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RNA sequencing and transcriptomal analysis of human monocyte to macrophage differentiation

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Abstract

Monocytes can be differentiated into macrophages *in vivo* and these cells play an important role in innate and adaptive immune responses. To reveal the global gene transcription change that occurs during monocyte to macrophage differentiation, we performed genome-wide RNA sequencing and analyses in human primary monocytes and monocyte-derived macrophages. We show that 1208 genes (with >twofold differences) were differentially expressed in macrophages compared with monocytes, including 800 upregulated and 408 downregulated genes. Gene ontology, pathway, and protein–protein interaction analyses indicated that the upregulated genes were related to macrophage functions in phagocytosis, metabolic processes, and cell cycle. The majority of downregulated genes comprised genes involved in the inflammatory response and locomotion. Genes encoding transcription regulatory factors, such as *FOXO1*, *RUNX3*, *NF-κB1*, and *C/EBP δ*, were highly expressed in monocytes and appeared to function in significant transcriptional repression, resulting in slight metabolic activity. Our transcriptome comparison between human monocytes and monocyte-derived macrophages using RNA sequencing revealed novel molecules and pathways associated with the differentiation process. These molecules and pathways may represent candidate targets involved in the pathophysiology of these important immune cells.

Keywords

Monocyte-derived macrophages; Monocytes; RNA-seq; Transcriptome; M-CSF

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1. Introduction

Monocytes and macrophages, which belong to the mononuclear phagocyte system, define innate immune-mediated processes that include clearance of cellular debris and microbial infections, control of cancer progression, and secretion of immunoregulatory bioactive factors including interferons, interleukins, growth factors, and chemokines (DeNardo et al., 2010; Serbina et al., 2008). Commitment to the mononuclear phagocyte lineage is determined at the stage of macrophage and dendritic cell progenitor (MDP), at which point erythroid, megakaryocyte, lymphoid, and granulocyte fates have been precluded. Cellular cloning and transplantation studies have shown that MDPs give rise to monocytes and common dendritic cell (DC) progenitors (Auffray et al., 2009; Fogg et al., 2006). Blood-circulating monocytes can migrate from blood vessel to most body tissues and become resident macrophages. Although monocytes and macrophages are known to be highly heterogeneous in phenotype, tissue distribution, and function (Taylor et al., 2003), the dynamic molecular, biochemical, and cellular events that underlie monocyte differentiation remain unclear.

In recent years, research efforts have focused on biochemical and genetic changes that control monocyte differentiation. Previous studies in mice have demonstrated the critical functions of the macrophage colony-stimulating factor (M-CSF) receptor and its two known ligands M-CSF and IL34 (Lin et al., 2008) during monocyte to macrophage differentiation since the M-CSF deficient mice or its receptor deficient mice have dramatic reduction of F4/80 cell density (Dai et al., 2002). Other cytokines and chemokines, such as GM-CSF, FLT3, CXCL12, and CCL18, have also been shown to control macrophage development and homeostasis (Brasel et al., 1996; Sanchez-Martin et al., 2011; Schraufstatter et al., 2012; Wiktor-Jedrzejczak et al., 1994). Genome-wide gene expression profiles associated with monocyte to macrophage differentiation have also been investigated. Serial analysis of gene expression (SAGE) (Hashimoto et al., 1999; Suzuki et al., 2000) and microarray (Li et al., 2007; Martinez et al., 2006; Rodriguez-Prados et al., 2010) data allow the study of thousands of genes that are clustered into different pathways during monocyte to macrophage differentiation or activation. However, these approaches do not offer precise gene sequencing information and lack direct quantification of gene expression.

Next-generation deep sequencing technology has been proven to be a powerful tool for transcriptome analysis (Balakrishnan et al., 2012). This direct sequencing methodology enables the simultaneous sequencing of millions of different DNA molecules without requiring prior annotation (Cloonan and Grimmond, 2008; Morozova and Marra, 2008). Sequencing-based methods generate absolute gene expression measurements and show many advantages over previous microarray-based assays. For example, sequencing-based methods generate an unbiased view of the transcriptome, are not limited by predictions of expressed transcripts used to determine array content, and are more sensitive in detecting low-abundant transcripts and small changes in gene expression (t'Hoen et al., 2008; Wilhelm et al., 2008).

