

Epigenetic regulation of established human type 1 versus type 2 cytokine responses

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Background: Multiple biologic factors influence maintenance of immunologic responsiveness. Here, we studied whether epigenetics has a regulatory function in maintaining pre-established T_H1-like and T_H2-like immunity in human beings. **Objective:** We focused on delineating the role of endogenous histone deacetylase (HDAC) activity in regulating cytokine recall responses.

Methods: Using RT-PCR and ELISA, the effect of increasing cellular acetylation on T_H1/T_H2 cytokine expression was systematically examined in 58 children by inhibiting HDAC activity with trichostatin A.

Results: Phytohemagglutinin activation selectively stimulates antigen-experienced CD45RO⁺ T cells, eliciting recall cytokine responses. Trichostatin A reduced HDAC activity by approximately 1/3. The resulting cellular hyperacetylation led to increased T_H2-associated (IL-13, 139%; IL-5, 168%; $P < .0001$) and reduced T_H1-associated recall responses (IFN- γ , 76%; CXCL10, 47%; $P < .0001$). IL-2 and IL-10 production were reduced 25% to 55% ($P < .0001$). These alterations in T_H2-associated and T_H1-associated recall responses were associated with increased expression of *Gata-3* and *sphingosine kinase 1*, a T_H1-negative regulator, independent of T-bet expression. Overall, inhibition of endogenous HDAC activity shifted T_H1:T_H2 ratios by 3-fold to 8-fold ($P \leq .0001$), skewing recall responses toward a more T_H2-like phenotype, independent of the stimulus used.

Conclusion: Endogenous HDAC activity plays a crucial role in maintaining the balance of pre-established T_H1-like and T_H2-like responses, inhibiting excessive T_H2 immunity.

Clinical implications: These findings (1) offer an epigenetic regulatory mechanism explaining how environment may influence the maintenance of T_H1/T_H2 immunity by modulating cellular acetylation levels, and (2) open the door to investigations of epigenetic mechanisms in midlife pathogenesis and ongoing maintenance of complex diseases that are characterized by immune dysregulation such as allergy and asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Human, PBMCs, T_H1/T_H2, cytokine

The molecular mechanisms that regulate maintenance of established immunity are the subject of intense investigation. Epigenetic contributions, including covalent modifications of chromatin structure via acetylation, methylation, phosphorylation, and ubiquitination of histones, and methylation of DNA, generate heritable patterns of gene expression not encoded by DNA.¹⁻⁴ From the late 1990s, there is considerable evidence indicating the importance of such modifications in initial commitment toward type 1 versus type 2 (T_H1-like vs T_H2-like) activation.⁵⁻⁷ Most evidence, largely from murine systems, centers on the importance of epigenetic events in T-cell fate determination. Our understanding of what role, if any, epigenetics contributes to maintenance of established immunity is scant. Although data from such highly polarized, homogeneous murine systems are instrumental in extending our appreciation of epigenetic regulation in immunity, how they relate to the heterogeneous responses of human beings is unclear. Significant differences are evident in, for instance, the plasticity of human versus murine T_H1/T_H2 clones in reorientation toward the opposite functional phenotype.^{5,8,9}

Histone acetylation is a major mechanism of epigenetic regulation. The extent of acetylation of core histones reflects a balance between opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs).^{1,10} Ten classic HDACs and 16 HATs have been identified,¹¹ suggesting that regulation of histone acetylation is of great physiological importance. With so many family members, it is technically challenging to study their function using knockdown or knockout approaches. Rather, most have used the strategy of inhibiting HDAC activity. Acetylation of histone tails plays a critical role in activation of gene transcription,^{12,13} enhancing chromatin accessibility¹⁴ and promoting recruitment of transcription activators and chromatin remodeling enzymes.^{2,3,12} Hence, it has generally been viewed as a positive regulator of gene transcription.

Importantly for immune memory, the histone modification profile at a given gene locus can be inherited through mitosis.^{4,8} In cloned human T cells, the polarized acetylation pattern is retained through more than 20 cell divisions in the absence of stimuli or polarizing cytokines.⁸ This suggests that epigenetic modifications play

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Abbreviations used

HAT: Histone acetyltransferase
 HDAC: Histone deacetylase
 PHA: Phytohemagglutinin
 SPHK1: Sphingosine kinase 1
 TSA: Trichostatin A
 TSST-1: Toxic shock syndrome toxin 1

an important role in maintaining pre-established patterns of cytokine memory.¹⁵

Here, using a polyclonal stimulus that selectively elicits pre-established cytokine responses, we investigated the role of endogenous histone acetylation in epigenetic regulation of recall type 1 and type 2 immunity in 58 children by interfering with endogenous HDAC activity then assessing alterations in human T_H1/T_H2 cytokine recall responses.

