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 Altering chromatin structure by blocking histone deacetylase activity with specific inhibitors such as trichostatin A can result in an up-regulation of gene expression. In this report, however, we show that expression of the ETS domain transcription factor PU.1 is down-regulated in cells following the addition of trichostatin A. The loss of PU.1 is seen at both the mRNA and protein levels in multiple cell lines and is reversible following removal of the drug. More importantly, we show that the loss of PU.1 results in a loss of PU.1 target gene expression, including CD11b, c-fms, Toll-like receptor 4, and scavenger receptor. Chromatin immunoprecipitation analysis of cells treated with trichostatin A showed a significant increase in the acetylation of histone H4, but not histone H3, across approximately 650 bp of the PU.1 promoter region. Our data suggest that the consequences of using drugs that inhibit histone deacetylase activity may be a loss of blood cell development and/or function due to a block in PU.1 gene expression. The Journal of Immunology, 2001, 167: 5160–5166.

PU.1 is an Ets domain transcription factor whose expression is required for normal hemopoietic development and formation of a functional immune system (1–4). This is best illustrated by studies on two PU.1 /−− mouse models (3, 4). One null model was embryonic lethal by day 18, and the PU.1 /−− knockout embryos showed a block in the development of monocytes, granulocytes, and B cells (3). In contrast, PU.1 /−− mice were born at a normal frequency in the second knockout model, but died shortly after birth (4). These null mice could be kept alive for up to 14 days if maintained on antibiotics. The second PU.1 /−− mice showed a complete loss of macrophages and B cells. The loss of macrophages probably results from the loss of expression of specific genes regulated by PU.1, including c-fms and CD11b. These mice also have an altered population of nonfunctional neutrophil-like cells (5). These cells do not express certain cell surface markers such as Mac-1 or many molecules involved in the innate immune response to bacteria, such as GP91phox (5). In both PU.1 /−− models T cell development was altered (4, 6). In the second model T cell development in the thymus was delayed until after birth, and T cell numbers in the thymus only reached a fraction of the numbers seen in normal wild-type mice (4). Thus, PU.1 can influence many aspects of blood cell development and function, even in cells where it is normally not expressed.

Just as PU.1 expression is critical for the proper development of the myeloid and lymphoid lineages, its expression can be detrimental to development of the erythroid population (7). PU.1 expression is normally turned off as myeloid/erythroid precursor cells commit to the erythroid lineage (8, 9). This occurs because the GATA1 transcription factor interacts with PU.1 and blocks its function, which promotes erythroid development (8, 9). When PU.1 expression is not extinguished and increases above baseline levels, as following integration of the spleen-focus forming virus into the PU.1 5′ regulatory regions, the erythroid cells are prevented from differentiating and continue to proliferate (10–12). These cells will sometimes mutate a second gene, such as the p53 gene, resulting in the formation of erythroleukemia. The knockout and spleen-focus forming virus models illustrate the importance of turning PU.1 expression on and off during hemopoiesis.

The mechanisms that control this tissue-specific expression of PU.1 are still unclear. Using transfection analyses, several reports have suggested that the proximal promoter can regulate PU.1 expression in specific lineages (13–15). The trans-acting factors suggested to be important, such as Sp1 and Oct1, are not restricted to the same lineages where PU.1 is found. PU.1 may potentially autoregulate its own expression, but it is not clear how PU.1 expression would be initially turned on in cells (14). Recent experiments from our laboratory revealed that cell lines expressing PU.1 contain specific DNase I-hypersensitive sites within the first intron (16). We also showed that the PU.1 locus was hypomethylated at specific CpG sites flanking exon I. Treatment of a nonexpressing T cell line with the drug 5-azacytidine, which blocks DNA methylation, resulted in the expression of PU.1. These data suggested that chromatin structure may play an important role in regulating the tissue-specific expression of PU.1.

