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Transcription Factor-Dependent Chromatin Remodeling of \textit{Il18r1} during Th1 and Th2 Differentiation

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The IL-18Rα-chain is expressed on Th1 but not Th2 cells. We have recently shown that Stat4 is an important component of programming the \textit{Il18r1} locus (encoding IL-18Rα) for maximal expression in Th1 cells. \textit{Il18r1} is reciprocally repressed during Th2 development. In this report, we demonstrate the establishment of DH patterns that are distinct among undifferentiated CD4 T, Th1, and Th2 cells. Stat6 is required for the repression of \textit{Il18r1} expression and in Stat6-deficient Th2 cultures, mRNA levels, histone acetylation, and H3K4 methylation levels are intermediate between levels observed in Th1 and Th2 cells. Despite the repressive effects of IL-4 during Th2 differentiation, we observed only modest binding of Stat6 to the \textit{Il18r1} locus. In contrast, we observed robust GATA-3 binding to a central region of the locus where DNase hypersensitivity sites overlapped with conserved non-coding sequences in \textit{Il18r1} introns. Ectopic expression of GATA-3 in differentiated Th1 cells repressed \textit{Il18r1} mRNA and surface expression of IL-18Rα. These data provide further mechanistic insight into transcription factor-dependent establishment of Th subset-specific patterns of gene expression. \textit{The Journal of Immunology}, 2008, 181: 3346–3352.

Materials and Methods

Mice

The generation of C57BL/6 \textit{Stat4−/−} mice was previously described (17). Wild-type (WT) C57BL/6 and BALB/c mice were purchased from Harlan Bioproducts for Science. BALB/c \textit{Stat6−/−} mice (18) were purchased from The Jackson laboratory. All experiments used C57BL/6 mice, except for BALB/c WT and \textit{Stat6−/−} mice as noted in figure legends. Mice were maintained in pathogen-free conditions in barrier facilities in the Laboratory Animal Resource Center (Indiana University School of Medicine). All experiments were performed following approval of the Indiana University Animal Care and Use Committee.

Th differentiation and analysis

CD4 cells were isolated from spleen and lymph nodes of mice using magnetic beads (Miltenyi Biotec). For Th differentiation, CD4 cells (1 × 10^6 cells/ml) were cultured with plate bound anti-CD3 (4 μg/ml), 0.5 μg/ml soluble anti-CD28, under Th1 (2 ng/ml IL-12 and 10 μg/ml anti-IL-4) or Th2 (10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ) skewing conditions and expanded after 3 days. After 5–7 days of culture, cells were harvested for FACS analysis with anti-IL-18Rα (R&D Systems), RNA isolation and quantitative PCR (performed as described) (19), or assays described below. Transduction with GATA-3-hCD4 expressing bicistronic retroviruses was

1 Abbreviations used in this paper: CNC, conserved non-coding sequence; WT, wild type; TSS, transcriptional start site; DH, DNase hypersensitivity; ChIP, chromatin immunoprecipitation.

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performed as previously described (20), except that cells had been differentiated toward the Th1 phenotype. Five days after transduction, hCD4+ cells were analyzed for expression of IL-18Rα or CXCR3. In parallel experiments, hCD4+ cells were purified from control and GATA-3 transduced cells, and RNA was isolated for analysis.

**DH analysis**

Isolation and DNase I digestion of nuclei was performed as described (21). In brief, CD4+ T cells were washed in cold PBS and resuspended in 0.3 M sucrose. Membranes were disrupted with 0.2% Nonidet P-40 and cells were incubated on ice for 10 min before Dounce homogenization. The cell suspension was layered onto 1.7 M sucrose cushion, and nuclei were pelleted by centrifugation at 13,000 rpm for 15 min. The pellet was resuspended in 5 mM CaCl2 and 1 mM MgCl2 and nuclei were digested with DNase I (Worthington) in 5 mM CaCl2 and 1 mM MgCl2 and 5% SDS, 100 mM EDTA, 20 mM Tris (pH 7.4), and 2 mg/ml Proteinase K. DNA was purified using phenol/chloroform and digested to completion with EcoRI or EcoRV as indicated. DNA was separated through 1% agarose and fragments were detected using Southern analysis. Sequences of primers used to generate probes for Southern blotting are available upon request.