In the present study, to understand the molecular mechanisms involved in monocyte to macrophage differentiation better, we employed Illumina mRNA sequencing (RNA-seq) to perform transcriptome comparative analysis between monocytes and monocyte-derived macrophages for the first time. cDNAs generated from both monocyte and macrophage mRNAs were subjected to deep sequencing and gene expression profiles between monocytes and macrophages were analyzed. Our findings provide a comprehensive view of the changes in the monocyte transcriptome following M-CSF-stimulated differentiation into macrophages. The results can improve our understanding of gene regulation during monocyte hematopoietic differentiation on a genome-wide scale.

2. Material and methods

2.1. Cell culture and RNA extraction

Monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from a healthy blood donor (American Red Cross Blood Service, Columbus, OH, USA) as previously described (Dong et al., 2007). Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over a Ficoll-Paque gradient. Primary monocytes were then isolated from PBMCs using Histopaque-Percoll gradient centrifugation. Cells were cultured in RPMI medium with 10% fetal bovine serum. To generate monocyte-derived macrophages, isolated monocytes were cultured in the presence of M-CSF (50 ng/mL; PeproTech) for 7 days. Total RNAs were extracted from freshly isolated monocytes or differentiated macrophages using a Qiagen RNeasy kit.

2.2. cDNA library preparation for RNA-seq

cDNA libraries were prepared according to the Illumina TruSeq RNA Sample Preparation Guide for TruSeq RNA Sample Prep Kit (FC-122-1001). Briefly, poly-A-containing mRNA molecules were purified from 4 µg of total RNA using poly-T oligo-attached magnetic beads, then fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers, followed by second-strand cDNA synthesis using DNA polymerase I and RNaseH. These cDNA fragments were then subjected to an end-repair process using a combination of T4 DNA polymerase, *Escherichia coli* DNA Pol I large fragment (Klenow polymerase), and T4 polynucleotide kinase. The blunt, phosphorylated ends were treated with Klenow fragment (3' to 5' exo minus) and dATP to yield a protruding 3-'A' base for ligation of Illumina adapters, which have a single 'T' base overhang at the 3' end. These products were purified and enriched by 15 cycles of polymerase chain reaction (PCR) to create the final cDNA library. Agencourt AMPure XP magnetic beads by Beckman Coulter were used at each step of the library-making process to purify the desired fragments. The final purified DNA was captured on an Illumina flow cell for cluster generation. Libraries were sequenced on HiSeq 2000, following the manufacturing protocols.

2.3. Data processing

The reads obtained by RNA-seq were compiled using Bowtie 2 read aligner software. Reads were aligned to the human mRNA reference sequence database (NCBI, ftp://ftp.ncbi.nih.gov/refseq/H_sapiens/mRNA_Prot/human.rna.fna.gz). Only uniquely mapped reads with less than two mismatches were used. The number of each read was further normalized to reads per kilobase of exon model per million mapped reads (RPKM); thus, the values were considered the final expression levels for each gene (Mortazavi et al., 2008).

2.4. Gene ontology (GO) functional enrichment analysis

We used the web-based GO analysis tool, GOrilla (<http://cbl-gorilla.cs.technion.ac.il>) to map all differentially expressed genes (DEGs) to terms in the GO database, searching for significantly enriched GO terms in DEGs compared with the genomic background. Our analysis was limited to categories with a P-value <0.001. Within the significant category, the enrichment factor was given by $(b/n) / (B/N)$, where b is the number of DEGs within the particular category, B is the total number of genes within the same category, n is the number of DEGs in the gene reference database list, and N is the total number of genes in the gene reference database list.

2.5. Pathway analysis of DEGs

Genes involved in different steps of a common pathway tend to overlap in their expression profiles. As such, pathway-based analysis can provide insights into the biological functions of genes. Pathways were constructed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and Path-Net was used to identify differential networks and core regulators involved in monocyte to macrophage differentiation (Yi et al., 2006).

2.6. Protein–protein interaction network analysis

Direct binding between different proteins can also reveal shared functionality. The online data analysis tool Ingenuity Pathway Analysis (IPA) version 7.1 (Ingenuity® Systems, www.ingenuity.com) was used to construct a network between the top 10 up- or downregulated genes and their mediators. Transcriptional-Net, a network map of differentially expressed transcription regulators, was constructed using Expression2kinase (X2K) software (<http://www.maayanlab.net/X2K>). Transcription factors likely to regulate gene expression were identified by X2K and transcriptional networks were built according to protein interaction databases, including BIND (<http://www.isc.org/software/bind>), BioGrid (<http://thebiogrid.org/>), and HPRD (<http://www.hprd.org/>).