The structure of the chromatin over a given locus will determine whether a gene is expressed in a cell. Genes that are actively transcribed are typically in decondensed regions of chromatin, making the locus accessible to trans-acting factors. Conversely, condensed chromatin blocks access to a locus, preventing gene transcription. Modulation of the chromatin structure occurs through enzymatic modification of core nucleosome histones. Although the N-terminus of the core histones can be modified through multiple enzymatic processes, perhaps the best understood modification is acetylation (17–22). The addition of an acetyl group by a histone acetyltransferase to specific lysine residues is thought to disrupt nucleosome-nucleosome interactions, leaving a locus more accessible to trans-acting factors. Reversing this process through histone deacetylase (HDAC)3 activity recondenses the locus and blocks gene expression. The importance of these two processes in gene

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3 Abbreviations used in this paper: HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; TLR, Toll-like receptor; TSA, trichostatin A.
regulation is highlighted by the interaction of many trans-acting factors with coactivator molecules that contain histone acetyl transferase activity (18–22). Likewise, many factors repress transcription because they interact with corepressor molecules that contain HDAC activity (21). More recent studies have shown that nonhistone trans-acting factors can also be modified by acetylation, potentially affecting their ability to regulate gene expression (22).

Blocking HDAC activity in mammalian cells results in the increased acetylation of histones. This effectivly alters the balance of acetylation/deacetylation in cells, which has been shown to have selective effects on gene expression (23). Although the majority of genes are not affected, most that do respond to HDAC inhibition increase their expression. Among these genes are the cyclin-dependent kinase inhibitor, p21, which can block the cell cycle and lead to apoptosis, as well as CD40 and MHC class I and class II molecules, which could influence an acquired immune response (24, 25). These results have prompted several groups to propose the use of HDAC inhibitors to treat cancer and other diseases (26–28). Studies have shown that HDAC inhibitors such as trichostatin A (TSA) can induce the differentiation and/or apoptosis of tumor cell lines (27, 28). Little is known, however, regarding any detrimental effects of these drugs on normal cells. In this report we show that treatment of cells with TSA blocks the expression of the ETS domain transcription factor PU.1. The loss of PU.1 expression is reversible following removal of the drug. We show that the loss of PU.1 results in the loss of PU.1 target gene expression, including the loss of CD11b, c-mfos, Trf4, and scavenger receptor gene expression in macrophages. TSA treatment increased the acetylation of histone H4 that is associated with the PU.1 promoter region, which may block PU.1 expression. These data suggest that the use of HDAC inhibitors to treat cancer may have the unwanted side effect of blocking blood cell development and/or function through the loss of PU.1 gene expression.

Materials and Methods

Cell lines and reagents

The murine macrophage cell lines P388D1, Raw264.7, and WEHI-3 were grown in DMEM (BioWhittaker, Walkersville, MD) with 10% Fetalclone I (HyClone Laboratories, Logan, UT). The murine T cell line 2052 was grown in RPMI (BioWhittaker) with 10% FBS (HyClone Laboratories) and penicillin/streptomycin. The murine myeloid cell line 503-PU.1, a gift from Dr. B. Torbett, was grown in IMDM (BioWhittaker) with 20% FBS, penicillin/streptomycin, 50 μM 2-ME, and 100 U/ml recombinant murine IL-3 (R&D Systems, Minneapolis, MN). All cells were cultured at 37°C with 7.5% CO2. TSA (Sigma, St. Louis, MO) was dissolved in ethanol to a stock concentration of 50 μM. Sodium butyrate (Acros Fine Chemicals, Pittsburgh, PA) was dissolved in water to a stock concentration of 1 M.

Whole cell extract preparation and Western blot analysis

Whole cell extracts were generated using standard protocols. Briefly, cell pellets were resuspended in lysis buffer (50 mM Tris·HCl (pH 7.4), 120 mM NaCl, 0.5% Nonidet P-40, protease inhibitor I, and phosphatase inhibitors I and II; Sigma) and snap-frozen in a dry ice/ethanol bath. After thawing on ice, extracts were centrifuged at 14,000 rpm for 15 min at 4°C. Soluble protein was removed and quantitated using the DC protein assay kit from Bio-Rad (Hercules, CA). Equal amounts of protein were electro- phoresed on 10% SDS-PAGE gels. Proteins transferred to nitrocellulose were blocked in PBS containing 5% milk and incubated with appropriate Ab dilutions. After washes, the blots were visualized using the ImmunStar detection system (Bio-Rad). The following Abs were used in these studies. Anti-PU.1 rabbit polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH Ab was purchased from Chemicon (Temecula, CA). Alkaline phosphatase-conjugated anti-rabbit secondary Ab was obtained from Rockland Immun Diagnostics (Gilbertsville, PA). Alkaline phosphatase-conjugated antimouse secondary Ab was purchased from Bio-Rad.

