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Global H4 acetylation analysis by ChIP-chip in SLE monocytes

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Abstract

Systemic lupus erythematosus is a polygenic disorder affecting approximately 1:1000 adults. Recent data have implicated interferons in the pathogenesis and the expression of many genes downstream of interferons are regulated at the level of histone modifications. We examined H4 acetylation and gene expression in monocytes from patients with systemic lupus erythematosus to define alterations to the epigenome. Monocytes from 14 controls and 24 SLE patients were used for analysis by chromatin immunoprecipitation for H4 acetylation and gene expression arrays. Primary monocytes treated with μ -interferon were used as a comparator. Data were analyzed for concordance of H4 acetylation and gene expression. Network analyses and transcription factor analyses were performed to identify potential pathways. H4 acetylation was significantly altered in monocytes from patients with systemic lupus erythematosus. Sixty three percent of genes with increased H4 acetylation had the potential for regulation by IRF1. IRF1 binding sites were also upstream of nearly all genes with both increased H4 acetylation and gene expression. μ -interferon was a significant contributor to both expression and H4 acetylation patterns but the greatest concordance was seen in the enrichment of certain transcription factor binding sites upstream of genes with increased H4 acetylation in SLE and genes with increased H4 acetylation after μ -interferon treatment.

Keywords

SLE; lupus; epigenetics; chromatin; interferon; IRF1

Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease affecting primarily young women. One of the most important recent advances in the understanding of the etiopathogenesis of SLE was the finding of increased expression of type I interferons

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and increased expression of genes downstream of type I interferons (1–3). The role of interferons has also been implicated by genome wide SNP array studies, which have identified functional polymorphisms in genes in the type I interferon pathway, that could contribute to susceptibility (4–6).

Monocytes were selected as a relatively homogeneous cell type which is easily obtainable and relevant to the SLE disease process. Monocytes contribute to the major cause of mortality in SLE patients, accelerated atherosclerosis, and the major cause of morbidity in SLE, renal disease (7–9). Monocyte dysfunction has been widely described in SLE and monocytes both produce and respond to type I interferons. Therefore, we focused our study on monocytes.

There is a growing appreciation of the role of epigenetics in human disease states. The epigenetic landscape of the cell sets the developmental program of the cell (10). By defining the set of genes which are competent for expression and the set of genes which are repressed, the character of the cell is determined largely by the epigenome (11). In the immune system, where cells are responding to environmental signals and redefining their responses as a consequence, the role of epigenetics appears to be particularly important. For example, Th1 and Th2 differentiation is largely determined by epigenetic changes to central genes (12) and the expression of proinflammatory genes is regulated at the level of chromatin, which determines the kinetics of the response to inflammatory stimuli (13, 14).

Developmentally regulated epigenetic changes can be associated with activating or repressive histone modifications, DNA methylation, or changes in three dimensional higher-order packaging of DNA (15–17). Collectively, the developmentally acquired epigenetic changes determine the set of transcribed genes and define the differentiation status of the cell. Histone modifications can also be transiently altered in response to acute stimuli, and these changes also regulate transcription. H4 acetylation (H4ac) specifically can be regulated developmentally, where it contributes to the differentiated character of the cell, and acutely, where it participates in the transcriptional regulation of inducible genes. In the latter case, the H4ac is transient, whereas developmental changes are durable.

We previously identified increased H4ac at the TNF α locus in patients with SLE (18). Data from other laboratories suggested a more global role for histone modifications and DNA methylation in SLE (19–24). Pathologic DNA demethylation in both primary SLE and drug-induced lupus appears to be a common feature (22, 23). In murine models and human cells, histone deacetylase inhibitors have led to disease-related changes *in vitro* and *in vivo*, supporting a role for functionally relevant histone modifications in the disease (19, 25). Acquired epigenetic changes could contribute to the chronicity of the disease.

