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CUTTING EDGE

Cutting Edge: Changes in Histone Acetylation at the *IL-4* and *IFN-γ* Loci Accompany Th1/Th2 Differentiation

Patrick E. Fields,* Sean T. Kim,* and Richard A. Flavell[1]*†

Peripheral T cell differentiation is accompanied by chromatin changes at the signature cytokine loci. Using chromatin immunoprecipitation we demonstrate that profound increases in histone acetylation occur at the *IFN-γ* and *IL-4* loci during Th1/Th2 differentiation. These changes in histone acetylation status are locus and lineage specific, and are maintained by the transcription factors Tbet and GATA3 in a STAT-dependent manner. Our results suggest a model of cytokine locus activation in which TCR signals initiate chromatin remodeling and locus opening in a cytokine-independent fashion. Subsequently, cytokine signaling reinforces polarization by expanding and maintaining the accessible state at the relevant cytokine locus (*IL-4* or *IFN-γ*). In this model, GATA3 and Tbet serve as transcriptional maintenance factors, which keep the locus accessible to the transcriptional machinery. ***The Journal of Immunology,*** **2002, 169: 647–650.**

Much recent work has focused on the programs of differentiation leading to the development and stability of the Th1 and Th2 phenotypes (1). Lineage commitment by T cells is directed by the cytokine environment in which the T cell encounters Ag. IL-12 and IL-4 can strongly drive differentiation of Th1 and Th2 cells, respectively, via STAT4 and STAT6 (2, 3). GATA3, expressed predominantly in Th2 cells, is critical for Th2 lineage development and may be the principal mediator of STAT6 function (4–6). Tbet is expressed predominantly in Th1 cells and influences IFN-γ production and Th1 development (7). GATA3 and Tbet may be "master regulators" of Th lineage determination. Both act as transactivators, can induce DNase I hypersensitivity changes in the cytokine loci, and possess negative regulatory functions; GATA3 inhibits IFN-γ expression, and Tbet inhibits Th2 cytokine expression (7–10).

Epigenetic events are important determinants of cytokine gene expression and lineage commitment. Selective changes in DNase I hypersensitivity and methylation in the *IL-4* and *IFN-γ* loci accompany Th differentiation (11, 12). Also, repositioning of the *IL-4* or *IFN-γ* loci to areas of silencing within the nucleus occurs during Th1 or Th2 differentiation, respectively (13). A causative relationship between chromatin remodeling and Th cell differentiation has not yet been established. The study of other events more closely tied to transcriptional regulation, such as enzymatic modification of histone proteins, might aid in our understanding of such a relationship.

Histones undergo an array of posttranslational modifications on tail domains, including acetylation, phosphorylation, and methylation (14). These modifications, as well as the primary sequence of the histone tails themselves, are highly conserved from yeast to man and have been closely linked to biological events (e.g., replication, transcription, and silencing). High levels of histone acetylation at particular loci correlate with transcriptional activity (15), whereas reduced levels correlate with silencing (16). Factors mediating acetylation and deacetylation serve as transcriptional coactivators and corepressors, respectively, suggesting a causative relationship between acetylation and transcription (17, 18).

We show that histones in the cytokine loci of naive T cells are unacetylated. Upon TCR stimulation, the loci are rapidly and progressively acetylated on histones H3 and H4. Early acetylation occurs in the *IL-4* locus irrespective of polarizing conditions, correlating with early transcription. The maintenance of acetylation depends on cytokine/STAT signaling. Tbet and GATA3 also contribute to the polarized acetylated state.

Materials and Methods

Mice

Four- to 8-wk-old C57BL/6J, BALB/c, STAT6$^{-/-}$, and STAT4$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Yale University Animal Resources Center (New Haven, CT).

In vitro T cell differentiation

Naive CD62L^{high}CD44^{low}CD4^{+} T cells were purified by flow cytometric cell (FACS) sorting as previously described (19). These cells were either used directly in chromatin immunoprecipitation (ChIP[2]; naive samples) or stimulated in 24-well plates under polarizing conditions as described with 1 μg/ml anti-CD3 mAb (145-2C11; American Type Culture Collection, Manassas, VA) plus 1 μg/ml anti-CD28 (37.5.1; American Type Culture Collection) (19). T cells were expanded under the same conditions as the primary cultures. Cytokines were measured (ELISA) from 2×10^5 T cells restimulated for 24 h by plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (2 μg/ml).

