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Temporal Induction Pattern of STAT4 Target Genes Defines Potential for Th1 Lineage-Specific Programming

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STAT4 is a critical component in the development of inflammatory adaptive immune responses. It has been extensively characterized as a lineage-determining factor in Th1 development. However, the genetic program activated by STAT4 that results in an inflammatory cell type is not well defined. In this report, we use DNA isolated from STAT4-chromatin immunoprecipitation to perform chromatin immunoprecipitation-on-chip analysis of over 28,000 mouse gene promoters to identify STAT4 targets. We demonstrate that STAT4 binds multiple gene-sets that program distinct components of the Th1 lineage. Although many STAT4 target genes display STAT4-dependent IL-12-inducible expression, other genes displayed IL-12-induced histone modifications but lack induction, possibly due to high relative basal expression. In the subset of genes that STAT4 programs for expression in Th1 cells, IL-12-induced mRNA levels remain increased for a longer time than mRNA from genes that are not programmed. This suggests that STAT4 binding to target genes, while critical, is not the only determinant for STAT4-dependent gene programming during Th1 differentiation. The Journal of Immunology, 2009, 183: 3839–3847.

The Th1 cell is responsible for cell-mediated immune functions including eradication of intracellular pathogens. STAT4 is required for the development of Th1 cells from naive CD4⁺ T cells and most IL-12-stimulated functions (1, 2). STAT4-deficient mice are highly susceptible to infection by intracellular pathogens but are resistant to T cell-mediated autoimmune diseases (1). In humans, STAT4 also mediates IL-12 functions and single nucleotide polymorphisms in the STAT4 gene correlate with susceptibility to autoimmune disease (3, 4). How STAT4 establishes the Th1 genetic program remains unclear.

Following activation, STAT4 binds to cis-regulatory regions of several genes to induce gene expression, though relatively few of these have been directly identified. In humans STAT4 has been shown to bind to IFNG and IL12RB2, and in mouse Ifng, Hlx1, and Il18r1 (5–10). However, a much larger set of genes appears to rely on STAT4 for expression in the Th1 differentiation program (9, 11, 12). It is possible that the ability of STAT4 to activate an inflammatory genetic program can be understood better by a genome-wide analysis of target gene promoters. Recently, a number of studies have used chromatin immunoprecipitation followed by microarray analysis (chromatin immunoprecipitation-ChIP⁴-on-chip) or large-scale sequencing of the bound targets (ChIP-seq) to identify, characterize, and analyze transcription of genes controlled by transcription factors (13, 14). These studies provide a global picture of transcription factor-target gene interactions in the physiological state. Such a ChIP-on-chip study may reveal STAT4-mediated transcriptional regulatory networks active in Th1 cell development.

In this report, we have performed ChIP-on-chip experiments for STAT4. Using informatics and biological interrogation of the data, we have identified additional aspects of the STAT4-dependent genetic program and activation patterns that predict potential programming of Th1-specific gene expression. Moreover, we identify additional STAT4 target genes that may play a role in the inflammatory cell phenotype. These data establish a framework for genome-wide understanding of the transcription factor network that regulates inflammatory immune responses.

Materials and Methods
T cell preparation and analysis
For ChIP-on-chip experiments CD4⁺ T cells from C57BL/6 mice were activated for 3 days with anti-CD3 and anti-CD28. Cells were washed and incubated in the presence or absence of 5 ng/ml IL-12 for 4 h before being processed for ChIP analysis by Genpathway. For analysis of gene induction and histone modification, wild-type and Stat4⁻⁄⁻ BALB/c mice were activated for 3 days as above before RNA was isolated from cells that were unstimulated or stimulated with 5 ng/ml IL-12 for 4 or 18 h. Generation of Th1 cultures and quantitative (real-time) PCR (qPCR) was performed as described (15). ELISA for TNF-α was performed using reagents from BD Pharmingen. DNA affinity precipitation assay and ChIP was performed as described (8). ERK immunoblots were performed using standard methods (8).

