Impact on Genetic Networks in Human Macrophages by a CCR5 Strain of Human Immunodeficiency Virus Type 1

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Human immunodeficiency virus type 1 (HIV-1) impacts multiple lineages of hematopoietic cells, including lymphocytes and macrophages, either by direct infection or indirectly by perturbations of cell networks, leading to generalized immune deficiency. We designed a study to discover, in primary human macrophages, sentinel genetic targets that are impacted during replication over the course of 7 days by a CCR5-using virus. Expression of mRNA and proteins in virus- or mock-treated macrophages from multiple donors was evaluated. Hierarchical agglomerative cluster analysis grouped into distinct temporal expression patterns >900 known human genes that were induced or repressed at least fourfold by virus. Expression of more than one-third of the genes was induced rapidly by day 2 of infection, while other genes were induced at intermediate (day 4) or late (day 7) time points. More than 200 genes were expressed exclusively in either virus- or mock-treated macrophage cultures, independent of the donor, providing an unequivocal basis to distinguish an effect by virus. HIV-1 altered levels of mRNA and/or protein for diverse cellular programs in macrophages, including multiple genes that can contribute to a transition in the cell cycle from G_1 to G_2/M, in contrast to expression in mock-treated macrophages of genes that maintain G_0/G_1. Virus treatment activated mediators of cell cycling, including PP2A, which is impacted by Vpr, as well as GADD45 and BRCA1, potentially novel targets for HIV-1. The results identify interrelated programs conducive to optimal HIV-1 replication and expression of genes that can contribute to macrophage dysfunction.

Human immunodeficiency virus type 1 (HIV-1) pathogenesis results from perturbations in complex cellular networks leading to global immune dysfunction and a spectrum of AIDS-defining illnesses. HIV-1 infection of macrophages, which are long-lived cells, can also lead to establishment of reservoirs for virus production and spread to other cell types and compartments (2, 3, 7, 25, 27, 30, 31, 40, 82). Viral infection of macrophages produces a global impact on macrophage physiology, including alterations in the cell cycle (78, 80), chemotaxis (1, 21), levels of tumor necrosis factor alpha and other Th2-type cytokines (16, 24, 56), phagocytosis (9, 20, 45, 59), and antigen presentation (42, 51). In particular, HIV-1 or envelope gp120 activates multiple signal transducer and activator of transcription (STAT) proteins that modulate the expression of multiple receptors and/or cytokines contributing to generalized immune dysfunction in hematopoietic cells (11, 43, 66).

Discerning interactive networks through analysis of single pathways has limitations. In contrast, global analysis of gene expression profiles provides a comprehensive approach to developing an integrated picture of the complex perturbations of cellular gene expression by virus treatment. Initial genomic

approaches to the study of HIV-1 infection have examined interactions between prototypic T-cell-tropic CXCR4 strains of HIV-1 and proliferating T-cell lines or primary peripheral blood mononuclear cells (PBMC) (28, 61, 63, 76) or between gp120 and PBMC or monocyte-derived macrophages (MDM) (16). We designed a unique study using the Affymetrix Gene-Chip system in combination with high-throughput Western blot analysis to develop a genetic profile of virus treatment by a prototypic macrophage-tropic CCR5 virus, HIV-1$_{JR-FL}$, over the course of 7 days in human macrophages from multiple donors. A primary objective of using a systems biology approach was to identify sentinel genetic events that would distinguish between virus and mock treatment of macrophages. Subsequent analysis focused on the expression of networks of genes involved in cell cycle regulation (33, 44, 64, 78, 80).

MATERIALS AND METHODS

Preparation of viral stocks and infection of MDM. A stock of HIV-1$_{JR-FL}$ (National Institutes of Health, AIDS Reference and Reagent Program, Rockville, Md.) was prepared in PBMC from a seronegative donor and calculated to have a titer of $10^{4.5}$ 50% tissue culture infective doses per ml (73). Supernatants for mock treatment of MDM cultures were prepared simultaneously from uninfected PBMC from the same seronegative donor.

Monocytes from four HIV-1- and hepatitis C virus-seronegative donors were isolated using RosetteSep (a positive-depletion monocyte enrichment method; Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's protocol, which produces cell populations that are >99% CD14$^+$ monocytes with <1% B or T cells (CD3$^+$). MDM from four donors provided a 90% chance