In an effort to gain information on the epigenome in patients with SLE, we obtained primary monocytes from patients with SLE and controls. Expression arrays and chromatin immunoprecipitation arrays (ChIP arrays) for H4ac were performed. H4ac is a histone modification which is associated with competence for transcription (26–28). Our data demonstrate that SLE patient monocytes have an altered epigenome with footprints of type I interferon exposure but a significant effect from other stimuli as well. The importance of this

finding is that epigenetic changes can be stable in the cell and perpetuate dysfunctional responses.

Methods

Samples

IRB approval was given for this study and all participants consented to the study. In the initial analyses, 14 controls and 24 patients were recruited. Peripheral blood monocytes were obtained from patients and controls and purified by Ficoll-Paque followed by adherence. Monocyte preparations were 90–95% pure by CD14 staining. The mean lupus activity index (LAI) was 0.35 (range 0–1) and the mean systemic lupus erythematosus disease activity index (SLEDAI) score was 0.67 (range 0–3) on the date of the visit. The mean prednisone dose was 4.7mg/day. Sixty two percent were on hydroxychloroquine. All but one of the patients in the initial group was female. Controls were comparable in gender and age. No patient was on high level immune suppression for disease activity. For interferon treated cells, primary control monocytes were treated with 500U/ml of $\alpha 2a$ interferon for 18 hours (PBL Interferon Source, Piscataway, NJ). Three independent monocyte samples were divided into treated and mock-treated cultures.

Arrays

The H4ac immunoprecipitation was performed as previously described (29). Purified DNA from the immunoprecipitation was amplified, cleaved, and labeled using the GeneChip WT double stranded DNA terminal labeling kit (Affymetrix, Santa Clara, CA). DNA preparation and hybridization were all performed according the recommendations for the GeneChip Human Promoter 1.0R array (Affymetrix). The U133A 2.0 platform was used for the expression analyses. cRNA was prepared according to the recommendations of the manufacturer (Affymetrix).

Bioinformatics

The labeled DNA for the ChIP arrays was hybridized in two batches of mixed control and SLE samples. All .CEL files from the Human Promoter 1.0R arrays were processed by MAT (30), which generated a .BAR file from each array. The MAT-processed H4ac scores from more than 4.4 million probes were retrieved from .BAR files by CisGenome (31) and used as input for R (www.R-project.org) for statistical analyses. The probes on Human Genome U133A 2.0 arrays were re-mapped to the current version of the human genome by AffyProbeMiner (32) and re-grouped into probe sets. Additional information is available in the Supplemental files. Network analysis was performed with Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com).

Results

H4 acetylation in SLE monocytes is globally altered

We had previously identified increased H4ac at the TNF α locus in monocytes from patients with SLE (18), consistent with the overproduction of this proinflammatory cytokine. To define epigenetic changes across the genome, we performed ChIP array studies. ChIP data

on 34 samples (10 healthy controls and 24 SLE patients) and 11,321 genes, were analyzed initially. We identified a group of 179 genes with significantly enriched promoter H4ac in SLE patients compared to controls (FDR<0.01%) (Supplemental data 1). We confirmed the specificity of our array findings by a ChIP followed by quantitative Real Time PCR for four target genes (Figure 1). Network analysis of these 179 increased H4ac genes revealed 5 networks with scores of 24. The two lowest scoring networks had nodes of HNF4A (regulated by inflammation) and TGF β 1 (similarly regulated by inflammation). The three highest scoring networks had significant nodes of ERK, p38, NF κ B, CREB1, and α IFN (Figure 1). Ingenuity analysis allows networked genes to be categorized according to biological functions and scores the enrichment within the networked sets. When analyzed according to anticipated effect on biological function, macrophage activation (p=0.001), cell proliferation (p=0.002), CNS toxicity (due to CREB1) (p=0.004), and antiviral immunity (p=0.015) were significant.

We examined which transcription factor binding sites (TFBS) were over-represented in this gene group using WG rVISTA (33). This tool evaluates human-mouse conserved TFBS within the 5kb upstream region of each gene. Among the 179 increased H4ac gene set, 124 TFBSs were identified with p < 0.005, corresponding to 44.6% of the total TFBS tested (Supplemental data 2). Some of the identified TFs are known to be important in monocyte development and activation such as IRF1, RFX1, and BLIMP1. More specifically, 113 of the 179 increased H4ac genes have potential IRF1 binding sites within the 5kb upstream region.

