T-Bet Dependent Removal of Sin3A-Histone Deacetylase Complexes at the Ifng Locus Drives Th1 Differentiation

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Forming and removing epigenetic histone marks at gene loci are central processes in differentiation. Here, we explored mechanisms establishing long-range H4 acetylation marks at the Ifng locus during Th1 lineage commitment. In Th0 cells, histone deacetylase (HDAC)-Sin3A complexes recruited to the Ifng locus actively prevented accumulation of H4 acetylation marks. Th1 differentiation caused loss of HDAC-Sin3A complexes by T-bet-dependent mechanisms and accumulation of H4 acetylation marks. HDAC-Sin3A complexes were absent from the locus in NOD Th0 cells, obviating the need for Th1 differentiation signals to establish histone marks and Th1 differentiation. Thus, Ifng transcription is actively prevented in Th0 cells via epigenetic mechanisms and epigenetic defects allow unregulated Ifng transcription that may contribute to autoimmunity. The Journal of Immunology, 2008, 181: 8372–8381.

ukaryotic genomes are organized into a higher-order structure, known as chromatin, which plays an essential role in gene activation and silencing. The nucleosome, composed of 146 bp of DNA wrapped around an octamer of histones, represents the core unit of chromatin (1). These histones are subject to a vast array of posttranslational modifications, including acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination, that regulate the function of chromatin, in particular the activation and silencing of transcription of associated genes (2, 3). The identification of enzymes that catalyze these modifications, and the recognition that inhibition of these enzymes or deletion of genes encoding these enzymes not only affects patterns of histone modifications but also changes transcriptional activity of underlying genes, has given rise to the histone code hypothesis, which proposes that establishing and maintaining histone marks at a gene locus play essential roles in determining developmental and cell-type specific expression of genes, which is central to cell-fate decisions (4). Enzymes both form and remove histone marks (5–8). For example, histone acetyltransferases (HATs) catalyze the acetylation of histone H4 and histone deacetylases (HDACs) catalyze the removal of this acetyl group. Similarly, separate enzymes catalyze the methylation of specific histone lysine residues and the demethylation of the same histone lysine residue. Taken together, these results indicate that the histone code can be highly dynamic and represents the balance of recruitment of enzymes that catalyze formation of a given histone mark and enzymes that catalyze removal of that histone mark at a specific gene locus.

Evidence is also emerging to indicate that the histone code is more complex at genes that exhibit developmentally complex or cell-type specific patterns of expression (9, 10). For example, plasticity of embryonic stem cells may be established by large domains of repressive methylation marks superimposed upon smaller domains of activating methyl marks (11). Removal of either repressive methylation marks or activating methyl marks, presumably by histone demethylases, could permit developmental regulation of transcription of cell-specific genes. Thus, removing histone marks may be as fundamental as forming histone marks to achieve cell-fate decisions. The relevance of these epigenetic mechanisms to not only development but also to disease mechanisms, including cancer and autoimmune disease, is becoming increasingly recognized (12).

The complexity of the histone code is also revealed by activation and silencing of genes exhibiting cell-type specific changes in expression, such as Ifng and Th2 cytokine genes, which are either activated or silenced during differentiation from naive T cells to Th1 or Th2 lymphocytes, respectively (13–18). It is generally considered that naive T cells have the potential to differentiate into either Th1 or Th2 lymphocytes and this cell-fate decision, governed by external stimuli, is determined internally by transcription factors. Stat4 and T-bet are key transcriptional activators of the Th1 cell-fate decision and Stat6 and GATA-3 are key transcriptional activators of the Th2 cell-fate decision (19–21). This plasticity is achieved by the relative paucity of histone marks at either the Ifng or Th2 cytokine gene locus in naive T cells and initiation of respective differentiation cascades sets off a series of complex epigenetic modifications giving rise to a differentiated lymphocyte capable of transcribing either Ifng or Th2 cytokine genes. Although multiple mechanisms have been proposed (22, 23), a general consensus has not been reached to explain underlying mechanisms by which key transcriptional activators that drive Th1/Th2 differentiation programs achieve these cell-fate decisions.

The Th1 differentiation program sets off a series of events whereby T-bet and Stat4 are recruited to multiple sites across the Ifng locus, which precedes histone H4 acetylation and H3K4 dimethylation, two marks associated with transcriptional activation (13, 16, 18). H4 acetylation across the Ifng locus requires both