Semiquantitative cycle RT-PCR and Northern analysis

Total RNA was extracted from cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH), as previously described (29). For analysis of gene expression by semiquantitative cycle RT-PCR, the RT reaction was performed using 1 μg total RNA and the Advantage RT for PCR kit using random primers, according to the manufacturer’s instructions (Clontech Laboratories, Palo Alto, CA.). Semiquantitative cycle PCR using Biolase polymerase (Midwest Scientific, St. Louis, MO) was performed using 10 μl of a 60-μl reaction at each indicated cycle number. PCR products were resolved on agarose gels and visualized using ethidium bromide staining. The oligonucleotide sequences used for the semiquantitative RT-PCR experiments are as follows: PU.1–5′, 5′-GGCGACGTATGGAGAAGAC; PU.1–3′, 5′-GACTTCTTCACCTCGCC; CD11b–5′, 5′-CTTAAAGCCTTCTGGTGACAAGCC; CD11b–3′, 5′-GTTTCCTCCAGTGTGTTGCGC; c-Fms-3′, 5′-ATGGAGTGGGGCCCTCCTGTGC; c-Fms-3′, 5′-AGCCCTTGCGGATATCGCCTGC; SR-A-3′, 5′-ATGACAGGAATCAGAGGCGTC; SR-A-3′, 5′-TGTTGACCAGATAATGTCTCTAC; CD40–5′, 5′-GTTAAGCTTCCGGATGCGA; and CD40–3′, 5′-CTCAAGGCTATTGCTGCTGT.

Flow cytometric analysis

Flow cytometric analysis was performed on a FACScan and analyzed using CellQuest software (BD Biosciences, Palo Alto, CA). The Abs used included PE-anti-CD11b, rat anti-CD40, and PE-goat anti-rat, all purchased from BD Pharmingen (San Diego, CA).

Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed with a ChIP assay kit according to the manufacturer’s protocol (Upstate Biotechnology) with the following modifications. Briefly, 1 × 106 cells were either untreated or treated with 50 nM TSA for 4 h and cross-linked in 1% formaldehyde for 7–10 min. Cells were washed in PBS, resuspended in 200 μl ChIP/SDS lysis buffer, and incubated for 10 min on ice. Following sonication to shear the chromatin, the cellular debris was pelleted. Supernatants were recovered and diluted with 1.8 ml ChIP dilution buffer, from which 40 μl was removed to serve as a 2% input sample. The diluted supernatants were precleared with 80 μl protein G (Upstate Biotechnology) for 30 min at 4°C with rotation. Following centrifugation to remove the protein G, 5 μg of the respective Abs was added to the supernatants and incubated for 18 h at 4°C with rotation. Forty-five µl of an overnight incubation, 60 μl protein G was added to samples for 1 h at 4°C with rotation. The remaining steps leading to purified DNA were performed as stated in the manufacturer’s protocol. The purified DNA samples were resuspended in 50 μl dH2O and used in subsequent PCR analysis. The PCR parameters used were 94°C for 2 min, followed by 28 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, and a 7-min extension period at 72°C. Thirty cycles were used for the input sample control experiments. PCR products were resolved on agarose gels and visualized using ethidium bromide staining. Oligonucleotide sequences used for the ChIP analysis are as follows: P1a, 5′-CAGCCTGGTGTAACCTGCGG; P1b, 5′-CTTGGCTAAGTAGTGTCGCCAG; P2a, 5′-GAGGCGCCTACAGGAAGAC; P2b, 5′-AGGAATCTTGCGCCGCTG; P3a, 5′-CTCTGGCGAGGAGGCTTGCGC; P3b, 5′-GGTGAGGATCAACGCGCAGTC; P4a, 5′-CTTGACCCTGCGTCGCC; and P4b, 5′-ACCCGATCACCGCCACCAGT.

