Azacytidine induces global DNA hypomethylation and increases cellular and nuclear SAMHD1 in feline lymphocytes

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Abstract: Sterile α motif and histidine-aspartate domain–containing protein 1 (SAMHD1) is a multifunctional protein that limits cellular dNTP availability, interacts with specific retroviral proteins to induce degradation. Regulation of dNTP availability is crucial for cell cycle regulation and DNA stability. Demethylating agents such as azacytidine are in clinical use for cancer therapy, and reduce methylation of the SAMHD1 promoter and SAMHD1 gene expression. Here, we evaluated the effect of azacytidine on global DNA methylation in feline lymphocytes, and specifically on the abundance and cellular distribution of phosphorylated and non-phosphorylated SAMHD1. Azacytidine increased cellular and nuclear SAMHD1 but did not increase phosphorylated SAMHD1. Phosphorylation is essential for SAMHD1 stability and function but is unaffected by demethylation. The findings suggest that treatment with azacytidine could increase viral restriction, and they lend support to development of in vivo models utilizing azacytidine to modulate SAMHD1 activity.

Keywords: Azanucleoside; DMSO; DNA methylation, epigenetics; phosphorylation; restriction factor; triphosphohydrolase; methyltransferase inhibitor

1. Introduction

Sterile α motif and histidine-aspartate domain–containing protein 1 (SAMHD1) was first discovered as an intracellular antiviral factor, and is now the subject of intense research due to multifaceted roles, in particular the differential regulation of dNTP availability at different cell cycle stages. The protein has three structural domains consisting of 1) a nuclear localization sequence (NLS); 2) a central catalytic (HD) domain and; 3) a regulatory C-terminus domain subject to phosphorylation by cyclins and other post-translational modifications [1]. The NLS sequence mediates predominant but not exclusive nuclear localization, while the HD domain conveys dNTP triphosphohydrolase activity, resulting in degradation of dNTPs into 2'-deoxynucleoside and triphosphate components [2]. The C-terminus has a threonine residue at position 592 (T592), which can be phosphorylated by the cyclin A1/CDK complex during the S phase of the cell cycle, and dephosphorylated by the protein phosphatase 2A (PP2A) during late mitosis [3]. Among the most critical roles of SAMHD1 is regulating dNTP availability for cellular processes through hydrolysis, an activity countered through dNTP biosynthesis by ribonucleotide reductases. SAMHD1 is most active in a tetrameric configuration, and while SAMHD1 expression is relatively consistent throughout the cell cycle, tetramer stability is reduced by phosphorylation of T592 and other posttranslational modifications [4]. The functions of the SAM domain are not well understood, but it contains a highly conserved protein-protein interaction motif, and mediates protein-DNA/RNA interactions [5].

A key function of SAMHD1 is restriction of dNTP availability in virally infected cells, which limits replication of viruses like HIV in non-cycling myeloid cells and memory T-lymphocytes. In cells with tetrameric non-phosphorylated SAMHD1, HIV reverse transcription was incomplete, and replication of DNA cytomegalovirus and poxviruses, and other retroviruses such as equine infectious anemia virus and feline immunodeficiency virus, was limited (FIV) [4,6-9]. Some viruses have evolved to encode proteins, such as Vpx of HIV-2, that bind SAMHD1 and direct proteasomal degradation [10].

The role of SAMHD1 is also of great interest in cancer biology. Loss of SAMHD1 activity results in dysregulated dNTP pools, which in turn has been associated with genomic instability and increased mutagenesis. Commensurate with this observation, SAMHD1 is mutated in many cancers, and reduced activity was associated with a greater proportion of cells in the G1 phase [11]. On the other hand, hypomethylating cancer drugs such as azanucleosides may bind to and be inactivated by SAMHD1 [12]. Gene expression is in large part related to the compaction ("condensation") state of chromatin [13]. Phosphorylation and acetylation of histones, and direct DNA methylation, affect DNA compaction and hence accessibility to transcription factors and polymerases. Histone modification affects gene expression

by directly or indirectly altering chromatin structure [14]. Methylated DNA is the most stable epigenetic modification, and defined as the covalent addition of a methyl group at the 5-C position of cytosine (5-methylcytosine, 5-mC); a process catalyzed by DNA methyltransferases (DNMTs) [15]. Concurrent flow cytometric assessment of cell cycle and methylation, combined with sequencing of specific gene regions, has allowed deciphering of the mechanism of action of demethylating agents such as azacytidine (5-AZA), and subsequent gene reactivation [14,16,17]. Methylation of a CpG island proximal to the first SAMHD1 codon repressed transcription in cutaneous T-cell lymphoma and other $CD4^+$ T-cell neoplasms, while in PBMCs of healthy individuals the SAMHD1 promoter was unmethylated (Figure. 1) [18,19]. Thus, treatment with hypomethylating agents such as 5-AZA may alter SAMHD1 gene transcription and in turn dNTP availability during the cell cycle and retroviral restriction.