Gene expression is altered in SLE monocytes

From the expression data of 28 samples (14 controls and 14 patients) and the same 11,321 genes, we identified 225 over-expressed genes in patients with a p value <0.01 (FDR=4.4%) (Supplemental data 3). This result is comparable to what others have seen in mixed peripheral blood mononuclear cell populations from SLE patients (1, 2). Twelve genes in this group are also included in the 179 genes with increased H4ac and 10 of them have potential IRF1 binding sites (Figure 2A,B). The 12 genes with coordinately increased H4ac and gene expression may represent acutely induced genes while the 167 genes with increased H4ac and stable gene expression could represent genes with have durably altered potential for expression. WG rVISTA identified 37 TFBSs over-represented upstream of the over-expressed genes (p<0.005) (Supplemental data 4). All but six of these TFBSs were also over-represented in the increased H4ac gene set, including IRF1 (Figure 2C). We reasoned that some of the implicated transcription factors could be over-expressed, leading to a feed forward loop. Of the 37 TFBS, cognate TF were over-expressed infrequently. Only three of the cognate TF were over-expressed. Among these were IRF1, LEF1/TCF1 and GATA3. Shared TFBS between decreased H4ac and decreased gene expression was not observed.

The spatial relationship of identified transcription factors and H4ac

Histone acetylation is associated with competence for transcription and histone acetyl transferases are recruited by many transcription factors. Therefore, we defined the spatial relationship of the peak of H4ac and the associated TFBS in 10 of the 12 genes with both increased H4ac and increased expression. Two genes were not included: FAM128A was not

recognized by WG rVISTA and CD7 has no potential TFBS. The 10 focus genes had 64 potential binding sites of three transcription factors, BLIMP1, IRF1, LEF1/TCF1 (Table 1). Visual inspection suggested the co-localization of these sites with H4ac peaks (Figure 3). We measured the distance of these TFBSs to the nearest H4ac peak. The average distance was reduced by approximately half (352 vs. 683 bases) when we compared the distance from the three TFBSs to the nearest H4ac peak with the average distance obtained by chance. The difference was statistically significant with $p = 0.008$.

Paired H4ac and gene expression studies

Histone modifications such as H4ac can be acquired developmentally in response to differentiation cues or can be acutely induced in parallel with gene expression. Biologically, this can reflect genes which are actively being expressed or genes which are competent for expression but have not yet acquired transcription factor binding. To further examine the relationship between altered gene expression and altered H4ac in patients with SLE, we utilized a subset of samples with paired H4ac and gene expression data available. This subset consisted of six female SLE patients on minimal medication (prednisone $<5\text{mg/day}$) and six female controls, with members of both groups between 30–50 years of age and with sufficient cells for paired CHIP and RNA studies. We examined the concordance of gene expression with H4ac. Gene sets corresponding to high H4ac with high gene expression (HH), high H4ac with low gene expression (HL), low H4ac with high gene expression (LH) and low H4ac with low gene expression (LL) were identified (Supplemental data 5A). The pattern of H4ac at most active promoters is a peak centered on the transcription start site (TSS). The pattern at some genes represented the inverse of this, with a nadir at the TSS (Supplemental data 5B). The nadir typically represents nucleosomes which have been remodeled off the promoter, i.e., a complete lack of histones. When H4ac was examined in gene sets filtered by expression, it became clear that the association of H4ac with expression extends to the region outside the classical peak (Supplemental data 5B). The expressed genes with low H4ac and a TSS nadir have higher surrounding H4ac than the unexpressed genes with low H4ac where the surrounding H4ac is also low.

