Research Article
Cytokine-Induced Monocyte Characteristics in SLE

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Received 15 January 2010; Accepted 14 May 2010

Academic Editor: George C. Tsokos

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Monocytes in SLE have been described as having aberrant behavior in a number of assays. We examined gene expression and used a genome-wide approach to study the posttranslational histone mark, H4 acetylation, to examine epigenetic changes in SLE monocytes. We compared SLE monocyte gene expression and H4 acetylation with three types of cytokine-treated monocytes to understand which cytokine effects predominated in SLE monocytes. We found that γ-interferon and α-interferon both replicated a broad range of the gene expression changes seen in SLE monocytes. H4 acetylation in SLE monocytes was overall higher than in controls and there was less correlation of H4ac with cytokine-treated cells than when gene expression was compared. A set of chemokine genes had downregulated expression and H4ac. Therefore, there are significant clusters of aberrantly expressed genes in SLE which are strongly associated with altered H4ac, suggesting that these cells have experienced durable changes to their epigenome.

1. Introduction

Systemic lupus erythematosus (SLE) has long been characterized as a disease associated with the overexpression of proinflammatory cytokines. The early studies focused on proinflammatory genes and identified increased serum levels of TNFα, IL-6, γIFN, and IL-10 [1–6]. When cells have been examined directly, either by flow cytometry or culture techniques, γIFN, IL-10, IL-17, and IL-6 have been found to be increased in SLE patients [7–12]. In contrast, studies have generally supported an underexpression of IL-12, which is classically considered a proinflammatory cytokine [13, 14]. More recently, studies have identified a signature of type I interferons in whole blood using gene expression arrays [15–19]. This has generated a sea change in the etiologic modeling of the disease and has led to a clinical trial using a neutralizing antibody [20].

Other studies have sought to identify altered expression of chemokines in SLE and these studies have demonstrated increased RANTES, and MCP-1 [21, 22]. Indeed, urine chemokine detection shows promise as an early biomarker of nephritis [23, 24]. Chemokines are often secondary regulators of cell migration, induced by cues from other cells.

This study defined gene expression alterations in monocytes from patients with SLE and correlated those changes with cytokine-induced gene expression changes. We further evaluated H4 acetylation (H4ac) changes, as H4ac is an epigenetic mark of transcriptional potential [25, 26]. Monocytes were selected for study because monocytes and their tissue counterpart, the macrophage, play an extremely important role in systemic lupus erythematosus (SLE). Macrophage infiltration into end organs is thought to be critical to the disease process and renal infiltration of macrophages is specifically associated with a poor prognosis [27]. Monocyte infiltration into glomeruli is driven by fractalkine and both fractalkine levels and monocyte numbers in glomeruli correlate with BUN, proteinuria, and GFR [28]. Additionally, increased macrophage migration inhibitory factor levels correlate with disease activity in lupus patients [29]. Additional well-characterized roles for monocytes in SLE include the clearance of apoptotic cells, participation in atheroma formation, and the elaboration of inflammatory cytokines [30–37].

Monocytes have long been recognized as exhibiting aberrant behavior in patients with systemic lupus erythematosus
CD16 compared to the αSLE monocytes have statistically significant differences in many markers. The cells were gated on physical parameters and CD14 expression. The SLE monocytes have statistically significant different expression of CD16 compared to the αIFN-treated cells.

An example of Figure 1: Cell surface markers are altered in SLE and cytokine-treated monocytes. Control monocytes were either mock treated or treated with the indicated cytokines for 18 hours (n = 3). The SLE cells were studied without any intervention (n = 4). In each case, the cells were gated on physical parameters and CD14 expression. The SLE monocytes have consistently significant different expression of CD16 compared to the αIFN-treated cells.