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of detecting a twofold difference between virus- and mock-treated cultures, based on analysis of variance using a randomized complete block design, a two-sided t test, and a standard deviation of 0.5. Cells were induced to adhere to plastic at a concentration of 2×10^6 per ml and were incubated in differentiation medium comprised of Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, Md.) with 15% human AB serum (Sigma-Aldrich Corp., Saint Louis, Mo.) and a single treatment of 10 U of granulocyte-macrophage colony-stimulating factor (Invitrogen, Carlsbad, Calif.) per ml. After 7 days, >70% of cells expressed CD4 and >80% were positive for CCR5 as measured by flow cytometry (data not shown). After differentiation, CD14+, Fcγ receptor+ macrophages were the only cell type detected; no CD3+ or CD19+ T or B lymphocytes, respectively, were detected (49, 73). MDM cultures were transferred to GM-CSF-free media and treated on day 0 with 2 ml of either HIV-1$_{JR-FL}$ (multiplicity of infection, ~0.02) or mock supernatants for 24 h, washed, and cultured in GM-CSF-free media. Levels of supernatant p24 antigen on days 2, 4, and 7 were assessed using a specific enzyme-linked immunosorbent assay kit (Coulter, Hialeah, Fla.). Aliquots of cells were washed twice with phosphate-buffered saline and lysed in a nonionic detergent buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 2.5 mM MgCl$_2$, 0.1 mg of gelatin/ml, 0.45% NP-40, and 0.45% Tween 20) containing proteinase K (100 μg per ml) (K buffer) for quantitation of infected cells by TaqMan real-time PCR assay using HIV-1 gag as the analytical parameter and human apoB as a genomic template control. The primers and probe used for quantitation of apoB were as follows: forward primer, 5'-TGAAGGTGGAGGACATTCCTCTA; reverse primer, 5'-CTGGAATTGCGATTTCTGGTAA; probe, VIC (Applied Biosystems)–CGAGAATCACCCTGCCAGACTTCCGT–6-carboxy-tetramethylrhodamine (TAMRA). Viral gag gene copies by TaqMan were quantified using forward primer (5'-ACATCAAGCAGCCATGCAAAT-3'), reverse primer (5'-ATCTGGCCTGGTGCAATAGG-3'), and probe (5'–6-carboxy-fluorescein (FAM)–CATCAATGAGGAAGCTGCAGAATGGGATAGA–TAMRA–3') (19). Amplification reactions consisted of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif.), 450 nM (each) forward and reverse primers, and 125 nM probe in a total volume of 50 μl. The PCR conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Thermal cycling and data acquisition were performed using a Prism 7700 Thermal Cycler (Applied Biosystems).

RNA isolation and hybridization. Total RNA for each sample was extracted and prepared for hybridization according to protocols in the GeneChip Expression Analysis Technical Manual (Affymetrix). Briefly, cells from virus-treated or mock-treated cultures at days 0, 2, 4, and 7 were washed two times in MDM rinse medium and resuspended in RLT RNA lysis buffer (QIAGEN, Valencia, Calif.) for total RNA isolation (RNEasy RNA isolation kit; QIAGEN). RNA aliquots (8 μg) were reverse transcribed from a (dT)$_{24}$ primer into double-stranded cDNA, which served as a template for in vitro transcription to produce biotin-labeled cRNA that was fragmented under conditions of high magnesium concentration (1 mM) and heat. The quality of the cRNA was first assessed using Test 3 Genechip arrays (Affymetrix) before hybridization to HG-U95A (version 2) arrays. The arrays were stained by a streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, Oreg.) using the Affymetrix GeneChip Fluidic Station and scanned by an Agilent argon-ion laser with 488-nm emission and 570-nm detection (GeneArray Scanner). High-resolution chip images were analyzed using Microarray Suite version 4 (Affymetrix). Intensity values from each probe cell were adjusted for background and multiplied by a scaling factor to facilitate comparisons across the arrays. Images were converted into both average difference values and numerical (n-fold) change data based on comparison of virus-treated and mock-treated samples.

RNA data analysis. A custom relational database was designed using Microsoft (Redmond, Wash.) Access 2000 on the Windows 2000 platform. The database contained all raw data, as well as links between unique gene identifiers for mRNA and protein, functional categories, and summary of function. Functional categories of genes were based on the Gene Ontology Consortium public domain database (http://www.geneontology.org/). Cluster analysis was performed to gain an overview of temporal gene expression patterns within the data set. Prior to clustering, filters were applied so that a robust set of genes could be analyzed. Genes were first filtered to remove any transcripts that were considered absent under all conditions. A subsequent filter identified genes in infected cultures that displayed >4-fold change (induced or repressed) in hybridization intensities relative to levels in uninfected cultures. Average linkage hierarchical agglomerative clustering with uncentered correlation was performed on the median values obtained for each probe set for all donors at each time point using the software package Cluster (M. Eisen, Berkeley, Calif.). Major branches in the dendrogram were defined by correlation coefficients of >0.75 (mean, 0.85; range, 0.75 to 0.96)
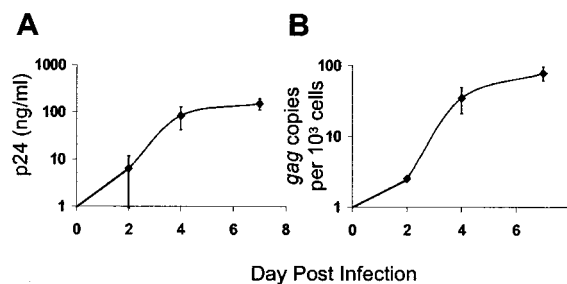

FIG. 1. Kinetics of HIV-1 replication and spread in MDM. (A) Supernatant p24 antigen. (B) Number of gag DNA copies per 10^3 cells. The data are means, with error bars representing standard errors of the mean for triplicate donors.

To identify subsets of genes that were expressed exclusively in HIV-1 or mock-treated cultures of MDM, queries were performed within GeneSpring version 4.1 (Silicon Genetics, Redwood City, Calif.). Genes from each treated population were subjected to three high-stringency statistical filters, which required that a gene be present in all donors, vary by no more than 0.5 standard deviations from the mean expression in all donors, and be induced at least twofold relative to the globally normalized background intensity for the entire data set. The combined effect of the filters identified a set of genes that was robust to outlier, interassay, and donor variability. All genes expressed in virus-treated cultures were compared to all genes expressed in mock-treated cultures by using Venn diagrams. Unique genes that were expressed by each population were used to create lists and import them into the custom relational database.