We examined whether the four patterns of H4ac and gene expression could reflect different functional categories of genes. DAVID (34) was used to characterize different functional categories from the control set (Supplemental data 6). The HH group in controls was characterized by genes related to cellular metabolism and host defense. When the functional categories unique to patients were examined, a variety of diverse categories appeared to be upregulated. The two most significant p values were the categories that contain genes involved in splicing and ribosomal function. The third group relates to innate anti-viral defenses. As expected, The LL group was also diverse. Patients were minimally different than controls. The HL group potentially represents genes that are competent for expression as defined by H4ac but were quiescent at the time of sampling. The HL categories unique to patients included predominantly zinc finger transcription factors. These four categories potentially represent genes with different regulatory mechanisms. HH genes with high expression and high H4 acetylation could correspond to housekeeping genes or to inducibly expressed genes. An examination of the HH patient-specific gene list in supplemental data 6 suggests that both housekeeping genes and inducibly expressed genes are included in this

list. The HL group represents genes with increased potential for expression. In this regard, it is of interest that most genes in this category, which are unique to patients, are transcription factors.

To determine whether the changes seen in SLE patients could be associated with any specific pathways, we performed a network analysis using Ingenuity. One strong node linking multiple genes unique to the SLE HH group was α IFN. NF κ B, proteasome, and Akt nodes were also seen (Supplemental data 6). In the SLE HL group, a new node centered on p53 was identified.

The role of α IFN in SLE monocyte gene expression and H4ac changes

The role of type I interferons in SLE is well established but it is unlikely that one cytokine is responsible for all the pathologic effects. To examine the magnitude of the effect of α IFN in SLE, we compared gene expression and H4ac from SLE monocytes with that of three sets of monocytes treated *in vitro* with α IFN (Figure 4). α IFN led to a significant increase in 1.3% of all probes after H4ac ChIP. Among the 179 genes identified as having increased H4ac in SLE patients, the frequency of significantly increased probes was increased to about 2% in the α IFN treated cells. The difference was significant ($p=1.4e-5$) according to Wilcoxon rank sum test performed on the proportion of significant probes at the gene level. The enrichment among over-expressed genes was even more significant. The global geometric average of expression change caused by α IFN was about a 2% increase. Among the 225 genes with increased expression in SLE, α IFN led to 28% increased expression on average. The difference was also significant with $p=4.2e-4$.

We directly compared gene sets with increased H4ac and increased expression after α IFN treatment. Of the 199 genes with increased H4ac and 114 genes with increased expression, 21 were common to both groups (Figure 4A). When directly comparing increased H4ac gene sets from SLE monocytes and α IFN treated monocytes, there was modest overlap, however, the degree of overlap was substantial among over-expressed genes (Figure 4 B,C). Finally, we investigated the potential for common TFBS among gene sets. Most genes with increased H4ac in both SLE monocytes and α IFN treated monocytes shared TFBS (Figure 4D). This was even more substantial than the degree of overlapping TFBS among over-expressed gene sets (Figure 4E).

To compare the H4ac profile of genes induced by α IFN and SLE, we selected 12 well-defined interferon inducible genes. The maps of those genes are shown in Figure 5. While many individual peaks differ, the majority of the H4ac peaks are similar between the α IFN treated cells and the SLE cells, and the expression is increased in both conditions, suggesting a common mechanism. In particular, IFIT1, IFIT2, IFIT3, and IFI44 have H4ac peaks which nearly overlap in the SLE monocytes and the α IFN-treated monocytes. In contrast, STAT2 has divergent H4ac peaks and gene expression in SLE monocytes and α IFN-treated monocytes.

Discussion

The rationale for examination of the epigenome in SLE is that it could provide valuable insights into gene sets with an altered competence for expression as well as the more typically defined gene sets with altered transcript levels as defined by expression arrays. Our study is the first to examine the epigenome in SLE patients and we found that the epigenome of monocytes is widely altered by the disease. Although it is possible or even likely that medications could contribute to the altered epigenome, patients were selected with minimally active disease and limited medications.

