed a reversal of immunotherapeutic effects at higher doses of 5A2 (30 μ M or higher). A recent clinical trial has been conducted using a relatively low dose 5A2 in CLL patients. While 5A2 alone demonstrated little cytotoxic effect on CLL cells the 10 mg/m² dosage was well tolerated; unfortunately however, the effects to T lymphocytes were not studied [25]. In a similar Phase I study of T-cell lymphomas 5A2 at 10 mg/m² reduced global DNA methylation in T lymphocytes by 2.5–6%, lending credence to the idea that sub-chemotherapeutic levels of demethylating agents may alter the methylation of the T cell compartment [26].

Currently, there is scant evidence regarding the regulation of IFNG in human T lymphocytes, however regulation in mice has been comprehensively studied by CB Wilson [22]. A single manuscript has previously identified the epigenetic repolarization of human tumor infiltrating lymphocyte clones using 5A2, however the scope of this study lacked a detailed analysis of the mechanism and molecular phenotype [23]. In prior published studies we identified a number of demethylation-induced immunotherapeutic effects which in total lead to an increased antigen presenting phenotype in CLL cells, a result which complements our current findings [27].

We have identified molecular signatures which parallel the increased IFN γ response. The molecular signals recapitulate an expected Th1 T cell including T-bet, pSTAT1, and decreased pSTAT6. To our knowledge we are the first to identify these molecular signature alterations in response to demethylating agents. In addition, we have identified a region of the IFNG locus which is actively demethylated by 5A2 and potentially induces expression in T cells. In previous reports using human T cells methylation of the CNS-6 kb region of the IFNG locus was shown to mirror T cell phenotype. Our studies clearly demonstrate that demethylation of the CNS-6 kb region is not necessary for IFNG expression and a Th1 phenotype. It remains unclear why the CNS-6 kb region was unchanged by 5A2 however it

is possible that this region may be progressively demethylated as a Th1 clone expands, further reinforcing Th1 polarity.

Our findings generate provocative questions regarding the efficacy of new immunotherapeutic strategies based around 5A2. Numerous studies have identified the induced expression of various cancer germline antigens in both solid and hematologic malignancy, including CLL [17]. A strategy which incorporated the restoration of T cell cytolytic capacity with the expression of potent immunotherapeutic antigens specifically within tumor cells would likely be attractive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.leukres.2011.02.007.

Fig. 1.

Intracellular staining reveals 5A2 induced IFN γ and reduced pSTAT6. Magnetic negatively selected CD4⁺ T cells from healthy donors were treated with 5A2 (30, 10, 3, 1, 0.3, and 0 μ M) or left untreated and stimulated in vitro prior to FACS analysis. (A) Intracellular levels of IFN γ and IL-4 after 12 h of PMA/ionomycin stimulation. (B) T cells were assayed for intracellular pSTAT6 at various timepoints after stimulation with α CD3 and α CD28 (15, 30, 45, 60, and 75 min) all percentage data were normalized to an untreated and unstimulated sample. Experiments were repeated three times with various healthy donors and error bars represent standard deviation of triplicate samples.

Fig. 2.

Th2 cytokine polarization is inhibited by 5A2 treatment. (A) IFN γ released by in-vitro polarized and 5A2 treated Th1 and Th2 T cells subjected to 24 h of stimulation with α CD3 and α CD28 was assayed via CBA. Error bars represent the standard deviation of four replicate samples. (B) Intracellular staining of IL4 and IFN γ in magnetically isolated 5A2 treated naïve CD4 T cells from a healthy blood donor subjected to stimulation with α CD3 and α CD28 along with strong Th1 or Th2 stimulation (indicated under title). Error bars represent standard deviation of the mean. Student's *t*-test *p* values are calculated versus untreated (note: *p* value for IL4 in panel B) – Th2 polarization compares untreated to all others).

Fig. 3.

T cells from CLL patients are phenotypically repolarized by 5A2. CD4 T cells were magnetically isolated from CLL patients, immediately subjected to 5A2 treatments, and assayed for alteration to phenotype. (A) T cells secretion of IFN γ was measured using CBA. Data represents the mean and standard deviation of triplicate samples of four independent CLL patients. (B) T cells from a CLL patient were assayed via FACS analysis for intracellular levels of IFN γ (top panel) and IL4 (bottom panel) in response to 5A2 treatment and PMA/ionomycin stimulation. For comparison histograms representing an unstimulated (red) and stimulated yet untreated (blue) sample are provided along with the relative percentage of positive cells for each histogram. (C) Proliferation of T cells from CLL patients in response to α CD28 and α CD3 along with 20 U/ml IL2 was interrogated using an MTX assay. Data are normalized to the untreated sample and the graph represents triplicate well from four independent CLL patients with error bars representing standard deviation. Student's *t*-test was used to calculate indicated *p* values versus the vehicle (0 μ M) control.

Fig. 4.

5A2 induces pSTAT1 and T-bet signaling in T cells. Negatively selected 5A2 treated CD4 T cells from a healthy blood donor (A) or a CLL patient (B) were assayed via Western blot for total cellular levels of pSTAT1 and T-bet protein. In-vitro polarized (C) Th2 and (D) Th1 T cells were assayed via western blot for pSTAT1. For comparison β -Actin and total STAT1 levels were also included. Western blots are representative of three independent experiments.

Fig. 5.

pSTAT1 signaling is induced by autocrine IFN γ . (A) Magnetic negatively selected CD4 T cells from healthy donors were treated with 5A2 and α IFN γ as indicated and subjected to Western blot for pSTAT1 and total STAT 1. (B and C) Total RNA obtained from magnetically isolated CD4 T cells from healthy donors were subjected to T-bet and IFN γ transcript specific qRT-PCR. Data depicted is representative of three independent experiments; Student's *t*-test *p* values indicated compare each sample to the vehicle (0 μ M) control.

Fig. 6.

5A2 specifically demethylates the IFNG promoter. (A) Synthetically methylated, unmethylated, and genomic DNA were assayed using methylation specific PCR primers demonstrating transcript specificity. (B) MSP was carried out on bisulfite treated DNA obtained from Th1, Th2, and CD4 T cells obtained from CLL patients treated with various concentrations of 5A2 in addition to synthetically methylated and unmethylated DNA samples. Student's *t*-test *p* values indicated compare each sample to the untreated (0 μM) control. (C) Bisulfite sequencing of the promoter region of the IFNG locus confirms MSP results. For methylation analysis the relative level of methylation for three independent experiments is depicted along with error representing the standard deviation. Note that the graphical range is abbreviated for graph clarity. (D) A graphical depiction of our proposed signaling model in which 5A2 induces ectopic demethylation, expression, and secretion of IFN γ resulting in autocrine IFNGR signaling via pSTAT1 α a known T-bet transcription factor resulting in T-bet expression.