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Stat4 and T-bet. Conversely, the Th2 differentiation program establishes repressive H3K27 dimethyl and trimethyl marks across the locus by recruiting the Polycomb protein, EZH2, to the Ifng promoter via a GATA-3 dependent mechanism (13, 14). Initially, repressive H3K9 dimethyl and trimethyl marks are established in developing Th1 and Th2 cells. These marks are sustained in Th1 cells but lost in Th2 cells, further demonstrating the dynamic nature of the histone code as it evolves at the Ifng locus in differentiating Th cells (14). CD8+ T cells, stimulated to differentiate into IFN-γ producers via IL-12 dependent mechanisms, also establish long-range H4 acetylation marks across the Ifng locus (17). Similar long-range H4 acetylation marks across the Ifng locus are formed in proliferating CD8+ T cells independent of IL-12 in the presence of specific HDAC inhibition (trichostatin A; TSA), which is also sufficient to induce Ifng transcription. Thus HDAC inhibition in proliferating CD8+ T cells may stimulate recruitment of lineage specific transcription factors to the Ifng locus, may enhance recruitment of HATs to the locus, or may alter the balance of activity of recruited HATs and HDACs at the locus. Here, we sought to determine whether HDAC inhibition also stimulated H4 acetylation at the Ifng locus in proliferating CD4+ T cells and to evaluate these hypotheses in CD4+ T cells. Our results support a model where both HATs and Sin3A-HDAC complexes are recruited to the Ifng locus in proliferating CD4+ T cells. The function of T-bet is to cause displacement of these Sin3A-HDAC complexes, which allows existing HATs already recruited to the locus to establish stable H4 acetylated marks across the locus. NOD CD4+ T cells fail to recruit Sin3A-HDAC complexes to the Ifng locus, which allows H4 acetylation across the locus and Ifng transcription in the absence of Th1 differentiation signals. Our results illustrate the dynamic nature of the histone code in differentiating lymphocytes and provide mechanistic insights into defects in the histone code in NOD CD4+ T cells that may contribute to autoimmunity.

Materials and Methods

Mice
C57BL/6, NOD, C57BL/6,Stat4−/−, and C57BL/6,Tbx21−/− were bred in the Vanderbilt University animal facilities, and used between 4–5 wks of age. Research using mice complied with all relevant institutional and federal guidelines and policies and was approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Cell purification and cultures
CD4+ T cells were purified by negative selection from spleens of 4–5 wk-old mice using specific mAbs essentially as previously described (16, 17). CD4+ T cells are between 90 and 95% CD45RB-high and CD44-low at this age, indicative of a naive phenotype. CD4+ T cells, 1 × 10^6 cells/ml, were stimulated with immobilized CD3 mAb and irradiated spleen cells (1 × 10^6 cells). Anti-CD3-coated plates were prepared by treating tissue culture plates with a 10 μg/ml coating solution overnight at 0 – 4°C (2C11 mAb; American Type Culture Collection). T cells were stimulated under Th0 conditions did not harbor detectable levels of H4 acetylation at these sites (Fig. 1A). In contrast, CD4+ T cells cultured with TSA acquired H4 acetylation marks across the locus in proliferating CD4+ T cells. The function of T-bet is to cause displacement of these Sin3A-HDAC complexes, which allows existing HATs already recruited to the locus to establish stable H4 acetylated marks across the locus. NOD CD4+ T cells fail to recruit Sin3A-HDAC complexes to the Ifng locus, which allows H4 acetylation across the locus and Ifng transcription in the absence of Th1 differentiation signals. Our results illustrate the dynamic nature of the histone code in differentiating lymphocytes and provide mechanistic insights into defects in the histone code in NOD CD4+ T cells that may contribute to autoimmunity.

Results

HDAC inhibition and H4 acetylation at the Ifng locus in T cells

We sought to evaluate epigenetic events at the Ifng locus in T cells that differentiate into IFN-γ producers via culture under standard Th1 conditions or under artificial conditions by culturing with the selective HDAC inhibitor, TSA. CD4+ T cells were stimulated with plate-bound anti-CD3 under Th0 (no additional cytokines or antibodies), Th1 (IL-12 and anti-IL-4), or Th2 conditions (IL-4 and anti-IFN-γ) in the presence or absence of TSA. In contrast to some Th1/Th2 nonpolarizing culture systems, anti-IFN-γ and anti-IL-4 were omitted from these Th0 cultures to permit measurement of IFN-γ and IL-4 protein levels early in the culture period. Cells were harvested after 3 days and histone H4 acetylation levels were determined by ChIP assay. Identical cultures were continued for 5 days, harvested, and restimulated with plate-bound anti-CD3, alone. To evaluate Th1 differentiation, cultures were harvested after an additional 48 h and Ifng mRNA levels were determined by quantitative real-time PCR and IFN-γ protein levels were determined by ELISA. We evaluated histone H4 acetylation levels at the promoter, transcribed region of Ifng, and at a series of evolutionarily conserved DNA elements (25, 26) spanning 70 kb upstream (5′ CNS) to 59 kb downstream (3′ CNS) of the Ifng transcriptional start site. Primers pairs were as previously described except for the primer pair at the ~22 kb CNS site, which was shifted ~200 bp 3′ to be better centered within this CNS. CD4+ T cells cultured under Th0 conditions did not harbor detectable levels of H4 acetylation at these sites (Fig. 1A). In contrast, CD4+ T cells cultured with TSA acquired H4 acetylation marks across the locus in proliferating CD4+ T cells. The function of T-bet is to cause displacement of these Sin3A-HDAC complexes, which allows existing HATs already recruited to the locus to establish stable H4 acetylated marks across the locus. NOD CD4+ T cells fail to recruit Sin3A-HDAC complexes to the Ifng locus, which allows H4 acetylation across the locus and Ifng transcription in the absence of Th1 differentiation signals. Our results illustrate the dynamic nature of the histone code in differentiating lymphocytes and provide mechanistic insights into defects in the histone code in NOD CD4+ T cells that may contribute to autoimmunity.