Monocytes have been extensively studied in SLE patients and exhibit abnormal behaviors (35–40). Macrophage involvement in atheroma formation contributes to the accelerated atherosclerosis seen in SLE (7, 41, 42) and renal macrophage infiltration is associated with a poor renal prognosis (9, 43). The longevity of the tissue macrophage could contribute to disease persistence and macrophage cytokine production could mold subsequent T cell responses. It is not currently known whether epigenetic changes acquired as a circulating monocyte are retained after differentiation into tissue macrophages. Retention of acquired changes to the epigenome could potentially modulate subsequent responses.

In general, H4ac was increased in SLE patients compared to controls. In order to index the data to derive biological insights, we sought for commonalities amongst over-expressed genes and increased H4ac genes. More importantly, when we sought pathways by network analysis, α IFN, NF κ B and MAP kinases were consistently implicated. The proteasome network identified in the HH gene set is significant because proteasome components are known to be elevated in SLE and treatment of murine lupus models with a proteasome inhibitor was therapeutic (44, 45).

When we sought pathways by bioinformatically filtering genes by potential TFBS, three transcription factors were identified as being themselves upregulated in SLE and having binding sites over-represented upstream of both increased H4ac genes and over-expressed genes. These three transcription factors, GATA3, IRF1, and LEF1/TCF1 can be implicated in some of the features seen in SLE monocytes. Although the IRF1 binding site is identical to that of other IRF proteins, IRF1 was also over-expressed in SLE monocytes and was implicated in networking analyses. We further attempted to identify disease-specific pathways by filtering the information based on p value. Ten focus genes, identified as having both significantly increased expression and increased H4ac, had a very high enrichment of BLIMP1, IRF1, And LEF1/TCF1 binding sites in their upstream region and these binding sites colocalized with H4ac peaks. BLIMP1 is critical for macrophage differentiation and it represses c-myc, leading to cessation of cell division (46). LEF1/TCF1 is a transcriptional regulator of the Wnt/ β catenin pathway, which is important for macrophage proliferation and survival (47). IRF1 was the most consistently implicated transcription factor in the cumulative SLE analyses. IRF1 is induced by interferons and TNF α and it acts as a weak transcriptional activator of genes involved in the antiviral response, immune modulation, and anti-tumor effects (48). IRF1 gene deletion ameliorates renal disease in a murine lupus model, supporting a potential mechanistic role (49). IRF1 cannot directly acetylate H4, but it can bind CBP which can then acetylate histones (50).

IRF1 is strongly induced by EBV, which has also been linked to SLE (51). Therefore, these data provide a rationale for further investigation of IRF1 specifically and a potential explanation for the consistent finding of upregulated genes downstream of type I interferon while type I interferons themselves are inconsistently seen in patients.

SLE is clearly a complex disorder and there is likely heterogeneity in the pathologic processes. The consistency in identifying IRF1 was surprising. To further investigate the impact of the interferon pathway in SLE, we treated control monocytes with α IFN. This *in vitro* model does not mimic the milieu of the host, however, it allows a simplified analysis of one variable. α IFN treatment was able to reproduce some of the gene expression and H4ac changes seen in SLE. One gene, IFIT3, was identified in the increased H4ac gene lists of both SLE and α IFN treated cells and in the increased expression gene lists of both SLE and α IFN treated cells. IFIT3 is known to be induced by type I interferons and has been demonstrated to have increased expression in both SLE and Sjogrens syndrome (52). These data confirm an important role for α IFN, but also demonstrate the complexity of the effects on cells. α IFN effects accounted for a significant portion of the gene expression changes seen in SLE patients but a relatively small portion of the H4ac changes. This could reflect the short time frame for α IFN treatment or the limitations of an *in vitro* system, however, it is likely that multiple stimuli converge on the monocyte to induce the disease-specific effects.

An important aspect of these analyses is the ability to leverage four types of data to improve the focus. In SLE, a straight comparison of genes with increased H4ac and increased expression revealed little commonality. Applying TFBS analysis revealed much more commonality. This was also true when α IFN treated cells were analyzed. The greatest enrichment of all analyses was found by analyzing TFBS overlap between increased H4ac in SLE monocytes and α IFN treated monocytes demonstrating that epigenetic studies can be powerful in identifying biological themes.