METHODS

Isolation of PBMCs and cell culture

After approval of this study by University of Manitoba Research Ethics Board, written informed parental consent was obtained from 723 children (of whom 58 were randomly selected for detailed analysis) within a general population-based case-control cohort. PBMCs were isolated by using Histopaque-1077 (Sigma, St Louis, Mo). Isolated cells (>99% viability) were cultured in RPMI 1640 medium (Sigma) at 3.5×10^5 cells per 200 μ L in 96-well round bottom plates (Nunc, Roskilde, Denmark), in triplicate. PBMCs were treated with trichostatin A (TSA; 4 nmol/L; Sigma) or medium alone for 45 minutes before stimulation with phytohemagglutinin-leucoagglutinin (PHA-L) from *Phaseolus vulgaris* (15 μ g/mL; Sigma) for 22 hours or with toxic shock syndrome toxin 1 (TSST-1; 10 ng/mL; Sigma) for 60 hours. For each individual studied, cultures were prepared in the absence of stimuli and in the presence of TSA alone. CD45RO⁺ T cells and CD45RA⁺ T cells were enriched from adult PBMCs by using the Pan T Cell Isolation Kit and CD45RA Microbeads (Miltenyi Biotec Inc, Auburn, Calif). In most experiments, ~95% enrichment was achieved in CD45RO⁺ and CD45RA⁺ T-cell isolations, as assessed by flow cytometry. The contaminating cells in both enriched populations were CD3⁺ and exhibited forward and side scatter characteristics of accessory cells. Enriched CD45RO⁺ and CD45RA⁺ T-cell populations were cultured at $3.5 \times 10^5/200 \mu$ L. Supernatants were harvested 22 hours poststimulation. Cell pellets were frozen in liquid nitrogen and stored at -70°C for RNA isolation.

Quantitation of cytokines in culture supernatants

Supernatants from triplicate cultures were assayed for type 1 (IFN- γ , CXCL10/IP-10), IL-2, type 2 (IL-13, IL-5), and IL-10 expression by ELISA.^{16,17} Antibodies and standards were from BD Pharmingen (San Diego, Calif), Biolegend (San Diego, Calif), PeproTech (Rocky Hill, NJ), and R&D Systems (Minneapolis, Minn), except for IFN- γ standard (1 U = 115 pg; National Institutes of Health Research Reference Reagent GX01-902-535). Sensitivities were 7.8 pg/mL (IL-5), 3.9 pg/mL (IL-13), 7.8 pg/mL (IL-10), 2 pg/mL (IL-2), 18 pg/mL (IFN- γ), and 7.8 pg/mL

(CXCL-10/IP-10). Concentrations were calculated from the linear portion of standard curves on each plate. Coefficients of variation (CVs) typically ranged from 2% to 15%.

RT-PCR

Total RNA was prepared by using an RNeasy Micro Kit 2.0 (Qiagen, Mississauga, Ontario, Canada), and treated with RNase-free DNase I (Invitrogen, Burlington, Ontario, Canada) before being subjected to reverse transcription with random hexamers (Sigma Genosys, Oakville, Ontario, Canada) and SuperScript reverse transcriptase II (Invitrogen). Resulting cDNA was evaluated in semiquantitative PCR with primer sets specific for *Il4*, *Il13*, *Ifn γ* , *T-bet*, *p21^{CIP1}*, *Sphk1*, *Gata-3*, and *Gapdh* (annealing temperature, 60.5°C). PCR products were analyzed in 2% agarose gels. *Ifn γ* , *Il13*, and *Gata-3* mRNA were also analyzed against *Gapdh* expression (as endogenous control) with Power SYBR-Green qPCR Master Mix (Applied Biosystems 7500, Foster City, Calif). The average threshold cycle (Ct) from duplicate wells (with a CV less than 10%) was used to calculate the relative change in *Ifn γ* , *Il13*, and *Gata-3* expression, using the culture media (CM) alone condition as a reference ($\Delta\Delta\text{Ct}$ program; Applied Biosystems). Primer sequences were designed by using Primer 3 program (www.primer3.com), verified by using the PCR program on the University of California Santa Cruz genome website (www.genome.ucsc.edu) and National Institutes of Health Entrez Tools to ensure that the primer pairs amplify only the specific targets, and synthesized by Sigma Genosys. PCR products had the expected lengths, with no nonspecific bands on agarose gels.

Flow cytometry

10^5 cells were stained with fluorochrome-conjugated or isotype-matched control antibodies in FBS-DPBS buffer (Dulbecco's PBS with 1% FBS; 2 mmol/L EDTA) 30 minutes on ice. Combinations of antibodies from Biolegend or BD Pharmingen were used to assess enrichment of CD45RO⁺ and CD45RA⁺ T cells. Antibodies were CD4 (RPA-T4), CD45RA (HI 100), CD45RO (UCHL1), CD3 ϵ (SP34), and CD8 (clone HIT8a). Cells were fixed immediately after staining in 0.5% paraformaldehyde and then analyzed with a Becton Dickinson FACSCalibur (BD Biosciences, Franklin Lakes, NJ) within 4 days. Live lymphocyte population was gated by using forward and side scatter. All analyzed events were saved for further analysis by using CellQuest Pro Software (BD Biosciences).