FactorPath ChIP-on-chip
Preparation of cells for ChIP-on-chip was performed at Genpathway (16). Cells prepared as above were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Cell lysates were sonicated to an average
Results

Generation and analysis of STAT4 ChIP-on-chip data

To identify STAT4 target genes, CD4+ T cells were activated for three days with anti-CD3 before washed cells were stimulated with IL-12 for 4 h and chromatin was immunoprecipitated with anti-STAT4 Ab. The preactivation was performed to allow cells to

DNA fragment length of 300–500 bp. Genomic DNA from aliquots was purified for use as input. Chromatin was precleared with protein A agarose beads (Invitrogen) and STAT4-bound DNA sequences were isolated using specific Abs (Santa Cruz sc-486). Complexes were precipitated with protein A agarose beads, washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65°C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. qPCR reactions were conducted in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). The resulting signals were normalized for primer efficiency by carrying out quantitative PCR for each primer pair using input DNA.

Analysis of ChIP DNA by Affymetrix tiling arrays was conducted by GenPathway. ChIP (multiple reactions) and input DNAs were amplified using ligation-mediated PCR. The resulting amplified DNA was purified, quantified, and tested by qPCR at the same specific genomic regions as the original ChIP DNA to assess quality of the amplification reactions. Amplified DNA was fragmented and labeled using the DNA Terminal Labeling Kit from Affymetrix, and hybridized to the Affymetrix GeneChip Mouse Promoter 1.0R Array at 45°C overnight. This array contains over 2.5 million 25-mer oligos tiled to interrogate over 28,000 mouse promoter regions (2.5 kb relative to the transcription start site). Arrays were scanned and the resulting CEL files were analyzed using Affymetrix TAS software that computed estimates of fold enrichment over hybridization with input DNA in linear scale. This metric is referred to as peak binding intensity. Thresholds were selected, and the resulting BED files containing lists of sequence intervals with significant binding detected were analyzed using Genpathway software that provides comprehensive information on genomic annotation, peak metrics, and sample comparisons for all peaks (intervals). Graphing of peak binding values were generated using the Affymetrix Integrated Genome Browser. The entire dataset is submitted to the NCBI GEO database (GSE16845; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16845).

Intersects of our dataset with previously published datasets were determined using Microsoft Excel. Published datasets that were used for analysis of common genes were from naive BALB/c mouse Th cells cultured under Th1 conditions for 0, 2, 6, and 48 h (MG-U74Av2 arrays containing 12,488 probes) (12), differentiated C57BL/6 mouse Th1 cells stimulated with IL-12 for 18 h (custom array with 24,000 probes) (11), differentiated human Th1 cells stimulated with IL-12 for 4 or 24 h (HuGeneFL array containing 6000 genes) (17), and PHA/IL-2 activated human T cells stimulated with IL-12 for 6 h (U133A and U133B arrays) (18).

Multiple expectation maximization for motif elicitation (MEME) analysis

The 1111 interval sequences with peak intensity >4, which were in close proximity to 1540 genes, were screened by MEME (19) to find over-represented motifs. The parameters for MEME included looking at the reverse complement sequence, a maximum motif width of 15, and a cutoff of 30 motifs ordered by increasing E-value. The analysis was run using three different approaches regarding the number of times the motif repeated, any number of repeats, zero or one repeat, and one or more repeats with virtually the same results each time. WebLogo (20) generated the motif logos using the aligned site occurrences for each motif given by MEME.

Gene ontology (GO) analysis

The GO analysis was performed by GOstat (21). Using an input list and a selected background, GOstat determines enriched GO terms based on a Fisher exact test. The p-value from the Fisher exact test is altered using a Benjamini correction. Only terms with a corrected p-value less than 0.1 are reported. The analysis was performed three times with the curated gene list as the input and the Affymetrix chip gene list as the background.