Protein extraction and PowerBlot screening. Monocytes (3×10^7 to 4×10^7) from each of four donors were plated in six-well plates (4×10^6 cells/well), differentiated for 7 days, and treated as previously described. Two of the four donors were assayed for both proteins and mRNA. After 48 h of virus treatment, the cells were lysed with boiling lysis buffer (10 mM Tris, pH 7.4, 1 mM sodium orthovanadate, 1% sodium dodecyl sulfate). Lysates were heated at 95°C for 30 s and passed 10 times through a 25-gauge needle to shear the DNA. Total protein yields were quantitated using the bicinchoninic acid protein assay (Pierce, Rockford, Ill.).

Immunoblot analysis of proteins was performed by BD Biosciences. Briefly, 5 mg of total protein per sample was loaded on a total of five gradient (4 to 15%) SDS-polyacrylamide gels and subsequently transferred to Immobilon-P membranes (Millipore). A total of 1,005 proteins were screened using different complex antibody cocktails. Digitally captured images of all blots from duplicate or triplicate runs of the two samples (HIV$_{JR-FL}$ and mock treated) were compared, and confidence values based on change and reproducibility were designated (semiquantitative). Only proteins with a confidence value of ≥3 were considered in the final analysis. Each protein was entered into our custom relational database and identified by locus link identification (ID) and Swiss Protein ID numbers, predicted and observed molecular weights, and functional categories. A query was designed in the database to link mRNA and protein data according to common locus link ID numbers.

RESULTS

CCR5-tropic viral replication and spread in MDM cultures. To relate gene expression to viral replication kinetics, the supernatant p24 antigen production and number of gag DNA copies were measured in MDM cultures over the course of 7 days (Fig. 1). In general, peak levels of supernatant p24 antigen (100 ng/ml) developed between days 4 and 7 following HIV$_{JR-FL}$ infection (Fig. 1A). Production of supernatant p24 levels paralleled virus spread based on cell-associated virus DNA copies (Fig. 1B). By 2 days posttreatment, ~0.2% of MDM harbored virus, based on one gag copy per cell, while at day 7, ~10% of macrophages were infected (Fig. 1B). Maximal infection of 10% of MDM by HIV-1$_{JR-FL}$ was consistent with other data from our laboratory and indicated that nearly 90% of macrophages in virus-treated cultures remained uninfected.
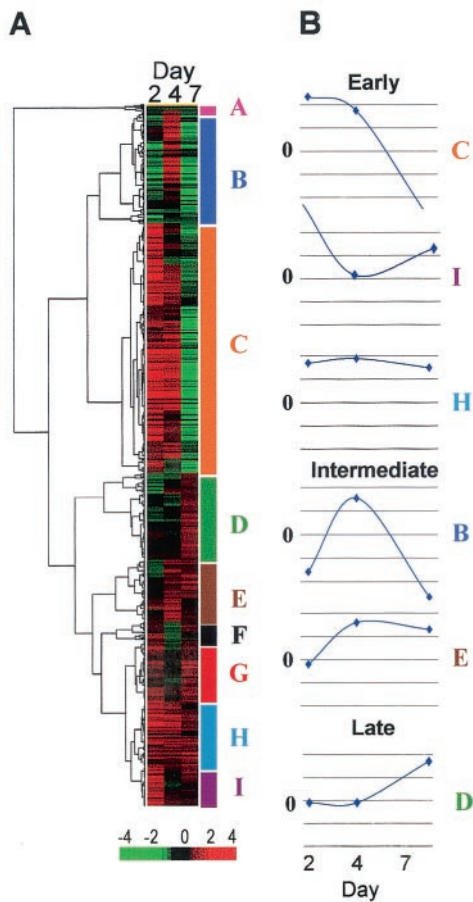
FIG. 2. Clustering of genes perturbed by HIV-1 infection of MDM. (A) Hierarchical agglomerative clustering of ~900 genes was based on expression patterns over time. Median induction (red) or repression (green) of expression ranged between +4 and −4 for each gene. Genes were distributed into nine cluster patterns designated A to I (color coded on the right [y] axis of the dendrogram). (B) Median induction or repression (*n*-fold) over time for ~800 genes in six clusters ranged between +2- and −2-fold. Three patterns of induction of gene expression were observed: early (day 2; clusters C, H, and I), intermediate (day 4; clusters B and E), and late (day 7; cluster D).
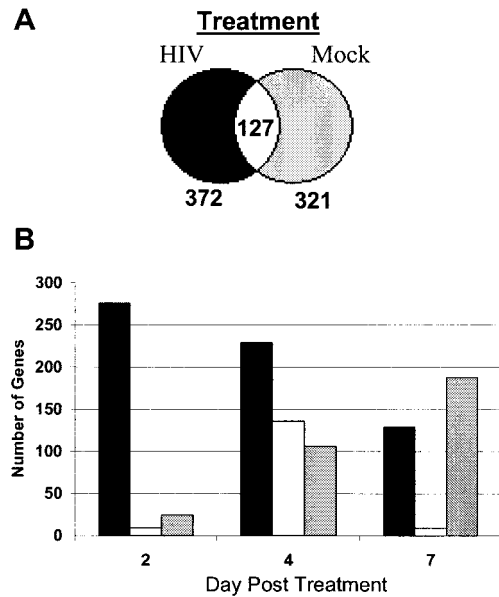

FIG. 3. Functional categories for genes expressed exclusively in infected or in uninfected MDM cultures. (A) Genes that were expressed only in virus-treated or mock-treated MDM cultures were identified by Venn diagram analysis. (B) Numbers of genes expressed exclusively in virus- or mock-treated cultures were identified on each day. Black bars, genes expressed uniquely in virus-treated cultures; gray bars, genes expressed uniquely in mock-treated cultures; white bars, coexpressed genes.