Patients with SLE have mildly altered monocytes as defined by cell surface markers [54, 55], however, cytokine expression is clearly aberrant [56, 57]. SLE monocytes are generally reported to overproduce IL-1RA [58], IL-6 [59, 60], and TNFa in vitro [55, 61], while IL-12 production in both humans and mice is diminished [62–64]. To better define disease effects on SLE monocytes, we examined gene expression changes in purified SLE monocytes and compared those to gene expression changes in control monocytes treated with different cytokines. To determine whether any of the gene expression changes could be mediated by altered chromatin, we examined H4ac, as a mark of transcriptional competence [65–67].

2. Methods

2.1. Cells and Reagents. The SLE monocyte gene expression and ChIP-chip data have been previously reported [68]. In both cases, healthy control donors were used to establish the baseline. The samples studied here are five controls and 9 patients. The patients had a very low SLEDAI score (mean score 0.6) and were on no immune suppressive medications at the time other than low dose prednisone. The cytokine-treated monocytes utilized the cells from a single donor for each set of cytokine treatments. The cytokine data were reported initially in a separate study (submitted). The data represent the averages of three different donors. The cells were purified by elutriation and were ≥95% pure by CD14 staining. The cells were treated with 50 ng/ml of IL-4, 50 ng/ml of γIFN (R&D Systems, Minneapolis, MN), or 500 IU/ml of αIFN (PBL Interferon Source, Piscataway, NJ) for 18 hours. Flow cytometry for cell surface markers utilized antibodies from BD Pharmingen and were run on a FacsCalibur instrument using appropriate isotype controls.

2.2. Microarray Experiments. The H4ac immunoprecipitation was performed as previously described [69, 70]. 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 to 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). The expression experiments included nine SLE patients, five healthy controls, and three samples of each cytokine treatment group. The H4ac experiments included four nonspecific GST controls, six patients, five healthy controls, and three samples of each treatment group. The data processing has been previously described for the coanalysis of expression and H4 acetylation data [68]. Additional information about the data processing and statistical methods is available in the Supplemental Methods.

3. Results

3.1. SLE Monocytes Exhibit Cell Surface Marker Expression Which Cannot Be Attributed to Single Cytokine Effect. To understand the biology of monocytes from SLE patients, we examined cell surface markers by flow cytometry. We selected a variety of cell surface markers which have been implicated in monocyte function. The cells were gated on physical parameters and CD14. CD16, CD23, CD32, CD64, CD80, CD197, CD206, and CCR2 expression levels were compared between SLE monocytes and control monocytes treated for 18 hours with cytokines. Cell surface markers significantly altered in SLE are shown in Figure 1. γIFN treatment was associated with increased expression of FcγRI (CD64) and CCR7 (CD197). IL-4 treatment was associated with increased expression of FcγRII (CD23) and the macrophage mannose receptor (CD206). The monocytes polarized with αIFN displayed a unique phenotype with increased CD64. CD80 expression was not significantly altered by any treatment. The SLE monocytes expressed cell surface markers are somewhat consistent with the monocytes treated for
18 hours with αIFN, but with a clear difference in CD16 expression. Although in vitro treatment with cytokines does not perfectly replicate chronic in vivo exposure, these data suggested that the phenotype could not be attributed to a single cytokine exposure. We wished to examine whether cytokines could be molding the phenotype of SLE monocytes more globally. With multiple reports of elevated cytokines in addition to type I interferons, we hypothesized that we would find footprints of other cytokine effects within the monocyte population.

3.2. Overlap between Cytokine-Induced Gene Expression and the SLE Gene Expression. We performed pairwise comparisons of gene expression between SLE monocytes or cytokine-treated monocytes and healthy or untreated control samples. We filtered the genes with $P$ values ($t$ test) <.05 and selected the top 200 genes with the highest or lowest log2 ratio of group means for further study. According to a permutation procedure, the false discovery rates of genes up- and down-regulated in SLE were 3.1% and 6.9%, respectively. To gain insights into the biological alterations as a result of these gene expression changes, we further performed Gene Ontology (GO) analysis through DAVID functional annotation [71]. Table 1 summarizes the top five nonredundant GO terms enriched in SLE or cytokine upregulated genes. The αIFN and γIFN GO terms were similar and partly overlapped the SLE terms, indicating that the SLE monocytes underwent some gene expression changes similar to the effect of interferon treatment. On the other hand, IL-4 terms had little similarity to SLE and interferon terms. This as expected because IL-4 has not been implicated in SLE and was included as a control.