FIGURE 1. STAT4 binding in target gene promoters. A, Wild-type CD4+ T cells were activated for 3 days and cultured in the presence or absence of IL-12 for 4 h. ChIP analysis was performed for STAT4 or control IgG and qPCR was performed to determine STAT4 binding at the indicated target sequences. The scale indicates relative binding that is normalized to input DNA. B, The number of genes was graphed against binding intensity. Intensity of 4669 intervals ranged from 2.2–32.4. The cut-off intensity for subsequent analysis was 4. C, Histograms of the frequency of STAT4 binding across the arrayed promoter sequences relative to the TSS. The curated list has all 1540 genes with binding intensity >4. The exact match list is a subset of 660 genes that contain a consensus STAT4 binding site in the promoter. D, Comparison of the frequency of subsets of genes in the curated list (binding intensity >4) and the frequency on the Affymetrix chip using GOstat to assign gene function. p values for significant differences are indicated. E, Venn diagram comparing the overlap of IL-12-induced genes from published microarray analysis (see text) with the ChIP-on-chip dataset.
acquire IL-12 responsiveness (22). IL-12 stimulation of activated cells resulted in 70–80% of cells in the population becoming pSTAT4-positive with a 6-fold increase in mean fluorescence intensity, as assessed by intracellular staining (data not shown). To confirm STAT4 binding to target genes, we tested Il18r1 promoter and the Il2ra PRRIII element DNA in the precipitate following IL-12 stimulation (8, 23) and observed a greater than 30-fold induction of STAT4 binding, compared with unstimulated cells (Fig. 1A). DNA from the ChIP was then hybridized to an Affymetrix array containing sequences spanning −7.5kb to +2.5kb of over 28,000 promoters in the mouse genome and enrichment was calculated based on comparison to an array hybridized with input DNA. In replicate experiments, the ChIP-on-chip analysis identified a total of 4,669 genes as near a STAT4 binding site in vivo. The peak binding intensities (see definition in Materials and Methods) of immunoprecipitated DNA hybridizing to the promoter oligonucleotides showed a wide range (2.2 to 32.4, with the highest value being the most intense binding) indicating the diverse nature of STAT4 binding to target genes. Mean and median of this data set were at 4.4 and 3.3, respectively (Fig. 1B). A manual inspection of the data showed many of the known STAT4 target and Th1-associated genes to have peak intensities of four or higher. Thus, we filtered the list of genes using a cutoff peak intensity of 4 (1111 associated genes to have peak intensities of four or higher. Thus, FIGURE 2. STAT4 binding to target genes. WT and Stat4−/− activated T cells were stimulated with IL-12 for 4 h before nuclei were isolated for chromatin immunoprecipitation with anti-STAT4. Results are shown as percent input as calculated using qPCR results of amplification with primers specific for the promoter region of each of the genes indicated. The peak intensity of STAT4 binding derived from the ChIP-on-chip dataset and the fold-enrichment of STAT4 binding in WT, compared with Stat4−/− cells, are indicated. STAT4 binding in the Stat4−/− cells was within 1 SD of the background (IgG control).

To determine whether STAT4 target genes were enriched for any specific category, we defined the enrichment of GO terms in the curated set compared with an Affymetrix mouse genome background using the Gostat program (21). The major statistically significant enrichments (p < 0.05) were in genes involved in cytokine binding, including factors with IL receptor activity and TNF receptor binding activity (Fig. 1D). There was also a significant enrichment for nucleic acid binding factors. These analyses demonstrate selectivity in STAT4 binding target genes associated with specific cellular functions.

A number of published reports have examined IL-12-induced gene expression using microarrays from human or mouse cells treated with IL-12 for various times during or after Th1 differentiation (11, 12, 17, 18). We generated a cumulative list of IL-12-induced genes from these studies and compared them to the ChIP-on-chip dataset. The Venn diagram in Fig. 1E identifies an overlap of 72 genes (Table S2A) between these two analyses.

