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Differential transcriptional regulation by human immunodeficiency virus type 1 and gp120 in human astrocytes

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Astrocytes may be infected with the human immunodeficiency virus type 1 (HIV-1) or exposed to the HIV protein gp120, yet their role in the pathogenesis of HIV dementia is largely unknown. To characterize the effects of HIV on astrocytic transcription, microarray analysis and ribonuclease protection assays (RPA) were performed. Infection of astrocytes by HIV or treatment with gp120 had differential and profound effects on gene transcription. Of the 1153 oligonucleotides on the immune-based array, the expression of 108 genes (53 up; 55 down) and 82 genes (32 up; 50 down) were significantly modulated by gp120 and HIV infection respectively. Of the 1153 oligonucleotides on the neuro-based array, 58 genes (25 up; 33 down) and 47 genes (17 up; 30 down) were significantly modulated by gp120 and HIV infection respectively. Chemokine and cytokine induction occurred predominantly by HIV infection, whereas gp120 had no significant effect. These results were confirmed by RPA. The authors conclude that profound alterations of astrocytic function occur in response to HIV infection or interaction with viral proteins, suggesting that astrocytes may play an important role in the pathogenesis of HIV dementia. *Journal of NeuroVirology* (2003) **9,** 358–371.

Keywords: astrocyte; chemokine; cytokine; gp120; HIV; microarray; RPA; transcription

Introduction

Astrocytes are the most numerous cell type in the brain. They are an important component of the blood-brain barrier and provide a critical stimulatory and supportive role that affect neuronal excitability and function. Additionally, astrocytes are immune-responsive cells and have the ability to produce a number of cytokines and chemokines. Thus, a disruption of astrocyte function could have devastating effects on cerebral function. The role of astrocytes in the pathogenesis of human immunodeficiency virus (HIV) dementia is poorly understood. Astrocytes may be infected with HIV. Viral replication peaks within 3 days and then gradually evolves into a latent infection (Tornatore *et al*, 1991). Besides direct infection of astrocytes, HIV can also indirectly modulate astrocyte function by the release of viral proteins from HIV-infected mononuclear cells or microglia (Nath and Geiger, 1998). We have previously shown that the HIV envelope protein gp120 can bind to unique receptors on astrocytes (Ma and Nath, 1997) and induce an influx of extracellular calcium (Holden *et al*, 1999). Hence, understanding the full array of direct and indirect effects of HIV infection on astrocytes is essential to better understand the pathogenesis of this disease entity. Identification of common underlying mechanisms may point to potential therapeutic targets.

To develop a better understanding of the direct and indirect mechanisms by which HIV may modulate astrocyte function, we either infected human astrocyte cultures with HIV or treated astrocytes with the HIV protein gp120 and analyzed the effect on

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transcription by microarray analysis and compared these observations to those obtained with ribonuclease protection assays (RPAs).

Results

Correlation between replicants and across treatment effects on astrocytes

Given the myriad of effects seen in astrocytes that have been attributed to HIV or its proteins, we undertook a cDNA microarray analysis to determine the effects that infection by HIV or exogenous treatment of gp120 had on gene expression of astrocytes. The pseudocolor images of representative arrays can be seen in Figure 1**A**. The coefficient of variance between the replicates was found to be approaching 1, as can be seen by in Figure 1**B**. With a P value of .05, the odds of finding significant genes at 1.5 s.d. by chance out of 1000 genes would be $6.25 \leq 10^{\leq 6}$ percent or $6.25 \leq 10^{\leq 3}$ genes. To add an extra level of stringency, we sorted the results that were altered by 1.5 s.d. using a two-tailed t test, reporting only those that had a P value of .001 or less. Therefore, the following results are altered by at least 1.5 s.d. and have a P value of .001 or less. We found that a 3-day infection with HIV or exogenous treatment of primary human astrocytes with the HIV protein gp120 did indeed have a sweeping and profound effect on gene regulation. However, each of these treatments regulated a unique set of genes, as can be seen in the following. Similar effects on a broad array of genes have been seen in HIV-infected lymphocytes at 3 days post HIV infection (Geiss *et al*, 2000). Another group has found that gp120 has similar effects on peripheral blood mononuclear cells and monocytes-derived macrophages (Cicala *et al*, 2002). Recently, 15 genes were found to be up-regulated in astrocytes following infection by HIV or gp120 treatment using a rapid subtraction hybridization technique (RaSH) (Su *et al*, 2002). Because gene accession numbers were