This study characterized a single histone modification using an array restricted to the promoters of annotated genes. H4ac at enhancers or other distant regulatory regions would not be identified by our study. Therefore, this study represents a minimal estimate of the full extent of the epigenetic changes seen in SLE monocytes. The importance of examining histone modifications in SLE is that they can serve to perpetuate pathologic gene expression patterns. In many cases, they are concordant with gene expression, but they can also reflect competence for expression and in so doing mold the character of a cell's behavior. These analyses confirm the importance of type I interferons and suggest that they may have a sustained effect by inducing transcription factors and by altering the epigenome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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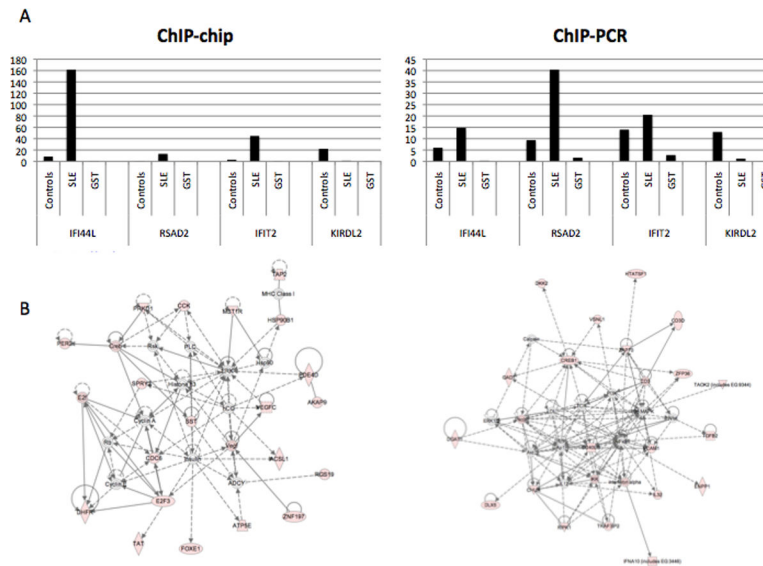


Figure 1. Analyses of genes with increased H4ac

A) H4ac normalized signals for the ChIP array data are expressed for comparison with the H4ac Real Time PCR signals. Three genes identified as having increased H4ac in SLE and one gene with decreased H4ac in SLE were examined. The graphs indicate the average of all samples included in this study. The pattern of the signals is identical. This confirms the validity of the ChIP array detection system in our study. B) Two of the significant networks are shown demonstrating nodes of α IFN, NF κ B, and ERK.

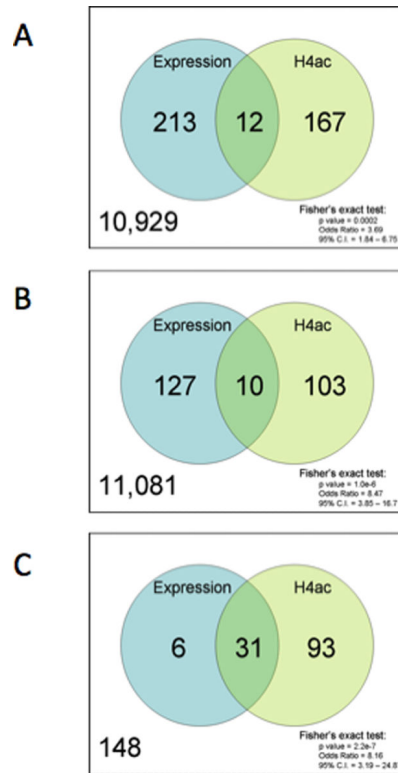


Figure 2. Concordance of increased expression and increased H4ac in SLE

A) Of the 179 genes with increased H4ac and the 225 genes with increased expression, 12 are shared. B) Of the 113 genes with increased H4ac and potential IRF1 binding sites and the 137 genes with increased expression and potential IRF1 binding sites, 10 are shared. C) The increased H4ac genes are enriched for 124 different TFBS. The increased expression gene set is enriched for 37 different TFBS. 31 of those potential transcription factors are shared.