2.7. Quantitative RT-PCR (qPCR) for confirmation of gene expression

Total RNA from monocytes and monocyte-derived macrophages was used for qPCR analyses. Reverse transcription with random hexamer primers was performed with the reverse transcription system (TaKaRa, Japan). Gene-specific primers were designed based on the gene sequences using Primer Premier software. qPCR assays were performed using SYBR® Premix Ex Taq™ (TaKaRa) with an Eppendorf RealPlex4 instrument under the following two-step conditions: denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 40 s for a total of 40 cycles. Hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) was used as an endogenous reference to assess the relative levels of mRNA transcripts. The relative gene expression changes were measured using the $-\Delta\Delta C_t$ method.

3. Results

3.1. RNA-seq of monocytes and monocyte-derived macrophages

Isolated monocytes were over 90% pure, as determined by surface marker CD14 staining, and did not acquire the morphological appearance of differentiated macrophages at day 0. When monocytes were cultured with M-CSF, these monocytes strongly adhered to the plates and displayed morphology characteristic of differentiated macrophages in vitro at day 7. These cells were enlarged with rough edges and distinct from small round, suspending monocytes.

To understand the transcriptional events associated with M-CSF-dependent monocyte to macrophage differentiation further, we performed high-throughput RNA-seq for two poly-A purified RNA samples using an Illumina Genome Analyzer. Table 1 shows that approximately 244 million and 146 million reads were obtained after eliminating low-quality reads, resulting in 188 million and 93 million reads from macrophages and monocytes, respectively. Of these high-quality reads, 188 million (77%) and 93 million (63%) reads were aligned to the human mRNA reference sequences and 26,266 and 25,870 transcripts were measured in primary macrophages and monocytes, respectively. RPKM was used to represent the expression level of each transcript (Mortazavi et al., 2008). Among the transcripts obtained, 1369 transcripts and their variants with P-values <0.01 and estimated absolute log₂-fold change >1 were annotated to 1208 DEGs (Supplementary data 1, 2, and 3).

The gene expression profiles derived from RNA-seq were also compared with the profiles generated from microarray analysis (Martinez et al., 2006). The RNAs of these two groups were obtained from both human primary monocytes and M-CSF-stimulated macrophages. Despite technological differences and sample variations, the correlations between these two groups were not low, with R-values of 0.66 and 0.67, for macrophages and monocytes, respectively (Fig. 1A). It suggested that our results were in accordance with the microarray data, as indicated by the significant number of genes determined in both techniques. We also observed that several genes with low expression levels could be detected by RNA-seq.

The top 10 upregulated and downregulated genes following monocyte to macrophage differentiation are shown in Fig. 1B. The list is headed by selenoprotein P (SEPP1), followed by complement component 1q subcomponent C (C1QC), triggering receptor expressed on myeloid cells 2 (TREM2), hepcidin antimicrobial peptide (HAMP), and spleen tyrosine kinase (SYK). The most downregulated mRNAs following differentiation included DNM2, IL-8, CCR2, and CCR7. The IPA web-based tool was used to identify known interactions between these most up-or downregulated genes and their interplayers. As shown in Fig. 2, the most significant interactions between these genes were related to inflammation, as demonstrated by TNF, IFN- γ , and IL-6 in the middle of the map. Downward of the map, the interactions between APOE and PPARG, two important proteins involved in lipid metabolism, were also evident. These results indicate that during monocyte to macrophage differentiation, significant mRNA expression changes in cell function are related to inflammation and lipid metabolism.