Quantitative RT-PCR assays

Total RNA was purified using TRI REAGENT (Applied Biosystems). Two micrograms of total RNA was reverse-transcribed using the SuperScript III first-strand cDNA synthesis kit (Invitrogen). For each sample, 100 ng of cDNA was used in replicate quantitative RT-PCR for each gene assay. The relative expression levels of Ifng and Gapdh were determined using TaqMan gene expression assays (Applied Biosystems) and detected on an ABI7700/SDS platform (Applied Biosystems). Results are expressed as

Chromatin immunoprecipitation (ChIP) assays

For ChIP assays, ~1 × 10^7 T cells were processed as previously described (16, 17). Briefly, cells were fixed with 1% paraformaldehyde and lysed in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1 (600 μl total volume). After sonication to achieve an average length of genomic DNA of ~500 bp, protein-DNA complexes (100 μl of the 600 μl total volume) were immunoprecipitated overnight at 4°C with specific Abs or normal rabbit IgG as control. Ab-protein-DNA complexes were purified with protein A agarose. Aliquots (40 μl of the 600 μl total volume) were processed without immunoprecipitation as input controls. DNA was purified after reversal of cross-links at 65°C and suspended to a total volume of 100 μl and 2 μl were used for PCR. PCR primers for the ChIP assay spanned non-repetitive, conserved (mouse and human) genomic sequences from ~70 kb upstream to ~70 kb downstream of the Ifng gene (14, 16). Sequence conservation was identified using the UCSC Genome Browser Website (18–20). Real-time PCR was used to determine quantitative amounts of DNA using SYBR green fluorescence. Dissociation curves after amplification showed that primer pairs generated single products and this was confirmed by analysis on agarose gels. Relative abundance of histone H4 acetylation, T-bet, Stat4, or Sin3A at the different positions was obtained by determining the amount of immunoprecipitated DNA from a standard curve generated by serial dilution of input DNA and expressing results as the fraction of input DNA. Standard curves produced from input DNA were identical among the different mouse strains used in the experiments. Quantitative ChIP assays were performed in triplicate and results from two or three independent experiments were averaged. The following Abs were used in the ChIP analysis: acetylated H4 (Millipore: 17–229), Sin3A (Millipore: 06-913, Abcam: ab3479), Stat4 (Santa Cruz Biotechnology: sc-73954), and T-bet (Santa Cruz Biotechnology: sc-21003).

Retroviral transduction

The control retroviral (RV) vector, GFP-RV, and T-bet expression vector were transfected into 293 (Phoenix-Eco packaging cell line) packaging cells (24). CD4+ T cells were stimulated as described above and infected with viral supernatants containing either control or T-bet RV vector on day 1. GFP-positive and negative cells were isolated on day 3 by cell sorting and processed for ChIP assay.

Western blot analysis

Cells were washed in HBSS (1X) and lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.1% SDS) in PBS with a protease inhibitor mixture. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Abs used for western blot analysis were anti-acetyl histone H4 from Millipore and HRP-labeled sheep anti-rabbit antiserum.

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FIGURE 1. H4 acetylation patterns across the Ifng locus and Ifng transcription after HDAC inhibition. CD4+ T cells were activated with plate bound anti-CD3 with or without TSA under Th0 (no other Abs or cytokines added) (A), Th1 (IL-12 and anti-IL-4) (B), or Th2 (IL-4 and anti-IFN-γ) conditions (C). After 3 d, cells were harvested and processed for ChIP assays using Abs specific for acetylated H4 or with rabbit IgG as control, and with PCR primers specific for various positions across the Ifng locus (horizontal axis, in kilobases from the Ifng transcriptional start). Values for the IgG control were less than 0.01 and were subtracted from the values for acetylated H4. P, promoter; TR, transcribed region; 5′ CNS, evolutionarily conserved sequences 5′ of the Ifng transcriptional start; 3′ CNS, evolutionarily conserved sequences 3′ of the Ifng transcriptional start. Results are expressed as the fraction of input DNA determined from a standard curve and are the average of three separate determinations from each of three separate experiments (error bars, S.D.). D, Cells were cultured for 5 days in the presence or absence of TSA as indicated, harvested, and restimulated with plate bound anti-CD3 for 2 d. Levels of Ifng transcripts relative to Gapdh transcripts were determined by quantitative RT-PCR (left panel) and IFN-γ levels were determined by ELISA. Results are expressed as the average of three separate determinations from each of three separate experiments (error bars, SD).
Ifng locus. T cells cultured under Th1 conditions also acquired H4 acetylation marks across the Ifng locus (Fig. 1B). In contrast to T cells cultured under Th0 conditions, inclusion of TSA in Th1 cultures had little effect upon the distribution of H4 acetylation marks across the locus or the quantity of H4 acetylation marks at the individual sites we interrogated. Further, the pattern of H4 acetylation marks in T cells cultured under Th0 conditions with TSA was similar to the pattern of H4 acetylation marks achieved by T cells cultured under Th1 conditions. CD4 T cells cultured under Th2 conditions did not acquire detectable H4 acetylation marks in the presence or absence of TSA (Fig. 1C). Levels of H4 acetylation marks across the locus correlated with Th1 differentiation as determined by levels of Ifng transcription and IFN-γ produced by restimulated cultures (Fig. 1D). These results argue that HDACs play a major role in preventing both H4 acetylation across the Ifng locus and Ifng transcription in Th0 cells. In contrast, we did not see