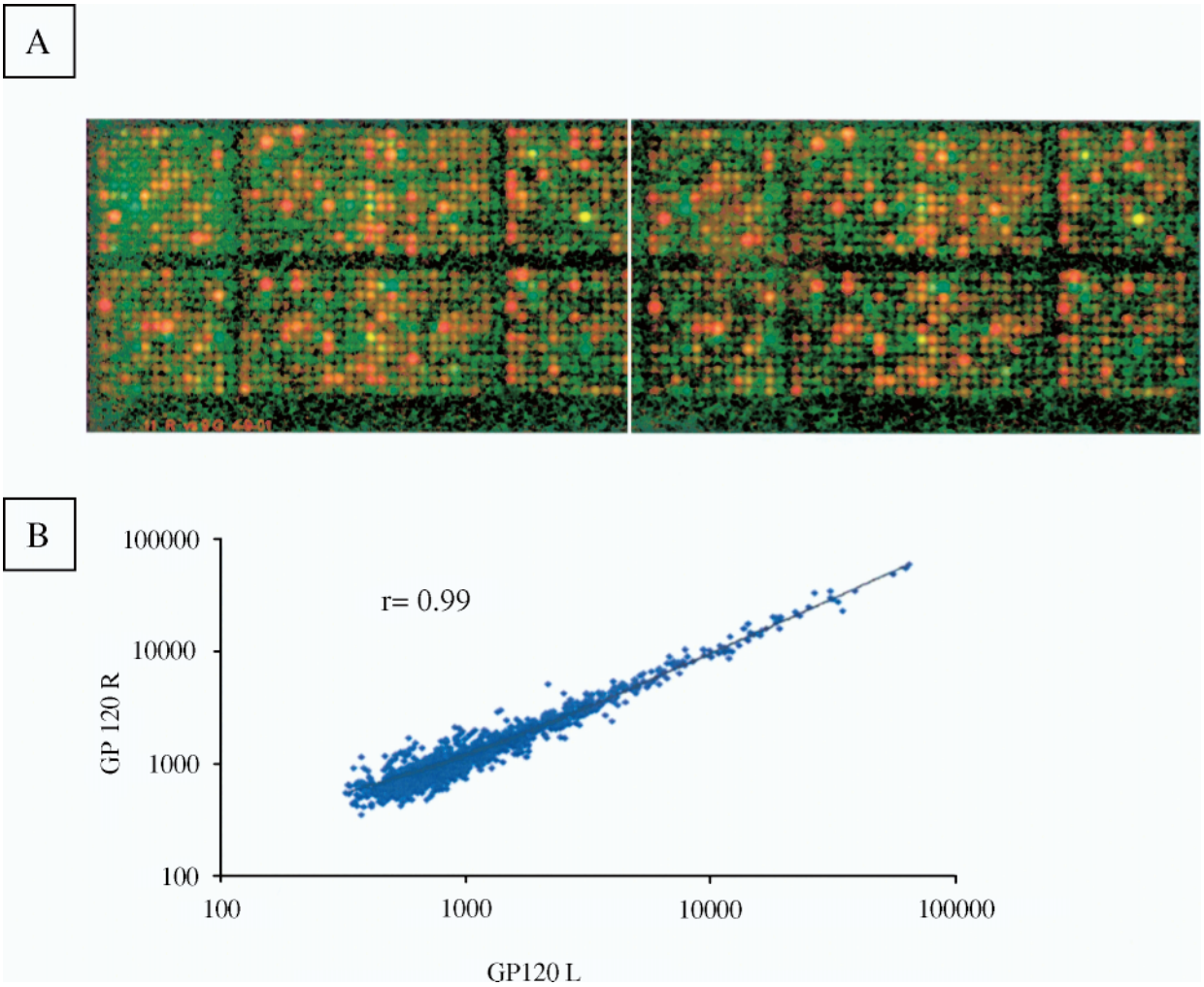

Figure 1 (**A**) Images of microchip arrays show superimposition of signals obtained from HIV-infected astrocytes (*red*) and gp120-treated astrocytes (*green*). Each array had duplicate sets of genes as shown separated by a white line. Excellent correlation is seen between the replicates. (**B**) Signals obtained from replicate samples from gp120-treated astrocytes are plotted against one another (L = left array; R = right array).

