Quantitative Proteomics Reveals a Role for Epigenetic Reprogramming During Human Monocyte Differentiation*§

Dequina Nicholas‡, Hui Tang§, Qiongyi Zhang¶, Jai Rudra§, Feng Xu¶, William Langridge‡, and Kangling Zhang‡§

The differentiation of monocytes into macrophages and dendritic cells involves mechanisms for activation of the innate immune system in response to inflammatory stimuli, such as pathogen infection and environmental cues. Epigenetic reprogramming is thought to play an important role during monocyte differentiation. Complementary to cell surface markers, the characterization of monocytic cell lineages by mass spectrometry based protein/histone expression profiling opens a new avenue for studying immune cell differentiation. Here, we report the application of mass spectrometry and bioinformatics to identify changes in human monocytes during their differentiation into macrophages and dendritic cells. Our data show that linker histone H1 proteins are significantly down-regulated during monocyte differentiation. Although highly enriched H3K9-methyl/S10-phos/K14-acetyl tri-modification forms of histone H3 were identified in monocytes and macrophages, they were dramatically reduced in dendritic cells. In contrast, histone H4 K16 acetylation was found to be markedly higher in dendritic cells than in monocytes and macrophages. We also found that global hyperacetylation generated by the nonspecific histone deacetylase HDAC inhibitor Apicidin induces monocyte differentiation. Together, our data suggest that specific regulation of inter- and intra-histone modifications including H3 K9 methylation, H3 S10 phosphorylation, H3 K14 acetylation, and H4 K16 acetylation must occur in concert with chromatin remodeling by linker histones for cell cycle progression and differentiation of human myeloid cells into macrophages and dendritic cells. Molecular & Cellular Proteomics 14: 10.1074/mcp.M113.035089, 15–29, 2015.

The linker histone H1s “beads-on-a-string” structure aids chromatin folding into highly compacted 30 nm chromatin fibers (1). Previous studies demonstrated that histone H1s are differentially expressed and incorporated into chromatin during embryonic stem cell differentiation and reprogramming to pluripotency (2). More than being accumulated after differentiation, the three histone H1 isoforms, H1.3, H1.4, and H1.5, are required for embryonic stem cell differentiation as demonstrated by in vivo H1.3/H1.4/H1.5 triple null experiments (3). Histone H1 null cells exhibit altered nucleosome architecture (4) which may cause epigenetic reprogramming (2), specific changes in gene regulation including repression of pluripotency gene Oct4 expression (3, 5), and cell growth (6, 7). In human blood or bone marrow, hematopoietic stem cells give rise to two major pluripotent progenitor cell lineages, myeloid and lymphoid progenitors, from which are derived mature blood cells including erythrocytes, megakaryocytes, and cells of the myeloid and lymphoid lineages. However, epigenetic regulation or reprogramming in this complex differentiation system has not yet been fully understood. As a follow up to our proteomics studies on epigenetic networks in U937 cell differentiation (8), we have performed proteomics studies on primary human monocyte differentiation. In this report, using proteomics and bioinformatics tools in lieu of microarray analysis of gene expression, we describe the presence of unique protein expression profiles, specifically the linker histones, in monocyte differentiation into macrophages and dendritic cells.

Differentiation of monocytes from primary leukemia cell lines or from human peripheral blood mononuclear cells into macrophages or macrophage-like cells using different differentiating reagents has been frequently used as a mimetic model for understanding the process of innate and adaptive immune responses to inflammatory stimuli, viral infection, and environmental cues. Either phorbol myristate acetate (PMA)¹ or granulocyte-macrophage colony-stimulating factor (GMCSF) has normally been used for differentiation of monocytes, though the former is generally for differentiation of primary monocyctic cell lines, while the latter for differentiation of hu-

¹ The abbreviations used are: PMA, phorbol myristate acetate; GMCSF, granulocyte-macrophage colony-stimulating factor; ACN, acetonitrile; HCD, higher-energy collision dissociation; PCA, principal component analysis; IPA, ingenuity pathway analysis.
man blood monocytes (9–11). In our experiments, CD14+ monocytes were treated with PMA, PMA + ionomycin, GMCSF, or GMCSF + IL4. After treatment, monocyte differentiation into macrophages or dendritic cells was monitored by mass spectrometry and bioinformatics analyses. We report here that monotypic cell lineages can be distinguished based on protein expression profiles, specifically, histone H1.4 and H1.5 expression patterns. We identified H3K9-methyl/S10-phos/K14-acetyl tri-modification forms in the monocyte and macrophages but not in dendritic cells. In addition, histone H4 K16 acetylation was low in monocytes and macrophages but significantly higher in dendritic cells. Our findings suggest a switch from H3 tri-modification and linker histone expression to histone H4 K16 acetylation occurs during the monocyteto-dendritic cell transition.

MATERIALS AND METHODS

Monocyte Isolation, Differentiation, and Flow Cytometry Analysis—Peripheral blood from healthy adult donors (age 18+) were collected from plasma apheresis filters from Lifestream blood bank, San Bernardino, CA, according to Loma Linda University IRB requirements (IRB number: 58168). Leukocytes were obtained by lysing the red blood cells using ACK lysis buffer (8.3g/L NH4CL, 1.0g/L KHCO3, and 3.7 mg/L EDTA Na) according to standard protocol (Invitrogen, Carlsbad, CA). CD14+ cells were isolated from the leukocyte fractions with anti-CD14 micro beads (Miltenyi Biotech, San Diego, CA). Monocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at a density of 10^6 cells per ml. In parallel, the monocytes were cultured in the RPMI 1640 medium with 1 μM PMA or 1 μM PMA plus 1 μM ionomycin for 72 h to obtain PMA- or PMA+macrophage cells. Additionally, monocytes were cultured in RPMI 1640 media with 50 ng/ml GMCSF for 6 days to obtain macrophages or cultured with 50 ng/ml GMCSF + 10 ng/ml IL-4 for 6 days to obtain dendritic cells, respectively (Fig. 1A). In parallel, as previously reported, the promonocyte/monoblast U937 cells were also cultured in RPMI 1640 media. U937 cells were differentiated with PMA for 72 h to monocytes/macrophages (8) (Fig. 1A). Monocytes were also cultured in 1μM Apicidin (Santa Cruz Biotechnology, Santa Cruz, CA).