ChIP

For ChIP analysis, $0.5–1.5 \times 10^7$ T cells were fixed with 1% formaldehyde for 10 min at 37°C, washed with PBS, and lysed in ChIP lysis buffer

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[2] Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; eGFP, enhanced GFP; HSS, hypersensitive site; IE, intronic enhancer.

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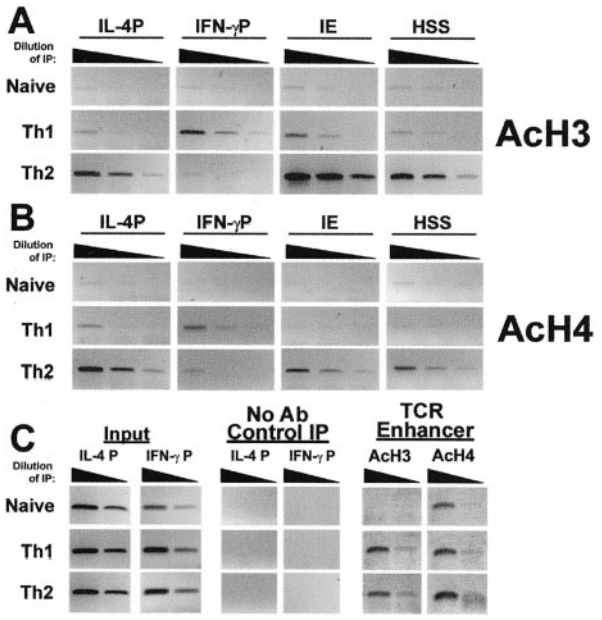

FIGURE 1. Locus- and lineage-specific histone acetylation changes accompany Th differentiation. Histones H3 (*A*) and H4 (*B*) acetylation of naive, Th1, and Th2 day 6 effectors. PCR amplification was performed on 5 μl from each 4-fold dilution (starting with undiluted sample). *C*, Control samples. Shown is a representative of six separate experiments.

(Upstate Biotechnology, Lake Placid, NY). DNA was sonicated by pulsing three to six times. Anti-acetylated histone H3 or H4 was added (4 μl per immunoprecipitation) and incubated overnight. Protein A-agarose beads (Upstate Biotechnology) were added for 1 h then washed once each with low-salt buffer, high-salt buffer, and LiCl buffer, and twice with TE (buffer ingredients are available in the Upstate Biotechnology catalog). Beads were eluted with 0.1 M $NaHCO_3$ and 1% SDS, and cross-links were reversed at 65°C. DNA was ethanol-precipitated in the presence of 20 μg glycogen. Aliquots of equal volume from each sample were used as input controls.

Polymerase chain reaction

PCR was performed on chromatin samples for 28 cycles under standard reaction conditions. Real-time quantitative PCR was performed using an icycler iQ (Bio-Rad, Hercules, CA). Samples were normalized against standard sheared input DNA. Concentrations were determined using the software provided by the manufacturer.

PCR primer sequences (Keck Oligonucleotide Synthesis Facility, Yale University) were as follows: IL-4P, 5′-ACTCATTTTCCCTTGGTTTC AGC-3′ and 5′-GATTTTTGTCGCATCCGTGG-3′; IFN-γP, 5′-CGTA ATCCCGAGGAGCCTTC-3′ and 5′-CTTTCAATGACTGTGCCGTGG-3′; intronic enhancer (IE), 5′-TCTGCTTGGACATCTCTCTTCCC and 5′-ACCACCCCACAGGTCTTTGTTC-3′; hypersensitive site (HSS), 5′-TT GGGGACAGAGGATGCCTTAC-3′ and 5′-GCCTTGCTGAGAGTTTC TTTTGC-3′; TCRα enhancer, 5′-AGATAGTGAATCAATAGCCAG-3′ and 5′-TTCAAAGGGGGACCTGTTT-3′.