Data analysis

Analysis of ELISA data was performed with Graph Pad Prism 4.0 (San Diego, Calif). Wilcoxon matched-pairs test was used to compare validated pairs cultured with and without TSA. Mann-Whitney tests were used to determine whether median values differed significantly.

RESULTS

Phytohemagglutinin selectively activates CD45RO⁺ T cells

Phytohemagglutinin (PHA) is a long-used, highly potent activator of T-cell cytokine production^{18,19} that has generally been assumed to activate most human T-cell subsets.²⁰ However, analysis of the stimulatory capacity of PHA on highly enriched naive CD45RA⁺ versus antigen-experienced CD45RO⁺ T cells (Fig 1) demonstrates that virtually all IL-13, IL-5, and IFN- γ cytokine responses evoked by PHA during the first 22 hours were

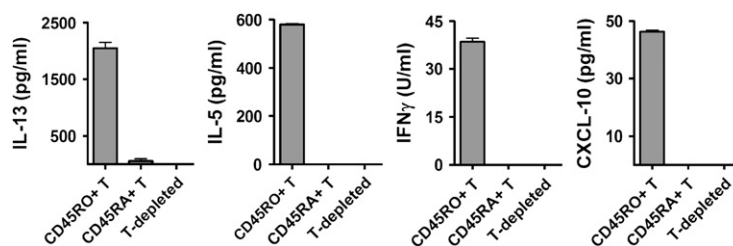


FIG 1. Polyclonal activator PHA selectively activates CD45RO⁺ T cells. CD45RO⁺ and CD45RA⁺ T-cell subsets were stimulated with PHA for 22 hours. Culture supernatants were assayed for IL-13, IL-5, IFN-γ, and CXCL-10 by using ELISA. Culture medium alone did not yield detectable amounts of cytokine/chemokine. Results (mean values of triplicates, with SEMs) are shown for 1 representative individual of 5 studied.

restricted to CD45RO⁺ antigen-experienced T cells. This provides a useful probe to assess selectively the role of epigenetic modifications on pre-established type 1 versus type 2 cytokine responses in human populations, directly *ex vivo*.

Inhibition of histone deacetylase activity augments T_H2 but reduces T_H1 cytokine transcripts

Active transcription of cytokine genes in murine and human *in vitro* polarized T_H1 and T_H2 clones is associated with multiple epigenetic events, including histone acetylation.^{5,6,21,22} We examined whether modulating endogenous histone acetylation by suppressing HDAC activity enhances cytokine expression in human cells directly *ex vivo*. TSA, an effective and reversible inhibitor of both class I and class II HDACs,²³⁻²⁵ was used to suppress endogenous HDAC activity in PBMCs.

Fresh human PBMCs were cultured in the presence of TSA before PHA stimulation. This *ex vivo* culture system allows examination of polyclonal recall immune responses characterized by strong type 1 and type 2 immunity with minimal manipulation of cells, circumventing the recognized functional and genetic effect of T-cell cloning on T-cell function.^{16,26} TSA under the conditions used (4 nmol/L, and as much as 100 nmol/L; data not shown) did not affect the viability of PBMCs. A marked increase in histone acetylation could be detected within 4 hours in cultures containing TSA (see this article's Fig E1, A, in the Online Repository at www.jacionline.org). As demonstrated in Fig 2, A, the transcripts of T_H1-associated (*Ifn*γ) and T_H2-like cytokines (*Il4*, *Il13*), a cyclin-dependent kinase inhibitor (*p21^{CIP1}*), and a housekeeping gene (*Gapdh*) were examined 22 hours poststimulation by using semiquantitative RT-PCR. Cellular *p21^{CIP1}* mRNA level provides a positive control for TSA action²⁷ and *Gapdh* transcripts, a loading control. TSA had no effect on *Gapdh* expression^{27,28} (Fig 2, A). TSA has a half life of ~14 hours. At the end of 22-hour culture, TSA still inhibited HDAC activity by ~33% (see this article's Fig E1, B, in the Online Repository at www.jacionline.org).

In the absence of PHA stimulation, low levels of *Il4*, *Il13*, and *Ifn*γ mRNA were detected in *ex vivo* PBMCs. Inhibition of HDAC activity with TSA resulted in

increases in cellular *Il4* and *Il13* mRNA and decreases in *Ifn*γ mRNA (Fig 2, A). These increases and decreases were more evident when the cells were stimulated with PHA. The concomitant presence of TSA with PHA led to a ~2 fold increase in *Il4* and *Il13* and a ~3 fold decrease in *Ifn*γ transcripts compared with PHA stimulation alone. When examined by using relative quantitative PCR, PHA stimulation resulted in 33-fold and 44-fold increases in *Ifn*γ and *Il13* mRNA, respectively (see this article's Fig E2 in the Online Repository at www.jacionline.org). Reduction of HDAC activity led to a 57% further increase in *Il13* and a 54% reduction in *Ifn*γ mRNA levels. This effect of TSA on actively transcribed cytokine genes demonstrates a crucial role for HDAC in enhanced expression of human cytokine mRNA, as suggested by previous murine studies of cytokine expression with T-cell clones.^{21,22,29,30} Our data also reveal an essential role for HDAC activity in maintaining human T_H1-associated cytokine gene expression. This suggests that histone acetylation makes an important contribution to regulating expression of pre-established T_H1-like and T_H2-like immunity in human beings.