The online version of this article contains supplemental material.
gene list. In the second approach, we used the expectation-maximization motif search algorithm, MEME (19) to identify motifs. We observed a number of motifs with low E-values. Sequences containing variants of a STAT4 consensus site were found in all STAT4 bound sequences (Fig. 3A, motif 2), further validating the dataset and suggesting that the false-positive rate is low. The percentage of the genes containing the consensus (motif 1) was highest in sequences that had high peak values, and decreased as peak values decreased (Fig. 3B). Although these data shows that some sequences containing the STAT consensus site were not included in the curated dataset (peak value less than 4), the problem of the exclusion of these false-negatives is offset by avoiding the inclusion of large numbers of genes (Fig. 1B). We functionally assessed binding to motif 3 using biotinylated oligonucleotides that spanned the motif. We detected STAT4 binding specifically to motif 3 that was competed by STAT consensus oligonucleotides to a greater extent than by nonspecific sequences (Fig. 3, D and E).

**STAT4 binds multiple genes associated with the Th1 phenotype**

Although the Th1 genetic program is known to be largely dependent on STAT4, and STAT4 binding to a subset of Th1 genes has been demonstrated, all targets of STAT4 required to establish Th1 differentiation are not clear. We examined the curated list for genes demonstrated in the literature to be functionally associated with the Th1 phenotype, or preferentially expressed in Th1 cells, and have demonstrated STAT4- or T-bet-dependence in their expression (Table S3). We identified binding to a set of genes (18/28 Th1 genes searched (Fig. 4A, Table S3); significantly enriched from the genome p < 10^{-8}) including Furin, Ifng, and Il18r1, genes that are known to be STAT4 targets (5, 6, 8, 26) as well as Th1 genes that are expressed independently of STAT4 including Egr2 and Ms4a4b. Using Affymetrix’s Integrated Genome Browser we mapped the peak intensities of binding in a subset of these genes (Fig. 4B). Although the microarray in these experiments is focused on promoter sequences,
STAT4 peak binding was largely localized to the gene regions immediately upstream of transcription initiation. The exceptions to this included Gadd45g, which had peaks both 5-prime and 3-prime of the gene, and Tnf that had intragenic peak binding, closely linked to STAT4 binding at the Lta promoter (Fig. 4B).

Several of these genes, while associated with Th1 function, had not been demonstrated to be STAT4-dependent. To test a requirement for STAT4 in Th1 expression of these genes, we differentiated wild-type and Stat4−/− CD4+ T cells under Th1 conditions and used quantitative PCR to examine relative expression. Gadd45g, which plays a role in IFN-γ production and potentially synergy between IL-12 and IL-18 (27), was decreased more than 5-fold in Stat4−/− Th1 cultures compared with wild-type cultures (Fig. 4C).

To test whether expression of these genes was affected by STAT4-deficiency, we differentiated wild-type and Stat4−/− Th1 cells and used qPCR to examine relative levels of gene expression, using Hlx1 as a STAT4-dependent control. Although expression of Prkcq and a number of other TCR signaling genes was not significantly decreased in Stat4−/− Th1 cells, Lcp2 expression in Stat4−/− cultures was decreased to one-half the level seen in wild type cultures (Fig. 4C). To determine whether there are consequences of this decrease in expression, we analyzed the TCR-induced phosphorylation of the ERK MAPKs and observed decreased ERK phosphorylation in Stat4−/− Th1 cells, compared with wild-type cells (Fig. 5C). These observations agree with previous data suggesting that strong stimuli minimize the defect in IFN-γ production by Stat4−/− Th1 cells (30), though it is difficult to test using IFN-γ as a readout because STAT4 plays an important role in programming Ifng for