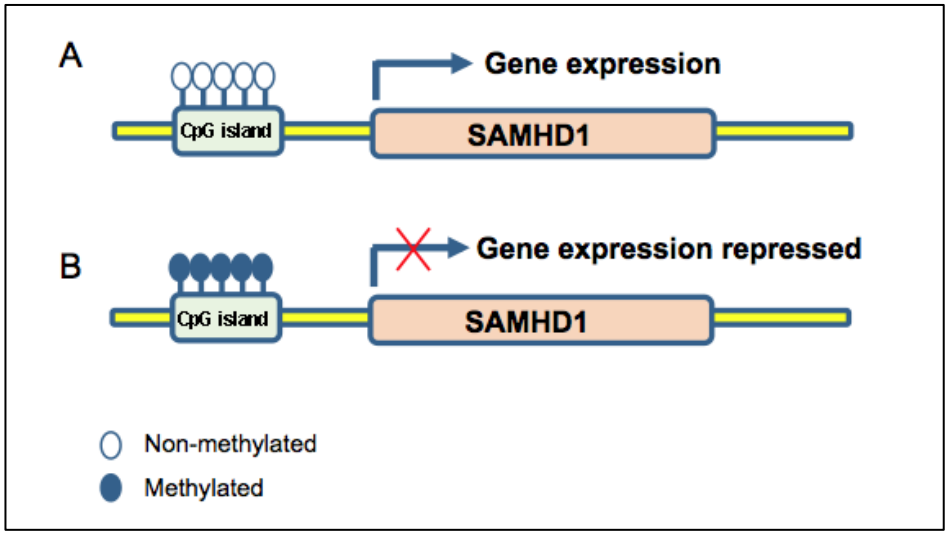

Figure 1. Promoter methylation regulates DNA expression. (A) When the promoter is non-methylated and accessible to transcription factor binding and recruitment of RNA polymerase, gene transcription is enabled (A). Once the promoter is methylated, it is inaccessible to binding by transcription factors, and gene expression is repressed (B).

We previously reported that SAMHD1 is widely expressed in diverse feline tissues, and particularly highly expressed in mucosal and hemolymphatic tissues [20]. Phosphorylation and nuclear translocation of SAMHD1 were increased in response to IFNγ stimulation of feline cells but differed in primary and immortalized cells [21]. Here, we addressed the hypothesis that DNA demethylation with the DNMT inhibitor azacytidine increases the amount of cellular but not nuclear SAMHD1.

2. Methods

2.1. Cell culture

The feline $CD4^+$ T cell line FeTJ was a kind gift of J. Yamamoto (University of Florida, Gainesville, FL). Cells (passage number <10) were cultured in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 50 μM of 2-mercaptoethanol (all from Invitrogen, Burlington, ON) at 5% CO_2 and 37° C.

Dimethylsulfoxide (DMSO) may affect in vitro cellular processes and epigenetic modifications. Since demethylating agents are dissolved in DMSO, the effect of the solvent by itself on cell viability and proliferation was first determined by culturing FeTJ cells in the presence of 0.025%, 0.05%, 0.1%, 0.5%, 1% of DMSO. Cells (10^6/mL) were plated for 72 hours, and then cells were counted, and viability was determined by flow cytometric analysis of 7-aminoactinomycin (7-AAD) exclusion. Based on detection of methylated cytosine, a concentration of 0.1% DMSO was chosen for all subsequent experiments (Figure 2).