Cells were stained for flow cytometry with the following antibodies: anti-CD14 APC (Miltenyi Biotech, San Diego, CA), anti-CD11b PE, anti-H1.5 (Abcam, San Francisco, CA), anti-H4K16Ac (Abcam), anti-H3K9Me3, anti-H3S10p, anti-H3K9Me3S10pK14Ac (supplemental Fig. S3), anti-Rabbit IgG FITC (Biolegend, San Diego, CA), anti-HL-DR PerCP (Becton Dickinson, Franklin Lakes, NJ), and fixable viability dye eFlour 450 (eBioscience, San Diego, CA) according to standard protocol. For intracellular histone staining, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with Odyssey blocking buffer (Licor, Lincoln, NE) + 0.2% Triton-X 100, then stained with 1 Åb followed by the 2 Åb at room temperature for 45 min each. Cells were then washed with PBS and resuspended in 195 μL PBS + 5 μL Propidium iodide (eBioscience) + 50 μg/ml RNase. Cells were run on a MACSQuant flow cytometer and data was analyzed using FlowJo software v.7.6 (Treestar, Ashland, OR).

Sample Preparation and Mass Spectrometry Analysis—The cell pellet was lysed in RIPA lysis buffer (Santa Cruz Biotechnology) with additionally added 1%Nonidet P40, PMSF (0.2 mM), and protease inhibitor mixture (Roche, one tablet per 10 ml) with the assistance of sonication (3 x 30 strokes) and incubation on ice for 2 h. The protein mixture was centrifuged and the supernatant transferred into a clean tube. This extraction procedure was carried out twice and the supernatants were combined. The protein concentration in the supernatant was determined by BCA assay. Approximately 60 μg of each protein sample was resuspended in 25 μl triethylammonium bicarbonate, pH 7.8. The protein solution was reduced by addition of 10 μl DTT and incubation at 50°C for 30 min, followed by carboxymethylation with 25 μl iodoacetamide in the dark for 1 h. The proteins were precipitated by addition of four volumes of −20°C precooled acetone and stored at −20°C overnight. The protein was pelleted by centrifugation at 14,000 rpm for 10 min and the supernatant was discarded. The protein pellet was dissolved in 25 μl triethylammonium bicarbonate buffer and digested by trypsin at a protein/trypsin enzyme ratio of 25:1 (by mass) for 10 h at 37°C. The TMT (Tandem Mass Tag) isobaric Mass Tagging Kit (Thermo-Fisher Scientific) was used to label the peptides following the manufacturer’s recommended conditions. Each TMT-labeled protein pool was acidified with 0.1% formic acid and fractionated with strong cation-exchange (SCX) chromatography on a Tiptop column (Poly LC, MD). For fractionation, the matrix was equilibrated with 0.1% formic acid in 20% acetonitrile (ACN) to facilitate peptide binding. After collection of the flow-through, 1 ml of each subfraction was sequentially eluted with 20% ACN, 0.05 M KCl, 0.2 M KCl, 0.35 M, 0.5 M KCl, and 5% ammonium hydroxide in 20% ACN. Next, the fractions were dried under vacuum to remove ACN, reconstituted in 1% formic acid, and then desalted using a Tiptop column with C18/hypercarb mixed materials (PolyLC, Columbia, MD). The eluted peptides were then again vacuum-dried, reconstituted in 30 μl of 0.1% formic acid, and then subjected to LC-MS/MS analysis. Quantitation of SCX fractionated TMT-6 labeled peptides was carried out on the Thermo LTQ-Orbitrap Velos Pro mass spectrometer. Peptides were separated by online reverse phase liquid chromatography using an Easy-nLC equipped with an autosampler (Thermo Scientific). A 10 cm, 75 μm id, 3-μm particle size, C18-A2 analytical column (Thermo) was used for the reverse phase liquid chromatography separations. Approximately 2 μg of peptide sample was injected. A precolumn (Thermo, 0.1 x 2 cm, 5 μm C18-A1) was brought inline with the analytical column and a 250-min gradient (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in ACN) from 5–30% solvent B was used for separating the peptides. The Orbitrap mass analyzer was set to acquire data at 60,000 FWHM resolution for the parent full-scan mass spectrum followed by data-dependent high collision-energy dissociation (HCD) MS/MS spectra for the top 12 most abundant ions acquired at 7500 resolution.

Quantification of Histone H1s by Selective Reaction Monitoring (SRM)—SRM experiments were run on the Agilent 6490 triple quadrupole mass spectrometer that was coupled with an Agilent 1200 microHPLC. Parameters for positive-ion electrospray were: ion source 250°C; nebulizer gas (N2) flow 14 L/h (42.0 psi in pressure); capillary voltage 3.5 KV; The SRM mode was chosen for qualitative and quantitative analysis of peptides with collision energies and fragmentor voltages optimized by a series of changes of parameters using standard peptides or test samples. HPLC condition: The separation was carried out on an XSelect HSS T3 30 μm C18 1.8 column (100Å, 2.5 μm, 4.6 mm x 150 mm) analytical column. The optimum mobile phase consisted of 0.1% formic acid in water as solvent A and 0.1% formic acid in ACN as solvent B. The gradient elution was employed according to the following program: at 0 min, 0% solvent B; at 8 min, 0% solvent B; at 20 min, 25% solvent B; at 25 min, 25% solvent B; at 26 min, 95% solvent B; at 30 min, 95% solvent B; at 31 min, 0% solvent B; and at 45 min, 0% solvent B. The flow rate was 0.3 ml/min throughout 45 min run. The column was maintained at 45°C and the auto sampler temperature was 5°C. Data acquisition and analysis was accomplished using Agilent MassHunter work station (version B.04.01). The injection volume was 5 μl.