Results

PU.1 protein expression decreases in response to histone deacetylase inhibition

Blocking histone deacetylation with specific inhibitors has been shown to increase the acetylation of histones, resulting in an open chromatin structure and increased gene expression. To determine whether disrupting the cellular deacetylation/deacetylation balance affected the expression of the Ets-domain transcription factor PU.1, the murine macrophage cell line P388D1 was treated with two different histone deacetylase inhibitors. Western blot analysis of whole-cell extracts from a dose response experiment showed

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that PU.1 protein levels decreased with increasing concentrations of either TSA or sodium butyrate (Fig. 1A). GAPDH levels were unchanged, which is consistent with previous studies (30). The amount of TSA or sodium butyrate needed to induce a loss of PU.1 protein corresponded well with the published effective concentrations of these compounds (31). PU.1 protein levels decreased significantly between 40 and 60 nM TSA and between 2 and 4 mM sodium butyrate. Since the only known enzymatic activity inhibited by TSA is deacetylation, we chose to perform all additional experiments with this compound.

To understand how rapidly PU.1 protein expression was lost, a 24-h time course experiment using 50 nM TSA was performed (Fig. 1B). Within 8 h, a significant loss of PU.1 protein expression occurred, and this loss was maintained for 24 h. Since TSA is a reversible inhibitor of HDAC activity, we treated cells for 15 h, followed by washing, and fresh medium without TSA was added for another 24 h to determine whether the loss of PU.1 expression was permanent. The results showed that the loss in expression was reversible, since PU.1 protein expression returned to normal levels (Fig. 1B, lane R). To show that the TSA-induced loss of PU.1 protein expression was not cell type specific, we treated the murine cell lines 2052 (pro-T cell) and A20 (B cell) with TSA and monitored PU.1 protein expression by Western blot analysis. TSA treatment resulted in a loss of PU.1 expression in both cell lines (data not shown). These data showed that blocking HDAC activity through the use of a specific inhibitor resulted in the loss of PU.1 protein expression.

**TSA treatment results in a loss of PU.1 mRNA expression**

We next determined whether the loss of PU.1 protein was the result of a decrease in PU.1 mRNA expression. Northern blot analysis showed that PU.1 mRNA levels decreased significantly within 5 h following treatment of the murine macrophage cell line P388D1 with 50 nM TSA (Fig. 2A). The loss of PU.1 mRNA was sustained through 15 h of treatment, followed by a slight recovery of PU.1 mRNA expression at 24 h. The increase in PU.1 mRNA levels by 24 h may be due to breakdown of the TSA compound. As was seen for PU.1 protein expression, PU.1 mRNA expression returned to normal levels following treatment with TSA for 15 h, followed by washing and a 24-h recovery period (Fig. 2A, lane R). These data suggest that the decrease in PU.1 protein expression was due to a loss of PU.1 mRNA expression.

To determine how quickly TSA caused a loss in PU.1 mRNA expression, we performed a 4-h time-course experiment. These data showed a significant loss of PU.1 mRNA expression within 3 h and a maximal loss after 4 h following the addition of TSA (Fig. 2B). The rapid decrease in PU.1 mRNA suggested that either transcription decreased from the PU.1 locus when cells were treated with TSA or that the stability of the PU.1 mRNA was affected. To rule out the latter possibility, we performed RNA half-life experiments to measure changes in PU.1 mRNA stability following treatment with TSA. No change in the stability of the PU.1 mRNA was detected (data not shown), suggesting that the effect seen was due to a change in transcription. These data show that the expression of PU.1 mRNA was negatively regulated when histone deacetylases were inhibited and that the loss of PU.1 expression was not due to a decrease in PU.1 mRNA stability.