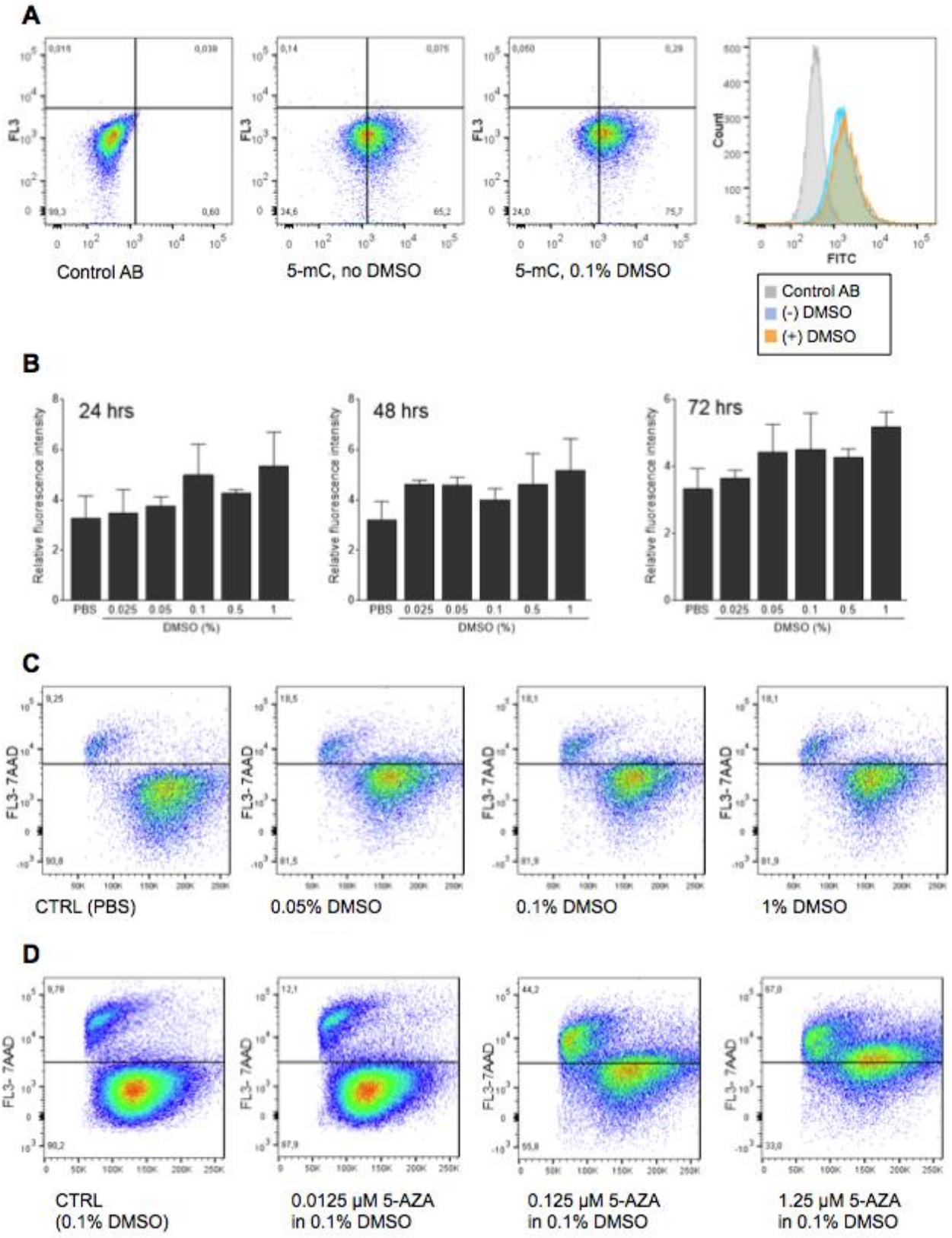

Figure 2. Culture with different concentrations of DMSO does not affect DNA methylation or cell viability. (A) DNA methylation was measured by flow cytometry with FITC anti-5-methylcytosine (5-mC) or isotype control antibody. (B) Analysis over time showed no significant differences after 24, 48 or 72 hours of treatment. (C) Different concentrations of DMSO had no significant effect on cell viability after 72 hours, as measured by 7-AAD fluorescence. (D) Viability was >50% in cells cultured for 72 hours with 0.0125, 0.0312, 0.0625, and 0.125 μM azacytidine in 0.1 % DMSO, but <50% at higher concentrations of azacytidine.

Next, the effect of azacytidine (5-AZA; Sigma-Aldrich, Oakville, ON) on cell viability was determined using the same flow cytometric method. Different concentrations of 5-AZA (0 to 10 μM) were prepared in 0.1% of DMSO and applied to the cells. Cells were cultured in RPMI media, and media containing fresh 5-AZA was replaced every 24 hours. Cells were collected 24, 48 and 72 hours after initiation of cell culture. Concentrations of 0.0125, 0.0312, 0.0625, and 0.125 μM 5-AZA resulted in cell viability >50%, and were assessed in each subsequent experiment (Figure 2).

2.2. Flow cytometry

DNA methylation in cells was quantified as previously described with some modifications [22,23]. Cells (1×10^6) were washed with Dulbecco's phosphate-buffered saline (DPBS), and stained with Zombie NIR fixable dye (BioLegend, San Diego, CA) for 30 min at room temperature, following the manufacturer's instructions. Cells were then washed and resuspended in 2% neutralized formalin for 10 min at 37 °C. After two washes with D-PBS containing 0.1% Tween 20 and 1% BSA (DPBT-BSA), cells were cooled and maintained in a 4 °C water bath for 10 min, and then permeabilized by adding 9 volumes of -20 °C methanol for 30 min. After three additional washes with DPBT-BSA, cells were incubated in 2 N HCl at 37 °C for 30 min and then with three volumes of 0.1 M borate buffer (pH 8.5) for 5 min. After three additional washes, 5-methylcytosine (5-mC) antibody (clone 5MC-CD, Abcam, Brantford, CT) conjugated to FITC was added, and cells were incubated for 20 min at room temperature (RT) in the dark. Controls consisted of cells were prepared in an identical manner, and incubated with isotype-matched CD55 antibody (clone MEM-118, Abcam), also conjugated to FITC.

The cells were washed, pelleted, and re-suspended in 400 μL of flow cytometry (FC) buffer (PBS containing 5 mM EDTA, 2 mM NaN_3 and 1% horse serum) light scatter and fluorescence was captured with a FACSCanto II (BD, Mississauga, ON) cytometer. Data were analyzed with FlowJo software (BD). Relative fluorescence intensity (RFI) was calculated as the ratio of the median fluorescence intensity (MFI) of cells analyzed with 5-mC antibody to the MFI of cells prepared with the isotype control antibody.

For detection of SAMHD1 and pSAMHD1, 1×10^6 cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and stained with Zombie NIR fixable dye (BioLegend, San Diego, CA) for 30 min at room temperature. Cells were washed with PBS and then fixed and permeabilized with Cytofix/Cytoperm Fixation/Permeabilization Kit, following the manufacturer's instructions (BD, Mississauga, ON). After washing and re-suspension in 100 μL of wash buffer (BD), cells were incubated with the relevant antibodies for 30 min at room temperature (RT) in the dark. The SAMHD1 antibody (clone OTI1A1, OriGene, Rockville, MD) was conjugated to Alexa Fluor 488 and the pSAMHD1 antibody (clone T592p, EMD Millipore, Billerica, MA) was conjugated to Pacific Blue (Zenon labeling kit, Molecular Probes, Thermo Fisher, Mississauga, ON). An antibody against human CXCR4 (clone 44747, R&D Systems) conjugated to the same fluorochromes was used as a non-binding isotype control. The cells were then washed, pelleted, and re-suspended in 500 μL flow cytometry (FC) buffer (PBS containing 5 mM EDTA, 2 mM NaN_3, and 1% horse serum). All samples were analyzed with the same cytometer, and data were analyzed with FlowJo software (BD).