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed as described (7). In brief, cells were fixed in formaldehyde and nuclei were isolated after cell lysis and centrifugation. Similar numbers of cells for each condition were used in an experiment to facilitate comparisons among samples. Following nuclear lysis, chromatin was fragmented by sonication. Extracts were pre-cleared with salmon sperm DNA, BSA, and protein A-agarose slurry. The supernatant was incubated overnight with Abs to acetyl-H3, acetyl-H4, acetyl-H3K9,
The IL-18R locus on mouse chromosome 1 contains the closely linked Il18r1 and Il18rap genes encoding, respectively, the IL-18Rα and IL-18Rβ receptor components, and the Il1rl1 gene, encoding the T1/ST2/IL-33R protein, within a 100 kb chromosome segment that is ∼70 kb telomeric from other IL-1R family genes (Fig. 1A). The IL-18R genes are expressed in undifferentiated CD4+ T cells and expression is dynamically modulated during Th1 or Th2 differentiation (Fig. 1B). Surface expression of IL-18Rα is decreased 24 and 48 h after activation in both Th1 and Th2 cultures, but while it continues to be expressed on only a small portion of Th2 cells, it is induced during Th1 differentiation and is expressed on the majority of differentiated Th1 cells (Fig. 1B). Similar patterns of expression during differentiation are observed in BALB/c and C57BL/6 cultures (Fig. 1B). Message levels assessed using quantitative PCR demonstrates a similar pattern of expression for both Il18r1 and Il18rap being repressed in Th2 and induced in Th1 C57BL/6 cultures, with similar expression patterns in BALB/c cells (Fig. 1C and data not shown).

To further explore the regulation of the Il18r1 gene, we performed DH analysis on regions near the transcriptional start site (TSS) (3). A genomic DNA probe (p1) was used to define DH sites of an EcoRV fragment that spanned the promoter, exon 1, and the 5′ end of intron 1 of Il18r1 (Fig. 2A). Th1 or Th2 cultures were differentiated for 1 wk and isolated nuclei were treated with increasing doses of DNaseI. One DH site (identified as DHI) was prominent in undifferentiated T cells and 1-wk Th1 and Th2 cultures, with similar expression patterns in BALB/c cells (Fig. 1C and data not shown).

Identification and histone modification of Il18r1 CNCs. A. VISTA plot of CNCs between human and mouse Il18r1 gene sequences. DH sites are indicated by arrowheads with roman numerals. CNCs are indicated by letters A-E. B, Chromatin from undifferentiated CD4+ T cells or cells differentiated under Th1 and Th2 conditions as described in Fig. 1B was isolated for ChIP analysis of AcH3, AcH4, 2mH3K4, and 3mH3K4 levels at the indicated CNC sites. C, CD4 T cells were activated with anti-CD3 and anti-CD28 for three days. Cells were washed and either left unstimulated or stimulated with IL-12 or IL-4 for 24 h. ChIP was performed as described in A. Results are expressed as percent input ± SD.

Results
Chromatin remodeling at the Il18r1 gene

The IL-18R remodeling on mouse chromosome 1 contains the closely linked Il18r1 and Il18rap genes encoding, respectively, the IL-18Rα and IL-18Rβ receptor components, and the Il1rl1 gene, encoding the T1/ST2/IL-33R protein, within a 100 kb chromosome segment that is ∼70 kb telomeric from other IL-1R family genes (Fig. 1A). The IL-18R genes are expressed in undifferentiated CD4+ T cells and expression is dynamically modulated during Th1 or Th2 differentiation (Fig. 1B). Surface expression of IL-18Rα is decreased 24 and 48 h after activation in both Th1 and Th2 cultures, but while it continues to be expressed on only a small portion of Th2 cells, it is induced during Th1 differentiation and is expressed on the majority of differentiated Th1 cells (Fig. 1B). Similar patterns of expression during differentiation are observed in BALB/c and C57BL/6 cultures (Fig. 1B). Message levels assessed using quantitative PCR demonstrates a similar pattern of expression for both Il18r1 and Il18rap being repressed in Th2 and induced in Th1 C57BL/6 cultures, with similar expression patterns in BALB/c cells (Fig. 1C and data not shown).