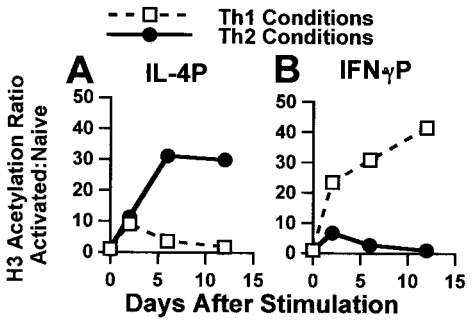

FIGURE 2. Time course of histone acetylation in the *IL-4* and *IFN-γ* loci. *IL-4* (*A*) or *IFN-γ* (*B*) locus acetylation over time. Quantitative, real-time PCR was performed on samples at day 0 (naive cells), or 2, 6, and 12 days after initiation of the cultures. Cells were restimulated at day 6. Relative amounts of DNA in each sample were determined using a standard curve of sonicated DNA of known concentration. Samples were normalized to input DNA. Fold-change from naive samples is plotted. Shown is a representative of three independent experiments.

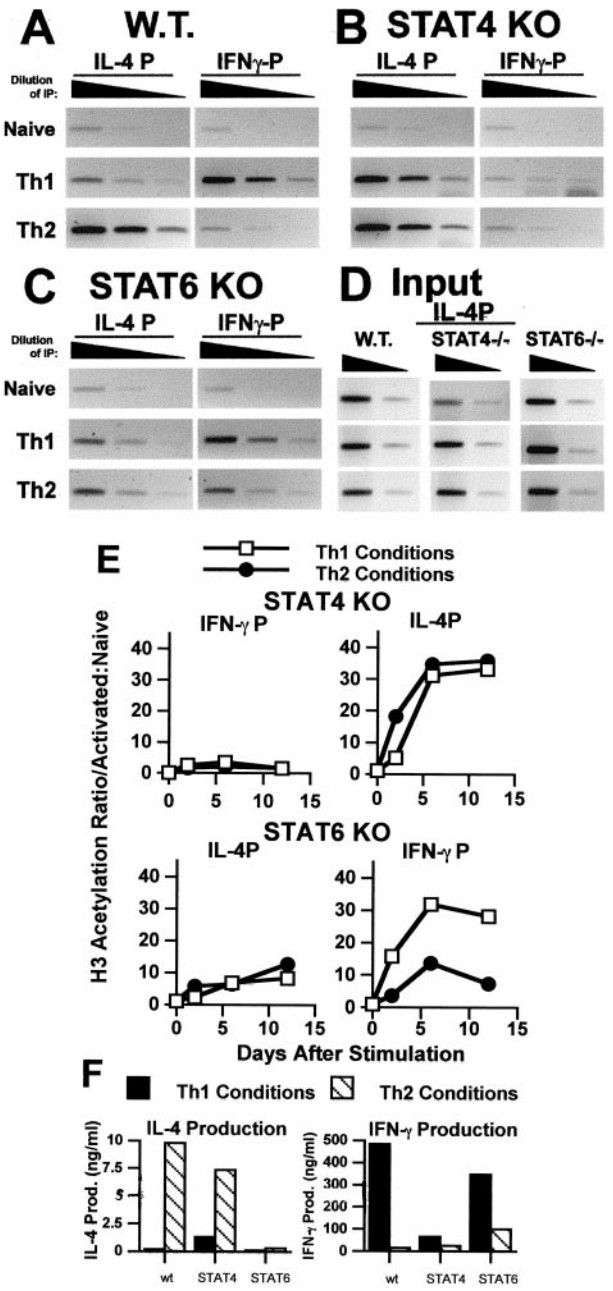

FIGURE 3. Maintenance of histone acetylation in the *IL-4* and *IFN-γ* loci is STAT dependent. Shown is H3 acetylation in wild-type (*A*), $STAT4^{-/-}$ (*B*), and $STAT6^{-/-}$ (*C*) naive, Th1, and Th2 effector T cells. ChIP was performed as described. *D*, Input controls for wild-type, $STAT4^{-/-}$, and $STAT6^{-/-}$ cells. *E*, Time course analysis of H3 acetylation in $STAT4^{-/-}$ (*upper panels*) and $STAT6^{-/-}$ (*lower panels*) T cells. At different times after stimulation (as in Fig. 2), IL-4P and IFN-γP H3 acetylation was examined by ChIP. Quantitative PCR was performed as in Fig. 2. Shown is a representative of three independent experiments. *F*, Cytokine production by restimulated wild-type, $STAT4^{-/-}$, and $STAT6^{-/-}$ T cells. T cells were polarized for 6 days as described. Cells (2×10^5) were restimulated for 48 h with anti-CD3 plus anti-CD28. IL-4 (*left panel*) and IFN-γ (*right panel*) were measured in supernatants by ELISA.

Real-time PCR sequences were as follows: IL-4P, 5′-TCTTGATAA ACTTAATTGTCTCTCGTCAC-3′ and 5′-GCAGGATGACAACTAGCT GGG-3′; IFN-γP, 5′-TCAGCTGATCCTTTGGACCC-3′ and 5′-CTCA GAGCTAGGCCGCAGG-3′.

Fluorogenic probes (Biosearch Technologies, Novato, CA) were as follows: IL-4P, 5′-ACGGGACAGAGCTATTGATGGGTCTCA-3′; IFN-γP, 5′-CTGACTTGAGACAGAAGTTCTGGGCTTCTCC-3′.

Retroviral transduction