An impact of virus treatment on genetic networks in MDM, including both infected and uninfected cells, was compared to gene expression by uninfected cells in mock-treated MDM cultures. A 7-day time course was analyzed to provide an overview of genetic networks during the course of spreading virus infection.

Temporal expression of genes in HIV-1-treated MDM cultures. Approximately 900 genes with defined functions were induced or suppressed by >4-fold between HIV-treated and mock-treated cultures from any donor at any time point. Based on hierarchical agglomerative cluster analysis of expression patterns over time, the genes fell into nine clusters (Fig. 2A, A through I). Median induction or repression for the entire data set was ~4-fold.

Approximately 800 of the 900 functionally annotated genes fell into six major clusters (B, C, D, E, H, and I). Patterns of expression over time for clusters of genes were represented as waveforms that displayed the median change for the population of genes within each cluster (Fig. 2B). More than 50% of the genes (465 of 808) were induced as early as 2 days following HIV-1 treatment and were included in clusters C, H, and I (Fig. 2B). About 28% of the genes (231 of 808; clusters B and E) were induced at the intermediate day 4 time point, while 14% of the genes (112 of 808; cluster D) were induced late, by day 7 following treatment (Fig. 2B). Although the genes were classified by temporal patterns of induction of expression, distinct patterns of gene repression over time were also apparent.

Genes expressed exclusively in virus-treated or mock-treated MDM cultures. Temporal analysis required that genes be expressed in both virus- and mock-treated MDM cultures and excluded genes that were expressed only under one or the other condition. To determine if genes might be expressed exclusively in virus- or mock-treated MDM, a series of statistical filters was applied to the data set and the results were summarized using Venn diagrams (Fig. 3A). Analysis identified 372 genes that were expressed exclusively in virus-treated macrophage cultures at all time points, 321 genes that were expressed exclusively in mock-treated cultures, and 127 genes that were expressed by MDM independent of treatment.

A second set of queries examined mutually exclusive gene expression on different days of treatment (Fig. 3B). A dramatic difference in the number of genes expressed exclusively by HIV- or mock-treated cultures was apparent as early as day 2 (Fig. 3B). The number of genes expressed exclusively in virus-treated cultures gradually decreased from 260 to 130 over 7 days (Fig. 3B). Simultaneously, the number of genes that were expressed exclusively in mock-treated cultures increased from <50 to 190 (Fig. 3B).

TABLE 1. Functional categories of genes with altered mRNA expression in MDM following HIV-1 treatment

Gene function[a]	No. of genes[b]			
	E	I	L	Total
Adhesion and extracellular matrix	31	15	6	52
Apoptosis	14	2	5	21
Cell proliferation	20	14	4	38
Cell stress	8	8	1	17
Cell-cell signaling	21	8	5	34
Chromatin structure	7	3	0	10
Cytoskeleton and cell motility	33	24	12	69
DNA binding protein	7	4	0	11
DNA synthesis and repair	14	8	3	25
Energy metabolism	42	25	14	81
Hematopoetic	3	5	8	16
Hormone or growth factor	23	10	4	37
Immune response	27	17	13	57
Inflammatory and coagulation	10	3	3	16
Ion and solute transport	28	12	7	47
Protein metabolism	31	20	10	61
RNA binding protein	16	3	0	19
Signal transduction	37	19	3	59
Transcription	52	14	5	71
Tumor related	21	7	3	31
Vesicle related	20	10	6	36
Total	465	231	112	808

[a] Gene function was assigned based on Gene Ontology Consortium categories.
[b] The numbers of genes within each category for each cluster are tabulated. The total number of genes for each category across all clusters is also tabulated in the final column. Only gene categories with ≥10 genes per category are included for six of the nine clusters, resulting in omission of some genes. E genes changed during the early (day 2) timepoint; I, intermediate (day 4) timepoint; L, late (day 7) time point.

Functional categories of genes modulated by HIV-1. More than 800 genes that were summarized by temporal expression (Fig. 2) were subsequently classified into 21 functional categories as defined by the Gene Ontology Consortium. About 17% of the genes (142 of 808) were classified in the energy or protein metabolism category (Table 1). More than 30% (258 of 808) were grouped into categories with related functions, such as apoptosis, cell proliferation, cytoskeletal and cell motility, signal transduction, or transcription, while 11% of the genes (89 of 808) were classified into the hematopoietic, immune response, or inflammation category.

Similar analysis of mutually exclusive expression profiles (Fig. 3) identified genes from multiple functional categories (data not shown). In particular, HIV-1 treatment altered the expression of genes in a subset of functional categories related to immune function, cell cycle proliferation, and cell stress. In contrast, macrophages in mock-treated cultures expressed few, if any, genes in these categories. Patterns of mutually exclusive expression of genes provided a signature for virus treatment.

HIV impacts similar genes in lymphocytes and macrophages. To determine if similar target genes were altered by HIV-1 treatment in different cell types, we compared our data from primary macrophages with those of other studies that analyzed an impact of HIV-1 on gene expression in transformed monocytic or lymphoid cell lines (Table 2) (8, 9, 13, 18, 26, 28, 29, 31, 33, 40, 42, 47, 52, 54, 61, 63, 64, 65, 67, 79, 81, 82, 83). Twenty genes, including 4 exact matches and 16 that were similar based on function or alternate isoforms of the same

gene product, were induced following virus treatment (Table 2). In addition, treatment with HIV-1 repressed 38 genes, including 11 that were exact matches and 27 that were similar by function (Table 2). For example, both CD4 mRNA levels and CD4 cell surface expression were down regulated by virus treatment in cell lines and MDM (13, 73). In addition, HIV-1 induced expression of ERF, BCL2, and IFRD2 genes involved in regulation of the cell cycle in both monocytic and lymphocytic cells, as well as macrophages (Table 2). Therefore, we focused our subsequent analysis to determine the extent to which cell cycle mediators might be impacted by virus in macrophages.