Data Analysis—Proteins were identified and quantified through the Proteome Discoverer 1.3 platform (Thermo) by using both Mascot searching engine (Mascot Daemon 2.2.2; Matrix Science, London, UK)
and Sequest HT (employing the International Protein Index (IP) Homo Sapiens database version 3.73, June 2010, containing 89,739 entries). Mascot (or Sequest) searching parameters were used as follows: Carbamidomethylation of cysteine and TMT-6 modification of peptide N terminus and lysine were set as fixed modifications and oxidation of methionine and deamination of asparagine and glutamine were set as variable modifications. Trypsin was the protease selected and up to two missed cleavages was used. Mass tolerance for the precursor ions was 10 ppm and for the MS/MS 0.2 Da. Only peptides with minimal length of four amino acids were considered and peptides were filtered for maximum false discovery rate of 1%. At least one unique peptide with posterior error probability of less than 0.05 was accepted for quantification using the TMT-reporter ions and proteins were grouped.

Bioinformatic Analysis—Multivariate statistics analysis using the “R” program has previously been utilized for metabolic profiling (12). We have adapted this method for protein expression statistics analysis. Multivariate statistics and associated graphics were performed in R, version 64.25.1. dendrograms were created from this pairwise distance data using the as. matrix distance calculation capability and the as. dendrogram function that utilizes the hierarchical agglomerative clustering method. Heat maps were drawn using the heatmap.2 function found in the gplots package. Ingenuity Pathway Analysis (IPA) of protein functions/pathways or upstream regulators was performed using individual or the combined protein expression data set from two PMA/PMAI experiments and two monocyte GMCSF treatment experiments. The activation z-scores (a numerical value representing the strength of the relationship between a set of proteins and a function/disease) of “Cell movements” and “Functions for Hematological System Development” from IPA analysis were extracted for multivariate statistics analysis to determine cell motility patterns associated with cell differentiation. Principal component analysis (PCA) was performed using the procpr function in R and the calculation was based upon a singular value decomposition of the centered and scaled data matrix. The first two principle components, PC1 and PC2, which contributed to the majority of the variance in the data set, were plotted using the built-in biplot function.

Statistical Analysis—Determination of statistical significance of histone H1 expression among treatment groups was completed using two-way ANOVA in GraphPad Prism v6. For TMT measurement of Histone H1s, five biological replicates were performed and for SRM measurement three biological replicates combined with multiple transitions were performed. A web based Java code based on the Satterthwaite equation (13) was written for calculation of the degrees of freedom (v or DF) and t-values that were used for calculation of p values. Significance was accepted at p = 0.05. In figures, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

Antibody Generation—A rabbit polyclonal antibody against histone H3 K9me3S10pK14Ac was made as described (14) using a synthetic peptide containing histone H3 sequence: KQTAR(K-Me3)(S-Pho)-TGG(K-Ac)APRKQC. Specificity of this anti-H3 tri-modification antibody was further tested by ELISA. K9me3S10pK14Ac peptide was conjugated to KLH as described by the manufacturer (Pierce, Thermo Scientific, Rockford, IL) and coated onto the microtiter plates. The specific or unspecific H3 competitor peptides were tested in the competition assay for their ability to inhibit binding of anti-H3 tri-modification antibody to K9me3S10pK14Ac-KLH conjugate.

RESULTS

Analysis of Protein Expression in Differentiated Monocytes—Monocytes isolated by CD14+ magnetic beads were differentiated into macrophages in culture using GMCSF (50 ng/ml) for 6 days or differentiated with either 1.0 μM PMA or 1.0 μM PMA + 1.0 μM ionomycin for 3 days. After GMCSF treatment, the cells were divided evenly. One half was harvested and presumed to be macrophage cells (MC) and the other half was further treated with interleukin-4 (IL4) (10 ng/ml) for an additional 18 h to differentiate them into dendritic cells (DC). Alternatively, monocytes were treated with GMCSF (50 ng/ml) and interleukin-2 (IL4) (10 ng/ml) for 6 days to differentiate them into dendritic cells (DC). The cells were stained for flow cytometry with anti-CD14 and anti-CD11b (integrin alpha M) to ensure greater than 97% CD14+ monocyte isolation purity and the success of differentiation (Data not shown). Fig. 1A illustrates differentiation experiments performed in this study. Whole cell proteins were extracted by RIPA buffer with
additional 1% Nonidet P-40 and extensive sonication to shear chromatin. Under such acute extraction conditions, nearly complete extraction of nuclear proteins was achieved as attested by histone H1.5 that was analyzed by WB in the whole cell protein extracts and in the histone extraction solution from the remaining pellets that underwent regular acid-precipitation (Fig. 1B). Histone proteins were abundant in the cell extracts and sequence coverage by mass spectrometry for most histones was over 90% (Fig. 1C). Equal amounts of proteins extracted from the cell lysates were digested by trypsin and labeled with TMT reagents. Monocyte peptides were labeled with TMT-126 and -127, and GMCSF- and PMA-treated cells with TMT-128 and -129, and GMCSF + IL4- and PMA + ionomycin-treated cells with TMT-130 and -131. The GMCSF- and PMA-treated cells were analyzed separately. About 2292 and 1551 proteins were, respectively, identified and quantified in two sets of GMCSF or GMCSF + IL4 differentiated cells and, 1433 and 1480 proteins were identified and quantified in the PMA or PMA + ionomycin (PMAI) differentiated cells. A total of 236 proteins that appeared in all four protein data sets as illustrated in the Venn diagram (Fig. 2A) were used for statistical analysis. Hierarchical agglomerative clustering analysis divided the four experiments into two major groups: PMA/PMA + ionomycin (PMAI) group and GMCSF (MC)/GMCSF + IL4 (DC) group, suggesting that PMA- and GMCSF-treatments yield distinct phenotypes, potentially cor-
responding to different stages of monocyte differentiation. Within each major group, there were two subgroups that were found to correspond to the two biological repeats. Common protein expression profiles were observed between PMA- and PMAI-treated cells as well as between GMCSF and GMCSF + IL4-treated cells as demonstrated by each pair being individually clustered in one subgroup (Fig. 2B). The correlation between PMA- and PMAI-treated cells, plotted from proteins (Fig. 2A) identified in all four experiments demonstrated a great similarity between PMA treatment with and without combination with ionomycin as judged by the R² linear regression, 0.821 (supplemental Fig. S1A) and 0.8453 (supplemental Fig. S1B). A lesser degree of similarity was observed between GMCSF- and GMCSF + IL4-treated cells based on an R² linear regression of 0.5185 (supplemental Fig. S1C) and 0.7283 (supplemental Fig. S1D). Two data sets from two biological repeats were clustered in one group, demonstrating a satisfactory reproducibility of the experimental processes starting from monocyte isolation, cell culture, and differentiation to sample preparation and mass spectrometry analysis.