For nuclear staining, 2×10^6 cells were washed in PBS and then treated for 2 minutes with 100 μL of 0.2 % Nonidet-P40 (Sigma-Aldrich, Oakville, ON) to dissolve the cell membrane. Cytoplasmic remnants were removed by washing nuclei twice, and then the nuclei were fixed, permeabilized, and stained as described above. The RFI of SAMHD1 and pSAMHD1 was calculated as above: MFI of cells stained with specific antibody)/MFI of cells stained with control antibody.

2.3. Immunofluorescent microscopy

Cytocentrifuge preparations were generated by spinning PBS-washed FeTJ cells onto charged slides at 500 *g* for 5 minutes using a cytocentrifuge (Cytopro, Wescor, Logan, UT). For cell fixation, slides were covered with acetone for 10 min at −20 °C. After two washes, cells were incubated with 7AAD for 15 minutes at 4 °C, and then washed. Slides then were incubated overnight with either 5-mC or CD55 antibody diluted 1:10 in PBS. Imaging was carried out using a multispectral imaging system (Quorum Technologies, Guelph ON Canada), which consists of the Nuance CRi multispectral camera mounted on an inverted Leica DMI 6000 brightfield microscope. Single filter images were merged and analyzed with Metamorph image analysis software (Molecular Devices Corp., California USA).

2.4. Statistical analysis

Each experiment was performed with at least two biological replicates and analysis of 10,000 viable cells. Differences between groups were tested by one-way analysis of variance and Dunnett's multiple

comparison test using SigmaStat 4.0 (Systat Software Inc., San Jose, CA). All results were expressed as mean ± standard deviation (SD), and $P < 0.05$ was considered significant.

3. Results

3.1. DMSO treatment does not significantly change global DNA methylation

DMSO was considered to be among the least reactive chemical solvents for biological research, but recent detailed analysis in fields such as epigenomics suggested that DMSO might affect dynamic regulation of DNA methylation and expression of certain proteins [24,25]. Immunofluorescence analysis of 5-MC antibody binding also showed that FeTJ cells had variable levels of methylated DNA (Figure 3). Therefore, the effect of different concentrations of DMSO on DNA methylation in FetJ cells was first evaluated. Flow cytometric analysis showed that 0.025 to 1% DMSO minimally affected DNA methylation in cultured FeTJ cells (Figure 2A and B). A concentration of 0.1% DMSO was used in subsequent experiments to dissolve 5-AZA.

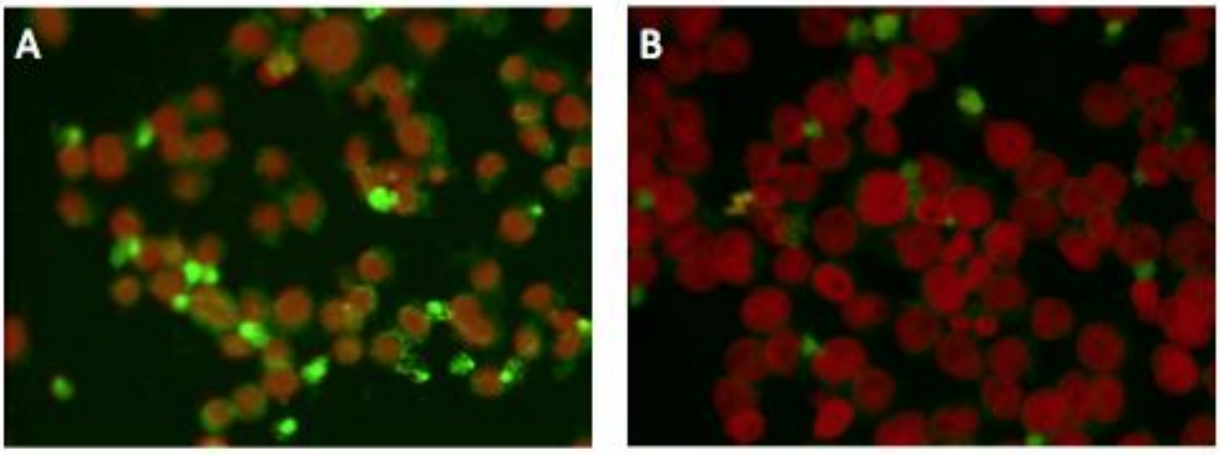

Figure 3. Detection of methylated DNA in untreated cells with a FITC-conjugated antibody to 5-mC shows variable degrees of fluorescence among lymphocytes (A). An isotypic control antibody yields minimal fluorescence (B). Nuclei were counterstained with 7-AAD.