HIV-1 impact on genes involved in regulation of the cell cycle in MDM cultures. Database queries were performed to focus analysis on genes in the cell cycle category that were preferentially impacted by HIV-1 in MDM. Twelve genes involved in regulating cell cycle transitions were expressed exclusively in mock-treated MDM cultures (Table 3). Seventeen other genes that are implicated in transitions through cell cycle checkpoints were expressed uniquely in HIV-treated cultures (Table 3). When ratios for expression of cell cycle genes in HIV- and mock-treated MDM cultures were evaluated, temporal patterns were apparent. Sixteen genes involved in cell cycling were induced in virus-treated cultures by day 2 (early) and subsequently were repressed or returned to near-baseline values (Table 3). Ten genes were induced exclusively by day 4 (intermediate time point), while four genes were induced by day 7 (late).

Qualitative identification of genes involved in cell cycle regulation checkpoints. A number of genes involved in regulating G_1/S or G_2/M checkpoints in the cell cycle were detected exclusively in mock-treated MDM cultures (Fig. 4). For example, CDC2, CDKN1B, and GAS1 genes are implicated in G_1/S transition, while CDC2 and PCTAIRE1 (a gene homologous to the CDC2 gene), CDC2-like kinase (CLK2), and cyclin B2 (CCNB2) genes are required for G_2/M transition. In contrast, none of these genes was expressed in virus-treated cultures. Expression by only four genes that are involved in the maintenance of G_1 (G_1/S transitioning) was detected uniquely in virus-treated cultures. Most of the genes uniquely expressed following virus treatment, including protein phosphatase 2A (PP2A), breast cancer 1 (BRCA1), and growth arrest and DNA-damage-inducible (GADD45) genes, were involved in arresting cell cycling at the G_2/M checkpoint (Fig. 4).

Altered protein expression in macrophages treated with a CCR-5-tropic virus. To determine the effect(s) on protein expression in MDM of treatment with a CCR5 strain of HIV-1, >1,000 proteins from virus- or mock-treated cultures were assayed and ranked based on changes. High-throughput Western blot screening with monoclonal antibodies evaluated levels rather than posttranslational modifications, including phosphorylation, that can be critical determinants of function for some proteins. Overall, 300 proteins had detectable differences of ≥1.5-fold in expression in virus-treated MDM from each donor, including ~50 proteins that can have a functional role in regulation of the cell cycle or cell proliferation. A subset of proteins is presented in Fig. 5. For example, ubiquitination protein (UbcH6 and UbcH7) levels were reduced, while the levels of retinoblastoma binding protein 4 (RBBP) and interferon-induced protein kinases (p38 MAPK and ERK) and a

TABLE 2. Genes induced or repressed by HIV-1

Status	Genbank no.	Description[a]	mRNA[b]	Protein[b]
Induced	M75099	FK506 binding protein[d]	61	
	NM005347	HSP70[d]	77	77
	U15655	**Ets repressor factor (ERF)[d]**	61	
	M13995	**BCL2[d]**	78	7
	AA427491	TCR alpha, c region	30	
	D87116	Map kinase kinase 3	61	
	J03909	Interferon-inducible protein (IP-30)	61	
	L27584	Ca^{2+} channel B3 subunit	61	
	M13755	Interferon-induced 17/15 kDa	61	
	M20681	GLUT3	25	
	M60485	FGF receptor	61	28
	M61827	Leukosialin (CD43)	61	50
	M62831	Transcription factor ETR101	61	
	M77476	Helix-loop-helix zipper protein	61	
	S63368	TNF receptor 75 kDa	61	12
	U09585	**IFRD2**	61	
	U09848	Zinc finger protein (ZNF139)	61	
	U24576	Breast tumor autoantigen	61	
	XM055937	HSP27	77	77
	NM001228	Caspase 8	8	53
Repressed	D13748	EF 4A1[d]	61	
	J02683	ADP/ATP carrier protein[d]	61	
	J03191	Profilin[d]	61	
	L15189	HSP 75[d]	64	
	L25080	rhoC[d]	61	
	M14630[c]	Prothymosin alpha[d]	61, 30	
	NM000616	CD4[d]	32	17, 76
	U90313	GST homolog[d]	61	
	X01060	Transferrin receptor[d]	61	62
	AR201243	MHC-11[d]	74	41
	AA401111	Glucose phosphate isomerase	30	
	AA455931	Adenylosuccinate lyase	30	
	AF006082	ARP2	61	
	D11086	IL-2-R gamma	61	39
	J03592	ADP/ATP translocase	61	
	L00160	Phosphoglycarate kinase	30	
	M27132	ATP synthetase beta subunit	61	
	M84326	ADP ribosylation factor 1	61	
	U36764	TGF-beta R-interacting protein 1	61	
	U94855	Translation initiation factor 3	61	
	X54473	COX Vib	61	
	X69549	Rho GDP-dissociation inhibitor 2	61	
	X79538	Translation initiation factor nuk34	61	
	Y08890	RanGTP binding protein 5	61	
	Z37166	Nuclear RNA Helicase	61	

[a] Regulators of cell cycle (boldface underlined). TCR, T-cell receptor; TNF, tumor necrosis factor; GST, glutathione S-transferase; MHC, major histocompatibility complex; IL-2, interleukin-2; TGF, transforming growth factor.