Consistent with hierarchical agglomerative clustering analysis, PCA of 236 proteins in the four experiments demonstrated that PMA and PMAI treatment of monocytes had a distinct expression profile from that of GMCSF- and GMCSF + IL4 (Fig. 2C). However, both hierarchical agglomerative clustering analysis and principle component analysis based upon the protein expression profiles of the 236 protein data set could not distinguish between PMA- and PMAI- treated monocytes or GMCSF- and GMCSF + IL4-treated monocytes.

IPA indicated that pathways involving cell movement and hematological system development and function are down-regulated for both PMA- and GMCSF-treated monocytes. These results support the notion that macrophages and dendritic cells move slower than their precursor-monocytes (Fig. 3) (15). Because the proteins regulated in these two pathways are related to cell motility, the activation z-scores from IPA analysis from these two pathways were combined for hierarchical agglomerative clustering analysis and PCA (Fig. 3). Similar to the clustering analysis of the whole protein data set.

Fig. 3. Selective protein expression profile in cell motility. The z-scores of combined cell movement and hematological system development and function from IPA analysis are plotted. A, Heat map of the z-scores of 33 types of cellular functions related to cell motility. Datasets for two biological repeats of PMA/PMAI- and GMCSF/GMCSF + IL4-treated are combined from z-score analysis by IPA and plotted. B, PCA of the z-scores. The relative variance in the data set reflected by the first four PCs is shown in the bar graph, and PC1 and PC2 for the z-scores corresponding to the 33 types of cellular functions as indicated in A, are plotted.
shown in Fig. 2, hierarchical agglomerative clustering analysis using the activation z-scores also divided cells into two major groups: PMA/PMAI and GMCSF (MC)/GMCSF + IL4 (DC) (Fig. 3A), further demonstrating that PMA- and GMCSF-treatments yielded distinct phenotypes of differentiated monocytes. The PCA revealed almost indistinguishable features of cell motility between PMA/PMA + ionomycin (PMAI), whereas a clear separation was detected between GMCSF- and GMCSF + IL4 treatment (Fig. 3B). GMCSF + IL4 induced dendritic cells showed less adhesion of immune cells, neutrophils, and phagocytes, but faster cell movement and migration, and higher levels of phagocytosis of myeloid cells in comparison with GMCSF induced macrophages (Fig. 3A).

Therefore, activation z-scores of cell motility could be used to distinguish between the two cell phenotypes related to GMCSF- and GMCSF + IL4-treated monocytes. Because surface antigen CD11b is a macrophage marker, we measured CD11b expression in monocytes and cells differentiated from monocytes by flow cytometry. As indicated in supplemental Fig. S2, CD11b was downregulated in PMA-treated cells while significantly up-regulated in GMCSF-treated cells, suggesting that under current experimental conditions, human monocytes could not be differentiated into macrophages by PMA but could be differentiated by GMCSF. Therefore, it was not surprising that the protein profiles of PMA-treated cells were significantly different from those of GMCSF-treated cells (Figs. 2 and 3). Next, we focused on pathway analysis based upon protein expression data of GMCSF- (macrophages) and GMCSF + IL4-treated monocytes (dendritic cells). As shown in the Venn diagram (Fig. 2A), 1078 common proteins were identified and quantified in two repeated experiments of monocytes isolated from two different patients. The hierarchical agglomerative clustering analysis of this subdata set clearly separated dendritic cells (DC) from macrophages as shown by the heat map (Fig. 4A). The two immune cell types were also well distinguished by PCA (Fig. 4B and 4C). The following proteins as shown in Fig. 4C, ZNF337 (#1069), TMEM106B (#969), and EPS15 (#298) were proteins significantly up-regulated only in DCs but not changed in macrophages. ZNF337 is a C2H2 zinc finger protein whose function is unknown. However, it is predicted to have transcriptional regulatory property based upon its structure. TMEM106B (transmembrane protein 106B) localizes to late endosomes and lysosomes, and its expression is inversely related to lysosomal activity (16, 17). EPS15 (Epidermal growth factor receptor substrate 15) is a clathrin-coated pit adaptor protein (18, 19). CALM (clathrin assembly lymphoid myeloid leukemia) (8), another adaptor protein, was up-regulated twofold in dendritic cells, but no change was observed in macrophages (supplemental Fig. S3). Taking these protein changes into consideration, it is logical that cell morphologies change upon macrophages differentiation into dendritic cells and that these changes in cell morphologies likely result in alterations in endocytosis pathways and endosomal/lysosomal macromolecule degradation pathways (20,
that ultimately influence cell-cycle and lipid metabolism (22).