3.2. Azacytidine decreases global DNA methylation

Global DNA methylation was quantified by measuring the fluorescence of bound 5-mC antibody in lymphocytes after 72 hours of culture in the presence of 5-AZA. Overall methylated DNA was decreased in a concentration-dependent manner (Figure 4). A significant decrease in DNA methylation was observed in the samples treated with 0.125 μM of azacytidine compared to cells treated with DMSO.

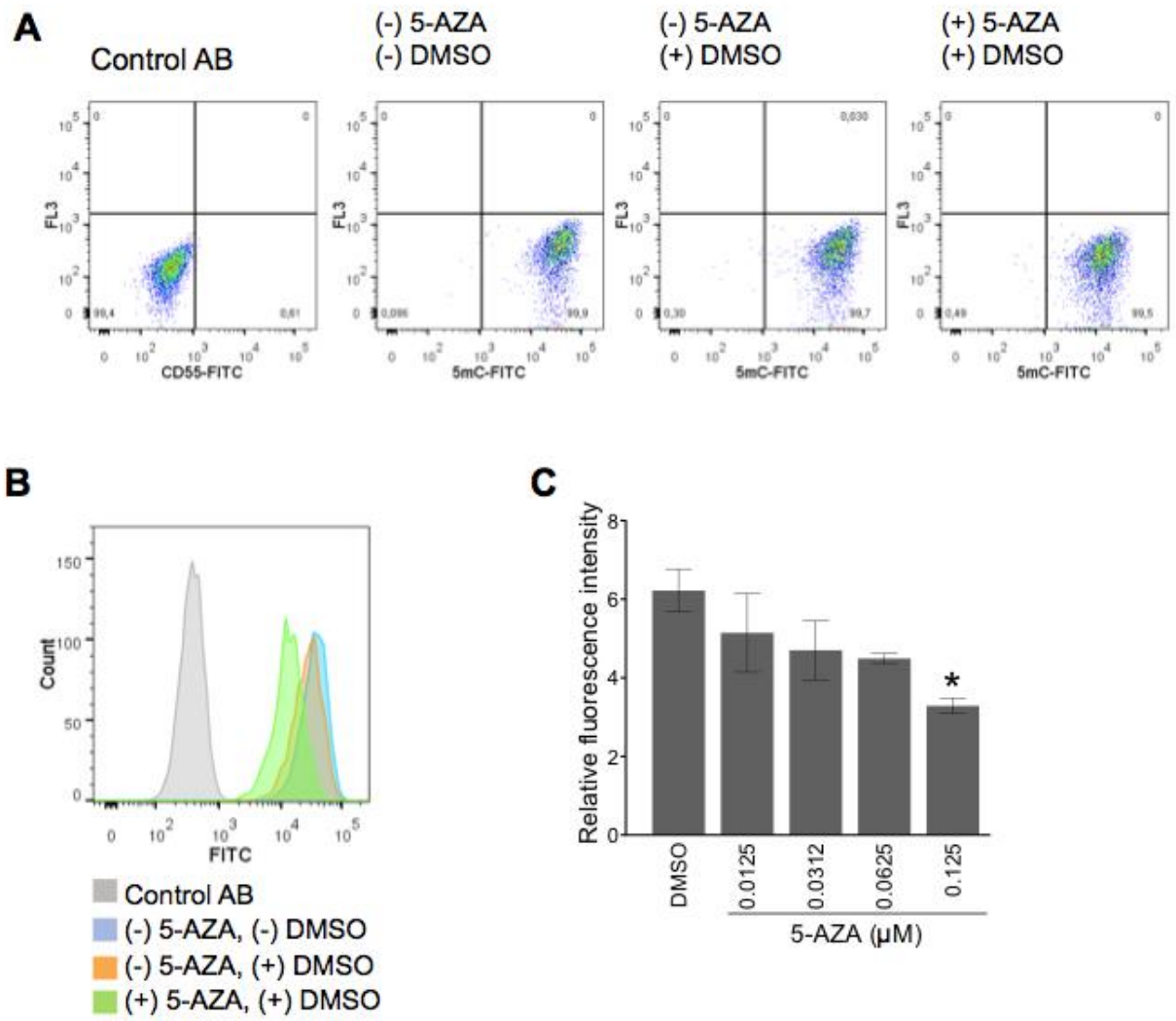

Figure 4. Azacytidine decreases DNA methylation in a concentration-dependent manner. (A, B) Azacytidine at 0.125 μM reduced 5-mC intensity in lymphocytes. (C) The effect was dose-dependent with a significant decrease (* $P = 0.015$) in methylation in cells cultured for 72 hours in the presence of 0.125 μM azacytidine.

3.3. Azacytidine increases cellular SAMHD1 but not pSAMHDI

Treatment of lymphocytes with different concentrations of 5-AZA increased total cellular SAMHD1 (Figure 5), with concentrations of 0.0625 and 0.125 μM resulting in a significant increase at 72 hours. On the other hand, detection of phosphorylated SAMHD1 did not differ significantly over the range of 5-AZA concentrations, suggesting minimal effect on post-translational modification of SAMHD1.