As interferons and IL-4 caused distinctive gene expression changes in monocytes, we carried out a gene clustering analysis on the cytokine data to identify a cluster of 187 genes that responded to both interferons, but not IL-4 (Figure 2(a)). In this cluster, DAVID analysis confirmed the enrichment of genes involved in immune system processes as sources for gene expression arrays [72]. The raw data from the study were downloaded from the GEO database (GSE10325) and processed with the same procedure used in this study. The top 200 upregulated genes were identified with the same criteria from each cell type and ten of them were included in the lists of all cell types examined, including our SLE monocytes. These ten genes were also highly induced by αIFN with a smaller effect seen in the γIFN-treated cells, but slightly down-regulated by IL-4 (Figure 2(b)). This analysis ensured that although our sample population had very low disease activity, the findings were generalizable.

3.3. H4 Acetylation in SLE. We have previously reported that H4ac was altered in SLE monocytes [68, 73]. We therefore reanalyzed the data to understand the role of the different cytokines in the altered H4ac landscape of the cells. The
H4ac mark was mapped using tiling arrays and all genes were aligned by their transcription start site (TSS). The H4ac content exhibits a forked peak pattern, centered on the TSS (Figure 3(a)) [66, 74]. When the SLE H4ac signal across the genome was compared to controls, SLE genes overall has a higher signal. Much of the difference between the patients and controls lies around the transcription start site, with relative hypoacetylation distant from the TSS (Figure 3(b)). H4 acetylation marks are typically placed by histone acetyltransferases recruited by transcription factors. We previously reported an increase of H4ac at potential binding sites of transcription factors like IRF1 and the expression change of IRF1-downstream targets [68]. We generalized our analysis in this study. All potential TFBSs in human genome conserved in human/mouse/rat alignment were downloaded from UCSC Genome Browser and mapped to the TSSs. The average H4ac at binding sites around TSSs had a distinctive pattern (Figure 3(c)). TFBSs located in the upstream promoter region had higher H4ac than average while those located immediately downstream of TSS had lower H4ac content. In SLE monocytes, the H4ac change at TFBSs had a similar pattern (Figure 3(d)). These observations suggest that H4ac at TFBSs is part of an expression regulatory network.

We considered whether increased competence for expression of the histone genes themselves could contribute to this picture. It may be seen that the H4ac at the H2, H3, and H4 gene clusters was generally increased in SLE monocytes (Figure 4). This could contribute to globally increased H4ac in the SLE monocytes but cannot be the complete explanation because the acetyl mark is placed posttranscriptionally. To understand whether histone acetyltransferases might have dysregulated expression as a mechanism to explain the globally increased H4ac, we examined the expression of the human histone acetyltransferases in SLE patients. The expression of these genes was not globally increased although several individual members were upregulated including HAT1 (log2 ratio = 0.79), KAT2B (log2 ratio = 0.77), MYST3 (log2 ratio = 0.74), and MYST4 (log2 ratio = 0.53). We examined whether this effect was replicated in any of the
cytokine-treated monocytes. Only KAT2B was upregulated in γIFN and αIFN-treated cells, which is consistent with the observation that H4ac is globally elevated in SLE monocytes but not in cytokine-treated cells.