To extend these observations to a larger population that better reflects heterogeneous genetic and environmental backgrounds, we tested the effect of inhibiting HDAC activities on T_H1-like and T_H2-like cytokine production in 58 individuals by using a quantitative assay (Fig 2, B). In the absence of stimuli or in the presence of TSA alone, production of IL-13, IL-5, IFN-γ, CXCL-10/IP-10, or IL-2 protein in culture supernatant was consistently low, below the sensitivity of the assays used (2-20 pg/mL). In response to PHA stimulation, both characteristic type 1 and type 2 immune responses were readily quantified by ELISA 22 hours poststimulation.

In accordance with the impact of TSA on T_H1/T_H2 immunity-associated cytokine mRNA expression (Fig 2, A), TSA-induced histone hyperacetylation consistently augmented IL-13 and IL-5 and reduced IFN-γ, CXCL-10/IP-10, and IL-2 production across this population (Fig 2, B). HDAC inhibition led to median IL-13 and IL-5 responses increasing by 1/3 (146 to 196 pg/mL; *P* < .0001) and 2-fold (149 to 275 pg/mL; *P* = .0002), respectively. Conversely, interfering with endogenous HDAC activity resulted in reductions of median IL-2, IFN-γ, and CXCL-10 responses by 1/3 (156 to 118 pg/mL), 5-fold (2667 to

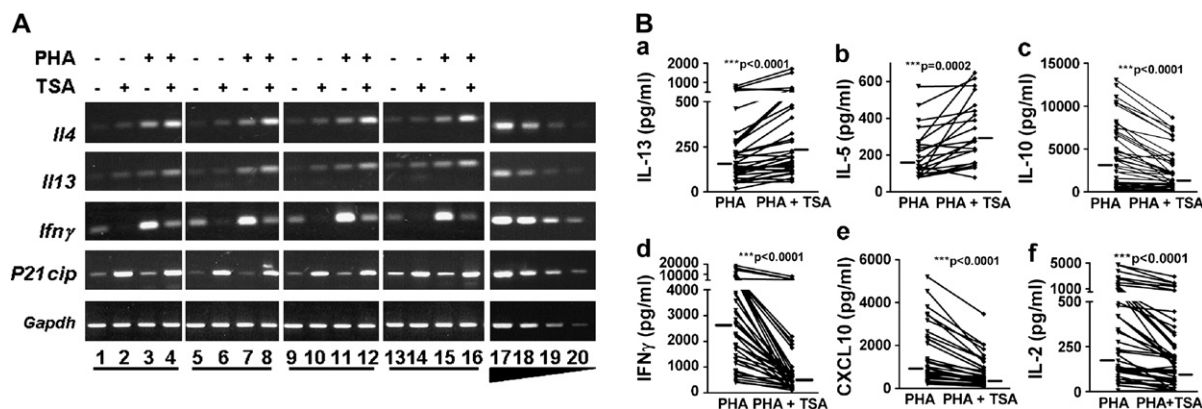


FIG 2. The effect of inhibiting HDAC activity on cytokine responses in PHA-stimulated *ex vivo* PBMCs. PBMCs were treated with TSA before PHA stimulation. **A**, At 22 hours poststimulation, cellular mRNA of *Il4*, *Il13*, and *Ifnγ* genes were examined with semiquantitative RT-PCR. cDNA from PBMCs treated with PHA and TSA (except for *Ifnγ*, where PHA-stimulated sample was used) were titrated to demonstrate the linearity of PCR. These 4 are representative of 12 individuals examined. **B**, Culture supernatants were assayed by ELISA for IL-5, IL-13, IL-10, IL-2, IFN-γ, and CXCL-10 at 22 hours.

538 pg/mL; $P < .0001$), and 2-fold (843 to 444 pg/mL; $P < .0001$), respectively. In parallel with the decline in T_H1 -associated cytokine and chemokine production, IL-10 production was also significantly reduced on interruption of endogenous HDAC activity (median, 2837 to 1300 pg/mL; $P < .0001$; Fig 2, B).

To analyze more broadly the effect of TSA on net immune balance, the ratio of recall T_H1 -associated (IFN-γ, CXCL-10, IL-2) versus T_H2 (IL-5 or IL-13) responses (PHA vs PHA plus TSA; Fig 3) was calculated for each individual. Inhibition of HDAC activity with TSA resulted in marked reduction of IFN-γ:IL-13, IFN-γ:IL-5, CXCL-10:IL-13, CXCL-10:IL-5, IL-2:IL-13, and IL-2:IL-5 ratios by 3-fold to 8-fold (** $P \leq .004$ in all cases). These findings demonstrate that interfering with HDAC activity during stimulation of human PBMCs shifts the resulting cytokine response toward a markedly more intense T_H2 -like phenotype.