3.2. Comparison of gene expression between monocytes and monocyte-derived macrophages by GO enrichment analysis

To characterize the functional consequences of gene expression changes associated with monocyte to macrophage differentiation, we performed GO enrichment analysis of DEGs based on the GO database. A total of 747 of the 800 upregulated genes and 372 of the 408 downregulated genes were obtained from 1208 DEGs after data mining, reflecting functional molecular changes during differentiation. The biological process of GOs in obtained results was the most robust; thus, we focused on biological processes defined at level 3 with a P-value <0.001. As shown in Table 2, upregulated and downregulated genes shared common GOs including responses to chemical stimulus and regulation of biological quality of GO analysis but appeared to have distinct gene expression patterns. The upregulated genes were mostly involved in metabolic processes, cell cycle, cell division, oxidation–reduction process, and plasma lipoprotein particle clearance. Of these, macrophage scavenger receptor 1 (MSR1), apolipoprotein C-I (APOC1), apolipoprotein E (APOE), scavenger receptor class B member 1 (SCARB1), and CD36 were the genes most enriched in the plasma lipoprotein particle clearance category. By contrast, downregulated genes were characterized with functions of regulation of biological process, locomotion, and cellular component movement. Genes involved in locomotion and cellular component movement numbered 42 and 34, respectively. The majority of the genes in these two categories encoded chemokines and their receptors, such as CCR2, CCR7, CXCR4, CXCR3, CCL5, CCL24, CXCL2, CXCL3, CXCL5, CXCL10, CXCL11, and XCL2. Other enrichment GOs for inflammatory response, apoptosis, oxygen response, and endocytosis were also analyzed. Among these GOs, 33 genes including ALOX5, *NF- κ B1*, *DEFB1*, and IL-8 functioned in inflammatory response and 19 genes including NR4A2, VEGFA, SOD2, and CYP1A1 were responsible for oxygen response. Our results suggest that monocytes gradually lose their abilities for cell trafficking and migration upon differentiation into macrophages but simultaneously gain cell metabolic activity and undergo proliferation, and phagocytosis.

3.3. Significant pathways involved in monocyte to macrophage differentiation

To characterize the functional consequences of gene expression changes associated with monocyte differentiation, we performed pathway analysis of DEGs based on KEGG database using the two-sided Fisher exact test. Significant signaling pathways included hematopoietic cell lineage, steroid biosynthesis, cell cycle, lysosome, peroxisome proliferator-activated receptor (PPAR) signaling pathway, cell adhesion molecules, chemokine signaling pathway, nucleotide oligomerization domain (NOD)-like receptor signaling pathway, and cytokine–cytokine receptor interaction.

The majority of DEGs annotated in the lysosome pathway were upregulated, except Lamp-3 (also known as CD63), one of the components of late endosomal/lysosomal membranes. As shown in Fig. 3, Path-Net was used to determine the interaction network of 24 significant pathways ($P < 0.05$) of macrophages in comparison with those of monocytes. Path-Net was built according to interactions between KEGG database pathways to permit systematic identification of interactions between significant pathways (Yi et al., 2006). The interactions between different pathways were illustrated by their common proteins, as indicated by the lines connecting to pathway labels in Fig. 3. Significant pathway interactions in Path-Net were identified among hematopoietic lineage, cell adhesion molecular, NOD-like receptor signaling, graft-versus-host disease, viral myocarditis, prion disease, and type I diabetes mellitus pathways with interactions 8 on the map. A small network was also obviously associated with metabolic process, including pyruvate metabolism, glycerolipid metabolism, propanoate metabolism, butanoate metabolism, tryptophan metabolism, and arginine and proline metabolism.

3.4. Protein–protein interaction and transcriptional network analysis

To identify the regulatory mechanism upstream of genome-wide differences in gene expression, X2K software was applied to identify the transcription regulators among DEGs (Supplementary data 4). The Z-score represents the ability of a gene to mediate, and Degree represents the gene–gene interaction number. The interaction network between these regulators, the so-called transcriptional-Net, was constructed using the BIND, BioGrid, and HPRD protein interaction databases within the software. Key genes with Degree of 30 included AR, EP300, SP1, CREBBP, HDAC1, HDAC3, SMAD3, and VDR (Fig. 4). AR, a member of the nuclear receptor superfamily, becomes activated upon androgen binding and translocates into the nucleus to modulate the expression of its target genes. Besides its central function in the growth and development of the normal prostate gland and in the proliferation and progression of prostate cancers, a recent study showed that monocyte/macrophage AR can modulate the inflammatory response by enhancing TNF production (Lai et al., 2009). Both EP300 and CREBBP have histone acetyltransferase activity and participate in the ESR1/SP pathway, with functions in regulating lipid metabolism (Li et al., 2001). HDAC1 and HDAC3, two histone deacetylases, are responsible for the deacetylation of lysine residues on the N-terminal part of core histones and several other non-histone substrates, resulting in transcriptional repression for transcriptional regulation, cell cycle progression, and developmental events (Hayashi et al., 2010). Our results suggest that during monocyte to macrophage differentiation, genome-wide gene expression is largely affected by chromatin epigenetic modification via histone acetylation or deacetylation.