[b] Numbers correspond to citation numbers of publications that have documented induction or repression of these genes at either the mRNA or protein level during HIV-1 treatment.

[c] Addition M26708.

[d] Exact matches based on GenBank accession number.

related signal transducer and activator of transcription factor, STAT 5A, were induced. Cell cycle control was modified following virus treatment of macrophages through increased levels of MCC (a negative regulator of cell cycling) (15, 69), in combination with repression of PP2A catalytic alpha subunit, the nuclear Dbf2-related (Ndr) family of protein serine-threonine kinases (negative regulators of CDK1 required for completion of the cell cycle) (70), and BM28 (a protein associated with cell division) (72).

The overall effects of HIV-1 in MDM on specific networks of cellular mRNA and proteins that promote transition from G$_1$ to G$_2$ from our data are integrated in Fig. 6. Global effects include G$_2$ arrest and suppression of mitosis through two path-

ways. One pathway, as expected, involved the PP2A complex, presumably through a Vpr-dependent mechanism (22, 23, 33). Both induction of the PP2A regulatory subunit and repression of the catalytic subunit can promote the enzymatic activity of Wee1 and inhibit the phosphatase CDC25 (41), resulting in hyperphosphorylation of CDC2 that leads to G$_2$/M arrest (46, 53). Inactivation of CDC25 prevents dephosphorylation of CDC2, thus inhibiting mitosis (32, 41). Induction of mRNA and protein levels of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein, epsilon polypeptide (YWHAE), which blocks CDC25, also promotes G$_2$ arrest (Fig. 6). The alternate pathway involves the nonphosphorylated form of the CDC2/CCNB2 complex. HIV-1 induces

TABLE 3. Differentially expressed genes involved in cell cycling

Unique genes[a]				Cluster analysis[b]					
Mock treated		Treated		Early		Intermediate		Late	
GenBank no.	Description	GenBank no.	Description	GenBank no.	Description	GenBank no.	Description	GenBank no.	Description
AF053305	BUB1L	M25753	CCNB	D43969	AML1	M14752	ABL	Y11284	AFX
AL080146	CCNB2	U47414	CCNG2	U77629	ASCL2	AF045581	BRCA1	U82759	HOX1
X05360	CDC2	U65410	MAD2L	L36844	INK4B	M29551	Calcineurin A2	A1018098	Integrin Beta 1
U02570	CDC42-GAP	AF053306	MAD3L	X06292	C-FES	X66364	CDK5	M26682	Rhombotin-1
L29218	CDC-like 2	X98263	MPP-6	X86371	HUGL1	AF049703	ESE2		
AI304854	CDKN1B	L07590	PP2A B	U59464	PTC1	L06895	MAX-BP		
AF048731	Cyclin T2	M69199	G0/G1 Switch	Y11306	TCF4	AB010419	MTG16		
Z36714	FBX1	X71440	CDK3	AJ000414	CIP4	AF059681	AIK3		
L13698	GAS1	X61123	BTG1	AF038198	Chordin	L19267	DM9		
U26727	INK4	M29551	Calcineurin A2	X70991	MADER	M15881	Uromodulin		
X66363	PCTAIRE1	L25876	CIP2	S72043	Metallothionein III				
D38305	TROB1	X06292	C-FES	U33822	TAX BP 181				
		X55504	NOP120	M31523	TCF3				
		X66363	PCTAIRE1	Z47363	TCF7				
		AB014561	RB-BP	X66362	PCTAIRE3				
		Z11773	ZNF187	AF071538	PDEF				
		D84557	MCM6						

[a] Specific genes unique to either virus- or mock-treated cultures are grouped.
[b] Genes were clustered into groups based on whether they are induced at early, intermediate, or late time points posttreatment.
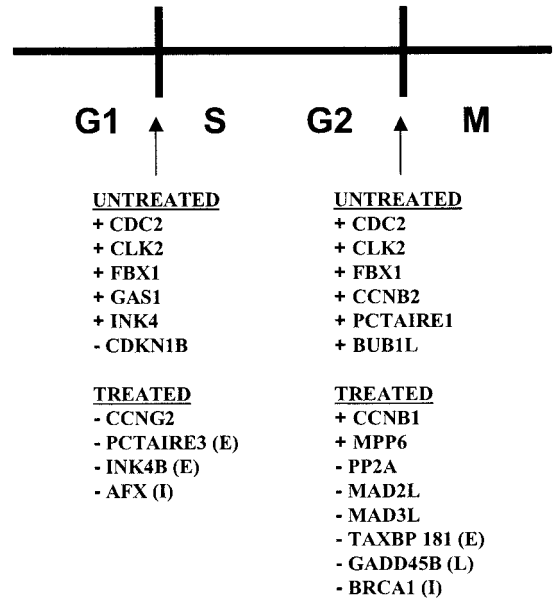

FIG. 4. Impact of HIV-1 infection on cell cycle genes in MDM. Schematic of the cell cycle, with G_1, S, G_2, and M indicated. Genes expressed differentially by cluster analysis or mutually exclusive analysis in mock- or virus-treated MDM cultures are listed below the G_1/S and G_2/M checkpoints. Genes marked + promote progression through cell cycle checkpoints. Genes marked − inhibit progression through cell cycle checkpoints. Time points at which genes were detected are indicated in parentheses: early (E), intermediate (I), and late (L).