There were distinctive surface marker/cytokine gene or protein expression profiles detected between GMCSF-treated monocytes (macrophages) and GMCSF/H11001 IL4-treated monocytes (dendritic cells) as indicated by previous genomic or flow-cytometry (FC) analysis (23, 24). Our mass spectrometry quantification data revealed that protein expression of CD209 (DC-SIGN) and CD1/H9251 was increased by 12- and 2.9-folds in GMCSF/H11001 IL4-treated monocytes versus 1.6 and 1.0 in GMCSF-treated monocytes. The expression of CD14 was decreased by 2.9-fold in GMCSF/H11001 IL4-treated monocytes versus 2.1-fold in GMCSF-treated monocytes. The protein expression profiles of these three proteins measured by mass spectrometry were in agreement with previously demonstrated FC and gene expression data of GMCSF + IL4-induced cell differentiation from human peripheral blood mononuclear cells (PBMC). These three markers are typically used for characterization of human monocyte-derived dendritic cells (23–27). IPA showed that the pathways involving small molecule/lipid metabolism were up-regulated for both GMCSF-treated and GMCSF + IL4-treated monocytes. Significantly up-regulated molecules in these pathways included low density lipoprotein receptor (LDLR), which is critical for cholesterol synthesis and N-acylsphingosine amidohydrolase (acid ceramidase) 1 (ASAH1), which catalyzes the synthesis and degradation of ceramide into sphingosine and fatty acids. Additionally, fatty acid binding protein 4 (FABP4) was significantly up-regulated (fourfold) in GMCSF + IL4-treated monocytes while slightly down-regulated (−1.7-fold) in GMCSF-treated monocytes. We used the IPA regulation z-score algorithm to identify biological functions associated with lipid metabolism pathways as previously described (28). Fig. 5A shows the activation z-score of lipid synthesis, degradation, transportation, and accumulation in GMCSF-treated and GMCSF + IL4-treated monocytes. Lipid transportation activity was significantly stronger in GMCSF + IL4-treated cells than in GMCSF-treated cells, which distinguished dendritic cells from macrophages. Analysis of upstream regulators indicated that IL4 and IL13 were significantly up-regulated (activation z-score > 2.0) and toll receptor 4 (TLR4) and its ligand lipopolysaccharide (LPS) (activation z-score < −2.0) was significantly down-regulated in GMCSF + IL4-treated cells. These results were consistent with the previously reported
phenotypes of dendritic cells with increased expression of CD209 (29–31). The overlay of mass spectrometry data onto interaction networks linking IL4 and LPS as shown in Fig. 5B revealed down-regulation of transcription factors CEBPA/B, JUN, STAT3, IRF8, and chromatin protein HMG1B, as well as up-regulation of NFkB1A in GMCSF + IL4-treated cells (dendritic cells). The predicted down-regulation of HMG1B by IPA concurred with down-regulation of the linker histone proteins measured by mass spectrometry. Both HMG1B and linker histones are correlated with repressive chromatin (32). However, linker histones have not previously been reported to be involved in the IL4 and LPS stimulatory pathways during human monocyte-to-dendritic cell differentiation. Other significantly up-regulated upstream regulators specific to GMCSF + IL4-treated cells include immunoglobulin, β-estradiol, PPARγ, TGFB, and TP53. These transcription factors have been shown to act cooperatively in the regulation of cell cycle progression and monocyte-derived DC differentiation (33, 34).

**Correlation Between Histone H1 Expression and Cell Lineage**—As shown in Fig. 2C, PCA identified six proteins that negatively correlated with monocyte differentiation. These proteins include histone H1.4, histone H1.5, high mobility group protein HMG-17, S100A8, S100A9, and lysozyme C. These proteins, located at the right bottom corner of the PC1-PC2 quartz-2D graph, were representative of the most significantly down-regulated proteins detected by mass spectrometry. The S100A8/9 complex (also known as calprotectin) was shown to be constitutively expressed in monocytes in various tissues associated with inflammatory processes while weakly expressed in tissue macrophages (35–38). The expression of this complex was shown to be regulated by lipopolysaccharide (LPS) (39). This result was consistent with our proteomics analysis results (Figs. 2C and 5B). Significantly higher levels of lysozyme C were detected in monocytes than in monocyte-derived macrophages and dendritic cells, which provides an explanation for the clinical outcome that lysozyme C secretion is significantly increased in the serum and urine of patients with myelomonocytic or monocytic-monocytic acute or chronic myeloid leukemias (40, 41). About one third of lysozyme is located in the lysosome, whereas two thirds secreted into the medium in U937 cells. In addition, lysosomal packaging of lysozyme is almost completely inhibited when the cells are treated with PMA (42). Because extremely altered expression of HMG-17 and H1.4/H1.5 proteins was observed in differentiated cells as revealed by PCA (Fig. 2C), we extracted the quantified expression levels of all the linker histone proteins.