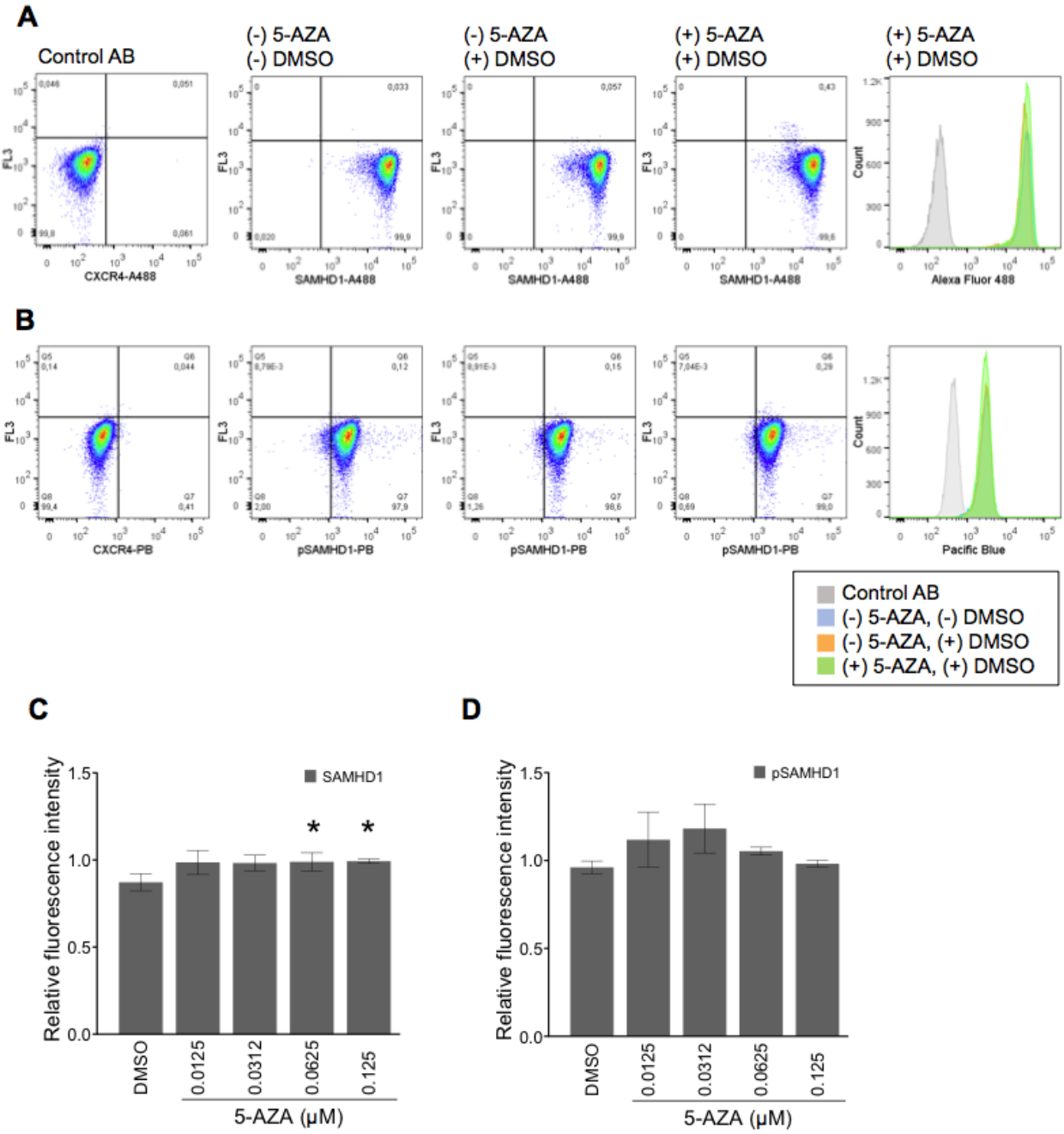

Figure 5. Treatment with AZA increases cellular SAMHD1 but not pSAMHD1. Flow cytometric detection of total (A) and phosphorylated (B) SAMHD1 in FeTJ cells cultured for 72 hours with 0.125 μM of AZA. Cells were stained with Alexa 488-conjugated isotype control or anti-SAMHD1 antibody (A); and with Pacific blue-conjugated isotype control or anti-pSAMHD1 antibody (B). Total SAMHD1 (C) was significantly higher in cells treated with 0.0625 ($P = 0.048$) and 0.125 μM ($P = 0.042$) AZA, while pSAMHD1 (D) was not significantly changed.

3.4. Azacytidine increases nuclear translocation of SAMHD1

SAMHD1 contains a NLS within the first 150 amino acids, which is required for translocation across the nuclear membrane. SAMHD1 with mutated NLS sequences was retained in the cytoplasm but nevertheless attenuated HIV-1 progression, presumably indirectly by limiting dNTP availability [26]. Hence, the relative cell distribution of SAMHD1 is important for viral restriction and cellular processes. Global lymphocyte demethylation with 5-AZA treatment increased nuclear SAMHD without a significant increase in the level of SAMHD1 phosphorylation (Figure 6).