**FIGURE 2.** HDAC inhibition does not increase T-bet and Stat4 localization to the Ifng locus. CD4^+^ T cells were activated with plate bound anti-CD3 under Th0 conditions ± TSA or under Th1 conditions for 3 d, harvested, and processed for ChIP assay using Abs specific for T-bet or Stat4 or with a rabbit IgG control. A, Binding of T-bet to the Ifng locus. B, Binding of Stat4 to the Ifng locus. Values for the IgG control were <0.001 and were subtracted from the values for T-bet or Stat4. Results are expressed as the fraction of input DNA determined from a standard curve and are the average of three separate determinations from each of three separate experiments (error bars, SD).

**FIGURE 3.** H4 acetylation and IFN-γ synthesis are independent of Stat4 and T-bet in HDAC-inhibited T cells. Wild type, Stat4-deficient, or T-bet deficient CD4^+^ T cells were cultured under Th0 conditions for 3 d, harvested, and processed for ChIP assay using Abs specific for acetylated-H4 or with a rabbit IgG control as in Fig. 1. A, Levels of H4 acetylation are expressed as the fraction of input DNA determined from a standard curve and are the average of three separate determinations from each of three separate experiments (error bars, SD). B, Cells were cultured for 5 d in the presence or absence of TSA, harvested, and restimulated with plate bound anti-CD3 in the absence of TSA. Cultures were harvested and analyzed for levels of IFN-γ by ELISA. Results are expressed as the average of three separate determinations from each of three separate experiments (error bars, SD).
increased production of IL-4 in Th0 cultures treated with TSA (not shown) suggesting that, at least under these experimental conditions, HDACs do not play a major role in preventing H4 gene expression and IL-4 production in Th0 cells.

HAT acetylate both histone and non-histone proteins, including transcription factors, and acetylation or de-acetylation also regulates the activity of these non-histone proteins (27–29). Therefore, induction of H4 acetylation across the Ifng locus by HDAC inhibition may result from inhibition of deacetylation of non-histone proteins or from inhibition of deacetylation of acetylated histone H4. Stat4 and T-bet transcription factors, recruited to multiple sites across the Ifng locus in effector Th1 cells, are necessary to achieve long-range histone acetylation across the locus and direct the Th1 differentiation program. Thus, TSA, by direct or indirect mechanisms, may induce H4 acetylation at the Ifng locus by increasing the level of Stat4 or T-bet recruited to the locus. To examine this possibility, we used ChIP assays to compare recruitment of T-bet and Stat4 to the Ifng locus in Th0 cells cultured with or without TSA to Th1 cells. Both T-bet and Stat4 were efficiently recruited to the Ifng locus in effector Th1 cells (Fig. 2, A and B). In contrast, neither T-bet nor Stat4 were recruited to the Ifng locus in Th0 cells in the presence or absence of HDAC inhibition. We conclude from these experiments that H4 acetylation at the Ifng locus in TSA-treated Th0 cells is not produced by increased recruitment of Stat4 and T-bet to the locus.

H4 acetylation across the Ifng locus and IFN-γ production in effector Th1 cells is dependent upon the transcription factors T-bet and Stat4 (16). Therefore, we also wanted to determine whether both H4-acetylation across the locus and IFN-γ synthesis were dependent upon T-bet and Stat4 in Th0 cells undergoing HDAC inhibition. We used ChIP assays to determine levels of H4 acetylation across the Ifng locus and ELISA to determine production of IFN-γ in wild type, Stat4 deficient, and T-bet deficient Th0 cells undergoing HDAC inhibition. Levels and patterns of H4 acetylation across the Ifng locus were very similar in TSA-treated wild type and in Stat4 deficient- and T-bet-deficient Th0 cells (Fig. 3A). Further, production of IFN-γ in TSA-treated Th0 cells was unaffected by the absence of Stat4 or T-bet (Fig. 3B). Taken together, these results demonstrate that both H4 acetylation patterns and levels across the Ifng locus and IFN-γ production in Th0 cells is independent of T-bet and Stat4 in the presence of HDAC inhibition.