STAT4 binds a subset of genes associated with TCR signaling

In a manual examination of the curated gene list, we defined sub-sets of genes that would have specific functional consequences for T cell differentiation or function. Among these clusters we observed a set of genes that are associated with TCR signaling identified from the literature (29) 7/23 TCR signaling genes searched (Table S2B); significant enrichment from the genome p = 1 × 10−4) including Cd3z, Prkcq/ PKCθ, and Lcp2/SLP-76 (Fig. 5A) that are important for the stimulation of T cells in response to specific Ag. To test whether expression of these genes was affected by STAT4-deficiency, we differentiated wild-type and Stat4−/− Th1 cells and used qPCR to examine relative levels of gene expression, using Hlx1 as a STAT4-dependent control. Although expression of Prkcq and a number of other TCR signaling genes was not significantly decreased in Stat4−/− Th1 cells, Lcp2 expression in Stat4−/− cultures was decreased to one-half the level seen in wild type cultures (Fig. 5B). To determine whether there are consequences of this decrease in expression, we analyzed the TCR-induced phosphorylation of the ERK MAPKs and observed decreased ERK phosphorylation in Stat4−/− Th1 cells, compared with wild-type cells (Fig. 5C). These observations agree with previous data suggesting that strong stimuli minimize the defect in IFN-γ production by Stat4−/− Th1 cells (30), though it is difficult to test using IFN-γ as a readout because STAT4 plays an important role in programming Ifng for

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**FIGURE 4.** STAT4 binding in Th1 genes. A, Heatmap of Th1 associated genes based on binding intensity of STAT4 to gene promoters. B, The Integrated Genome Browser (IGB) used BAR files generated from the ChIP-on-chip data to identify binding peaks relative to annotated genes. The graphs indicate the significance (p-value probability) of enrichment at promoter-arrayed sites. C, Wild type and Stat4−/− CD4+ T cells were cultured under Th1 conditions for five days before RNA was isolated for qPCR using the indicated primers. RNA levels are relative to wild type Th1 cells.
Th1 expression (31). To avoid this problem, we analyzed TNF-α production from wild-type and Stat4<sup>−/−</sup> Th1 cultures stimulated with either anti-CD3 or PMA and ionomycin. Although TNF-α production following anti-CD3 stimulation is decreased in Stat4<sup>−/−</sup> cultures compared with wild-type, stimulation with PMA and ionomycin, which bypasses the requirement for proximal signaling molecules such as SLP-76, resulted in similar levels of TNF-α production (Fig. 5D). Thus, Stat4 targets genes associated with TCR signaling and contributes to the potency of Ag receptor stimulation.

**Distinct patterns of Stat4-dependent gene induction among Stat4 target genes**

From the dataset of genes bound by Stat4, we wanted to determine whether there was a relationship between the intensity of Stat4 binding to a gene and the level of gene induction particularly because the overlap between our ChIP-on-chip targets and the public domain expressed gene lists was limited (Fig. 1E). To test this, we analyzed mRNA levels of over 30 genes, selected manually based on involvement in Th differentiation, immune responses or gene expression, in wild-type and Stat4<sup>−/−</sup> activated T cells stimulated for four or 18 h with IL-12. In Fig. 6A, an analysis of genes that demonstrated Stat4-dependent IL-12 induction indicated that there is no correlation between binding intensity and level of gene induction. This is exemplified by Ifnγrap, which was one of the highest intensity Stat4 binding genes, but was induced only 2-fold at 4 h, while Il18r1, which bound with less intensity, was induced 5-fold. The three genes that showed over 20-fold induction, Furin, Ifng, and Il24, had intensity ranging from 7.5 to 21. We also performed this analysis using an IL-12-induced microarray dataset (18), and observed a similar pattern when the published fold-induction was graphed against our binding intensity values (Fig. 6B).