Table 1 Genes modulated in astrocytes by HIV proteins and HIV infection

	gp120		*HIV*	
	Up	*Down*	*Up*	*Down*
NeuroArray (1153 genes)	25	33	17	30
ImmunoArray (1153 genes)	53	55	32	50

not provided, we are unable to make direct comparisons between the two studies.

Of the 1153 oligonucleotides included on the neuro-based array, gp120 treatment had the most profound effect, which modulated 58 genes, whereas HIV infection affected only 47 genes. Of the 1153 oligonucleotides included on the immune-based array, HIV infection of astrocytes modulated 82 genes, whereas gp120 treatment of astrocytes modulated 108 genes. Interestingly, with both of the treatment conditions and with each array, nearly half of the affected genes were up-regulated and an equal number were down-regulated (Table 1). Analysis of individual genes modulated by each of the treatments showed that clear differences emerged between the groups. The categories of genes modulated varied from structural genes, immune-specific modulators, and adhesion molecules to transcriptional machinery and signaling pathways. All the genes modulated by at least 1.5 s.d. and having a P value of $\leq$.001 are listed in Tables 2A to 2D.

Despite the disparate changes with each of the treatments, there were only 27 genes that were modulated by both treatments as determined by Z-ratios greater than 1.5 s.d. and having a P value of .001 or less. Of these, two genes were up-regulated (Table 3A), whereas 15 were down-regulated by both treatments (Table 3B). Interestingly, a number of genes showed a reciprocal relationship between the exogenously applied gp120 versus HIV infection. Two genes were activated by the viral protein gp120, but down-regulated by HIV infection (Table 3C), whereas another eight genes were suppressed by gp120 treatment while up-regulated by HIV infection (Table 3D).

Ribonuclease protection assay

To confirm the results obtained by the microarrays, we analyzed the effect of each of the treatment paradigms on select genes by ribonuclease protection assay and compared the results to those obtained from the microarrays. The multiprobe sets used in the analysis detect several chemokines and cytokines. These were chosen due to the potential effects that the proteins have in the pathogenesis of HIV-associated dementia.

Out of the 16 genes assessed in the two assays (Figure 2), chemokine and cytokine transcription