To examine the biological processes anticipated to be altered as a result of the SLE H4ac landscape, we utilized DAVID for the GO analysis of the 200 genes with the highest H4ac due to cytokine treatment or SLE (Table 2). This strategy collapses the H4ac data into functional categories. In this analysis, the finding of increased H4ac in SLE was seen in processes related to basic cell biology, including basic metabolic processes. Besides the GO terms listed in

### Table 1: DAVID analysis of SLE gene expression.

<table>
<thead>
<tr>
<th>Gene List</th>
<th>GO Term</th>
<th>Count</th>
<th>P value</th>
<th>Fold Enriched</th>
</tr>
</thead>
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<td>SLE UP</td>
<td>Immune system process</td>
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<td>6.46E-15</td>
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<td>Leukocyte activation</td>
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<td></td>
<td>Cytokine receptor activity</td>
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<td></td>
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<td></td>
<td>Defense response</td>
<td>15</td>
<td>2.16E-02</td>
<td>1.94</td>
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<tr>
<td>AIFN UP</td>
<td>Immune system process</td>
<td>57</td>
<td>2.34E-23</td>
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<td></td>
<td>Response to virus</td>
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<td>4.02E-22</td>
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<tr>
<td></td>
<td>Defense response</td>
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<td></td>
<td>Induction of apoptosis</td>
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<td>5.04E-03</td>
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<td></td>
<td>Interferon type I production</td>
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<td>7.90E-03</td>
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</tr>
<tr>
<td></td>
<td>Lymphocyte proliferation</td>
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<td>6.09E-04</td>
<td>6.61</td>
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<tr>
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<td></td>
<td>Ribosome biogenesis and assembly</td>
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<td>3.25E-04</td>
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<td></td>
<td>Cell communication</td>
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<td></td>
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<td>4.13E-03</td>
<td>5.60</td>
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</table>

### Table 2: DAVID analysis of H4ac gene sets.

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<th>Gene List</th>
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<th>Count</th>
<th>P value</th>
<th>Fold Enriched</th>
</tr>
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<td></td>
<td>Nucleic acid binding</td>
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<td>Metabolic process</td>
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<td></td>
<td>Chromatin assembly</td>
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<td>4.56E-03</td>
<td>5.44</td>
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<tr>
<td></td>
<td>Ribosome biogenesis and assembly</td>
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<td>9.61E-03</td>
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</tr>
<tr>
<td>AIFN UP</td>
<td>Response to virus</td>
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<td>1.10E-08</td>
<td>9.16</td>
</tr>
<tr>
<td></td>
<td>Immune system process</td>
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<td>3.27E-08</td>
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<td></td>
<td>Structural constituent of ribosome</td>
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<td>Defense response</td>
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<td>2.03</td>
</tr>
<tr>
<td></td>
<td>Interferon type I production</td>
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<td>1.20E-02</td>
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<td>Late endosome</td>
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<td>Immune system process</td>
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<td></td>
<td>Golgi vesicle transport</td>
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<td>2.90E-02</td>
<td>3.45</td>
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</table>
Table 2, DAVID also found increased H4ac of the Kruppel-associated box (KRAB) family of transcriptional repressors in SLE monocytes \((P = 8.1e^{-9})\). These typically function in hematopoietic cell differentiation \([75]\). When DAVID was used to examine the inferred biological processes altered in SLE compared to those in yIFN and aIFN-treated cells, there was little overlap, suggesting that the SLE monocytes are more fundamentally altered than can be explained by brief cytokine exposure.