Retroviral transduction of T cells was performed as described (20). Retroviral vectors (K. Murphy, Washington University, St. Louis, MO, and L. Glimcher, Harvard University, Boston, MA), allowed expression of GATA3 or Tbet plus enhanced green fluorescent protein (eGFP) (7, 21). At 24 h after stimulation, cells were infected with retroviral supernatant. At days 5–6, cells were sorted into eGFP-negative and eGFP-positive populations, expanded for 4 days, and either restimulated or used in ChIP experiments. The Phoenix-ECO packaging cell line was a gift of Dr. G. Nolan (Stanford University, Palo Alto, CA).

Results and Discussion

Histone acetylation in the *IL-4* and *IFN-γ* loci in naive and effector Th cells was assessed using ChIP with Abs specific for acetylated histones H3 or H4. We analyzed the IL-4 and IFN-γ promoter regions (IL-4P and IFN-γP) as well as two *IL-4* locus regulatory regions, the DNase I HSS (CNS-1) and the IE (HS2) (11, 22). These regions coordinately function as enhancers in the *IL-4* locus (19, 23). In naive cells, low levels of IL-4P, IFN-γP, HSS, or IE were coimmunoprecipitated with acetylated histones H3 or H4. In contrast, both H3 and H4 acetylation were substantially greater in day 6 Th1 and Th2 effectors (Fig. 1, *A* and *B*). In Th1 cells, IFN-γP, but not the *IL-4* locus, was hyperacetylated. Conversely, in Th2 cells, the IL-4 regulatory regions were hyperacetylated, whereas IFN-γP was not. These acetylation changes persisted in cells rested for 14 days, conditions where no cytokine transcription occurred (data not shown). Acetylation changes appeared to affect the entire *IL-4* locus, because identical patterns were observed at distal sites, including the region of the *IL-5* gene (data not shown). Input controls demonstrated that equivalent amounts of material were added to each immunoprecipitation (Fig. 1*C*). As an immunoprecipitation control, TCRα enhancer acetylation was analyzed. Identical results were observed in C57BL/6 (Fig. 1), BALB/c (Figs. 2 and 3), and AND TCR-transgenic mice (data not shown), establishing the generality of the findings. Identical patterns of H3 and H4 acetylation suggested that their regulation in the cytokine loci might be similar. Subsequent studies focused on H3 acetylation.