**Sin3A-HDAC localization and displacement at the Ifng locus in differentiating T cells**

Above results demonstrate that recruitment of T-bet and Stat4 to the Ifng locus does not explain induction of H4 acetylation patterns and IFN-γ production observed in Th0 cells in the presence of HDAC inhibition. An alternative possibility is that absence of H4 acetylation across the Ifng locus and Ifng transcription in Th0 cells actually reflects the balance of HATs and HDACs recruited to the locus and that Th1 differentiation results in the loss of HDACs from the Ifng locus rather than recruitment of HATs. HDACs exist in stable multicomponent complexes of proteins (7). Both HDAC1 and HDAC2 associate with Sin3A and Sin3A associated proteins, such as SAP18 and SAP30, to form a stable complex that deacetylates histones (30). Sin3A bridges HDAC1 and HDAC2 with different transcription factors and is critical for recruitment of HDAC1 and HDAC2 to chromatin to achieve HDAC-mediated repression of transcription. Therefore, we used Sin3A as a surrogate to evaluate recruitment of an HDAC complex to chromatin and ChIP assays to determine levels of Sin3A at the Ifng locus in...
Th0 and Th1 cells. We found that Sin3A associated with one major site and several minor sites at the \( \text{Ifng} \) locus in Th0 cells (Fig. 4). The major site was the \(-22\) kb CNS site, which has been previously shown to bind T-bet, become H4 acetylated in effector Th1 cells, and to be required for efficient \( \text{Ifng} \) transcription (31). Minor sites of Sin3A association were also found at the promoter (\(-0.4\) kb), and other distal sites. Association of Sin3A with the major site (\(-22\) kb CNS) was markedly reduced upon differentiation of Th0 cells into effector Th1 cells. Association of Sin3A at the promoter was also partially reduced in effector Th1 cells but not as dramatically as at the \(-22\) kb CNS site. We interpret these results to indicate that high levels of an HDAC complex are present at the \(-22\) kb CNS site in Th0 cells, preventing formation of stable H4 acetylation marks, and that differentiation into Th1 cells is accompanied by loss of this major HDAC complex from the \( \text{Ifng} \) locus, permitting formation of stable H4 acetylation marks across the locus.

Next, we determined whether key transcription factors that direct the Th1 differentiation program, Stat4 and T-bet, play a role in controlling association of Sin3A with the \( \text{Ifng} \) locus. We used ChIP assays to evaluate levels of Sin3A associated with the \( \text{Ifng} \) locus in wild type, Stat4-deficient, or T-bet deficient CD4\(^+\) T cells cultured under Th1 conditions. We found that Th1 differentiation in Stat4 or T-bet-deficient T cells did not result in loss of Sin3A from the \(-22\) kb CNS and the minor site at the promoter (Fig. 5). This indicates that loss of Sin3A from the \( \text{Ifng} \) locus in differentiating effector Th1 cells is an active process that requires Stat4 and/or T-bet. To evaluate this possibility further, we used RV transduction methods to directly introduce T-bet into Th0 cells. Introduction of T-bet into Th0 cells was sufficient to reduce levels of Sin3A at the \(-22\) kb CNS site almost to the level observed in effector Th1 cells (Fig. 6A). Association of Sin3A with the promoter was also reduced following RV transduction. Levels of Sin3A association with the \( \text{Ifng} \) locus in Th0 cells transduced with empty vector controls were similar to uninfected Th0 cells. Loss of Sin3A binding to the \( \text{Ifng} \) locus was associated with gain of H4 acetylation across the locus in Th0 cells transduced with T-bet.

![Figure 6](http://www.jimmunol.org/)
FIGURE 7. Epigenetic events at the \textit{Ifng} locus in NOD CD4$^+$ T cells. NOD CD4$^+$ T cells were activated with plate bound anti-CD3 under Th0, Th1, and Th2 conditions. After 3 d, cells were harvested and processed for ChIP assays using Abs specific for acetylated H4 (A), Stat4 and T-bet (B), or Sin3A (C) and analyzed as described in Figs. 1, 2, and 4. D. After 5 d of culture, cells were harvested and restimulated by plate bound anti-CD3 for 2 d. IFN-γ levels were determined by ELISA. Results are expressed as the average of three separate determinations from each of three separate experiments (error bars, SD). E. Cell-lysates from NOD ($n = 6$) and C57BL/6 ($n = 3$) CD4$^+$ T cells (3 d after activation, Th0 conditions) were analyzed for total levels of acetyl-histone H4 by western blotting.
Levels of H4 acetylation across the Ifng locus were somewhat lower than observed in effector Th1 cells but the overall pattern of H4 acetylation was similar. As reported previously, T-bet transduction stimulated IFN-γ production in Th0 cells, albeit to a lower level than observed in effector Th1 cells (Fig. 6C). We conclude from these studies that a key event in the Th1 differentiation program is displacement of Sin3A-HDAC complexes from Th0 cells and that T-bet plays a key role in this displacement.