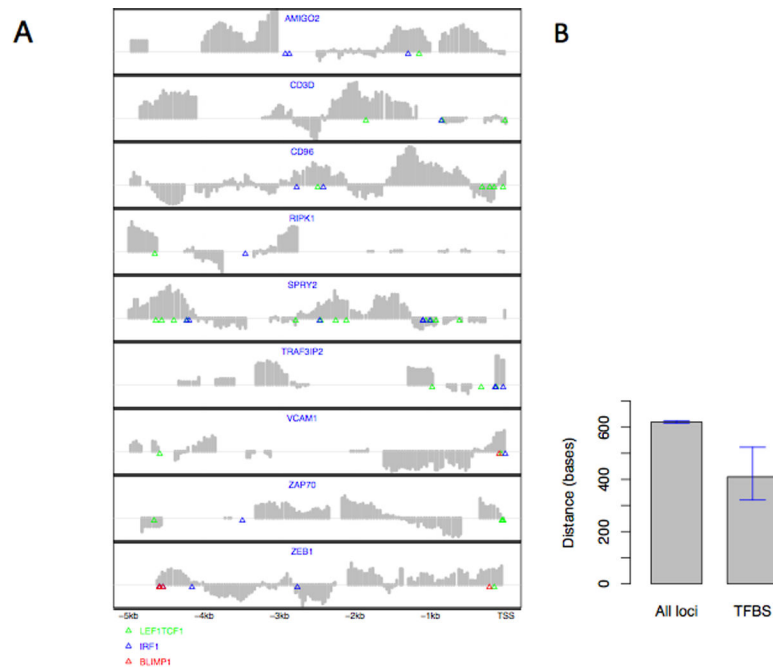


Figure 3. Colocalization of TFBS and H4ac

A) The y-axis indicates the overall H4ac change in patients over controls (scale: -0.75 to $+1.0$). The 5kb upstream region of 10 focus genes includes 64 potential binding sites of 3 transcription factors. B). The first bar indicates the (geometric) average distance of all bases within the 5kb upstream region of 10 focus genes to the nearest H4ac up-regulated site and its standard error. The second bar indicates the same parameters of the 64 potential TFBS.

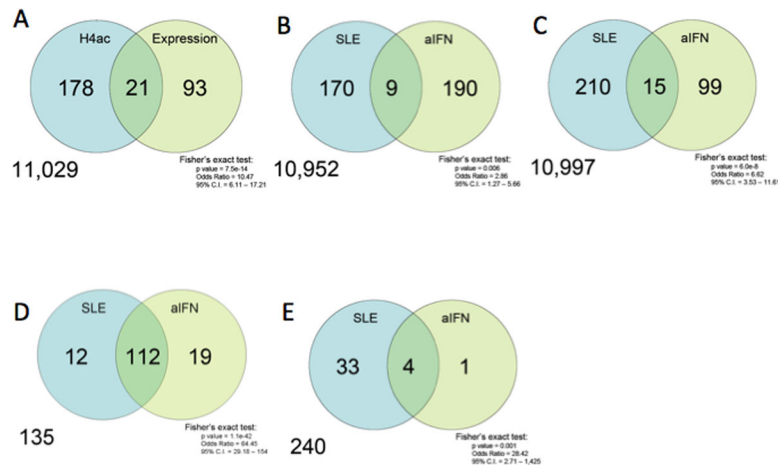


Figure 4. Concordance of α IFN treated cells and SLE monocytes

A) In the monocytes treated with α IFN, genes with increased H4ac and increased expression had minimal but significant overlap. B) Of the 179 genes identified as having increased H4ac in SLE and the 199 genes identified as having increased H4ac in α IFN treated monocytes, there were nine genes in common. C) Of the 225 genes identified as having increased expression in SLE and the 114 genes identified as having increased expression in α IFN treated monocytes, there were 15 genes in common. D) Of the 124 potential TFBS identified in the increased H4ac gene set in SLE patients and the 131 potential TFBS identified in the increased H4ac gene set from α IFN treated monocyte, there were 112 potential TFBS which were in common. E) Of the 37 potential TFBS identified upstream of the genes with increased expression in SLE and the 5 potential TFBS identified upstream of the genes with increased expression in the α IFN treated monocytes, 4 were common to both sets.