Kinetics of H3 acetylation was examined by ChIP with quantitative real-time PCR. In Th2-polarized cells, IL-4P acetylation increased at day 2, reaching near-maximum levels by day 6 (Fig. 2*A*). IL-4P acetylation was also observed in Th1-cultured cells at day 2, decreasing by day 6. This observation correlates with early and transient differentiation condition-independent IL-4 transcription (13, 24). Similar patterns at IE and HSS were observed (data not shown). IFN-γP acetylation patterns resembled those of the *IL-4* locus (Fig. 2*B*). Acetylation increases were detectable under both polarizing conditions as early as day 1 (data not shown). Acetylation continued to increase after day 2 in Th1 cultures while decreasing over time in Th2 cultures (Fig. 2*B*). Unlike the *IL-4* locus, acetylation at the *IFN-γ* locus in Th2 cells was substantially less than that in Th1 cells at all time points examined. Early and non-lineage-specific IFN-γ transcription also occurs, but with earlier and more transient kinetics (13). The "window" of acetylation in the *IFN-γ* locus may be transient and thus was less prominent in these studies. The reduction in acetylation could reflect active deacetylation under opposite polarizing conditions or selection of a population that fails to maintain an acetylated locus. The earliest phase of locus activation occurs independently of cytokine signaling and might be mediated by nonspecific entities triggered by TCR/CD28 stimulation, such as chromatin remodeling complexes (25).

IFN-γP and IL-4P acetylation was significantly lower in STAT4- and STAT6-deficient T cells cultured under Th1 or Th2 conditions (Fig. 3, *A–C*). Acetylation never reached levels observed in wild-type cells, even after restimulation (Figs. 2 and 3*E*). Interestingly, IL-4P acetylation was significantly increased in Th1-cultured, STAT4-deficient T cells (Fig. 3, *B* and *E*). Likewise, IFN-γP was hyperacetylated in Th2-cultured, STAT6-deficient T cells (Fig. 3, *C* and *E*). Thus, STAT signaling (i.e., IL-12, IL-4) might negatively affect Th1 or Th2 development by inhibiting cytokine loci accessibility. In the absence of STAT signaling, *IL-4* or *IFN-γ* loci hyperacetylation and transcription proceeded under normally suppressive conditions (Th1 or Th2, respectively). The mechanism by which this cross-regulation occurs is unknown. One possibility is that it is mediated by the coactivators, GATA3 and Tbet, because extinction of their expression is mediated by IL-12

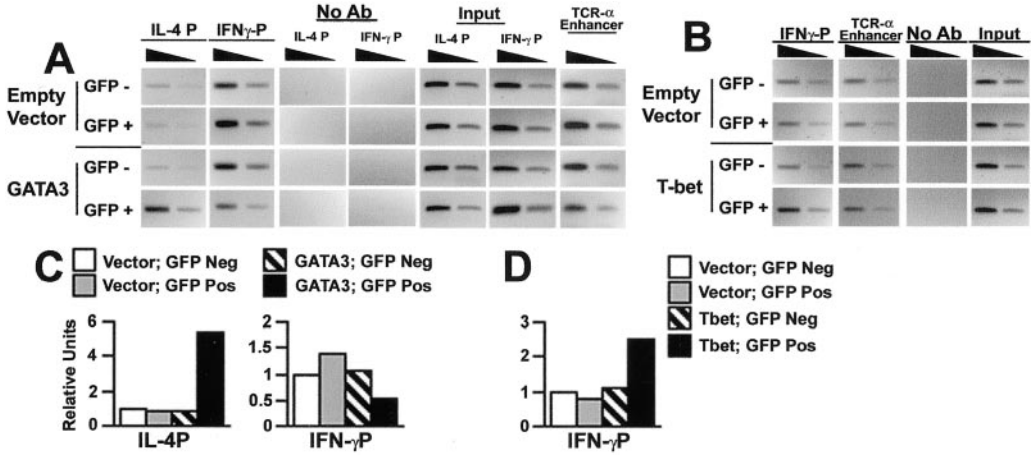

FIGURE 4. Changes in histone acetylation at the *IL-4* and *IFN-γ* loci induced by GATA3 and Tbet in STAT-deficient T cells. GATA3 (*A*) or Tbet (*B*) were introduced by retroviral transduction into STAT6$^{-/-}$ or STAT4$^{-/-}$ T cells stimulated under Th2 or Th1 conditions, respectively. H3 acetylation of the *IL-4* or *IFN-γ* loci was determined by ChIP on sorted green fluorescent protein (GFP)$^{-}$ and GFP^{+} populations. Real-time quantitative PCR analysis of GATA3 (*C*) or Tbet (*D*) transduced cells was performed as in Fig. 2. Relative units were calculated by normalizing the value for each sample to that of the empty vector/GFP-sample.