**Sin3A-HDAC is not recruited to the Ifng locus in NOD T cells**

In the majority of strains of mice, CD4⁺ T cells require both a TCR stimulus (either Ag or a mitogen such as anti-CD3) and an inflammatory stimulus, such as IL-12, to efficiently differentiate into effector Th1 cells capable of transcribing Ifng at high rates. CD4⁺ T cells from the NOD strain are an exception to this rule and efficiently differentiate into effector T cells capable of transcribing Ifng at high rates following exposure to only a TCR stimulus (32, 33). In contrast, the Th2 differentiation program will not only induce Ifng transcription but will also repress Ifng transcription in NOD CD4⁺ T cells. We evaluated H4 acetylation patterns across the Ifng locus in NOD CD4⁺ T cells cultured under Th0, Th1, or Th2 conditions by ChIP assay. H4 acetylation patterns were similar in NOD CD4⁺ T cells cultured under Th0 and Th1 conditions (Fig. 7A). The pattern and levels of H4 acetylation at the different CNS sites across the locus were similar to that found in C57BL/6 effector Th1 cells. In contrast, H4 acetylation across the Ifng locus was absent in NOD effector Th2 cells, similar to results observed in NOD CD8⁺ T cells. We interpret these results to indicate that mechanisms to repress Ifng transcription under Th2 culture conditions, H3K27 methylation, are intact in NOD T cells, but that mechanisms to prevent Ifng transcription in NOD T cells cultured under Th0 conditions are defective in NOD T cells.

As outlined above, recruitment of Stat4 or T-bet to the Ifng locus in NOD T cells or absence of Sin3A-HDAC complexes at the Ifng locus could produce the observed H4 acetylation across the locus in NOD T cells cultured under Th0 conditions. We used ChIP assays to distinguish between these two possibilities. Stat4 and T-bet were not recruited to the Ifng locus in NOD T cells cultured under Th0 conditions (Fig. 7B). Stat4 and T-bet were recruited to the Ifng locus in NOD effector Th1 cells and this pattern was relatively similar to that observed in C57BL/6 effector Th1 cells (not shown). Therefore, we addressed the alternate possibility, that Sin3A-HDAC complexes were not recruited to the Ifng locus in NOD Th0 cells. The Sin3A-HDAC complex did not associate with the major −22 kb CNS site or to the minor site at the promoter in NOD Th0 cells (Fig. 7C). Detectable levels of Sin3A were seen at the −70 CNS site but these were extremely low relative to the levels detected at the −22 CNS site, making it uncertain whether these are biologically relevant. Lack of Sin3A-HDAC complexes at the Ifng locus in NOD Th0 cells correlated with increased synthesis of IFN-γ (Fig. 7D). In contrast, total levels of acetyl-histone H4 were similar in NOD and C57BL6 Th0 cells (Fig. 7E). Our interpretation of these results is that the ability of NOD Th0 cells to establish H4 acetylation patterns across the Ifng locus and efficiently transcribe Ifng is directly attributable to a specific failure to recruit Sin3A-HDAC complexes to the locus rather than a global failure to deacetylate histone H4.

**Discussion**

T cells differentiating along the Th1 pathway acquire activating H4 acetylation and H3K4 dimethyl histone marks spanning at least 100 kb of genomic DNA across the Ifng locus via a Stat4 and T-bet dependent mechanism (13, 16, 18). In contrast, T cells differentiating along the Th2 pathway acquire repressive H3K27 dimethyl and trimethyl marks by recruiting the Polycomb protein, EZH2 to the promoter via a Stat6 and GATA-3-dependent mechanism (14). T cells activated to divide following TCR stimulation alone (Th0 cells) do not have detectable positive or negative histone marks across the Ifng locus and transcribe Ifng only at low levels (14, 16). However, inhibition of HDAC in Th0 cells stimulates both acquisition of H4 acetylation marks, Ifng transcription, and Th1 differentiation suggesting that the balance of HATs and HDACs stimulated by Th1 differentiation pathways control both the level of H4 acetylation at the Ifng locus, Ifng transcription, and Th1 differentiation.

HATs and HDACs acetylate and deacetylate both histones and non-histone proteins, including transcription factors, thus regulating their activities. The Stat family members and YY1 are two examples of transcription factors whose activities are regulated by acetylation and deacetylation and which are known to regulate Ifng transcription (34–37). Thus, inhibition of HDACs in Th0 cells could produce H4 acetylation marks across the Ifng locus and stimulate transcription by directly or indirectly affecting the ability of lineage specific transcription factors, T-bet or Stat4, to be recruited to the Ifng locus and stimulate HAT recruitment or directly by inhibiting the activity of HDACs already recruited to the Ifng locus via non-lineage specific mechanisms. Our results demonstrate that, in contrast to effector Th1 cells, T-bet and Stat4 are not recruited to the Ifng locus in Th0 cells in the presence or absence of HDAC inhibition. Further, H4 acetylation across the Ifng locus is achieved in Stat4 or T-bet deficient Th0 cells treated with a HDAC inhibitor. Our interpretation of these results is that it is unlikely that HDAC inhibition, directly or indirectly, alters the activity of these lineage specific transcription factors required to achieve long-range H4 acetylation across the Ifng locus.

In contrast to Th0 cells, HDAC inhibitors fail to induce H4 acetylation across the Ifng locus and Ifng transcription in developing Th2 cells. The likely explanation for this is that Th2 differentiation stimulates formation of repressive H3K27 methylation marks across the locus (13, 14). These dominant histone marks are involved in the irreversible silencing of genes, such as in X-chromosome inactivation (2) and it is generally believed that these marks produce a chromatin environment inhospitable to recruitment of other histone-modifying machinery and formation of other histone marks. In this model, Th2 differentiation would expel both HATs and HDACs from the Ifng locus.