To confirm that the loss of PU.1 mRNA expression was not unique to the P388D1 macrophage cell line, Northern blot analysis was performed on RNA isolated from two macrophage cell lines, RAW 264.7 and WEHI-3, and the pre-T cell line 2052 treated with 50 nM TSA for 4 h. In all three lines, a significant loss in PU.1 mRNA expression was seen after treatment with TSA (Fig. 3). These data suggest that the loss of PU.1 mRNA expression due to the inhibition of HDAC activity is not cell line or cell lineage specific.

**TSA inhibition of histone deacetylation blocks PU.1 target gene expression**

PU.1 regulates the expression of many genes critical for the development and function of macrophages. To show the consequences of the loss of PU.1 expression in macrophages, we determined whether TSA treatment affected the expression of several...
PU.1 target genes, including CD11b (32) and c-fms (33). We initially performed semiquantitative cycle RT-PCR using cDNA generated from untreated and TSA-treated P388D1 cells to measure PU.1 expression and PU.1 target gene expression after 4 h of TSA treatment. As shown, this technique revealed a loss of PU.1 mRNA at 4 h, in agreement with previous data (Fig. 4A, compare cycle 26 for untreated and TSA-treated samples). The expressions of CD11b and c-fms, however, were only modestly affected at 4 h, as measured by this semiquantitative approach (data not shown). Since the loss of PU.1 protein expression was not maximal until 8–12 h following the addition of TSA, we hypothesized that any effects on target gene expression would have delayed kinetics. Therefore, we measured the expression of CD11b and c-fms mRNA expression following treatment of P388D1 cells with TSA for 18 h. At this time point, a significant loss of expression was seen for both CD11b and c-fms mRNA using semiquantitative cycle RT-PCR (Fig. 4B). To extend these findings, we examined the expression of an additional gene regulated by PU.1, scavenger receptor A (34). The expression of this gene was also reduced significantly after 18 h (Fig. 4B). To confirm that the effects on PU.1 target gene expression were due to the loss of PU.1, we used this technique to study the expression of a gene previously shown to be induced following the addition of TSA. As shown, TSA treatment resulted in an induction of CD40 expression in the P388D1 macrophage line (Fig. 4B). These data showed that HDAC inhibition resulted not only in a loss of PU.1 expression, but also a loss in PU.1 target gene expression.

To independently confirm the results seen by our semiquantitative cycle RT-PCR approach, we performed Northern blot analysis to monitor the changes in PU.1 and PU.1 target gene expression. Northern analysis confirmed that PU.1 as well as CD11b expression decreased after 12 h of TSA treatment (Fig. 5). In addition, we showed that the expression of another PU.1 target gene, Toll-like receptor 4 (TLR4) (35), decreased after 12 h. Conversely, our Northern blot data showed an increase in p21Waf1/Cip1 mRNA expression, as has been previously shown (24). These data suggest that the loss of PU.1 expression following inhibition of deacetylase activity results in the specific loss of PU.1 target gene expression.

CD11b cell surface expression is reduced on P388D1 cells following TSA addition

To show that the TSA-induced loss of PU.1 target gene expression resulted in changes at the protein level, we used flow cytometric analysis to study CD11b cell surface expression following TSA treatment. As seen, treatment of the P388D1 cells with TSA for 24 h resulted in a significant loss of CD11b surface expression. In contrast, CD40 surface expression increased following treatment with TSA (Fig. 6). These data showed that the loss of CD11b mRNA expression was followed by a significant decrease in CD11b protein expression.

TSA negatively regulates PU.1 expression through the PU.1 genomic locus

We next assessed whether the loss of PU.1 target gene expression was due to TSA blocking PU.1 expression or TSA directly inhibiting the expression of a PU.1 target gene. The 503-PU line is a population of cells derived from the 503 PU.1−/− null cell line in which PU.1 was re-expressed via retroviral transduction (36). Thus, PU.1 expression is uncoupled from its own genomic locus, and we can study whether TSA directly affects the expression of PU.1 target genes. Northern blot analysis showed that TSA treatment of 503-PU cells for 12 h with 50 nM TSA resulted in an increase in PU.1 expression (Fig. 7A). In contrast to the other cell lines studied, CD11b expression was not affected (Fig. 7B). These data suggest that the loss of PU.1 target gene expression was due to the loss of PU.1 expression and not to a direct effect of HDAC inhibition on the expression of PU.1 target genes. In addition, these data support the conclusion that TSA’s negative effects on PU.1 expression were mediated through the endogenous PU.1 genomic locus.