To further characterize this subset of genes, we divided genes into categories based on the patterns of gene induction by IL-12. “Noninduced” genes had <2-fold induction at four or 18 h; “Transient” genes had >2-fold induction at 4 h but <2-fold induction at 18 h; and “Sustained” genes had >2-fold induction at 4 and 18 h. For each subset we graphed the fold IL-12-induced gene expression vs the basal gene expression normalized to the endogenous control β2-microglobulin. We observed a general trend that genes with lower basal expression had greater induction, and observed that those Stat4 target genes not induced by IL-12 were among the most highly expressed genes that we analyzed (Fig. 6C). This suggested that the noninduced genes did not lack induction because they were repressed in T cells, but rather that expression was high and induction by IL-12 might have a more limited effect. To determine whether IL-12 was having effects on the noninduced genes we compared histone modifications at the promoters of three sustained genes and four noninduced genes. We observed that IL-12 stimulation increased H3 acetylation and decreased H3K27 methylation at both sets of genes, but had significantly greater effects at the promoters of the induced genes (Fig. 6D). These data, coupled with the demonstration that Stat4 binds to the promoter of Irf1 (Fig. 2), one of the noninduced genes, suggests that they are bona fide Stat4 targets, but that the effects of IL-12 are limited at these promoters for reasons that may include high basal expression or lack of appropriate promoter context for induction.

Upon examining the genes that were in each group, we observed some interesting patterns (Fig. 6E). Within the noninduced set were Foxp3 and Gzmb, genes not expressed in Th1 cells, and Ms4adb, a Stat4-independent Th1 gene. The transiently induced set included receptors such as Il2ra and Cd40l, and transcription factors such as Irf4, Irf8, and Bcl6 where transient expression might confer temporary function to the cell. The sustained subset contained many of the genes that require Stat4 to program high expression in Th1 cells including Ifng, Furin, Il18r1, and Gadd45g. We analyzed two additional genes that Stat4 is known to program but were not found on the ChIP-on-chip list, Etv5 and Hils1, and found that they also were in this category. Thus, the induction kinetics of Stat4 target genes following IL-12 stimulation of activated T cells is a strong predictor of the requirement for Stat4 in programming expression for Th1 cells.

**Discussion**

Stat4 is a critical regulator of adaptive inflammatory responses, yet there is still little known as to how it performs these functions. In previous work, we have shown that Stat4 binds to specific target genes and mediates programming for high levels of expression in Th1 cells. It is still not well understood what genes are Stat4 targets on a genome-wide scale and how selectivity in activation is achieved. Previous microarray analyses have not provided significant insight into gene programs activated by Stat4.
In this report, we have used a ChIP-on-chip approach to identify STAT4 target genes and characterized STAT4 activity at these targets.

One of the important issues in examining a large dataset such as the one in this report is the level of false positives and false negatives identified. As we demonstrated in Fig. 3A, sequences from 60% of the genes in our curated list contained a consensus STAT binding site, and 100% were identified with a match to a motif similar to a STAT consensus. This suggests a low rate of false-positives in the list, which is further confirmed by showing that STAT4 binds to targets across a broad range of peak intensity values (Fig. 2). However, using the cut-off of peak value 4 for our curated list might omit some valid STAT4 targets. When divided into ranges of peak values, the percent of sequences that contain a STAT consensus decreases with decreasing peak value (Fig. 3B). In the peak value 4.0 – 4.9 set the percentage is 50%, but decreases further to 40% from 3.0 – 3.9 and 32% from 2.2 – 2.9, with an average of 43% for the entire list. Thus, there might be STAT4 targets below our cut-off. However, the large number of genes in the lower ranges (Fig. 1B) would greatly skew subsequent analyses. For example, while MEME identified a STAT-related site in 100% of the genes in our curated list with a cut-off above 4, the algorithm identified 10% of genes with a similar motif when a list with a cut-off of three was used, resulting from a large number of genes that lacked the site and decreased the enrichment potential for that motif. Regardless, this is a limitation in the interpretation of this and related datasets.

The Th1 genetic program involves many genes, which are either STAT4-dependent or STAT4-independent (9, 11). The 28 genes we considered functionally linked to the Th1 phenotype are shown in Table S3 and the STAT4 target genes in this dataset include cytokines (Ifng, Tnf, Lta), receptors (Il18r1, Il18rap, Il12rb2), and signaling factors (Gadd45b, Gadd45g, Myd88) (Fig. 7). STAT4 also bound Furin, which has critical roles in maintaining tolerance, a function that suggests STAT4 might have both pro- and anti-inflammatory activities (32). It is interesting that 6 of the 20 known STAT4-dependent Th1 genes (Table S3), including Hlx1 and Etv5, were not in our dataset. This likely represents either indirect activation of genes by STAT4, or STAT4 binding to target genes of other transcription factors. 