In order to make meaningful conclusions regarding the observed expression changes of HMG-17 and H1.4/H1.5 proteins in GMCSF and GMCSF + IL4 treated monocytes, we analyzed three additional bioreplicates and combined them with the extracted data from the previous two bioreplicates used in this study. We found that HMG-17 and histone H1 isoforms (H1.0, H1X, H1.2, H1.3, H1.4, and H1.5) were significantly down-regulated in both GMCSF- and PMA-differentiated cells (Fig. 6A, 6B, and supplemental Table S2). An overlap display of Fig. 6A and 6B as shown in Fig. 6C clearly show a progressive down-regulation of expression of histone H1s, typically H1.4 and H1.5, from PMA-differentiated cells to GMCSF-differentiated cells. Furthermore, we quantified histone H1 proteins by SRM of unique H1 peptides distinguishing between different histone H1 isoforms including H1.0, H1.2, H1.3, H1.4, H1.5, H1.x, and HMG17 (supplemental Table S3). The H1 protein expressions were normalized to the expression level of 40S ribosomal protein S28 because it did not change as determined by the TMT method. H1 analysis by SRM confirmed the results obtained by TMT and revealed a significant (with p value <0.005) expression difference of H1.2, H1.3, H1.4, H1.5, and H1.x between macrophages and dendritic cells (Fig. 6D). Together, the data suggest that each phenotype of differentiated cells has a unique histone H1 expression profile that possibly affects the degrees of plasticity of chromatin structures confined by the linker histone proteins. Presumably, cells with low levels of histone H1 expression (e.g. GMCSF induced dendritic cells) have loosely confined chromatin structures that could affect cell morphology (for example, monocytes are round, whereas both macrophages and dendritic cells are flat) and potentially cell motility. Loosely confined chromatin would have increased chromatin accessibility to transcription factors and histone modification enzymes including histone acetyltransferases.

**Identification of K9-methyl/S10-phos/K14-acetyl Tri-modification Form on Histone H3 Tail in Human Monocytes**—The same set of mass spectrometry data for protein expression analysis was reanalyzed by resetting the searching parameters including removal of TMT-6 labeling to lysine but addition of dynamic modification of acetylation and methylation to lysine and phosphorylation to serine and threonine. Under these new search parameters, protein acetylation and phosphorylation sites were identified and specific attention was paid to histone modifications. Though the purpose of this project was not originally established for identification of histone modifications, through analysis of the protein data, we found that most of the major histone proteins were identified and quantified with higher than 90% sequence coverage (Fig. 1C). The labeling of peptide N terminus with TMT-6 not only increases the peptide size by 229 Daltons but also the peptide hydrophilicity, thereby enhancing the retention of histone N-terminal hydrophilic peptides on a regular C18 column thus, facilitating the identification of histone modifications. As a result, the majority of known modification sites on human histone H3 were reconfirmed by mass spectrometry on whole cell lysates from monocytes and their derivatives (supplemental Table S1). Surprisingly, Mascot searching reported the identification of two H3 peptide sequences with K9-dimethyl-ation/S10-phosphorylation/K14-acetylation and K9-trimethyl-ation/S10-phosphorylation/K14-acetylation. These tri-modifi-
cation peptides were eluted in the 0.35 M KCl fraction of a strong-cation-exchange (SCX) fractionating system, indicating that SCX had an additional enrichment effect of phospho-peptides that are beneficial to their identification because of the reduction of sample complexity. To further confirm these new modification forms, we extracted the mass spectra from the raw data for manual analysis with the help of Protein Prospector MS-Product (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct). As shown in Fig. 7A, the MS/MS spectrum of the precursor ion [M+3H]3+ at m/z 427.5692, the signature fragmentation ions were the neutral-loss of phosphoric acid ion [M+3H−H2PO4]3+ at m/z 126.0913, establishing the peptide sequence as TMT-9Kme210SpTGG14KAcAPR where the first lysine was dimethylated (H3K9-dimethyl), the second lysine was acetylated (H3K14-acetyl), and serine was phosphorylated (H3S10-phos). The mass accuracy, in terms of the deviation of mass spectrometry measured monoisotopic mass from the theoretical monoisotopic mass for both precursor and major neutral-loss-of-H3PO4 fragmentation ions, was within 5 ppm. Similarly as shown in the Fig. 7B, the MS/MS spectrum of the precursor ion [M+2H]2+ at m/z 647.8569 established the peptide sequence as TMT-9Kme310SpTGG14KAcAPR where the first lysine was trimethylated (H3K9-trimethyl), the second lysine was acetylated (H3K14-acetyl), and serine was phosphorylated (H3S10-phos). As specifically noted, in addition to the neutral-loss of phosphoric acid ion [M+2H−H3PO4]2+ at m/z 598.8705, significant fragmentation ions observed were the neutral-loss of trimethyl amine ion [M+2H−N(CH3)3]2+ at m/z 618.3213, unique for tri-methylated lysine, and neutral-loss of both phosphoric acid and trimethyl amine ion [M+2H−H3PO4−N(CH3)3]2+ at m/z 569.3331, clearly demonstrating the coexistence of H3K9-trimethyl and H3S10-phos together with H3K14-acetyl as the unique tri-modification form. Neither H3K9-monomethyl/S10-phos/K14-acetyl nor H3K9-acetyl/S10-phos/K14-acetyl tri-modification forms was identified. However, significant amounts of H3K9-dimethyl/K14-acetyl di-modification and H3K9-acetyl/K14-acetyl forms were detected.

Fig. 6. Histone expression profile of human monocytes that were treated with the two differentiation protocols, PMA/PMA + ionomycin (PMAI) and GMCSF (MC)/GMCSF + IL4 (DC). A, Bar graphs of histone expression in monocytes treated with PMA or PMA + ionomycin (PMAI) are representative of three biological repeats. Statistical significance was assessed between PMA/PMAI treated cells and monocytes. B, Bar graphs of histone expression in monocytes treated with GMCSF (MC) or GMCSF + IL4 (DC) are representative of five biological repeats. Statistical significance was assessed between MC/DC and monocytes. C, Comparison of relative histone expression between PMA/PMA + ionomycin (PMAI)- and GMCSF (MC)/GMCSF + IL4 (DC)-treated monocytes. D, Selected-reaction-monitoring (SRM) analysis of H1 proteins. Data is normalized to 40S ribosomal protein S28 (RPS28). Normalized data allowed for statistical comparison between MC and DC histone expression. Statistical analysis was performed using two-way Anova. n = 5. Statistical analysis - Determination of statistical significance among treatment groups was completed using Two Way ANOVA in GraphPad Prism v6. Significance was accepted at p = 0.05. In figures, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
Taken together, the data presented here demonstrates that phosphorylation of H3S10 was selective for the H3K9 di- and tri-methylated peptides in human monocytes.