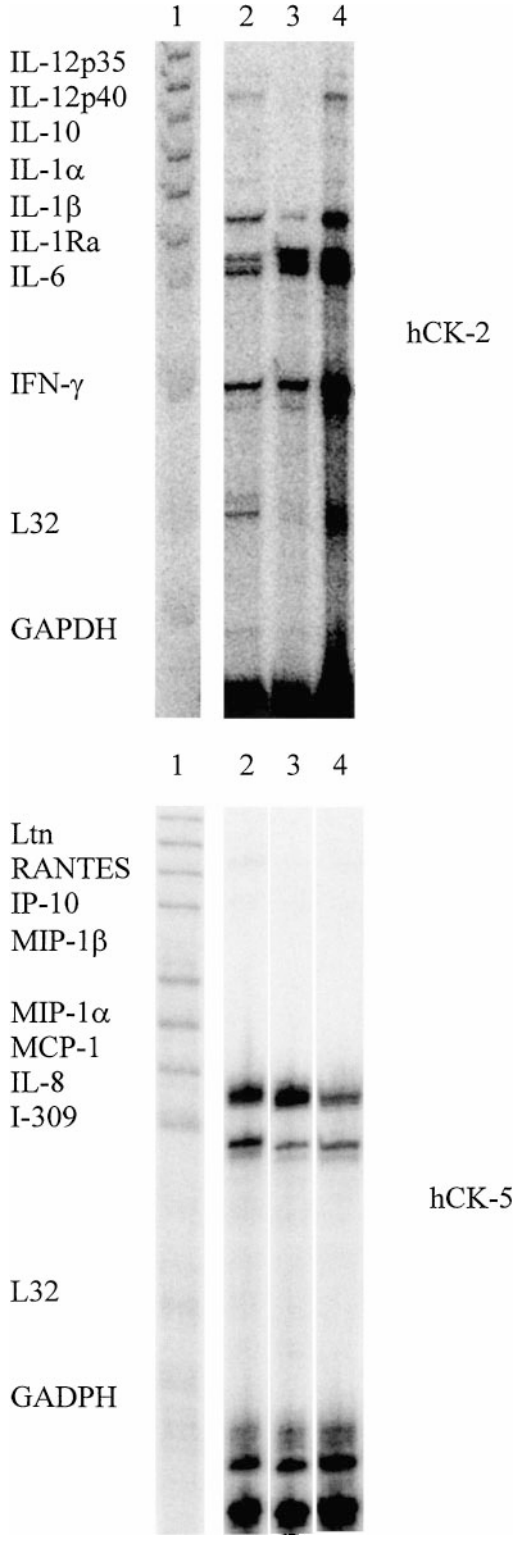

Figure 2 Representative images of ribonuclease protection assay gels derived from use of the Pharmingen hCK-2 and hCK-5 multiprobe sets and RNA derived from the following treatment groups. Bands are marked according to their respective transcripts from the lane containing only template. Lane 1: nonhybridized probes; lane 2: 3-day infection group; lane 3: 250 nM gp120 treated; lane 4: untreated.

Table 2A Genes up-regulated from NeuroArray*

	Abbreviation	*Accession no.*	P *value*	Z-*ratio*
gp120				
Gamma-aminobutyric acid (GABA) A receptor, pi	GABRP	AA102670	1.554E ≤ 15	2.920027
Destrin (actin-depolymerizing factor)	ADF	AA424824	0	2.784284
Histidine triad nucleotide-binding protein	HINT	T57556	0	2.754984
MORF-related gene 15		T90438	2.023E ≤ 07	2.72391
Protease, serine, 2 (trypsin 2)	PRSS2	AA284528	2.22E ≤ 16	2.136004
Syndecan-binding protein (syntenin)	SDCBP	AA456109	9.496E ≤ 05	2.044939
Protein tyrosine kinase 9	PTK9	AA019459	2.125E ≤ 06	2.032953
Protein phosphatase 2, regulatory subunit B (B56), beta isoform	PPP2R5B	AA129171	5.99E ≤ 06	2.013272
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITGA3	AA424695	1.815 ≤ 07	1.91515
Catenin (cadherin-associated protein), alpha 1 (102 kDa)	CTNNA1	AA676957	0.0004831	1.887905
Signal sequence receptor, alpha (translocon-associated protein alpha)	SSR1	AA099394	1.878E ≤ 05	6406
Protein kinase C–binding protein 1	PRKCBP1	AA480906	1.064E ≤ 07	1.85846
Heme oxygenase (decycling) 2	HMOX2	AA626370	5.59E ≤ 05	1.842608
Histone acetyltransferase 1	HAT1	AA625662	3.355E ≤ 05	1.830326
RNA helicase-related protein	RNAHP	T56281	2.951E ≤ 08	1.785728
Protein kinase, X-linked	PRKY	W24161	4.015E ≤ 06	1.770934
MAP kinase phosphatase-1		H86755	2.036E ≤ 13	1.758744