3.5. Validation of antiviral DEGs data by qPCR

Monocytes and macrophages contribute directly to the immune defense against microbial pathogens. To validate several antiviral DEGs identified by RNA-seq, we selected six genes suspected to interfere with HIV-1 replication during differentiation for qPCR confirmation (Dyer et al., 2008). These genes included interferon-stimulated gene ISG15, two genes encoding tripartite motif proteins (TRIM22 and TRIM47), and other three proteins (TUBA,

NUP98, and APOBEC3A). The HPRT1 product was used as an internal control. Primers for qPCR are listed in Supplementary data 5. The mRNA expression of the six genes determined by qPCR was consistent with the changing trends of gene expression from RNA-seq analysis (Fig. 5), supporting the reliability of RNA-seq results. For example, we detected more TRIM22 (9.9-fold), ISG15 (6.2-fold), and NUP98 (3.5-fold) in monocytes compared with those in macrophages using qPCR, whereas RNA-seq showed that there were 6.5-fold, 10.5-fold, and 9.6-fold more for TRIM22, ISG15, and NUP98 in monocytes, respectively. These results indicate that the susceptibility of HIV-1 to monocyte/macrophage is due to changes in the expression of several antiviral proteins during differentiation.

4. Discussion

Monocytes originating from bone marrow hematopoietic progenitors are capable of differentiating into morphologically and functionally heterogeneous effector cells. These biological processes are linked to specific sets of cellular functions regulated as a result of cell differentiation. M-CSF is an important factor for monocyte survival and differentiation. However, changes in signal transduction and the subsequent gene expression profile are not well characterized. In this study, we determined how RNA-seq-based high-resolution transcriptome data could be used to understand the biology of monocyte to macrophage differentiation via M-CSF stimulation further.

In vitro monocytes adhere to plastic surfaces and undergo spontaneous differentiation even without exogenous M-CSF (Kaplan and Gaudernack, 1982). We obtained monocyte-derived cells with morphologies resembling those of macrophages cultured with M-CSF. M-CSF-stimulated macrophages reportedly maintain the inactive phenotype (M0), which can be further differentiated into classically activated (M1) or alternatively activated (M2) cells, depending on the stimulus (Dai et al., 2002; Gordon and Martinez, 2010). In our study, expressions of a certain subset of macrophage activation-induced cytokine genes (*IL-8*, *IL-1β*, and *IL-15*), M1-specific genes (*Nos2* and *IL12b*), and M2-specific gene (*Arg1*) were constantly expressed at low levels or dramatically decreased with M-CSF stimulation, except the M2-specific gene CD163, which showed an increase of over 10-fold in expression (Supplementary data 1 and 2). Since only 8% transcriptional profile variance exists in the shift from M0 to M2 (Martinez et al., 2006), the cells we generated were inactive M0 macrophages. Furthermore, the interaction network of most up/downregulated proteins in Fig. 2 showed significant association with inflammation, as evidenced by the TNF-α, IFN-γ, and IL-6 expression profiles. The expressions of inflammatory cytokine gene *IL-1β* and pro-inflammatory cytokine genes (*IL-6*, *IL-12*, and *IL-15*) were very low, supporting the inactivation of M-CSF-stimulated macrophages with blunt inflammatory responses. Previous studies exploring the global gene profile change that occurs during human monocyte to macrophage differentiation have been limited to SAGE or commercially available microarrays (Hashimoto et al., 1999; Lehtonen et al., 2007). The major advantages of high-throughput RNA-seq are significantly improved detection accuracy, ability to identify alternative splicing without probe dependency, and de novo analysis of novel transcripts and long non-coding RNAs. Total reads were mapped to 25,870 and 26,266 transcript sequences in the human reference database for monocytes and macrophages and differentially expressed transcripts were identified according to P-value and absolute log2 RPKM change. The correlations between RNA-seq and microarray data were fairly well, ranging from 0.66 to 0.67 as previously reported (Marioni et al., 2008). While our comparison features several limitations, including technique difference, bioinformatic algorithms, standardization, and sample variance, it is consistent with a previous study because a large proportion of the genes were determined by two methods (Martinez et al., 2006). Furthermore, qPCR validation of RNA-seq confirmed the reliability of our RNA-seq. RNA-seq is able to detect a wide range of gene expressions, as exemplified by the

significant enrichment of the solute carrier family in this study, which controls the uptake and efflux of crucial compounds such as sugars, amino acids, nucleotides, inorganic ions, and drugs. This family regulates transcription through DNA-binding proteins and metal response elements, enzyme activity, including metallo-proteases, superoxide dismutase, inducible NO synthase, endosomal fusion, and metabolism. We observed 35 of DEGs for solute carriers measured by RNA-seq.