FIGURE 6. Patterns of STAT4-dependent gene induction. A, Wild-type and Stat4−/− CD4+ T cells were activated for 3 days and stimulated with IL-12 for 4 or 18 h before RNA was isolated for qPCR. Graphs are plot of STAT4 binding intensity vs fold IL-12-stimulated gene induction at 4 h for genes that showed STAT4-dependent gene induction. B, Graph of STAT4 binding intensity vs fold-IL-12 induction of the genes identified as overlap between the ChIP-on-chip data and published microarray data (18) using fold-induction values from the microarray data. C, Graph of gene expression relative to βm vs fold induction as determined in A. Genes that showed no induction or STAT4-dependent induction were divided into subsets based on the kinetics of expression (minimal induction, ≤ 2-fold induction at 4 or 18 h; transient, >2-fold induction at 4 h, ≤ 2-fold induction at 18 h; sustained induction, >2-fold induction at 4 and 18 h). D, ChIP assay for acetylated H3K9/18 or trimethylated H3K27 in purified CD4 T cells activated for three days and cultured with or without IL-12 for 4 h. qPCR for the promoters of induced genes (Furin, Ifng, IL18r1) and noninduced genes (Foxp3, Irf1, Mbd2, Ms4a4b) is expressed as the fold-increase in acetylation or the percent decrease in H3K27 methylation from unstimulated cells. * significantly different from induced genes (p < 0.05) as determined by Student’s t test. E, Expression pattern of genes examined in A at 4 and 18 h after stimulation with IL-12 divided based on criteria in C. A partial list of gene names in each subset is indicated to the right of the graph.
out of the promoter regions analyzed in this study, the latter highlighting the limitation of a promoter- vs a genome-wide ChIP-on-chip. Regardless, Etn5 and Hlx1 are clearly STAT4-dependent Th1 genes with induction kinetics similar to other STAT4-dependent genes identified in the array (Fig. 6) (9). Conversely, 2 of the 18 STAT4-bound Th1 genes (Fig. 4A) are not dependent on STAT4 for expression in Th1 cells, at least under standard in vitro differentiation conditions. The Th1 lineage-promoting factor T-bet is also partially dependent upon STAT4 (9), though Tbx21 was not identified in the STAT4 ChIP-on-chip dataset. As we recently demonstrated, many of the genes in the Th1 program are dependent on both STAT4 and T-bet, including at least 7 of the 18 STAT4-bound Th1 genes (Fig. 4A and Table S3). The T-bet-dependence of all the genes in the STAT4-bound set has not been determined but these preliminary data suggest that there is at least a 30% overlap in the target genes of these two factors.

Although STAT4 is clearly required for the establishment of the Th1 genetic program, how it mediates programming is still unclear. STAT4 binds to target loci including Iifng, Hlx1, and Il18r1, and mediates recruitment of Brg1-containing complexes, induces histone acetylation, and decreases histone methylation and the association of repressive enzymes such as DNMT3a and EZH2 (6, 8–10). All of these events contribute to increased gene transcription and programming. In this report we identified distinct sets of genes that are either transiently induced or have sustained induction. Moreover, there was a correlation between sustained gene induction and programmed expression in Th1 cells. We have previously analyzed an example of each of these gene categories; Il2ra is transiently induced, while Il18r1 is programmed for expression in Th1 cells (8, 23). At this point it is not clear whether there are specific chromatin modifications that distinguish transient from sustained induction. However, having established many of the STAT4-dependent events that occur during Th1 differentiation (9), and finding a set of genes, including Il2ra, that is only transiently induced, it will be possible to determine specific factors or histone modifications that distinguish the effects of STAT4 at various gene promoters.