Because, unlike most polyclonal activators, PHA has been reported to evoke a relatively T_H2 -biased immunity,²⁶ we speculated that the marked shift toward a T_H2 -like response induced by histone hyperacetylation may be unique to the mode of activation used. Hence, we next examined the effect of TSA-mediated HDAC inhibition on TSST-1 stimulation of human PBMCs (Fig 4). TSST-1 elicits a strongly T_H1 -dominant response, characterized by weak T_H2 -like immunity.²⁶ In this population, acute TSST-1 stimulation elicits intense IFN-γ production (median, 79,600 pg/mL) and weak IL-13 expression (median, 92 pg/mL; Fig 4, A, TSST-1 alone). However, recapitulating the results seen on stimulation with PHA, median IFN-γ production was reduced to half and median IL-13 production was enhanced by 2-fold after TSST-1 stimulation in the presence of TSA (Fig 4, A). Again, in the presence of TSA alone, production of IL-13 or IFN-γ was below the detection sensitivity. Thus, inhibition of HDAC activity with TSA led to a significant

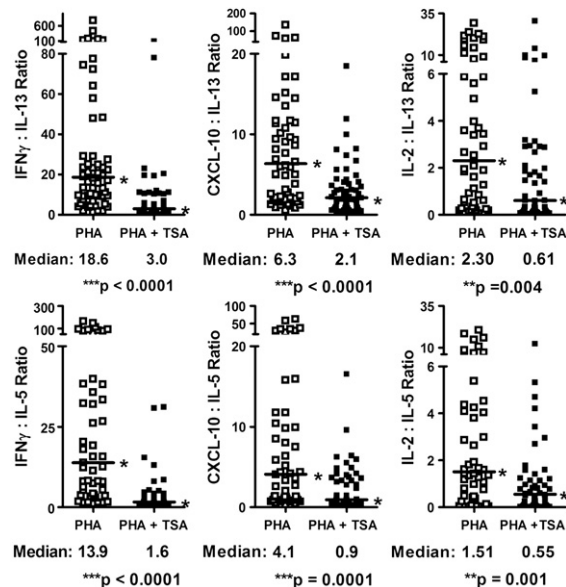


FIG 3. The effect of inhibiting HDAC activity on the balance of T_H1 / T_H2 -associated responses to PHA stimulation. TSA effect on the balance of T_H1 / T_H2 -associated responses was calculated as ratios of T_H1 (IFN-γ, CXCL-10, or IL-2) versus T_H2 (IL-13 or IL-5) cytokine production. Each dot represents an individual studied, with medians (* and bars) also indicated (n = 58).

reduction of the IFNγ:IL-13 ratio by 3.2-fold (* $P = .01$; Fig 4, B). Taken together, these data demonstrate that the effect of inhibition of HDAC activity on T_H1 / T_H2 immune balance is independent of the nature of the stimulus used to activate human PBMCs in that either mode of activation leads to a marked shift of human recall responses toward a T_H2 -like bias.

Trichostatin A modulation of T_H1 -associated and T_H2 -associated recall responses is strongly linked with

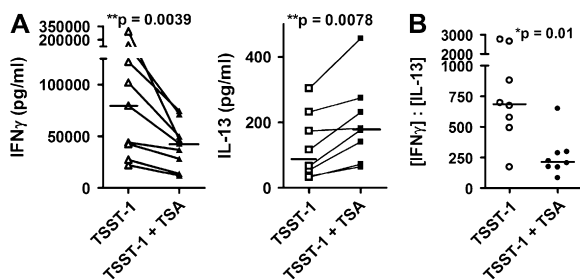


FIG 4. Inhibition of HDAC shifts TSST-1-stimulated T_H1 -like recall responses toward T_H2 -like immunity. PBMCs were incubated with TSA before TSST-1 stimulation. **A**, Culture supernatants were assayed for IFN- γ and IL-13. **B**, The ratio of IFN- γ versus IL-13 was calculated as in Fig 3. Each dot represents an individual studied, with medians also indicated ($n = 8$).

enhanced expression of both a T_H2 -positive regulator and a T_H1 -negative regulator.

Trichostatin A-mediated inhibition of HDAC activity is strongly associated with increased cellular histone acetylation and enhanced gene expression.³¹ However, TSA alone had little effect on *Il4* and *Il13* mRNA (Fig 2, A) and no effect on IL-5 or IL-13 protein production (data not shown). This suggests that in the presence of PHA stimulation, TSA-mediated increases in T_H2 -associated cytokine expression (Fig 2) are the result of both increased histone acetylation at *Il4*, *Il5*, and *Il13* loci and enhanced expression of a T_H2 -positive regulator or regulators. GATA-3 is a key regulator of T_H2 -associated cytokine,³² and forced ectopic GATA-3 expression has been shown to increase T_H2 cytokine expression.⁹ Therefore, the effect of TSA on *Gata-3* expression was examined by using quantitative PCR (Fig 5, A). In all samples, *Gata-3* mRNA was normalized to *Gapdh* expression. *Gata-3* expression in PBMCs treated with culture media alone was used as a reference (with the value 1.0). The presence of TSA alone enhanced median *Gata-3* mRNA by 1.8-fold (* $P = .013$). With PHA stimulation alone, median *Gata-3* expression was increased by 6.1-fold (*** $P = .0008$). Furthermore, the concomitant presence of TSA and PHA resulted in further enhanced *Gata-3* expression (5.8-fold; *** $P < .0003$). Thus, the increases in T_H2 -associated cytokine expression are tightly associated with enhanced *Gata-3* expression.