Our results reveal that locomotion-related gene change is associated with monocyte to macrophage differentiation (Table 2). Most DEGs encoding chemokines *CCL5*, *CCL24*, *CXCL2*, *CXCL10*, *CXCL3*, *CXCL5*, and *CXCL11* and their receptors *CCR2*, *CCR7*, *CXCR4*, and *CXCR3*, except *CCL2* and *CCL13*, were downregulated. *CCR2* expression on the surface of inflammatory monocytes and binding with CC chemokines *MCP-1(CCL2)*, *MCP-3(CCL7)*, and *MCP-4(CCL13)* can direct monocyte recruitment to inflamed tissue sites (Franci et al., 1995). This effect accounts for why monocytes tend to migrate to inflammatory sites and be differentiated into tissue-specific macrophages. With decreased *CCR2*, monocytes lose their ability for trafficking but retain the ability to recruit immune cells because of the expressions of *CCL2* and *CCL13*. The chemokine expression profile determined in our study is also different from that of polarized macrophages (Martinez et al., 2006), where macrophage activation is associated with upregulated chemokines, which contribute to their pathophysiology.

The phagocytic capacity of macrophages is central to their function. This capacity principally involves the clearance of cellular debris during physiological homeostasis and the removal of exogenous particles, including microorganisms. We observed that genes encoding phagocytosis receptors, such as *MSR1*, mannose receptor (*MR*), Fcγ receptors (*FcγR*), and *CD36*, were upregulated, enabling macrophages to uptake apoptotic cells and foreign pathogens (Anderson et al., 1990; Febbraio et al., 2001). Subunits of vacuolar type proton ATPase (*ATP6V1H*, *ATP6V0E2*, and *ATP6V0D2*) and acid hydrolase cathepsins (*CTSK*, *CTSF*, *CTSL*, *CTSK*, *CTSW*, and *CTSC*), which are required for the maintenance of the acidic milieu, markedly increased, suggesting greater degradation capacity in the phagosomes of differentiated monocytes.

Of the biological processes identified among the upregulated genes during monocyte differentiation, metabolic processes are among the most prominent (Table 2). These processes include not only lipid, steroid, and fatty acid metabolic pathways, as previously described (Liu et al., 2008; Martinez et al., 2006), but also amino acid and alcohol metabolic pathways. Proteins important in lipoprotein homeostasis, such as *APOE* and *APOC1*, are significantly induced in macrophages, consistent with another study that employed SAGE and utilized short sequence tag oligonucleotides and M-CSF (Hashimoto et al., 1999). Receptors important for the binding of modified lipoproteins, such as *MSR1*, *SCARB1-3*, and LDL-related protein, were all highly expressed. Genes including lipase A (*LIPA*), which promotes chylomicron formation and atherosclerosis (Kodvawala et al., 2005), *FABP3*, which regulates cholesterol export (Hansson et al., 2003), and *FADS1*, a member of the fatty acid desaturases, were also upregulated in macrophages. Protein network and Path-Net analysis revealed that the PPARγ (*PPARγ*) pathway, particularly *PPARγ* and acyl-CoA cholesterol acyltransferase 1 (*ACAT1*), was significantly affected during differentiation (Figs. 2 and 3). *PPARγ* is known to induce lipid uptake and storage (Quinn et al., 1987) and activate *CD36* expression (Nagy et al., 1998), thereby promoting intracellular cholesterol accumulation and foam cell formation. *ACAT1* is an intracellular enzyme that biosynthesizes cholesteryl esters from long-chain fatty acyl-CoA and cholesterol in various tissues. *CYP27A1* is a molecule responsible for producing 27-hydroxy-cholesterol for the alternative bile acid synthesis pathway. *ACAT1* and *CYP27A1* were both upregulated in the PPARγ pathway, in accordance with a previous study (Quinn et al., 1987). Therefore, the

PPAR γ pathway significantly contributes to the robust activity of lipid metabolism during macrophage differentiation.