Protein phosphatase 1, catalytic subunit, beta isoform	PPP1CB	R26434	0.0007444	1.71157
Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	PKIA	AA281667	0	1.698109
Folylpolyglutamate synthase	FPGS	R44864	0.0003955	1.679839
Ring finger protein 5	RNF5	AA402960	3.56E ≤ 08	1.655064
Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	SLC10A1	T68568	0.0007047	1.581188
Cell adhesion molecule with homology to L1CAM (close homologue of L1)	CHL1	R40400	0	1.579101
Protein tyrosine phosphatase, nonreceptor type 2	PTPN2	AA428195	8.145E ≤ 06	1.513367
Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM1	R77293	4.441E ≤ 16	1.509401
HIV				
Cyclic nucleotide-gated channel (photoreceptor), cGMP-gated2(beta)		H82535	1.73E ≤ 12	4.456159
Ubiquitin-conjugating enzyme E2M (homologous to yeast UBC12)	UBE2M	AA449119	0	2.85946
Rap1 guanine-nucleotide-exchange factor directly activated by cAMP	EPAC	AA453498	0.000484	2.748702
Intercellular adhesion molecule 3	ICAM3	AA478647	2E ≤ 06	2.724976
Recoverin	RCV1	AA074224	1.32E ≤ 05	2.42287
Human beta-1D integrin mRNA, cytoplasmic domain, partial cds		W67174	3.56E ≤ 05	2.239339
Developmentally regulated GTP-binding protein 1	DRG1	AA488466	0	2.071153
Seb4D		AA459588	1.5E ≤ 08	2.030693
TGF beta receptor–associated protein-1	TRAP-1	H22171	3.64E ≤ 09	2.026114
Chloride channel 6	CLCN6	H08188	1.96E ≤ 08	1.991755
Cullin 4A	CUL4A	AA598836	5.32E ≤ 06	1.955316
Transforming growth factor, beta receptor III (betaglycan, 300 kDa)	TGFBR3	H62473	0.000791	1.757171
Ras-related associated with diabetes	RRAD	W84445	1.18E ≤ 10	1.751459
Integrin, alpha M	ITGAM	AA436187	0	1.746724
Human guanine nucleotide-binding regulatory protein (Go-alpha) gene		R43320	0	1.662366
T-box, brain, 1	TBR1	H10054	8.87E ≤ 05	1.607488
Mitogen-activated protein kinase kinase kinase kinase 2	MAP4K2	R35283	1.4E ≤ 06	1.513208