3.4. Coanalysis of H4ac and Gene Expression. To understand whether cytokines could alter the chromatin landscape of monocytes and to correlate the H4ac changes seen in SLE monocytes with those induced by cytokines, we examined the agreement between H4ac and expression data of 9553 unique genes measured by both array platforms and the results are summarized in Table 3. The first part of Table 3 (with GST controls) compares the absolute measurements of expression and H4ac in six sample groups, after the GST control was used to remove nonspecific array signals from the H4ac data. The global correlation between expression and H4ac was consistently around 0.4 in all groups. We picked the 200 top and bottom genes from each group based on their expression and H4ac level and checked the overlap between the two lists. The numbers of overlapped genes and the odds ratios calculated by Fisher’s test are also listed in Table 3. The genes with high expression had relatively less overlap with genes with high H4ac, compared to the down-regulated pair, suggesting that high H4ac is not sufficient to ensure high gene expression. The overlapping at the lower end was much more prominent. The second part of Table 3 (the last four lines) presents the same results based on the relative change of expression and H4ac within each experimental group. The relative change in expression was calculated from the reference for each group, given in the second column. The global expression-H4ac correlation was notably lower as most genes were not affected by SLE or cytokine treatment. The overlap between genes with increased expression and H4ac was more significant, especially genes responding to interferons.

We identified two clusters of genes with an unusually high correlation of H4ac and gene expression. A cluster of chemokine genes that map to chromosome 4 had markedly repressed expression in SLE and this was associated with a markedly diminished H4ac. Interestingly, these genes were repressed by all three cytokines. These chemokines regulate neutrophil function, monocyte migration, and angiogenesis \([76–80]\). The interferon-responsive genes had a strong association of H4ac and gene expression in SLE and this was replicated in part by exposure to both types of interferon (Figure 5).

To more directly link gene expression change to histone modification, we summarized the average H4ac change of the genes identified above as differentially expressed in SLE or after cytokine treatment (Figure 6). On average, genes with upregulated expression in SLE had a dramatic increase in H4ac. However, as the H4ac was generally increased in SLE, the H4ac around the TSS of down-regulated genes was also increased slightly although H4ac was notably decreased in upstream promoter region. Genes up- or down-regulated by cytokines had a more consistent H4ac change. An interesting observation in Figure 6 is that the genes upregulated in SLE seemed to have lower baseline H4ac by average, suggesting that those genes may be repressed in healthy monocytes.

4. Discussion

The concept of an altered epigenome in SLE is an attractive model because the epigenome could contribute to the disease perpetuation by molding pathologic gene expression. One of the best-characterized epigenetic changes in SLE is the hypomethylation of DNA in T cells \([81]\). This finding is consistent in murine models \([82]\) and more recent studies have linked demethylation of DNA with the drug-induced lupus seen with procainamide and hydralazine \([83, 84]\). Induced demethylation alters the expression of a number of genes, which could contribute to the pathophysiology of lupus \([85–87]\).

Several groups have utilized histone deacetylase (HDAC) inhibitors in lupus models in an effort to reregulate aberrant gene expression. Trichostatin (TSA) or the chemically related compound, SAHA, was used to treat MRL/lpr mice \([88]\). These agents increase H4ac through inhibition of histone deacetylases. This murine model of SLE is characterized by increased expression of yIFN, IL-12, IL-6, and IL-10. \textit{In vitro} and \textit{in vivo} treatment with an HDAC inhibitor decreased RNA and protein levels for all four overexpressed cytokines. In addition, administration of TSA led to less active renal disease \([88]\). HDAC inhibitors are immunosuppressive \textit{in vivo} and therefore to better understand the epigenome in SLE, we directly characterized the epigenome in SLE \([89]\). Our previous study of H4ac in SLE monocytes found that many of the changes could be due to overexpression of the transcription factor IRF1 \([68]\). In the current study,
we directly examined whether gene expression and H4ac alterations in SLE monocytes could be attributed to cytokine exposure. The SLE literature is replete with studies demonstrating overexpression of a broad range of cytokines, not just type I interferons. The finding of specific features attributable to cytokine exposure would have a significant impact on the conceptualization of new treatments. A caveat of the system we used is that the cytokine-treated cells used as comparators represent an artificial in vitro system that clearly cannot replicate the complex chronic exposures seen in a disease state. Nevertheless, this study demonstrates that such attribution is possible and can be used to examine both the epigenetic changes in SLE as well as gene expression.