Compared with macrophages, monocytes lack active gene expression for metabolic processes. However, we detected a number of transcription factors including *FOXO1*, *RUNX3*, *NF- κ B1*, *CCAAT/enhancer binding protein δ* (C/EBP δ), *FOSB*, and nuclear receptor family NR1H3, which were all significantly expressed in monocytes (Supplementary data 4). For example, *FOXO1*, a forkhead box class-O transcription factor was recently shown to be an inhibitor of lipogenic gene expression (Deng et al., 2012). Both *RUNX1* and *RUNX3*, which function by recruiting co-repressors and histone deacetylases, are involved in transcriptional repression and gene silencing during T-cell development (Durst and Hiebert, 2004). *NF- κ B1* is capable of inhibiting transcription via histone deacetylase *HDAC1* (Zhong et al., 2002). Thus, these genes contribute to transcriptional repression in monocytes, resulting in slight metabolic processes. Transcriptional-Net, which was used to dissect the regulatory mechanism of genome-wide gene expression, indicated that global gene expression is also controlled by histone acetylation or deacetylation during chromatin epigenetic modification in monocyte to macrophage differentiation (Fig. 4). The detailed mechanism of modulation of general cellular metabolic activities during macrophage differentiation remains unclear.

In agreement with earlier studies, our data indicate that a number of cell cycle-related genes, including cyclin proteins, cyclin-dependent kinase (CDK), and cyclin-CDK inhibitors, are significantly changed to drive the cell cycle (Table 2). Although human mononuclear phagocytes, unlike mouse macrophages, are generally considered terminally differentiated non-proliferating cells, reports of human macrophage proliferation have been published (Cheung and Hamilton, 1992). In this study, we found that DNA checkpoint-related genes *DNA-PK*, *MAD2L*, and *CHEK2* were upregulated in macrophages, indicating the subtle quality control of cell proliferation. We also observed that *STMN1* and *RSK2*, two key components in ERK-1/2 signaling pathway, were upregulated. M-CSF triggers ERK-1/2 activation and inhibition of this pathway results in arrested bone marrow macrophages at the G0/G1 phase (Valledor et al., 1999). Finally, we observed that *C/EBP δ* expression decreased, the over-expression of which can result in G0/G1 proliferative arrest and a moderate increase in apoptosis in K562 cells (Gery et al., 2005). Thus, human monocyte-derived macrophages appear to favor proliferation rather than remaining in the G0 quiescent state.

Monocytes and macrophages serve as sentinels involved the eradication of various pathogens. It has been well known that monocytes are an important HIV-1 reservoir and potential contributors to viral latency (Ellery et al., 2007; Lambotte et al., 2000). Despite the expression of HIV-1 receptors, undifferentiated monocytes are resistant to HIV-1 infection in vitro, whereas monocyte-derived macrophages and DCs are permissive for productive HIV-1 infection. Therefore, the identification of the restricting factors during monocyte differentiation would be interesting. Our results confirm that several proteins with antiviral functions are highly expressed in monocytes and downregulated with macrophage differentiation (Fig. 5), including *TRIM22*, *TRIM47*, *ISG15*, *TUBA*, *APOBEC3A*, and nuclear pore complex protein *NUP98*. However, we did not detect an expression change in *SAMHD1*, which was recently identified as an HIV-1 restriction factor in myeloid cells (Goldstone et al., 2011). The correlations between the expression of these factors and HIV-1 infection during monocyte differentiation must be further characterized.

In conclusion, we showed that critical gene expression profiles change during monocyte to macrophage differentiation, including those genes involved in inflammation, phagocytosis, metabolism, cell cycle, and transcriptional regulation. Downregulated transcriptional

repression during monocyte differentiation can result in increased metabolism and activated cell cycles, which promote macrophage differentiation. Transcriptome comparison of the genes selectively expressed in human blood monocytes and M-CSF-induced macrophages will provide useful information in defining the ontogeny, development, and function of cells in monocyte and macrophage lineages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MDP	dendritic cell progenitor
SAGE	serial analysis of gene expression
RNA-seq	Illumina mRNA sequencing
PBMCs	peripheral blood mononuclear cells
RPKM	reads per kilobase of exon model per million
DEGs	differentially expressed genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
IPA	Ingenuity Pathway Analysis
X2K	Expression2kinase
GO	gene ontology

Fig. 1. RNA-seq data analysis. (A) Validation of gene expression from RNA-seq with microarray data. For RNA-seq, the LOG2 RPKM values were used; for microarrays, LOG2 fluorescence intensity values were used. The correlation between microarray and the RNA-seq from Illumina was shown as indicated for each comparison. Pearson's correlation ($P < 0.01$) coefficient is indicated. (B) List of 10 most up/down-regulated genes during monocyte to macrophage differentiation. The y-axis represented the listed gene symbols and x-axis represented the gene expression change normalized with Log2 RPKM fold changes.