*Expressed tagged sequences (ESTs) and duplicate genes have been deleted from the lists.

increased predominantly with HIV infection, but gp120 had no significant affect on any of the cytokines or chemokines tested by RPA. Induction of interleukin (IL)-6 was increased significantly in the HIV infection group (3–7≤; $P < .05$). However, the alteration of this gene by HIV from the microarray analysis was 0.83 s.d., which falls below the threshold of 1.5 s.d. and therefore does not appear in any of the tables. In comparison with the microarray data, the RPA data detected higher levels of mRNA for many of these genes (Figure 3). The *Z*-ratio is converted to approximate traditional fold induction using the equation $e^{(Z\text{-ratio}/0.9)}$ = traditional fold so that the data can be graphed against each other for comparison.

Discussion

We demonstrate that HIV and the viral protein gp120 may have profound effects on astrocyte function. The virus modulates a wide variety of genes and these genes differ depending on the mechanisms by which HIV interacts with astrocytes. One way in which genes were differentially regulated depended upon whether the virus infected the astrocytes or its envelope protein (gp120) interacted with uninfected astrocytes. Gp120 had a more profound effect than HIV infection. The discrepancy between the treatments may be due to effects from other HIV proteins, such as the transactivating protein Tat. Also, it is important to note that we may have underestimated