With its widely observed role in enhancing gene expression, it seems counterintuitive that inhibition of HDAC activity resulted in significant reduction in IL-2, IFN- γ , and CXCL-10 production (Fig 2). To explain this seemingly contradictory observation, we hypothesized that TSA facilitates enhanced expression of negative regulators of T_H1 cytokine responses. Sphingosine kinase 1 (SPHK1) is a recently discovered negative regulator of T_H1 cytokine responses.³³ With semiquantitative PCR, Fig 5, B, demonstrates that *Sphk1* expression was augmented by TSA or PHA stimulation alone, and that the presence of both TSA and PHA enhanced its median mRNA expression more than 30-fold, measured by quantitative PCR (data not shown). This

suggests that the inhibition of T_H1 responses seen on HDAC inhibition is a result of superfluous SPHK1 production.

DISCUSSION

Extensive literature indicates the importance of histone acetylation in regulating lineage decisions and consequent cytokine gene expression in murine models and T-cell clones, but little is known of the role of histone acetylation in regulating human cytokine gene expression. Here, examining a broad cross-section of activated recall responses in a population of 58 children, we demonstrate that the level of cellular histone acetylation, specifically endogenous HDAC activity, plays a key regulatory role in maintaining both the intensity and balance of ongoing T_H1 -associated versus T_H2 -associated immune responses in human beings. Increasing cellular acetylation levels by inhibiting the normal endogenous HDAC activity in human PBMCs markedly shifts immune responses toward T_H2 -like phenotypes, a consequence of both enhanced type 2 and reduced type 1 cytokine gene expression (Figs 2-4). This effect of inhibiting HDAC activity is independent of the nature of the stimulus used to activate human PBMCs in that either mode of activation leads to a shift of recall immune responses toward type 2 bias. Thus, endogenous HDAC activity, in addition to its role in influencing the quantity of gene expression and T_H1/T_H2 lineage commitment, as strongly suggested by previous murine studies, plays a pivotal role in preventing pre-established cytokine responses (ie, to pathogens and environmental antigen stimuli) from deviating toward undesirable, excessive T_H2 -like immunity.

Inhibition of HDAC activity affects cellular acetylation (see this article's Fig E1 in the Online Repository at www.jacionline.org) and is reported to affect either directly or indirectly the expression of 1120 genes (<5% of all elements examined) in human monocyte-derived macrophages.³⁴ Hence, to pinpoint the detailed molecular mechanisms in how inhibition of HDAC activity leads to increases and decreases in cytokine gene expression can be a tremendous challenge. Here, we examined the effect of TSA on the expression of key T_H1 and T_H2 regulators (Fig 5).

Because HDAC inhibition had very little effect on *Il4* and *Il13* expression (Fig 2, A), we speculate that the significant increases of T_H2 -associated cytokine mRNA and protein expression (P values $\leq .0002$) mediated by TSA during PHA stimulation are mainly contributed by positive regulators of T_H2 cytokine genes that are induced by PHA.³² Ectopic expression of *Gata-3*, a key regulator of T_H2 -associated responses, demonstrates increases in T_H2 -associated and decreases in T_H1 -associated cytokine expression.⁹ Here, we show that *Gata-3* expression is slightly increased by TSA alone (1.8-fold; Fig 5, A) and is further enhanced by the concomitant presence of both TSA and PHA (Fig 5, A), in parallel with the increases in *Il4*, IL-5, and IL-13 expression (Fig 2).

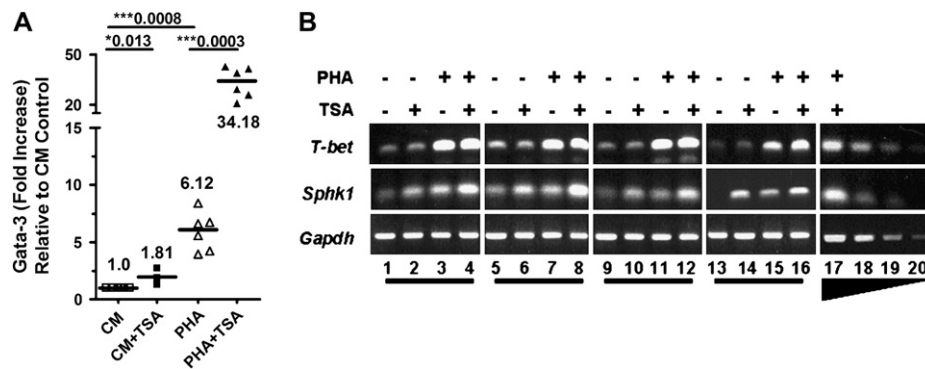


FIG 5. Inhibition of HDAC activity increases *Gata-3* and *Sphk1* expression. **A**, *Gata-3* expression was analyzed against endogenous *Gapdh* with real-time RT-PCR (Applied Biosystems) with culture media as a reference ($n = 6$). Medians are shown in the graph. **B**, *T-bet* and *Sphk1* transcripts were examined with semiquantitative RT-PCR. cDNA from PBMCs treated with PHA and TSA were titrated to show the linearity of PCR. These 4 are representative of 12 individuals examined.