and IL-4, respectively (8, 26). When restimulated, STAT6-deficient T cells cultured under Th2 conditions and STAT4-deficient T cells cultured under Th1 conditions produced detectable amounts of IFN-γ and IL-4, respectively (Fig. 3*F*), consistent with earlier reports (2, 27). These results indicate that the polarizing cytokine milieu can specify cytokine expression in two ways. First, STAT function can maintain cytokine locus accessibility. Second, STATs can inhibit locus accessibility of the opposing cytokine. Whether these putative functions are mediated by direct STAT binding or indirectly by STAT-induced factors remains to be determined.

GATA3 and Tbet are activators of IL-4 and IFN-γ production, respectively. To determine whether these factors affect cytokine locus acetylation, we introduced by retroviral transduction GATA3 or Tbet into STAT6- or STAT4-deficient T cells cultured under Th2 or Th1 conditions, respectively. Transduction of empty virus into STAT6 knockout T cells had no effect on *IL-4* locus acetylation (Fig. 4*A*). In contrast, GATA3 significantly enhanced acetylation in the locus (Fig. 4*A*, *lower panel*). These data correlate with IL-4 transcription in GATA-transduced cells (20, 21). GATA3 induced a slight decrease in acetylation of IFN-γP (Fig. 4*A*) while also reducing IFN-γ expression (8). Tbet induced a small but reproducible increase (2- to 3-fold) in IFN-γP acetylation (Fig. 4*B*), consistent with an ability to induce IFN-γ expression (7, 9). In contrast, Tbet did not affect IL-4P acetylation, despite a strong inhibitory effect on IL-4 production (10- to 50-fold; Ref. 7 and data not shown). These results were confirmed by real-time PCR analysis (Fig. 4, *C* and *D*). Thus, GATA3 and Tbet appear to have a direct effect on acetylation of the *IL-4* and *IFN-γ* loci in the absence of STAT6 and STAT4 signaling. We have not addressed the likely possibility that GATA3 and Tbet might cooperate with other factors, such as NFAT or STATs, to induce optimal *IL-4* and *IFN-γ* locus acetylation and transcription.

The relationship between acetylation and transcription in these cytokine loci is distinguishable in two ways. First, acetylation is spread over much of the loci, if not the entire loci. This contrasts with acetylation patterns of other gene loci, such as *IFN-β*, where activation-induced acetylation is restricted to promoter-proximal nucleosomes (28). Second, *IL-4* and *IFN-γ* loci acetylation persists when there is little if any transcription. In contrast, *IFN-β* locus acetylation occurs during acute stimulation and rapidly dissipates upon loss of the stimulus (28). Thus, acetylation in the *IL-4* and *IFN-γ* loci correlates more closely with transcriptional competence than with acute transcriptional activity, reminiscent of the β-globin locus (15). We postulate that this type of acetylation (widespread and persistent) is a mark of differentiation rather than of transcriptional activation.

Our data support a multistep model of *IL-4* and *IFN-γ* gene activation whereby locus accessibility (as measured by histone acetylation) regulates cytokine expression (29). In the first step, T cell activation results in acetylation (and transcription) of *IL-4* and, to a lesser degree, *IFN-γ*, irrespective of the cytokine environment. This phase is likely mediated by signals generated by TCR/CD28 stimulation. Maintenance and expansion of the accessibility of the relevant cytokine locus, as well as suppressive events preventing the expression of the opposing cytokine, combine to reinforce polarized Th1/Th2 populations. Positive and negative regulation is mediated by STAT proteins as well as GATA3 and Tbet.

Thus, T cell differentiation is characterized by dynamic changes in cytokine locus histone acetylation. Upon initial T cell stimulation, these changes enable early transcription of cytokines, possibly contributing to the cytokine milieu in which T cell differentiation can proceed. Second, they help to establish heritable and stable patterns of cytokine locus accessibility in effector cells, enabling rapid and robust secondary responses. Maintained acetylation would also provide locus and lineage specificity, because transcription factor binding would highly favor an acetylated locus.

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