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To define alterations in chromatin structure in the central portion of the Il18r1 locus, we performed similar experiments to examine DH in a second EcoRV fragment that spanned introns 1–7 (probe p2). We observed a number of DH sites in this fragment that we termed DHII-DHIV (Fig. 2C). DHII was observed most prominently in Th1 cultures after 2 wk of culture but was detected in undifferentiated T cells and 1-wk Th1 and Th2 cells (Fig. 2C). DHIII was most apparent in Th2 cultures, particularly after 2 wk of culture. DHIV was prominent in undifferentiated T cells, and was similar in Th1 and Th2 cells, though after 2 wk of culture it was less sensitive to DNaseI in Th2 cultures. DHV was not a strong DH site and was equally seen in Th1 and Th2 cells (Fig. 2C). As was observed in the 5′ end of the locus, the establishment of the Th1 chromatin configuration was independent of Stat4.
We examined DH sites in the 3' end of the Il18r1 using an EcoRI fragment that spanned exon 8 to the 3' end of the locus but did not observe any DH sites in that region (Fig. 2D). We further examined whether acute stimulation with IL-12 would affect the DH pattern. WT and Stat4−/− Th1 and BALB/c Stat6−/− Th2 cells differentiated for 5 days as described in Fig. 1A. Shaded histograms indicate control Ab staining; open histograms represent staining with anti-IL-18Rα. Mean fluorescence intensity for cells in each population is shown in the bar graph on the right. B, RNA was isolated from cells of the indicated genotypes cultured under Th1 or Th2 conditions as shown and was used for quantitative PCR to determine Il18r1 mRNA levels relative to levels observed in Th2 cells. C, ChIP analysis was performed for AcH3, AcH4, and dimethyl- or tri-methyl H3K4 with cells cultured as in A. Quantitative PCR was performed for CNC regions, and results are expressed as percent input ± SD.

II18r1 histone modifications during Th differentiation

Since histone acetylation of the Il18r1 locus is Stat4-dependent, but DH patterns appeared to be independent of Stat4 activity, we next wanted to determine whether the differences in chromatin structure among undifferentiated Th1 or Th2 cells were also reflected by altered histone acetylation in that region. We used ChIP followed by quantitative PCR with primers spanning the TSS and DHI to assess histone acetylation and methylation of the H3K4 residue, modifications associated with active gene expression (22). While the levels of all modifications were significantly increased following Th1 development, changes between undifferentiated cells and Th2 cells were more modest, showing only slight decreases in acetylated H3 levels and H3K4 methylation (Fig. 3).

To determine whether the DH pattern corresponded to CNCs, we overlayed the identified DH sites with a VISTA plot of Il18r1 highlighting CNC between mouse and human sequences. DHI, DHIIV, and DHV corresponded to CNC A, B, and C, respectively (Fig. 4A). DHI or DHI did not correspond to CNC, and CNC D and E did not correspond to observed DH sites. To determine whether the CNC had functional impact on chromatin structure, we examined histone acetylation in these regions using ChIP from undifferentiated Th, Th1, and Th2 cells. As with the DHI region (Fig. 3), there were significant increases in histone acetylation in the CNC A-D regions (including DHIII-V) in Th1 cells compared with undifferentiated cells (Fig. 4B). Similar increases in H3K4 methylation were also observed (Fig. 4B). Modifications of CNC E were present at a lower level than those at other CNC regions. Following Th2 differentiation, there were generally lower levels of histone acetylation and H3K4 methylation compared with undifferentiated cells, though patterns differed slightly among the CNC regions, with acetyl-H4 differing less in the CNC C and D regions, and tri-methyl H3K4 differing less in the CNC B and C regions (Fig. 4B).