Loss of PU.1 expression is correlated with a significant increase in histone H4 acetylation at the PU.1 promoter region

To begin to understand the mechanism controlling the loss of PU.1 expression following inhibition of HDAC activity, we determined
whether TSA treatment resulted in an increase in histone acetylation. Whole cell extracts from P388D1 cells treated with TSA for 4 h vs untreated cells were subjected to Western blot analysis using Abs to acetylated histone H3 and acetylated histone H4. As shown, a significant increase was seen in the acetylated forms of both histone H3 and histone H4 following treatment of P388D1 cells with TSA (Fig. 8A). ChIP analyses were next used to determine whether treatment of cells with TSA resulted in changes in the acetylation of histones associated with the PU.1 genomic locus. As shown using four sets of primers that cover approximately 650 bp of the PU.1 promoter region, acetylation of H3 within this region of the PU.1 locus was relatively unchanged following the addition of TSA (Fig. 8B). In contrast, ChIP analysis using Abs to acetylated histone H4 showed significant increases in H4 acetylation along this entire region of DNA. PCR of input starting material with the P1 primer set suggested that the differences seen were not due to variable amounts of starting material (Fig. 8C). These data suggest that the treatment of cells with TSA results in increased acetylation of histone H4 that is associated with the PU.1 promoter, and this correlates with the loss of PU.1 gene expression.

Discussion

In this report we have studied how altering the balance of acetylation in cells affects the expression of the Ets domain transcription factor PU.1. Surprisingly, the expression of PU.1 was negatively regulated following inhibition of HDAC. Treatment of the murine macrophage cell line P388D1 with two different HDAC inhibitors, either TSA or sodium butyrate, led to a dramatic reduction in PU.1 protein and mRNA levels. This effect was reversible, as PU.1 expression returned to basal levels following removal of the inhibitors. The loss of PU.1 expression was also seen in two additional macrophage cell lines, Raw 264.7 and WEHI-3, as well as the pre-T cell line 2052. The reduction in PU.1 protein levels following the inhibition of HDAC activity resulted in the loss of PU.1 target gene expression. These genes included CD11b, c-fms, TLR4, and scavenger receptor A, all of which are important for the development and function of macrophages. In contrast, the expression of p21 and CD40 increased following addition of TSA in these cell lines, which is consistent with previous reports (24, 25). Addition of TSA to 503-PU cells, in which a retroviral long terminal repeat regulates PU.1 expression, resulted in an increase in PU.1 expression and no effect on PU.1 target gene expression. These data suggested that the loss of PU.1 expression was mediated through elements intrinsic to the PU.1 genomic locus, and that the loss of PU.1 target gene expression was solely due to the loss of PU.1. ChIP analyses of the PU.1 promoter revealed that significant changes occurred in the acetylation of histone H4 in this region of the PU.1 genomic locus in response to TSA treatment. These data show that inhibition of HDAC activity and increased levels of acetylation negatively regulate the expression of PU.1. The consequences of this are the loss of PU.1 target gene expression, which may affect blood cell development and the ability to generate a functional immune response.