Because deacetylation has generally been viewed as promoting gene silencing, the finding that inhibition of HDACs by TSA represses T_H1 responses (Figs 2-4) appears counterintuitive. Recent studies reveal a more complex picture in which inhibiting HDAC activity can also lead to markedly reduced gene expression.^{34,35} The mechanisms by which increasing chromatin accessibility can lead to decreased gene expression are unclear. Deliberate downmodulation of a T_H1 -positive regulator, T-bet expression in murine T_H1 effectors, impaired IFN- γ expression.³⁶ Here, T-bet expression was not significantly affected by TSA (Fig 5, B). Rather, GATA-3 has been shown to inhibit IFN- γ expression via inhibiting signal transducer and activator of transcription 4, independent of T-bet.³⁷ In this study, increases in *Gata-3* transcripts (Fig 5, A) parallel the decreases in *Ifn γ* transcripts and IL-2, IFN- γ , and CXCL-10 protein production (Fig 2). Thus, increased GATA-3 expression can also contribute to reduced T_H1 -associated responses.

In addition, we identified a clear association between repression of IFN- γ and IL-2 gene production in healthy human PBMCs and increased expression of a T_H1 -negative regulator, *Sphk1* (Fig 5, B). In the study by Yang et al³³ of SPHK1 function, *Sphk1* expression was induced on activation of cytokine-polarized T_H1 clones. Furthermore, overexpression of *Sphk1* reduced IFN- γ and IL-2 mRNA independent of demonstrable effects on *Il4* or *T-bet* expression. Here, human *Sphk1* expression is stimulated in *ex vivo* PBMCs and augmented by inhibition of endogenous HDAC activity (Fig 5, B). TSA markedly suppressed T_H1 immunity-associated cytokine/chemokine mRNA and protein responses (Figs 2-4; P values $\leq .008$) without evidence of reduced *T-bet* expression (Fig 5, B). Hence, excessive *Sphk1* expression that results from reduced HDAC activity may also play an important role in selectively repressing human IL-2, IFN- γ , and CXCL-10 expression. However, the effect of transfected *Sphk1* or of siRNA knockdowns was not restricted to T_H1 clones,³³ and further studies to demonstrate causality will be required.

The finding that the endogenous HDAC activity markedly influences both the intensity and nature of immune responses suggests a potential mechanism through which environment can alter the balance of T_H1/T_H2 recall responses by modulating endogenous HDAC activity. Although little is known of putative associations between HAT and HDAC activity, hence the level of cellular acetylation and clinical phenotype, recent data suggest potential links between differential HDAC expression and severe asthma³⁸ or chronic obstructive pulmonary disease.³⁹ We have begun a well powered investigation of the role of HDAC activity in modulating recall immune responses in pediatric populations with current mild/moderate asthma versus nonatopic controls for a future publication. Multiple environmental factors (eg, viral product, bacterial toxin and diet) that activate or affect immune responses can also influence cellular epigenetic regulation.⁴⁰⁻⁴² For example, LPS, a main component of Gram-negative bacterial cell wall that activates cells via a Toll-like receptor 4 pathway,^{43,44} augments cellular HAT activity in alveolar macrophages.^{35,45} Reactive oxygen species from cigarette smoke also reduce HDAC-2 expression and activity, enhance cytokine expression, and inhibit glucocorticoid action in alveolar macrophages.⁴⁶ In addition, adenoviral protein early region 1A product of adenovirus 5 inhibits p300 HAT activity in various transformed cancer cell lines.^{47,48} Thus, our findings suggest that the presence of environmental factors that can increase cellular acetylation (eg, reactive oxygen species, LPS) may perpetuate or enhance recall responses toward T_H2 -like phenotypes, characteristic of several complex diseases such as allergies or asthma.

In summary, this study identifies a critical role for endogenous HDAC activity in maintaining the level and balance of cytokine gene transcription in human beings.

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METHODS

Study population

After approval of this study by University of Manitoba Research Ethics Board, written informed parental consent was obtained from 723 children (of whom 58 were randomly selected for detailed analysis) within a general population-based case-control cohort. This 1995 birth cohort (SAGE, Study of Allergy Genes and Environment) was created from the provincial health care registry. It identified the 16,320 children born that year in Manitoba. Of these, 2340 children moved out of the province or died over the next 7 years and were excluded from the cohort at the time of its construction. A 1-page survey was mailed to the households of all children to inquire about their health and home environment exposure and for permission to be contacted for future study. From the returned surveys, children were stratified according to the presence of asthma ($n = 398$), the presence of allergic rhinitis or food allergy ($n = 192$) and neither ($n = 3200$). Consented children and parents were assessed by pediatric allergist (Drs Allan Becker or Joel Liem) to characterize the individuals studied in this publication. For this cross-sectional population analysis, 58 participants (41% girls and 59% boys) were studied in detail, of whom 35 were healthy and 23 had a history of allergy with or without asthma.