One of the subsets of genes identified in this study was involved in TCR signaling. Earlier reports had suggested that TCR signaling might be compromised in Stat4−/− T cells, both from increased growth in vitro, and the ability of strong stimuli, like anti-CD3 plus anti-CD28 or PMA plus ionomycin, to minimize the difference in IFN-γ production between wild-type and Stat4−/− Th1 cells (30). Although we observed binding to a number of STAT4-bound TCR signaling genes in our dataset, we only identified one, Lcp2 encoding SLP-76, that showed dependence on STAT4 for expression in Th1 cells. We did not observe decreased expression of Lcp2 in Stat4−/− naive T cells or Th2 cells (data not shown). As SLP-76 plays a central role in TCR signaling (29), multiple pathways could be affected. We demonstrated that anti-CD3-induced ERK activation was diminished in Stat4−/− Th1 cells. However, activation of downstream signaling molecules using PMA and ionomycin bypasses proximal signaling molecules and minimized the differences in cytokine production between wild-type and Stat4−/− Th1 cells. Thus, Stat4−/− T cells not only have a defect in the acquisition of the Th1 phenotype, but also in T cell activation of cells differentiated in a Th1-promoting environment.

Several important features of STAT4 binding vs gene regulation were also realized in this study including the lack of correlation between the intensity of binding and the fold-induction of gene expression. Although one might expect that more binding would result in greater transactivation, there are several reasons that may explain a disconnect between binding and gene induction. First, the context of the promoter may dictate activity, demonstrating that even in the context of a consensus-binding site, binding is not the sole predictor of biological function. The focused time frame of our analysis might prevent observing induction of genes that require additional cofactors that were not present in IL-12-stimulated activated T cells. There may also be tissue-specific factors required for STAT4 function to regulate these genes in other cell types. Although no function for STAT4 in testis has been determined, we found 113 genes enriched in a testis microarray shared with the STAT4-bound dataset (Table S2C) that are unlikely to be induced in T cells. Second, the basal transcription of the gene may determine the sensitivity of the gene to induction. We demonstrated that of the genes we tested where IL-12 had minimal induction, all had high expression, compared with the control gene (Fig. 6C). Finally, STAT4 may bind to redundant consensus sites that mediate gene activation by other STAT proteins. For example, Irf1 was bound by STAT4 but was not induced by IL-12. In contrast, IFN-γ/STAT1 induce Irf1 in a number of cell types. Similar results were observed for T-bet targets, where T-bet was found to bind but not regulate gene targets (33).

The benefit of genome-scale experiments is the opportunity to identify clusters of genes involved in a biological process within the activated gene regulatory network that contribute to specific cellular functions or phenotypes. Apart from the Th1 cluster, we identified a TCR signaling gene (Lcp2) that STAT4 programs for increased Ag receptor sensitivity and an IL-18 response cluster (Il18r1, Il18rap, Gadd45b, Gadd45g, Myd88) (Fig. 7) that supports previous observations on the requirement for STAT4 in IL-18 sensitivity (28). A large number of IFN-induced genes were also identified (Fig. 7), though the genes examined were not induced to the same levels reported for IFN-γ stimulation in other cell types (34). Recently, two groups have analyzed high-throughput data from STAT1 ChIP (14, 35). Although these experiments were done in cultured cells (HeLa S3 or NIH3T3), and STAT1 has differing spatial and temporal specificities of expression from STAT4, the proportion of chromatin immunoprecipitated STAT1 target sequences that contain a match to the consensus-binding sequences was similar to our studies.

In this report, using a combination of in silico and in vivo techniques, we have identified STAT4 target genes in primary T cells.
Subsets of STAT4 targets mediate specific functions in Th1 cells. Analysis suggests that STAT4 target genes programmed for Th1 expression have distinct activation kinetics, suggesting that it may be possible, using bioinformatics analysis, to identify genes that play a role in inflammatory cell development and function.

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Disclosures

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References