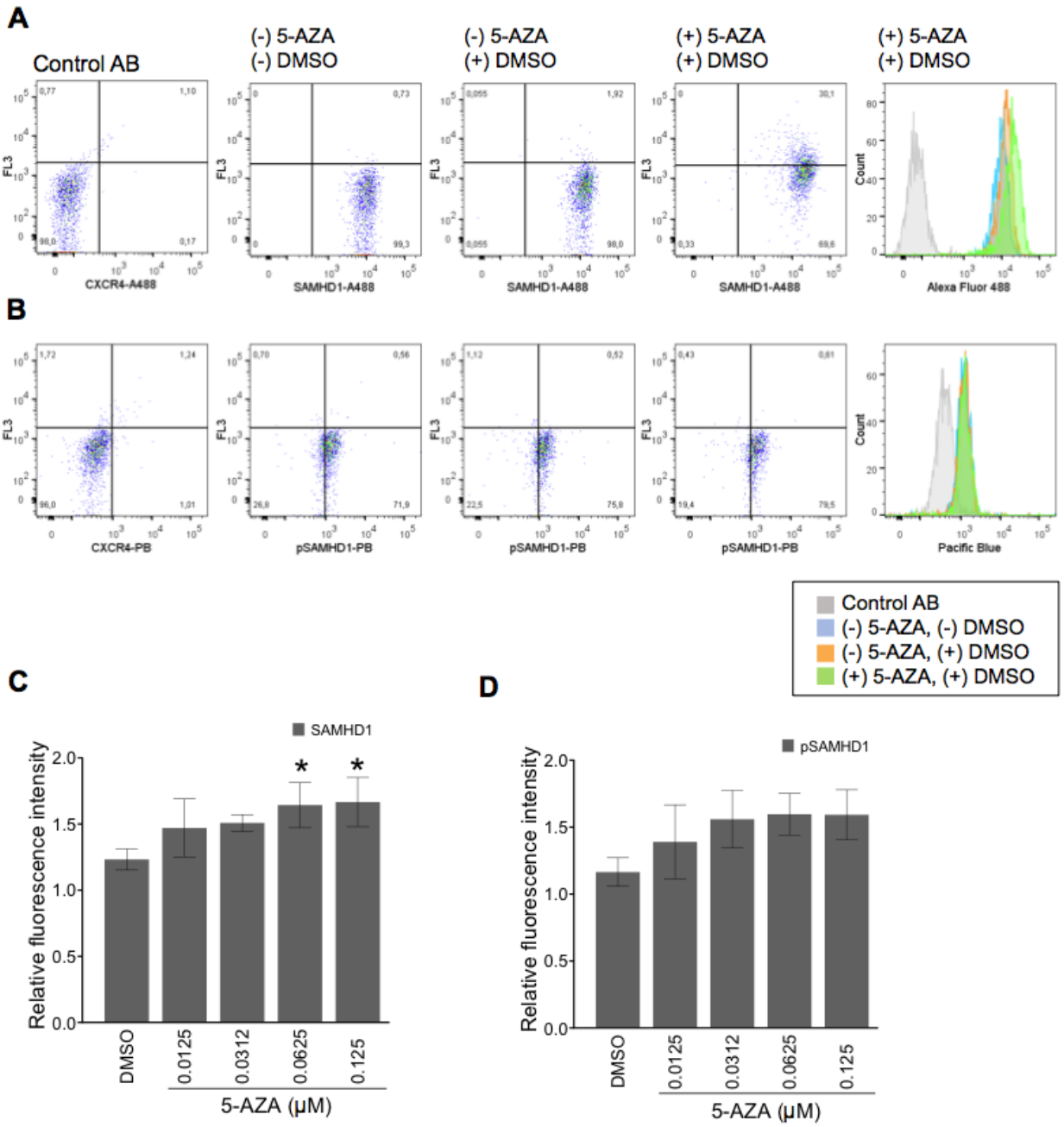

Figure 6. Treatment with AZA also increases nuclear SAMHD1. Flow cytometric assessment of total (A) and phosphorylated (B) SAMHD1 in lymphocyte nuclei after treatment with different concentrations of AZA. Cells were prepared and assessed as described above. (C) Detection of nuclear SAMHD1 was significantly increased after treatment with 0.0625 and 0.125 μM of AZA while (D) pSAMHD1 was not significantly increased.

4. Discussion

SAMHD1 has multiple distinct roles during the cell cycle and in curtailing the cellular progression of viral infections, and has also been recognized as having tumor suppressing activity. The tumor suppressing activity is mostly mediated by SAMHD-hydrolysis limiting cellular dNTP concentration. In contrast, methylation of the SAMHD1 promoter reduces SAMHD1 expression and therefore increases dNTP levels [11]. Azacytidine is a nucleoside-based antimetabolite triphosphate hypomethylating drug used to treat certain cancers, such as acute myeloid leukemia (AML) [27]. Treatment with 5-AZA is expected to increase SAMHD1 gene expression, which in turn may cause increased hydrolysis of 5-AZA. Thus, there is a need to carefully balance SAMHD1 repression and activation in combination with other chemotherapeutic agents. The effect of 5-AZA on SAMHD1 in the context of viral infection is also of great importance, since limited dNTP availability can abrogate cellular viral progression.