An underlying hypothesis is that monocytes “polarized” by the disease can contribute to ongoing inflammation or may mold the end organ involvement. Supporting this is the finding that in murine models of SLE as well as human disease, macrophage infiltration into the kidney correlates with the severity of the renal disease and the prognosis [27]. Aberrant regulation of chemokines, as demonstrated in this study, would be predicted to alter migration and potentially alter disease manifestations. If some of these alterations can
be traced to a specific signaling pathway, a novel therapeutic target would be identified.

This study examined a specific cell type in which dysfunction has been well characterized in humans. Murine lupus models also exhibit aberrant monocyte function, suggesting it is a consistent feature of the disease. Both monocyte uptake of apoptotic cells and DNA are abnormal [90–92]. Indeed, monocyte apoptosis itself may contribute to the disease process [93]. Many SLE murine models exhibit a monocytosis, and the monocytes may amplify the inflammatory process [94–97]. In MRL/lpr mice, macrophage expression of γIFN is required for the expression of the renal disease [98]. Additional studies demonstrated that engagement of TLR7 aggravated renal disease, characterized by infiltration of monocytes [98, 99]. In fact, inhibition of macrophage recruitment into the kidney, markedly attenuated the phenotype in MRL/lpr mice [100]. In the NZB × NZW system, signaling in myeloid cells through FcγR is critical to the inflammatory process and macrophages are critical for anti-dsDNA production.

Figure 6: The change in gene expression in SLE or cytokine-treated samples was matched by H4ac changes in the same samples. (a) Upregulated genes seen in SLE or after cytokine treatment were examined for H4ac content. The top 200 genes with the highest expression change caused by SLE or cytokines, as was identified by previous analysis were included. (b) Down-regulated genes seen in SLE or after cytokine treatment were examined for H4ac content. The 200 genes most down-regulated were examined. The black contour indicates the average H4ac in control samples and the colored regions correspond to the amount of H4ac increase (red) or decrease (green) in SLE or cytokine-treated samples.

Table 3: Concordance between gene expression and H4ac content.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 0</th>
<th>Correlation (r)</th>
<th>Num_gene (up)</th>
<th>Top 200 overlap*</th>
<th>Num_gene (down)</th>
<th>Odds Ratio (up)</th>
<th>Odds Ratio (down)</th>
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<td>Healthy</td>
<td>GST</td>
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<td>13</td>
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<td>19.76</td>
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<tr>
<td>GIFN</td>
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<td>18</td>
<td>4.98</td>
<td>23</td>
<td>6.73</td>
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were many other effects that could underlie many of the aberrant functions. There are many other effects that did not trace to the three cytokines used in this study. Potential caveats include the short exposure to cytokines in our model system, the effects of other cytokines or stimuli and the potential for the cells to have an aberrant differentiation pathway in the presence of active SLE.

This study hypothesized that many of the changes in both gene expression and H4ac would be attributable to type I interferons since their effects have been well characterized in SLE [16, 51, 105]. Indeed, many effects in gene expression and H4ac could be traced to IFNα, although γIFN led to similar changes. There are several lines of evidence suggesting that monocytes have been molded by a complex set of exposures. Our cytokine attribution found that the interferon-responsive genes cluster was upregulated 36.3% in SLE monocytes, thus leaving a significant gene set unexplained by interferon exposure. The association was even less robust for H4ac. The finding that H4 acetylation was globally increased and this increase appeared to map to TFBSs suggests a globally altered epigenome with a complex etiology. Therefore, monocytes are significantly impacted by both αIFN and γIFN exposure, however, our data suggest that additional cytokines and other exposures contribute to the aberrant monocyte behavior observed in SLE patients.

Acknowledgment

This work was supported in part by NIH R01 AI 0511323 and R01 ES 017627. The authors would like to thank Dr. Micheal Petzl.

References

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