We then assessed the ability of cytokine stimulation to directly alter histone acetylation across the locus. CD4+ T cells were activated with anti-CD3 and anti-CD28 in the absence of any skewing cytokines for 3 days. Cells were then washed and incubated for 24 h with IL-12, IL-4, or in the absence of any cytokine before ChIP. While IL-12 only had minor effects at the TSS and DHI sites, IL-12 induced acetylation of H3 and H4 at all CNC sites except CNC E (Fig. 4C). In contrast, IL-4 did not decrease acetylation of H3 or H4 at any of the CNC regions (Fig. 4C).
FIGURE 6. GATA-3 represses IL-18Rα. A, ChIP analysis of Stat4 and GATA-3 binding to the promoter, DHI, or CNC A-E regions of IL18r1 in differentiated Th1 and Th2 cells. Quantitative PCR was performed and results are expressed as fold increase over control IgG ChIP. B, CD4 T cells were activated with anti-CD3 and anti-CD28 for 3 days. Cells were washed and either left unstimulated or stimulated with IL-12 or IL-4 for 24 h. ChIP was performed as described in A. Results are expressed as fold enrichment over control IgG ChIP. C, CD4 T cells were differentiated under Th1 conditions for 1 wk. Cells were then transduced with a control or GATA-3-expressing bicistronic retroviral vector that also expresses hCD4. Four days after transduction, IL-18Rα and CXCR3 expression was examined on hCD4-positive cells by flow cytometry. D, Cells prepared as in C were sorted for hCD4 expression and RNA was isolated for analysis of IL18r1 expression.

STAT protein regulation of IL18r1 expression and histone modification

While the expression of IL18r1 is vastly different in Th1 and Th2 cells, Stat4 is required for the induced expression in Th1 cells and Stat6 is required for the repression observed in Th2 cells (Fig. 5, A and B). The intermediate level of inactivated IL18r1 expression in Stat4−/− Th1 and Stat6−/− Th2 is observed at the level of mRNA (Fig. 5B) and surface expression of IL-18Rα where these populations have an intermediate staining intensity (Fig. 5A).

To determine whether STAT proteins have a role in establishing chromatin modifications in the CNC regions, we differentiated C57BL/6 WT and Stat4−/− Th1 cultures and BALB/c WT and Stat6−/− Th2 cultures and examined the level of histone acetylation at each of the CNC regions. As expected, levels of acetyl-H3 and acetyl-H4 were decreased in Stat4-deficient Th1 cultures compared with WT Th1 cultures at CNC regions A-D (Fig. 5C). Acetyl-H3 and acetyl-H4 levels were very low in WT Th2 cells but were increased in the absence of Stat6 at CNC regions A-C (Fig. 5C). There was no significant difference between levels of histone acetylation in WT Th2 cells derived from C57BL/6 or BALB/c mice (data not shown).

We then tested whether there were differences in the levels of H3K4me2 and H3K4me3 at the IL18r1 CNC regions. Di-methyl-H3K4 and tri-methyl-H3K4 were detected at CNC regions A-D in WT Th1 cultures (Fig. 5D). In contrast to the patterns of acetylated histones, H3K4 methylation was not decreased in the absence of Stat4, correlating with the diminished but detectable IL18r1 mRNA in Stat4−/− Th1 cells. Methyl-H3K4 levels were lower in Th2 cells but were increased in Stat6−/− Th2 cultures to levels observed in Stat4−/− Th1 cultures (Fig. 5D). Thus, H3K4 di- and tri-methylation is reduced in Th2 cultures, coincident with the repression of IL18r1 mRNA.