Measurement of HDAC activity

Histone deacetylase activity was measured by using a colorimetric HDAC activity assay kit (BioVision, Mountain View, Calif),

following the manufacturer's protocol. Briefly, HDAC substrate (Boc-Lys[Ac]-pNA) was deacetylated by incubation with 20 μg nuclear extract from PBMCs for 2 hours at 37°C and then combined with lysine-developer to generate a measurable chromophore. The amount of chromophore generated is proportional to the deacetylation activity by HDAC in the nuclear extract and was analyzed by using an ELISA plate reader. TSA was included in 1 of the samples to control for the specificity of the measurements. Direct addition of TSA into nuclear extracts resulted in 85% to 95% suppression of HDAC activity.

Western blot

Nuclear lysates (25 μg), prepared from *ex vivo* PBMCs and PBMCs cultured in media alone or with TSA were digested with DNase I (Invitrogen, Burlington, Ontario, Canada) and enriched for histone H4 with antibodies specific for histone H4 (5 $\mu\text{g}/\text{mL}$; ab12843; Abcam, Cambridge, Mass) and Fast-Flow Sepharose beads (Amersham Biosciences, Piscataway, NJ). The resulting lysates (1 $\mu\text{g}/\text{lane}$ for histone H4; 3 $\mu\text{g}/\text{lane}$ for acetylated histone H4) were analyzed on a 12% SDS-PAGE gel and blotted for histone H4 (1 $\mu\text{g}/\text{mL}$; ab12843; Abcam) and subsequently for acetylated histone H4 (1 $\mu\text{g}/\text{mL}$; 06-866; Millipore, Upstate Biotechnologies, Billerica, Mass). Secondary antibodies were purchased from Upstate Biotechnologies, and the ECL developing reagents were purchased from Amersham Biosciences.

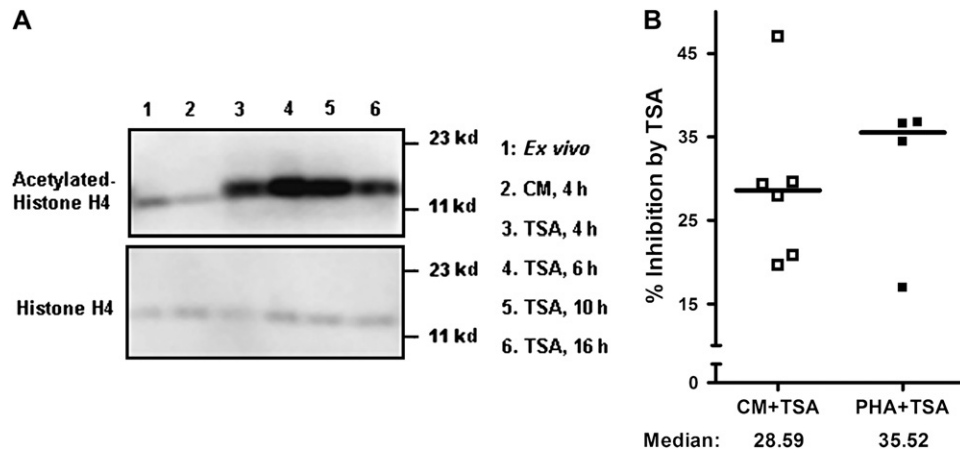


FIG E1. Effect of TSA on cellular histone acetylation and endogenous HDAC activity. **A**, Histone H4-enriched nuclear extracts, prepared from human PBMCs treated with TSA or media alone, were blotted for acetylated histone H4 and unmodified histone H4. **B**, HDAC activity in nuclear extracts of PBMCs treated with or without TSA were measured 22 hours postculture. Percentage inhibition was calculated with reference to PBMCs not treated with TSA.

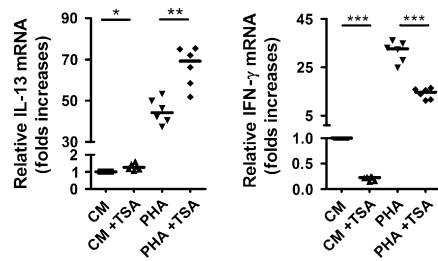


FIG E2. Quantification of changes in *Ifnγ* and *Il13* mRNA expression on TSA inhibition of cellular HDAC activity. RNA was extracted 22 hours poststimulation. Transcripts of *Ifnγ* and *Il13* were analyzed against endogenous *Gapdh* expression with real-time RT-PCR. Fold increases in *Ifnγ* and *Il13* transcription were calculated with the $\Delta\Delta$ Ct program (Applied Biosystems, Foster City, Calif) by using CM as a baseline reference. * $P = .01$; ** $P = .0018$; *** $P < .0001$; $n = 6$.