BRCA1 and subsequently GADD45 mRNA levels, which results in the inactivation of CDC2 and G_2/M arrest (38, 77). BRCA1 is a downstream target of Rad3-related protein (ATR) phosphorylation, and HIV-1 Vpr activates ATR (5, 60, 84). In addition, HIV-1 induces a CDK inhibitor, p21$^{\text{Cip1/Waf1}}$ protein, which blocks the CDK-activating kinase (CAK) phosphorylation of the cyclin D/CDC2 complex (10, 71), promoting G_2 arrest. Activation of ERKs by phosphorylation can induce p21$^{\text{Cip1/Waf1}}$ (6, 58), and activation of MEK/ERK is reported to occur in T-cell lines following infection with HIV-1 (57).

DISCUSSION

The overall impact of HIV-1 on gene expression patterns in MDM is global and complex. Perturbations by virus clearly distinguished between HIV-1 and mock-treated cultures even though <0.2% of MDM treated with virus were infected by day 2 and only 10% were infected by day 7. The limited frequency of infected macrophages ex vivo resembles restricted HIV-1 infection of lymphoid tissue macrophages or alveolar macrophages in vivo (12, 50, 62). The number of infected MDM was estimated based on the premise of one viral DNA copy per cell, although cells in vivo can be infected by more than one virus (39). Even if estimates of infected MDM in our study exceed the actual numbers of cells that harbor virus, interpretation of the data would remain the same. Even though low levels of supernatant p24 antigen or cell-associated viral DNA were detected 2 days following HIV-1 treatment, expression of >400 genes at the mRNA or protein level changed relative to mock treatment. In addition, mutually exclusive
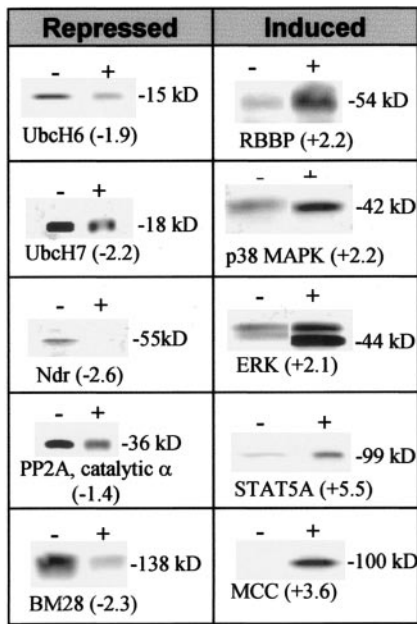
FIG. 5. Altered protein expression in MDM. Expression levels of representative proteins determined by Powerblot analysis from mock (−)- or HIV$_{JRFL}$ (+)-treated monocyte-derived macrophages. Digitally captured images of all blots from duplicate runs were semiquantitated for changes compared to relative controls (indicated in parentheses). Proteins with changes of >1.5 were confirmed on duplicate verification blots.

expression at day 2 of ~250 genes distinguished virus- from mock-treated MDM. The number of genes in macrophages altered by treatment with a CCR5 strain of HIV-1 in our analyses was similar to the number of genes in primary peripheral blood mononuclear cells or MDM that were altered by CCR5 gp120 treatment (16) but greater than the number of genes in lymphocytic or monocytic cell lines identified by treatment with CXCR4 strains of HIV-1 (28, 61, 63). Differences between primary cells and cell lines could reflect methodologies for measuring changes in mRNA or the types of viruses, as well as the possibility that transformed cell lines could have elevated basal levels of expression by genes that are altered in primary cells by HIV-1.

The number of genes expressed exclusively in mock-treated MDM increased over time, suggesting that macrophages in culture are dynamic rather than static. Increases in the number of genes expressed in the absence of virus could reflect actual changes induced by culture conditions or apparent changes reflecting down regulation of genes over time in virus-treated macrophages. Loss of cell viability is an unlikely explanation, because macrophage viability is >90% for 14 to 21 days following differentiation and infection (73) in contrast to rapid depletion of CD4 T lymphocytes (74). Either way, the net effect would be an increase in the number of genes expressed exclusively in mock-treated MDM over time. Clear differences between HIV-treated and mock-treated cells occur on day 2, which corresponds to the completion of first-round replication. The results agree with our earlier data showing that signal cascades are initiated by early virus-cell interactions (43). Expression of mutually exclusive genes over the course of the study provided sentinel biomarkers that characterized the effects of virus treatment on macrophage populations and dis-
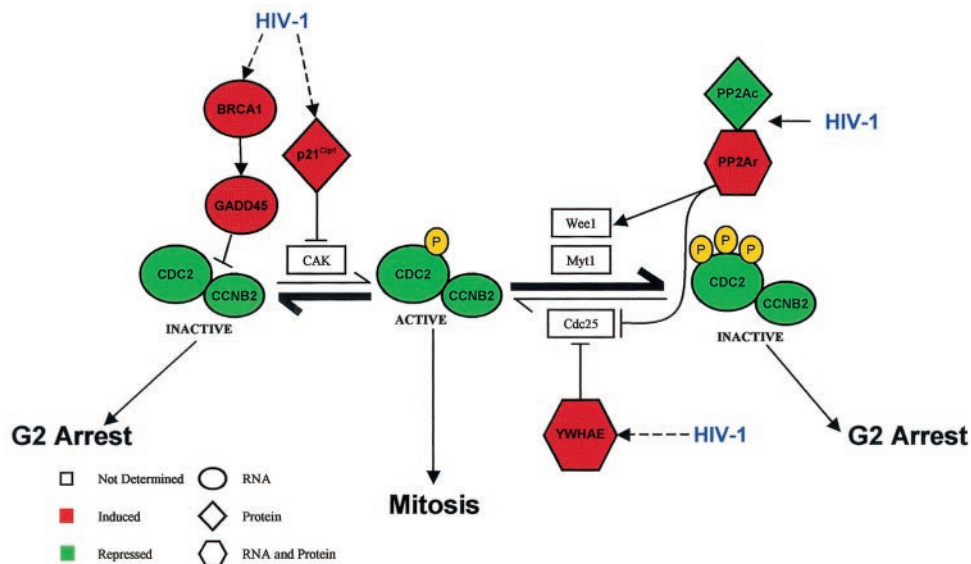