Transcription factor binding in the IL18r1 locus

In previous work, we identified that Stat4 binds to the IL18r1 promoter (7). To test whether factors that activate, or repress IL18r1 expression bind to specific regions of the locus, we performed ChIP for Stat4, Stat6, and GATA-3, and performed quantitative PCR for the promoter and CNC regions. Stat4 was observed binding to the promoter region of Th1 cells but had lower levels of binding at DHI and the CNC regions and minimal levels detected in Th2 cells (Fig. 6A). Conversely, GATA-3 binding was detected primarily in Th2 cells with maximal levels in the CNC A region (Fig. 6A).

To test whether these factors bound in response to cytokines during the differentiation process, CD4 T cells were activated for 3 days and left unstimulated or stimulated with IL-4 or IL-12 for 24 h. Stat4 binding to the promoter following IL-12 stimulation was increased 150-fold (Fig. 6B). Stat4 was also detected at the CNC A and CNC B sites where binding was increased 80- to 200-fold with only modest binding at the DHI site (Fig. 6B). In contrast to Stat4 binding which was 10-fold over control binding in unstimulated cells, Stat6 binding was increased less than 5-fold over control at any of the regions in unstimulated or IL-4 stimulated cells (data not shown). GATA-3 binding was not detected in the promoter but was significantly enriched at the CNC A and CNC B regions although IL-4 had only modest effects on GATA-3 binding (Fig. 6B).

These results suggested that IL-4 signaling and the induction of GATA-3 would repress IL18r1 expression in Th1 cells. Transduction of GATA-3 into differentiating cells would divest them to a Th2 phenotype where IL-18Rα would be low and it would be difficult to discern direct effects of GATA-3 from indirect effects on differentiation of the cells. To avoid this problem and examine
II18r1 regulation in absence of altered differentiation, we first differentiated cells under Th1 conditions for 1 wk before transduction with control-hCD4 or GATA-3-hCD4 expressing bicistronic retroviruses. After 5 days of culture, hCD42− cells were analyzed for expression of IL-18Ra. Expression of GATA-3 reduced IL-18Ra expression in Th1 cells and II18r1 mRNA levels (Fig. 6, C and D). To confirm that cells transduced with GATA-3 did not convert to a Th2 phenotype, we also analyzed expression of CXCR3, a chemokine receptor expressed on Th1 cells. GATA-3 did not have an effect on CXCR3, demonstrating specificity in the effects on IL-18Ra expression (Fig. 6C).

Discussion
Gene expression is dynamically regulated during cellular differentiation. This process has been studied in the development of Th subsets primarily by examining cytokine genes that are differentially expressed in Th1 and Th2 subsets. In previous work, we examined the II18r1 gene, a receptor protein that is expressed in and functionally linked to Th1 cells. We identified Stat4-dependent mechanisms for high IL-18Ra expression in Th1 cells that involved Stat4-induced changes in histone modification and regulation of the level of DNA methylation (7). In this report, we examine chromatin structural changes by assessing DH across the locus. Several DH sites correspond to CNCs and show distinct patterns of sensitivity in Th1 or Th2 cells. We further show that these DH/CNC regions are targets of histone modification following cytokine stimulation and during differentiation. Moreover, Stat4 and GATA-3 are associated with these regions during Th1 and Th2 development, respectively, and ectopic expression of GATA-3 represses IL-18Ra expression, suggesting that both factors directly affect II18r1 gene expression.