In this study we evaluated the effect of 5-AZA on SAMHD1 protein expression, posttranslational modification and cell distribution in FeTJ feline lymphocytes. First, we determined the effect of DMSO, an organic polar aprotic solvent, by itself, on DNA methylation. DMSO does not have an acidic hydrogen, and has been used as an inducer of cell differentiation, free radical scavenger and as a component of cryopreserving media [25]. There is also increasing evidence that DMSO may affect diverse cellular functions such as inflammation, lipid metabolism, apoptosis, cell cycle, enzyme activity, scavenging of reactive oxygen species, cell polarization, radioprotection, and autophagy [28-30]. The effect of DMSO on the epigenome in epithelial and osteoblast cells suggested that DMSO might not be biologically inert [24,31]. Hence, since DMSO was used as the solvent for 5-AZA, we first evaluated its effect on DNA methylation. Feline T lymphocytes (FeTJ) were cultured without and with a range of DMSO concentrations, and the level of CpG methylation was measured by flow cytometry. Methylated DNA increased with increasing DMSO concentration, but the effect at concentrations up to 2% (v/v) was negligible over 48 hours, and only the highest concentration of DMSO (2%) after 72 hours induced significantly increased methylation (Figure 2). These findings were agreed with the immunofluorescence analysis (Figure 3). Both flow cytometric and immunofluorescence results showed that untreated cells contain variable amounts of methylated DNA, and that exposure to DMSO has a minimal effect. This finding is consistent with CpG islands not being distributed evenly throughout the genome, and differential methylation at different cell stages [32].

We next determined that 5-AZA reduces FeTJ cell DNA methylation in a concentration dependent manner (Figure 4). The effect of DNA methyltransferase inhibitors is cell type-dependent. For example,

THP-1 monocytic cells, HuT/CCR5 $CD4^+$ T cells, and MyLa cutaneous T cell lymphoma cells have similar patterns of SAMHD1 promoter methylation but different responses to 5-AZA [18]. Therefore, concentrations of 5-AZA were chosen to induce demethylation while minimizing cytotoxicity [18,33,34]. Our findings showed that 5-AZA treatment reduced DNA methylation and increased SAMHD1 expression (Figure 5). Most cells constitutively expressed SAMHD1, and incubation with 0.625 and 0.125 μM of 5-AZA significantly increased SAMHD1, while detection of phosphorylated SAMHD1 was minimally affected. Phosphorylation is a posttranslational modification that regulates SAMHD1 activity. SAMHD1 has several phosphorylated residues but phosphorylation at T592 destabilizes tetramer formation [3] and modulates HIV-1 restriction activity [35,36]. Azacytidine treatment does not affect phosphorylation of SAMHD1, therefore the observed increase in total SAMHD1 is due to increased SAMHD1 transcription, and reflects an increased ratio of non-phosphorylated to phosphorylated SAMHD1. Since the non-phosphorylated form is the active form of the SAMHD1, it is expected that 5-AZA treatment of FeTJ cells will result in a decreased dNTP pool and increased viral restriction by SAMHD1.

DNA methyltransferase inhibitors have been considered to non-specifically inhibit DNA methylation [37]. Hypomethylation and anti-cancer effect of 5-AZA were explained four decades ago [38,39]. Despite substantial effort to identify and develop novel DNA methyltransferase inhibitors, still azanucleosides consider as the most potent gene reactivators [40]. The azanucleosides 5-azacytidine (aza-cytidine) and 20-deoxy-5-azacytidine (decitabine) are approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS) [41]. These derivates of the nucleoside cytidine can incorporate into DNA and RNA. Following cellular uptake, a portion of 5-AZA is converted to dNTP by ribonucleotide reductase and incorporated into DNA [42]. Decitabine incorporates directly into DNA, and its activity is more specific and less toxic than that of 5-AZA [43]. The azanucleosides incorporated into DNA trap methyltransferase enzymes, prevent DNA synthesis, and subsequently lead to cytotoxicity [42-45]. Azacytidine as a ribose nucleoside competes with cytosine for incorporation into RNA during transcription, and therefore alters RNA function and subsequently protein synthesis [46]. Transient exposure to low dose azacytidine resulted in promoter DNA methylation and gene re-expression in cultured colon cancer [37]. In this study, 5-AZA decreased global DNA methylation in lymphocytes. The effect of methyltransferase inhibitors is relatively non-specific; hence results do not allow distinction between demethylation in gene bodies from changes in CpG islands. Methylation has a complex relationship with gene expression where methylation in promoter regions may block interaction with activating transcription factors, but the role of gene body methylation remains to be clarified.

As determined by immunochemistry, SAMHD1 was present in both the cytoplasm and nucleus of almost all feline tissues and several feline cell lines [20]. Nuclear translocation is mediated by karyopherins and is not essential for retroviral restriction [10,47]. Treatment with 5-AZA increased nuclear SAMHD1, which likely is also due to enhanced SAMHD1 transcription (Figure 6). Phosphorylated SAMHD1 was not increased; emphasizing that 5-AZA does not affect the phosphorylation or dephosphorylation of SAMHD1. A limitation of this study is that the effect of 5-AZA treatment was assessed in lymphocytes and not also in myeloid origin cells. However, it is likely that the functions of SAMHD1, and regulation of gene transcription by DMT inhibitors, are similar in most cell types.

In summary, the findings reported here provide a basis for further evaluation of the effect of 5-AZA in vivo as a potential indirectly acting antiretroviral agent. Feline SAMHD1 restricts primate lentiviruses through binding of a C-terminal domain site to HIV-2 Vpx, and subsequent proteosomal degradation [9]. Hence, functions of SAMHD1 appear highly cross-species conserved, and the cat-FIV model may serve as a valuable in vivo system to study the diverse restricting- and nucleotide-regulating properties of SAMHD1.

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Conflicts of Interest: The authors declare no conflict of interest.

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