FIG. 6. Summary of impacts of a CCR5 strain of HIV-1 on cell cycle regulators in MDM. Progression through G$_2$ is regulated by phosphorylation states of the CDC2-cyclin B2 complex. Reversible phosphorylation reactions of the CDC2-cyclin B2 complex determine active (phosphorylated) or subsequent inactive (hyperphosphorylated) states. Hyperphosphorylated CDC2-cyclin B2 complex can be reactivated through dephosphorylation catalyzed by CDC25. HIV-1 exposure induces the expression of cell cycle regulators (PP2A, BRCA1/GADD45, p21^{Cip1}, and YWHAE) involved in arresting MDM in G$_2$. Inactive CDC2-cyclin B2 complex is promoted in HIV-1-treated cells (boldface arrows). Red indicates factors that were induced, and green indicates factors that were suppressed. Ovals, diamonds, and hexagons indicate whether the differential expression was detected at the level of mRNA, protein, or both, respectively.

tinguished unequivocally between mock- and virus-treated macrophages.

Using a systems biological approach, we identified substantial changes in mRNA and protein levels within the HIV-1-treated cultures that might occur exclusively in the small subset of macrophages that were infected directly, in the uninfected bystander cells, or in both types of cells. The possibility of residual CD4$^+$ T lymphocytes in the MDM cultures was ruled out by flow cytometry and an inability of MDM cultures to support entry or replication by non-macrophage-tropic viruses (75). A difference between direct and bystander effects of virus in macrophage cultures at early time points would be difficult to evaluate, because so few macrophages were infected and strategies to separate infected from uninfected macrophages could alter mRNA or protein profiles. Nonetheless, the fact that a distinct subset of genes was detected in mock-treated MDM cultures suggested that uninfected cells not exposed to virus differed from uninfected cells in virus-treated MDM. Bystander effects could be mediated by cytokine expression from infected cells that would influence neighboring uninfected cells in an exocrine fashion. Alternatively, soluble viral components, such as gp120, could mediate changes in signal transduction cascades that impact downstream gene expression in the absence of infection (14, 17, 36, 43, 65).

A number of genes that were reported in other studies as induced or repressed at the mRNA or protein level in monocytic or lymphocytic cell lines infected by CXCR4 strains of HIV-1 were also detected in our biological systems approach involving primary macrophages treated by a CCR5 strain of HIV-1 (8, 13, 28, 29, 47, 61, 78, 79, 81, 86). Similarities among genes that were induced or repressed during treatment of primary human macrophages or cell lines by CCR5 or CXCR4 strains of virus, respectively, suggest that elements of virus-host cell interactions are highly conserved regardless of the viral envelope, the coreceptor preference, or even the cell type infected, which could have implications for developing novel therapeutics.

Normal macrophage differentiation involves G$_1$ arrest (83), which would explain preferential expression of genes mediating the maintenance of G$_1$ phase in mock-treated MDM in our studies. Even though differentiated cells are typically defined as nondividing or nonproliferating, differentiated cells can maintain a capacity to shift in their cell-cycling state following adequate stimulation (48). Our data are consistent with studies that indicate alteration of the cell cycle stage in macrophages by HIV-1 infection and suggest that the intracellular environment of cells no longer in G$_1$, rather than cell division itself, plays a role in virus replication in nondividing cells, such as macrophages (33, 64, 80). G$_2$/M arrest in lymphocytes is critical for optimal gene expression by long terminal repeats (4, 85), while transition from G$_1$ to G$_2$ in MDM correlates with enhanced HIV-1 replication (35, 68). An ability by HIV-1 to mediate transition from G$_1$ to G$_2$ could contribute to the fitness of viruses in MDM (34), although G$_2$-dependent enhancement of long-terminal-repeat-mediated gene expression in macrophages is unclear. The theme of virus-induced cell cycle arrest, specifically in G$_2$/M, is not unique to HIV-1. For example, reovirus and herpes simplex virus also mediate G$_2$/M arrest via the CDC2-cyclin B pathway (23, 37, 55). A virus-dependent transition from G$_1$/S to G$_2$/M in macrophages could

impact MDM physiology and function and contribute to immune dysfunction. The potential for more extensive involvement with the cell cycle by HIV-1, beyond the known role of Vpr, warrants further examination.

Patterns of induced or repressed genes identified within virus-treated MDM cultures have dynamic implications for a cascade of downstream effects that can alter cellular physiology. Our studies focused on global differential gene expression induced in association with the viral replication cycle, similar to the global effects associated with gp120-macrophage interactions (16, 78). In contrast to approaches that analyze single genes or pathways, global analysis provides an overall view of networks and interactions between known and novel genes and can provide insight into macrophage physiology and immune deficiency.

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