Table 2B Genes down-regulated from NeuroArray*

	Abbreviation	*Accession no.*	P *value*	Z-*ratio*
gp120				
Cyclic nucleotide-gated channel (photoreceptor), cGMP-gated2(beta)		H82535	1.174E ≤ 06	≤5.26183
Human beta-1D integrin mRNA, cytoplasmic domain, partial cds		W67174	0	≤5.19546
Vimentin	VIM	AA487812	5.732E ≤ 05	≤3.33303
Cytochrome *c*-1	CYC1	AA447774	0	≤3.24339
Protein phosphatase 1, catalytic subunit, alpha isoform	PPP1CA	AA443982	0	≤3.19841
Insulin-like growth factor–binding protein 3	IGFBP3	AA598601	0	≤3.09652
Glial fibrillary acidic protein	GFAP	AA069414	7.225E ≤ 13	≤3.09263
A kinase (PRKA) anchor protein (gravin) 12	AKAP12	AA478542	0	≤3.00195
RAB4, member RAS oncogene family	RAB4	H93459	9.622E ≤ 05	≤2.94504
Major vault protein	LRP	AA158990	1.708E ≤ 13	≤2.93662
Membrane metalloendopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	MME	R98851	0	≤2.9322
Hypothetical protein PRO2987	LAMR1	AA629897	5.934E ≤ 05	≤2.66713
Tubulin, alpha 2	TUBA2	AA626698	0	≤2.65745
Cytochrome *c* oxidase subunit VIIa polypeptide 2 like	COX7RP	R10896	8.155E ≤ 05	≤2.52219
Insulin-like growth factor–binding protein 2 (36 kDa)	IGFBP2	H79047	9.786E ≤ 05	≤2.40221
Trinucleotide repeat–containing 3	ERDA3	N59721	0	≤2.25698
Protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	PPP2CB	AA490696	4.895E ≤ 06	≤2.16334
Cytochrome *c* oxidase subunit VIb	COX6B	N71160	2.35E ≤ 12	≤2.12499
Profilin 1	PFN1	AA521431	0	≤2.12261
Ubiquitin-activating enzyme E1 (A1S9T and BN75 temperature sensitivity complementing)	UBE1	AA598670	2.744E ≤ 09	≤2.0757
LIM binding domain 2	LDB2	H74106	0.0002842	≤2.04959
Collagen, type V, alpha 1	COL5A1	R75635	2.272E ≤ 07	≤1.90886
Transcription factor Dp-1	TFDP1	W33012	0	≤1.75732
Reelin	RELN	R19878	0	≤1.75697
Guanylate kinase 1	GUK1	AA490902	0	≤1.71074
Protein tyrosine phosphatase, receptor type *N* polypeptide 2	PTPRN2	AA464590	0	≤1.71
cAMP phosphodiesterasem RNA, 3′ end		H65034	0.0002488	≤1.69628
Tubulin, alpha 1 (testis specific)	TUBA1	AA180912	5.118E ≤ 12	≤1.66618
Activated leucocyte cell adhesion molecule	ALCAM	R13558	4.661E ≤ 13	≤1.64324
Collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	COL2A1	N66737	5.032E ≤ 13	≤1.62362
RAP2A, member of RAS oncogene family		W32660	0	≤1.60029
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	NM_000090	1.418E-08	≤1.51327
Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 1	SLC9A3R1	AA425299	0.0002014	≤1.5041
HIV				
Glial fibrillary acidic protein	GFAP	AA069414	0	≤6.73838
Fibulin 1	FBLN1	AA134871	8.17E ≤ 05	≤3.86561
KIAA0080 protein	KIAA0080	AA446147	0.000109	≤3.2514
A kinase (PRKA) anchor protein (gravin) 12	AKAP12	AA478542	0	≤3.21894
Cadherin 11 (OB-cadherin, osteoblast)	CDH11	AA136983	6.72E ≤ 06	≤3.11478
Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 1	SLC9A3R1	AA425299	0	≤2.83264
Protease, serine, 2 (trypsin 2)	PRSS2	AA284528	0	≤2.79543
Vascular cell adhesion molecule 1	VCAM1	H07071	0	≤2.77831
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	T98612	1.13E ≤ 11	≤2.67315
Guanine nucleotide–binding protein (G protein), beta polypeptide 2-like 1	GNB2L1	R96220	6.37E ≤ 05	≤2.63642
Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	FGR	AA256231	5.7E ≤ 05	≤2.49531
Protein phosphatase 1, catalytic subunit, alpha isoform	PPP1CA	AA443982	2.19E ≤ 05	≤2.46475
Basic transcription factor 3	BTF3	R83000	1.23E ≤ 05	≤2.40977
Activated leucocyte cell adhesion molecule	ALCAM	R13558	9.56E ≤ 06	≤2.39915
Insulin-like growth factor–binding protein 7	IGFBP7	T53298	1.8E ≤ 12	≤2.3551
Hexabrachion (tenascin C, cytotactin)	HXB	T77595	0	≤2.33342
P450 (cytochrome) oxidoreductase	POR	T73294	2.21E ≤ 12	≤2.30322
Glutathione-*S*-transferase like; glutathione transferase omega	GSTTLp28	AA441895	7.77E ≤ 05	≤2.29102
GTP-binding protein ragB	RAGB	N73499	1.57E ≤ 08	≤2.16488
Filamin A, alpha (actin-binding protein-280)	FLNA	AA598978	0	≤2.07445
Membrane metalloendopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	MME	R98851	2.05E ≤ 05	≤2.00327
Paxillin	PXN	AA430573	1.17E ≤ 08	≤1.99956
Succinate dehydrogenase complex, subunit D, integral membrane protein	SDHD	AA035384	2.09E ≤ 05	≤1.87524
Latent transforming growth factor beta–binding protein 2	LTBP2	AA424629	0.000627	≤1.87522
Gamma-aminobutyric acid (GABA) A receptor, gamma 2	GABRG2	R40790	4.55E ≤ 12	≤1.78232
Syntaxin 3A	STX3A	AA436871	5.15E ≤ 08	≤1.64938
Insulin-like growth factor 2 receptor	IGF2R	T62547	0.000969	≤1.62348
Protein phosphatase, EF hand calcium-binding domain 1	PPEF1	H18855	0	≤1.61637
Cytochrome *c* oxidase subunit VIIc	COX7C	AA629719	2.64E ≤ 08	≤1.5605
Neuronal pentraxin I	NPTX1	H22481	9.14E ≤ 08	≤1.52817