Fig. 2. Network of 10 most up/down-regulated genes and their potential inter-players. The online data analysis tool Ingenuity Pathway Analysis (IPA) version 7.1 (Ingenuity® Systems, www.ingenuity.com) was used to construct a network between the top 10 up- or down-regulated genes and their mediators. The red-colored nodes represent up-regulated genes and the green-colored nodes represent down-regulated genes. The intensity of the color indicates the level of gene expression change. Network shape was labeled in the box.

Fig. 3.

Path-Net of the significant pathways in the macrophage versus monocyte libraries. 24 significant pathways (Fisher exact testing, $P < 0.05$) were chosen according to KEGG database and Path-Net was used to identify differential networks and core regulators involved in monocyte to macrophage differentiation (Yi et al., 2006); The lines represent the interaction of the pathways.

Fig. 4.

The interaction network of upstream transcription regulation factors. Transcriptional-Net was constructed by X2K software transcription factors and protein network modules. Potential transcription factors among DEGs during differentiation were identified by X2K Chip-X database with the cut-off $P < 0.01$. Then the interactions between these identified factors were further mapped by protein interaction database and visualized by yED graph editor. Orange nodes represent transcription factors; yellow nodes represent intermediators and green nodes represent kinases respectively; area sizes represent Degree, and links represent interactions between the gene products.

Fig. 5. qPCR validation of antiviral DEGs. Relative qPCR was used to measure changes in target gene expression on day 0 for monocytes and on day 7 for macrophages. For each gene, the expression fold change was measured with the comparison of lower expression level on day 0 or day 7 which was set as 1 in the figure. Results were means and SDs, representative of three independent experiments.

Table 1

RNA-seq.

	Monocytes	Macrophages
Total reads	146×10^6	244×10^6
Mapped reads	93×10^6	188×10^6
Measured transcripts	25,870	26,266
DEGs	1208	

Table 2

List of GO enrichment analysis of up/down-regulated genes at biological process level 3.

No.	Go category	P value	Enrichment (N, B, n, b)
<i>Up-regulation</i>			
1	Small molecular metabolic process	2.20E-22	2.02 (13,479, 1664, 747, 186)
2	Response to chemical stimulus	8.92E-10	1.57 (13,479, 1888, 747, 164)
3	Cellular process	1.94E-09	1.15 (13,479, 8744, 747, 558)
4	Cell cycle	1.10E-07	1.87 (13,479, 714, 747, 74)
5	Regulation of biological quality	2.95E-07	1.54 (13,479, 1528, 747, 130)
6	Primary metabolic process	6.70E-07	1.23 (13,479, 4961, 747, 330)
7	Microtubule-based process	7.34E-06	1.87 (13,479, 272, 747, 34)
8	Response to stress	3.21E-05	1.40 (13,479, 1692, 747, 131)
9	Oxidation-reduction process	3.71E-05	1.90 (13,479, 408, 747, 43)
10	System process	1.19E-04	1.70 (13,479, 552, 747, 52)
11	Cell division	4.20E-04	2.65 (13,479, 293, 747, 43)
12	Cellular component organization	8.06E-04	1.24 (13,479, 2510, 747, 173)
13	Plasma lipoprotein particle clearance	9.72E-04	6.01 (13,479, 15, 747, 5)
<i>Down-regulation</i>			
1	Response to biotic stimulus	4.41E-23	5.09 (13,479, 377, 372, 53)
2	Response to chemical stimulus	1.85E-18	2.25 (13,479, 1888, 372, 117)
3	Regulation of biological process	1.24E-12	1.04 (13,479, 6350, 372, 242)
4	Response to external stimulus	7.59E-09	2.42 (13,479, 734, 372, 49)
5	Locomotion	7.20E-07	2.25 (13,479, 677, 372, 42)
6	Regulation of biological quality	2.26E-05	1.64 (13,479, 1528, 372, 69)
7	Cellular component movement	5.46E-05	2.11 (13,479, 599, 372, 34)