Alternate hypotheses to explain the dynamics of H4 acetylation across the Ifng locus in differentiating Th1 cells are that either HDACs are recruited to the Ifng locus in Th0 cells and expelled in effector Th1 cells or that HATs are deficient in Th0 cells and are recruited to the Ifng locus by the Th1 differentiation program. Our results indicate that there are not vast differences in HAT recruitment to the Ifng locus in Th0 cells and effector Th1 cells (data not shown). In contrast, we clearly demonstrate that major changes in the recruitment of HDAC complexes to the Ifng locus occur during the differentiation of Th0 cells to effector Th1 cells. One multi-component histone deacetylase complex consists of HDAC1, HDAC2, Sin3A, and Sin3A associated proteins, such as SAP18 and SAP 30. Using chromatin immunoprecipitation assays and Sin3A as a surrogate for the HDAC complex, our results demonstrate that Sin3A is recruited to one major and several minor sites across the Ifng locus in Th0 cells. The major site, the −22 kb CNS, clearly plays a major role in activating Ifng transcription in effector Th1 cells and NK cells (31), and our results indicate it also plays a key role in recruiting HDAC complexes to the Ifng locus in Th0 cells and preventing H4 acetylation and Ifng transcription. Our results also demonstrate that the major HDAC complex at the −22 CNS site is lost upon Th1 differentiation by a Stat4 and T-bet dependent mechanism. Minor HDAC complexes are also lost at
the promoter upon Th1 differentiation. At this point, we cannot be certain whether T-bet directly displaces the HDAC complex from the −22 kb CNS site or whether T-bet binding to multiple sites across the Ifng locus leads to HDAC complex displacement by indirect mechanisms. In cell lines, T-bet directly antagonizes Sin3a recruitment to the human IFNG proximal promoter supporting our hypothesis that T-bet displaces the Sin3a-HDAC complex from the −22 kb CNS site (38). Alternatively, T-bet regulates expression of other transcription factors, such as HLX and Runx3, which contribute to the Th1 differentiation program (39, 40). Because these transcription factors may also interact with the Ifng locus, they may, either alone or in cooperation with T-bet, contribute to displacement of the HDAC complex from the Ifng locus. Nevertheless, our results clearly demonstrate that T-bet is both necessary and sufficient to drive the major HDAC complex from the −22 CNS site and from minor sites, such as the promoter.

HDAC-Sin3A complexes are recruited to chromatin by multiple mechanisms. For example, methylated DNA CpG residues bind the protein MeCP2, which can recruit the HDAC-Sin3A complexes to DNA (41). This seems an unlikely mechanism to explain the recruitment of HDAC-Sin3A complexes to the Ifng locus because the −22 kb CNS site at the Ifng locus is the major site of HDAC-Sin3A recruitment, but this site lacks methylated CpG residues in naive CD4⁺ T cells, Th1 and Th2 cells (13, 42). An alternative possibility is that HDAC-Sin3A complexes are recruited to the −22 kb CNS site via transcription factors that bind to the site and also bind Sin3A serving as a bridge between DNA and the HDAC complex. An example of such a transcription factor is YY1. YY1 can activate or repress transcription by its ability to recruit HAT or HDAC complexes to DNA, respectively (42). Evidence from a number of analyses demonstrates that YY1 is recruited to the Ifng promoter and represses Ifng promoter activity in reporter assays (37, 43). In fact, in silico analysis predicts multiple YY1 binding sites exist within the −22 kb CNS site. Additional experimentation will be necessary to determine whether recruitment of Sin3A-HDAC complexes to the CNS’s across the Ifng locus is dependent upon YY1 or other transcription factors with similar properties.