Dynamic Histone Acetylation, Methylation, and Phosphorylation in Human Monocyte Differentiation—Histone modifications can be relatively quantified by ratios of TMT reporter ions. As indicated in Fig. 7A and 7B, the H3K9Me2/S10pK14Ac tri-modification form was enriched in monocytes while reduced in GMCSF-treated (macrophages) and was almost nonexistent in the GMCSF/H11001 IL4-treated cells (dendritic cells). However, histone H3K9Me3K14Ac (sequence: TMT-9Kme3STGG14KAcAPR) and H3K9Me3K14Ac (sequence: TMT-9Kme3STGG14KAcAPR) changed very slightly (Fig. 8A and 8B), whereas histone H4K16Ac was significantly higher in dendritic cells than in macrophage cells and monocytes (Fig. 8C).

We were able to generate an antibody specific to the H3K9Me3S10pK14Ac tri-modification form enriched in monocytes while reduced in macrophages (22.8%) and dendritic cells (4.82%) displaying a pattern similar to the mass spectrometry data as shown in Fig. 8C. Similarly to H1.5, H3S10p was also highly enriched in monocytes (98.3% cells positive) and dramatically reduced in macrophages (2.34%) and dendritic cells (2.28%). The same decreasing pattern was seen for H3K9Me3 from monocytes (24.8%) to macrophages (0.76%) and dendritic cells (1.61%). Consistent with the mass spectrometry data, H3K9Me3S10pK14Ac tri-modification was enriched in monocytes (8.82%), slightly reduced in macrophages (5.15%), and dramatically reduced in dendritic cells (0.7%). In contrast to histone H3 methylation and phosphorylation, H4 lysine K16 acetylation (H4K16Ac) was slightly increased in macrophages (0.51%) and fourfold increased in dendritic cells (1.34%) as compared with in monocytes (0.35%), which showed the same pattern as the mass spectrometry data (Fig. 8C). The data revealed that a switching from H3 methylation, phosphorylation, and linker histones to histone acetylation occurred during monocyte-to-dendritic cell differentiation.

![Fig. 7. Higher-energy C-trap dissociation (HCD) spectra of TMT tagged histone H3 peptides with K9-methyl/S10-Phos/K14-acetyl tri-modifications.](image-url)
Histone H1 Expression in Human Monocyte Differentiation

Proper Regulation of Histone Acetylation is Required for Monocyte Differentiation into Macrophages and Dendritic Cells—The H4 K16 specific acetyltransferase is hMOF/MYST1 (43) and HDAC3 deacetylates H4 K14 acetylation in mammalian cells (44). Histone deacetylase inhibitors, Vorinostat (SAHA) and Apicidin, have demonstrated promising anti-proliferation activity toward myeloid malignancies and solid tumors through acetylation of histones (45–47). Apicidin is a specific HDAC3/NCoR inhibitor (48). When monocyte HL60 cells were incubated with 100 nm Apicidin, global histone acetylation increased to the maximum level at 48 h (45). Because we observed that H4 K16 acetylation increased during monocyte differentiation with GMCSF + IL4 (Fig. 8C and 9B), we investigated the role histone H4 K16 acetylation plays in monocyte differentiation by treating monocytes with 1 μM Apicidin in order to manipulate histone acetylation. As expected, we detected hyperacetylation of histone H4K16 by both flow cytometry analysis (supplemental Fig. S7A) and Western-blot analysis (supplemental Fig. S7B) until the cells became arrested at the at the G0/G1 phase after six-day incubation (supplemental Fig. S6) (49). Brightfield microscopy clearly showed that the morphology of Apicidin-treated monocytes changed into a M2 macrophage-like phenotype (Fig. 10A) with the characteristics of a reduction of monocyte surface marker CD14 and an augmentation of macrophage surface marker CD11b (Fig. 10B). However, the surface marker CD11c decreased significantly in Apicidin-treated monocytes (Fig. 10B) revealing a phenotype of CD14medCD11bhiCD11clo, which is distinguishable from the phenotype of CD14hiCD11bhiCD11chi belonging to GMCSF (M1 macrophage) or GMCSF + IL4 (DC) treated monocytes (Fig. 10B). M2 polarized macrophages that were found in high-fat-diet-induced obese mice were characterized as CD11c− (51). Such polarized M2 macrophages were also obtained in HDAC3 knock-out mice (52). Judged by the cell morphology and the expression of cell surface markers (Fig. 10), the Apicidin-treated monocytes observed in this experiment were likely differentiated to polarized M2 macrophages but blocked for differentiation into M1 macrophages and dendritic cells.

Analysis of public ChIP-chip microarray data of lymphocytes using anti-H4K16Ac, H3K9Ac, and H3K9me3 showed that these PTMs differentially regulated transcription of CD14, CD11c, and CD11b (53). Acetylation of H4K16 specifically increases transcription at the CD11b gene, acetylation of H3K9 specifically increases transcription at the CD11c and CD11b genes, and tri-methylation of H3K9 increases transcription at the CD11c gene but decreases transcription at the CD11b gene (supplemental Fig. S7C). Because Apicidin non-specifically induces global hyperacetylation of H3 (including K9, K18, and K23) and H4 (including K5, K8, K12, and K16) (49), an equilibrium among H3 K9 tri-methylation, H3 K9 acetylation, and H4 K16 acetylation determines the expression levels of CD14, CD11b, and CD11c (Fig. 10) during monocyte differentiation. Therefore, a drug that specifically acetylates H4 K16 but deacetylates at the other H3 and H4 sites would be a promising agent for monocytes to be differentiated to dendritic cells using anti-H4K16Ac, H3K9Ac, and H3K9me3.