Unlike cytokine genes that have been the focus of previous work on chromatin differences involved in Th1 and Th2 programming, II18r1 is expressed in undifferentiated CD4 T cells (Fig. 1). During the differentiation period, II18r1 expression is induced in Th1 cells and repressed in Th2 cells at the level of mRNA and surface expression (Fig. 1). The pattern of DH sites I, II, and IV is similar between undifferentiated T cells and Th1, suggesting that overall chromatin structure does not change significantly during Th1 development, perhaps accounting for the lack of an effect of Stat4 deficiency on DH pattern (Fig. 2). In contrast, histone modifications are impacted more dramatically during Th1 development, with histone acetylation and methylation being decreased only modestly in Th2 cells, compared with undifferentiated CD4 T cells (Figs. 3 and 4). However, there are also differentiation independent changes in H3K4 methylation, presumably resulting from T cell activation and expansion, that are observed in Stat4−/− Th1 and Stat6−/− Th2 cells at similar levels to those observed in Th1 cells (Fig. 5). Together, these data provide a sequence of remodeling events during programming in Th1 differentiation. II18r1 exists in a poised state in undifferentiated CD4 T cells (Figs. 2–4). Following T cell activation, and in the absence of Stat6− and GATA-3-dependent repression, there is an increase in H3K4 methylation that correlates with the establishment of gene expression patterns intermediate between Th1 and Th2 cells (Figs. 3–5). IL-12 induces increased histone acetylation as early as 24 h following stimulation (Fig. 4). Stat4 binds to II18r1 following IL-12 stimulation and is required for the induction of histone acetylation, decreased DNA methylation, and establishment of the high level of gene expression observed in Th1 cells (Figs. 5 and 6) (7). Thus, gene induction during Th1 development is controlled by several distinct chromatin remodeling events.

Similarly, several processes are involved in gene repression during Th2 differentiation. The DH pattern changes during Th2 development, with DH sites I and IV becoming less sensitive, and the appearance of DHIII (Fig. 2). These changes are most significant after 2 wk of culture suggesting progressive changes correlating with increased commitment to the Th2 phenotype. While IL-4 did not have acute effects on altering chromatin modifications (Fig. 4), Stat6 is required for repression of the locus during Th2 development. In the absence of Stat6, there are increases in H3K4 methylation and histone acetylation, compared with undifferentiated CD4 T cells or WT Th2 cells that results in II18r1 expression increased to an intermediate level (Fig. 5). The precise function of Stat6 in this process is unclear. We did not detect significant increases in Stat6 binding to the II18r1 locus in the regions we examined. It is still possible that Stat6 binds to another region of II18r1 or to sites in adjacent genes. However, gene repression may be indirect, through the Stat6-mediated induction of GATA-3. GATA-3 was bound to the II18r1 locus in Th2 cells, most significantly in the CNC A and B regions, where CNC A corresponds to DHIII a Th2 site, and CNC B is DHIV, which is less sensitive to DNase in Th2 cells (Figs. 2, 4, and 6). GATA-3 is expressed in naive CD4 T cells, but is induced 10- to 20-fold during Th2 development, and it is likely that the induced level is required to mediate II18r1 repression. While GATA-3 is predominantly considered as an activator of the Th2 genetic program, it can repress gene expression during cellular differentiation (23–25). Importantly, we demonstrate that ectopic expression of GATA-3 in differentiated Th1 cells decreases IL-18Ra surface expression and II18r1 mRNA expression, without affecting CXCR3, another Th1-expressed receptor (Fig. 6). The fact that IL-18Ra expression is not reduced to a greater extent in retrovirus-transduced cells may be due to the presence of T-bet in Th1 cells, which is known to have antagonistic activity upon GATA-3 (26), as well as differences between the half-life of II18r1 mRNA and IL-18Rα protein. Together, these data define a mechanism for the repression of a locus that is active in undifferentiated CD4 T cells and repressed during Th2 differentiation.

Th cell differentiation provides a model for understanding changes in gene architecture that culminate in gene expression or repression. Much of the analysis of gene expression in Th1 and Th2 cells has been on cytokine genes. However, the changes that occur in these genes might be unique as they are inducible genes that are strictly controlled by stimulation from Ag or cytokine receptors. In a previous report and in this report, we have described the programming of the II18r1 locus, a gene that is highly expressed in Th1 cells and is rapidly repressed during Th2 development. We have shown that Stat4 is required for maximal expression and that Stat6 is required for maximal repression, possibly through a mechanism involving GATA-3 binding to the locus and repressing expression. This suggests that direct repression of genes during differentiation by a phenotype promoting transcription factor, in addition to interference with the expression or function of reciprocal program promoting factors, may be an important component of the ability of transcription factors to program lineage decisions.

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Disclosures
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