An emerging theme is that complex interplay among multiple sites across the Ifng locus is necessary to achieve proper transcriptional control of Ifng and that the dynamics of histone modifications across the locus are central to this process (13, 31, 44–46). Several distal CNS across the Ifng locus exhibit functional properties in various assay systems: −54 kb CNS, −34 kb CNS, −22 kb CNS, −6 kb CNS, −2 kb CNS, +18−20 kb CNS, and +46 kb CNS relative to the Ifng transcriptional start site. The CNS 6 kb upstream of the Ifng start site, −6 kb CNS (also called CNS1), possesses a DNase hypersensitivity site in naive T cells, suggesting it is a site critical for early events in Ifng remodeling (44, 47–49). The transcription factors T-bet, STAT5, and NFAT1 bind to the −6 kb CNS site. Within 24–72 h of Th1 cell development, a Jak3-dependent cytokine signal, probably IL-2, stimulates recruitment of STAT5 to the −6 kb CNS site. This STAT5 signal promotes T-bet binding to the promoter and histone acetylation. How −6 kb CNS functions in a differentiated Th1 cells is unclear. A +18–20 kb CNS site (located 18–20 kb upstream of the Ifng start site, also called CNS2) does not develop DNase hypersensitivity until Th1 or Th2 polarization, suggesting a role after the initial differentiation signal (49) and does not have enhancer activity in various reporter assays. Rather, the +18–20 kb CNS augments the enhancer activity of the −6 kb CNS. It appears that the +18–20 kb CNS functions by enhancing the activity of −6 kb CNS after the Th1 differentiation signal. The −22 kb CNS-22 is necessary for IFN-γ production by Th1 cells in an in vivo reporter model (31). This CNS lacks a DNase hypersensitivity site in naive CD4⁺ cells, but acquires a very strong hypersensitivity site upon Th1 or Th2 differentiation. So, this CNS has a regulatory role specific for events after the initial differentiation signal. It is a strong T-bet dependent enhancer in vitro (13, 31). In vivo, T-bet binds to CNS-22 in both resting and unstimulated Th1 cells. Because the site contains a permissive chromatin environment in both Th1 and Th2 cells, and strongly reacts with T-bet in an activation-independent manner, it is theorized that the −22 kb CNS functions to create an environment favorable to transcription in Th1 cells. The promoter, CNS-34, and CNS-54 also possess T-bet binding sites and have T-bet-dependent enhancer functions in reporter assays, in vitro (13, 16). Thus, numerous CNS, existing across large distances of genomic DNA within the Ifng locus, are capable of stimulating transcription by a T-bet dependent mechanism. Boundary functions of some of these distal CNS have also been described. Of the multiple CNS sites, the −22 kb CNS contributes dual functions. Not only is it necessary for Ifng transcription in Th1 cells, but it is also necessary to prevent Ifng transcription in Th0 cells.

In NOD mice, β-cell destruction is mediated, at least in part, by IFN-γ and NOD mice lacking IFN-γ, significantly delaying the onset of diabetes (50, 51). Thus, effector T cells from NOD mice appear to differentiate, in vivo, into producers of IFN-γ in the apparent absence of an inflammatory stimulus and clearly differentiate into IFN-γ producers in the absence of an inflammatory stimulus in tissue culture models. A key component of this differentiation pathway is the ability to establish long-range H4 acetylation across the Ifng locus in both CD4⁺ and CD8⁺ T cells. Our results demonstrate, at least for CD4⁺ T cells, that NOD Th0 cells fail to recruit the Sin3A-HDAC complex to the −22 kb CNS site and the promoter. Thus it seems a likely interpretation is that failure to deacetylate H4 across the locus permits accumulation of H4 acetylation marks catalyzed by HATs recruited to the locus in NOD Th0 effector cells to stimulate Ifng transcription. Further experimentation will be required to determine whether this defect is a result of differences in genomic sequences at the Ifng locus between NOD and C57BL/6 strains, strain-dependent differences in recruitment of specific transcription factors to the Ifng locus, or strain-dependent differences in levels of HDACs, Sin3A or Sin3A associated proteins, SAP18, and SAP30. However, our results clearly support the notion that failure to recruit the Sin3A-HDAC complex to the Ifng locus in NOD Th0 cells contributes to deregulated epigenetic modifications at the Ifng locus and Ifng transcription. We speculate that these defects may also contribute to excessive production of IFN-γ, in vivo, β-cell destruction and diabetes in NOD mice.

Stat4 and T-bet play key roles in directing the Th1 lineage decision, in part by associating with the Ifng locus and stimulating accumulation of positive histone marks across the locus. Our results demonstrate that displacement of Sin3A-HDAC complexes from the Ifng locus is a major mechanism by which this is achieved. This type of model also provides a mechanism to generate long-lived effector Th1 cells that do not require the continued presence of the transcription factors that determine the initial lineage decision to efficiently transcribe Ifng in response to Ag stimulation. Thus, sufficient HATs are recruited to the Ifng locus in Th0 cells to establish H4 acetylation by TCR-activation signals alone but HDAC activity that is also present removes these marks, leaving a locus without detectable H4 acetylation marks. The major function of the Th1 differentiation program is to drive away Sin3A-HDAC complexes from the locus to shift the HAT:Sin3A-HDAC balance in favor of HATs. Once H4 acetylation is established at nucleosomes, it can be sustained via TCR-activation signals. H4 acetylated histones also represent high-affinity binding sites for HATs through the HAT bromodomain (7). This may contribute to sustain H4 acetylation across the locus in the absence of
the transcription factors that were initially required to establish the H4 acetylation marks across the locus. This may also serve as a general model to ensure plasticity of the adaptive immune response before lineage commitment.

A variety of HDAC inhibitors are under commercial development, primarily for the treatment of solid and hematological malignancies (52). HDAC inhibitors also have activity in various animal models of inflammation and can inhibit different functions of the innate immune system (27, 52, 53). Our results indicate that HDAC inhibitors also have the potential to be proinflammatory. This proinflammatory potential may contribute to their activity against malignancy but may also induce or worsen inflammatory diseases where IFN-γ production by the adaptive arm of the immune system plays a critical role.

Disclosures

The authors have no financial conflict of interest.

References