CONCLUSION AND DISCUSSION

Using TMT-6 labeling and mass spectrometry analysis, we have compared the protein expression profiles of human monocytes during their differentiation using two different sets of differentiation reagents, PMA or PMA + ionomycin and GMCSF or GMCSF + IL4. These two types of differentiation
protocols generated two different phenotypes of differentiated cells from their precursor-monocytes as revealed by clustering analysis and PCA (Figs. 2 and 3). Clustering analysis using the activation z-scores obtained from IPA revealed no observable differences between PMA and PMA + ionomycin differentiated cells while GMCSF induced macrophages differed substantially from GMCSF + IL-4 induced dendritic cells based on their cell motility patterns (Fig. 3). These differences were manifested by differential expression of linker histone proteins, typically histone H1.4 and H1.5 (Fig. 6A–6C). Although ionomycin is normally used in combination with PMA for monocyte in vitro cell line differentiation, our protein expression data suggest that the calcium ionophore did not enhance PMA induced differentiation in primary human monocytes as indicated in the previous report by Pedrinaci et al. (54). This difference suggested that PMA differentiation protocols might not be as effective on primary cells as they are on cell lines such as U937 cells. The expression of H1.4/H1.5 was significantly increased in monoblast and pre-monocyte U937 cells after 72 h treatment with PMA (8). However, H1.4/H1.5 expression was markedly decreased in macrophages and dendritic cells differentiated from monocytes, suggesting that expression of these proteins might be a dynamic process (supplemental Fig. S5). The linker histone proteins H1s define chromatin structures and are normally considered to be chromatin repressors (1, 55). Chromatin in monocytes was associated with highly expressed H1s and differed from chromatin in dendritic cells, which was associated with low levels of histone H1 expression. This effect might be related to chromatin plasticity with a tightly confined structure found in the monocytes in comparison with loosely confined structures in dendritic cells that might affect cell morphology and cell motility.

Unique tri-modification forms, H3K9-dimethyl/S10-phos/K14-acetyl and H3K9-trimethyl/S10-phos/K14-acetyl, were identified in both human monocytes and their GMCSF-induced macrophages but was absent in dendritic cells (Fig. 7). This undeniable identification by mass spectrometry was con-
Histone hyperacytelation is necessary for proper monocyte differentiation into M2-like macrophages. A, Brightfield 100x microscopy images of monocytes, macrophages, and dendritic cells after 6 days of differentiation in the presence or absence of apicidin. B, Cells were stained for CD14, CD11c, and CD11b. The amount of surface expression of each marker is displayed as the geometric mean fluorescence intensity (Geom. MFI) or as percent of cells positive for the indicated marker. Bar graphs (mean ± S.E.) and microscopy images are representative of four different patient samples in sextuplicate. Statistical analysis was performed using Two-way Anova.

Histone H3 K14 acetylation, and H4 K16 acetylation (62), was low in monocytes and macrophages while significantly increased in dendritic cells (Figs. 8, 9, and supplemental Fig. S7A). Clearly, the cross-talk between inter- and intra-histone modifications that include H3 K9 methylation, H3 S10 phosphorylation, H3 K14 acetylation, and H4 K16 acetylation in concert with chromatin remodeling by linker histones is one of the major epigenetic regulatory machineries that regulate the processes of the two closely related cell functionalities, cell cycle progression and differentiation of human myeloid cells (63). Inhibition of histone deacetylase HDAC3 by Apicidin increased the acetylation of histone H4 K16 and decreased histone H3 K9 methylation, S10 phosphorylation, and H1.5 expression (data not shown), resulting in monocyte differentiation (Fig. 10). This PTM pattern suggests histone acetylation is required for monocyte initial differentiation. However, Apicidin most likely induced monocyte differentiation into the macrophage M2 lineage while arresting the cells at the G0/G1 phase. This cell cycle arrest indicates that Apicidin also targets pathways less relevant to monocyte differentiation such as the PI3K/AKT/mTOR cascade associated with cell growth and cell cycle progression (44, 52). In addition, Apicidin inhibits the expression of CD11c (Fig. 10B). Therefore Apicidin is not a proper small molecule to be used to obtain dendritic cells from monocytes. However, Apicidin could be of benefit in the treatment of cancer and inflammatory diseases by arresting cell growth. Drug design of small molecules that specifically manipulate H4 K16 acetylation by either activating H4 K16 specific acetyltransferase hMOF or inhibiting H4 K16 specific deacetylase is extremely important.

The information generated by proteomics analysis on histone modifications and the expression of linker histones during human monocyte-to-dendritic cell differentiation would help to elucidate epigenetic remodeling/reprogramming mechanisms that underlie human myeloid cell differentiation. Continued investigation of epigenetic factors that regulate immune cell differentiation and induction of gene expression of key inflammatory cytokines such as TNF α (64, 65) may uncover epigenetic targets that facilitate the development of therapeutic agents for the treatment of human immune system disorders. Further development of epigenetic drugs, such as histone acetyltransferase activators or histone deacetylase inhibitors that specifically modulate histone H4 K16 acetylation, may open up a new avenue for dendritic cell immune therapy.

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[This article contains supplemental Figs. S1 to S7 and Tables S1 to S3.]

To whom correspondence should be addressed: Department of Pharmacology and Toxicology, UTMB at Galveston, TX 77554. Tel.: 409-772-9650; E-mail: kazhang@utmb.edu.

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