Global hyperacetylation of histones and other proteins following inhibition of HDAC would be predicted to promote a general increase in gene expression (21). As chromatin structure opened, trans-acting factors would have greater access to their cognate binding sites, enhancing the recruitment of coactivators and basal transcription factors. Treatment of cells with HDAC inhibitors such as TSA, however, does not lead to increased expression of most genes (23). The reason for such a selective effect on
gene expression is not known. Of the select few genes whose expression does change as a result of HDAC inhibition, most show the expected increase in expression. Several reports have shown that blocking HDAC activity in transformed cell lines results in the increased expression of the critical cell cycle kinase inhibitor, p21Waf1/Cip1 (24, 30, 37). This can lead to cell cycle arrest in some of these cell lines. The increased transcription of the p21 gene appears to be due to an accumulation of acetylated histones in select regions of the p21 genomic locus (24) and the activity of the Sp1 and Sp3 transcription factors (37). This could result in an open chromatin structure providing access to these trans-acting factors, but the exact mechanism for the increased expression of this gene has not yet been elucidated. Based on the ability to up-regulate p21 expression and induce cell cycle growth as well as induce the differentiation of tumor cells, TSA and other HDAC inhibitors are now being studied for a possible role in cancer therapy (26, 27).

Alternatively, two reports have shown that the addition of TSA increased the expression of genes involved in the immune response to tumor cells. One study has shown an increase in the expression of the costimulatory molecule CD86 in acute myeloid leukemia cells (38). Another study showed that HDAC inhibition resulted in increased expression of MHC class I and II and CD40 on a wide variety of tumor cell lines (25). These studies suggested that HDAC inhibitors could alter the ability of the immune system to present and respond to tumor Ags, providing another avenue for HDAC inhibitors could alter the ability of the immune system to present and respond to tumor Ags, providing another avenue for HDAC-mediated loss of PU.1 expression. It is possible that another trans-acting factor is acetylated, and this results in a loss of PU.1 expression.

A second explanation for the loss of PU.1 expression is that blocking HDAC activity and increasing acetylation of histones associated with the PU.1 promoter cause the movement of nucleosomes on the PU.1 locus. This nucleosome movement might mask critical cis-acting sites and lead to the loss of a critical trans-acting factor, resulting in the loss of PU.1 transcription. Nucleosomes have been shown to move and be remodeled on specific loci, including the lysozyme gene (42) and the IL-12 p40 gene (43). Our data showed a significant increase only in the levels of acetylation of histone H4, but not histone H3, that are associated with the PU.1 promoter region. This may be due to how the nucleosomes are positioned on the PU.1 promoter, and the increase in H4 acetylation seen following the inhibition of HDAC activity may result in an altered chromatin structure. Why this would negatively affect the expression of PU.1 but not other genes is unclear. Further analysis will be required to determine the exact mechanism leading to the loss of PU.1 gene expression.

Since PU.1 is an important regulator of development for several blood cell lineages, including macrophages, neutrophils, and B
lymphocytes, loss of PU.1 due to inhibition of HDAC activity could have dramatic effects on blood cell development and the immune response. One consequence could be a decrease in blood cell production. In PU.1 knockout mice, blood cell development is impaired, and the bone marrow shows hypocellularity. Thus, long term treatment with drugs such as TSA of cancer or other disease might result in problems for the blood system. In macrophages the loss of expression of specific genes such as TLR4 could affect the ability of an immune system to respond to bacterial pathogens. Likewise, loss of c-fms or CD11b could affect the ability of neutrophils to develop and attack bacteria. Since the loss of PU.1 is irreversible, therapies using TSA could be designed to minimize any negative effects due to the loss of PU.1. Conversely, the ability to block PU.1 target gene expression might have some benefits. Macrophages use scavenger receptors to engulf lipids and form foam cells. These cells are necessary for the development of atherosclerosis (44). A selective loss of PU.1 and its target genes in a subset of macrophages could prevent the development of this disease. Understanding the mechanism through which HDAC inhibitors exert their negative effect on PU.1 expression could potentially lead to the development of inhibitors that would specifically inhibit the expression of PU.1, leaving unaffected the expression of other genes.

In this report we showed that inhibition of HDAC activity selectively blocks the expression of the Ets domain transcription factor PU.1. This results in the loss of expression of PU.1 target genes. This may have an effect on blood cell development as well as immune responses to pathogens. Understanding how the modulation of acetylation and deacetylation alters PU.1 gene expression may open up opportunities to treat many diseases in which PU.1 regulates the expression of genes important for their development.

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