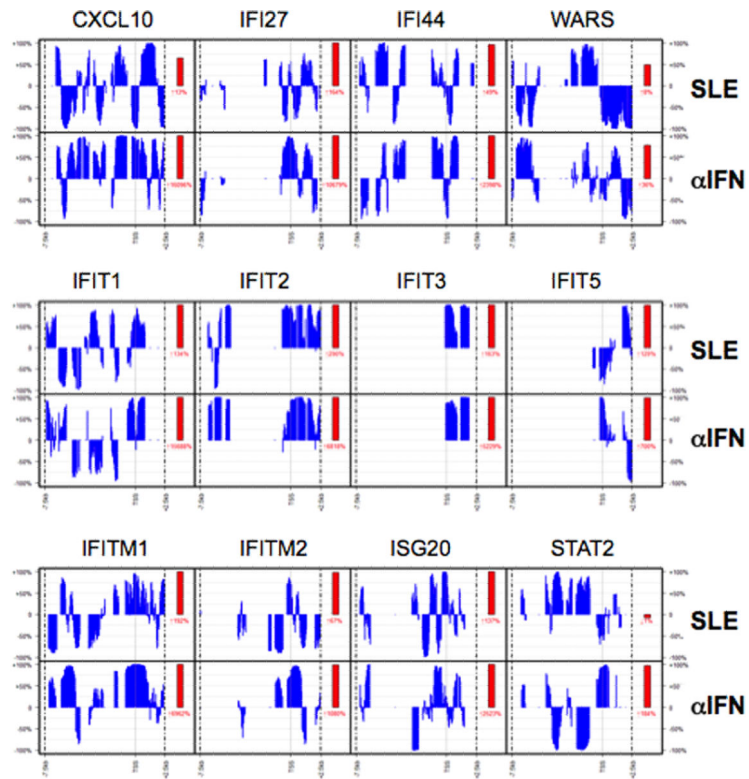


Figure 5. Comparison of H4ac patterns in SLE monocytes and α IFN treated monocytes
 These representative genes demonstrate both the diversity and the consistency of effects. The blue peaks indicate the change in H4ac and the red bar represents the change in gene expression over control samples. To show the variables on the same scale, all values were transformed to percentiles relative to global change, calculated separately for up- and down-regulated genes and H4ac and expression data.

Table 1

The 12 focus genes with increased expression and H4ac in SLE are significantly enriched for IRF1, BLIMP1, and LEF1/TCF1 binding sites

Symbol	H4ac data			Expression data			Binding sites				
	Total_probes	Up_probes	p_value (binomial test)	Average change (%)	p_value (ANOVA)	IRF1	BLIMP1	LEF1+TCF1			
AMIGO2	221	16	3.14E-07	76.42	1.54E-04	3	0	1			
CD3D	195	18	1.33E-09	166.11	1.07E-03	1	0	3			
CD7	250	24	1.13E-12	71.44	1.33E-04	0	0	0			
CD96	244	23	4.81E-12	68.94	2.38E-03	2	0	5			
FAM128A	165	18	8.66E-11	54.68	6.65E-06	N/A	N/A	N/A			
IFIT3	50	15	5.86E-16	162.72	1.03E-04	4	0	0			
RIPK1	160	17	4.32E-10	59.13	7.97E-06	1	0	1			
SPRY2	223	17	6.48E-08	124.22	1.22E-04	6	0	12			
TRAF3IP2	150	15	1.04E-08	62.83	6.34E-05	3	0	3			
VCAM1	169	15	5.16E-08	63.86	3.92E-03	1	1	2			
ZAP70	191	18	9.55E-10	75.87	2.55E-03	1	0	4			
ZEB1	196	20	2.73E-11	92.69	2.05E-04	5	4	1			
IRF1	234	8	2.59E-02	50.18	4.96E-04						