*Expressed tagged sequences (ESTs) and duplicate genes have been deleted from the lists.

the magnitude of changes with HIV infection because only 10% of the cells are infected 3 days following exposure to virus, as determined by visual quantification of yellow fluorescent protein–positive (YFP+) astrocytes (Figure 4). Within each paradigm, nearly equal numbers of genes were down-regulated as were up-regulated. Previous studies on effects of HIV proteins on astrocytes have focused only on a few genes involved in immune regulation that have been up-regulated (Nath, 1999). Our studies suggest

Table 2C Genes up-regulated from ImmunoArray*

	Abbreviation	*Accession no.*	P *value*	Z-*ratio*
gp120				
Villin 2 (ezrin)	VIL2	NM_003379	0.000253	2.623478
Major histocompatibility complex, class II, DQ beta 1	HLA≤DQB1	NM_002123	1.55E ≤ 07	2.554188
Lymphocyte antigen 64 (mouse) homolog, radioprotective, 105 kDa	LY64	NM_005582	0	2.497003
Major histocompatibility complex, class II, DM beta	HLA≤DMB	NM_002118	8.58E ≤ 05	2.49366
MAD homolog interacting protein, receptor activation anchor	MADHIP	NM_004799	2.1E ≤ 07	2.443154
CDC28 protein kinase 1	CKS1	NM_001826	1.3E ≤ 06	2.349632
Integrin, alpha M (complement component receptor 3, alpha)	ITGAM	NM_000632	7.65E ≤ 11	2.319783
Nonmetastatic cells 2, protein (NM23B) expressed in	NME2	NM_002512	6.86E ≤ 05	2.281797
G protein-coupled receptor 37 (endothelin receptor type B-like)	GPR37	NM_005302	0.00035	2.228295
Phosphogluconate dehydrogenase	PGD	NM_002631	2.11E ≤ 07	2.215645
Interleukin-7 receptor	IL7R	NM_002185	9.45E ≤ 09	2.165172
DNA-binding transcriptional activator	NCYM	NM_006316	0	2.148795
Nuclear transcription factor Y, alpha	NFYA	NM_002505	0	2.146055
Proteasome (prosome, macropain) subunit, alpha type, 1	PSMA1	NM_002786	1.73E ≤ 07	2.111627
Defensin, beta 1	DEFB1	NM_005218	2.41E ≤ 12	2.106281
B7-H1 protein	B7-H1	NM_014143	0	2.08118
Complement component (3d/Epstein Barr virus) receptor 2	CR2	NM_001877	2.89E ≤ 05	2.026556
Thioredoxin	TXN	NM_003329	2.96E ≤ 06	2.005055
SAC2 (suppressor of actin mutations 2, yeast, homolog)-like	SACM2L	AL031228	5.21E ≤ 09	1.996212
Bruton agammaglobulinemia tyrosine kinase	BTK	NM_000061	3.55E ≤ 15	1.992553
Ribosomal protein S25	RPS25	NM_001028	5.29E ≤ 05	1.908532
Proplatelet basic protein	PPBP	NM_002704	2.38E ≤ 12	1.900672
Protein kinase, cAMP-dependent, catalytic, beta	PRKACB	NM_002731	0	1.888425
Suppressor of Ty (S.cerevisiae) 4 homolog 1	SUPT4H1	NM_003168	0.000759	1.88139
Sialyltransferase 1 (beta-galactoside alpha-2,6-sialytransferase)	SIAT1	NM_003032	0	1.880238
Defensin, alpha 6, Paneth cell–specific	DEFA6	NM_001926	1.23E ≤ 09	1.877471
Annexin A6	ANXA6	NM_001155	1.9E ≤ 11	1.869928
Signal transducer and activator of transcription 1, 91 kDa	STAT1	NM_007315	4.48E ≤ 11	1.869684
Interleukin-18 (interferon-gamma-inducing factor)	IL18	NM_001562	3.85E ≤ 06	1.842086
Ras homolog gene family, member	ARHD	NM_014578	0.000104	1.796194
Primase, polypeptide 2A (58 kDa)	PRIM2A	NM_000947	8.72E ≤ 05	1.779806
Activin A receptor, type IB	ACVR1B	NM_004302	4.98E ≤ 05	1.760671
Major histocompatibility complex, class II, DR beta 1	HLA-DRB1	NM_002124	6.3E ≤ 06	1.742368
Apoptosis-associated tyrosine kinase	AATK	NM_004920	0	1.719727
Neuroblastoma RAS viral (v-ras) oncogene homolog	NRAS	NM_002524	0.000959	1.687639
Chemokine (C-X3-C) receptor 1	CX3CR1	NM_001337	0	1.68049
Interferon-induced protein with tetratricopeptide repeats 4	IFIT4	NM_001549	1.32E ≤ 06	1.676404
Fibroblast growth factor 12	FGF12	NM_021032	2.88E ≤ 05	1.673762
Interleukin-10	IL10	NM_000572	1.97E ≤ 08	1.657587
Excision repair cross-complementing rodent repair deficiency, complementation group 2	ERCC2	L47234	4.5E ≤ 06	1.647789
Small inducible cytokine subfamily A (Cys-Cys), member 17 (TARC)	SCYA17	NM_002987	7.44E ≤ 13	1.641096
Sterol regulatory element–binding transcription factor 1	SREBF1	NM_004176	0	1.631181
Lyphotoxin alpha (TNF superfamily, member 1)	LTA	NM_000595	6.79E ≤ 06	1.630593
cAMP responsive element–binding protein-like 1	CREBL1	NM_004381	7.19E ≤ 09	1.601362
Ephrin-A5	EFNA5	NM_001962	0.000435	1.598929
Burkitt lymphoma receptor 1, GTP-binding protein	BLR1	NM_001716	0.000113	1.582451
E2F transcription factor 3	E2F3	NM_001949	0	1.563808
Amyloid beta (A4) precursor protein	APP	NM_000484	0.00097	1.562166
EphB3	EPHB3	NM_004443	2.93E ≤ 06	1.556845
Thyroid-stimulating hormone receptor	TSHR	NM_000369	6.44E ≤ 15	1.52707
Src kinase–associated phosphoprotein of 55 kDa	SKAP55	NM_003726	1.6E ≤ 06	1.521267
Interleukin-15	IL15	NM_000585	1.04E ≤ 05	1.511444
COP9 (constitutive photomorphogenic, arabidopsis, homolog) subunit 5	COPS5	U65928	1.73E ≤ 05	1.51004
HIV				
Insulin-like growth factor–binding protein 3	IGFBP3	NM_000598	0.000422	4.66374
Opioid-binding protein/cell adhesion molecule-like	OPCML	NM_002545	0	3.671639
Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	CACNA1A	NM_000068	0	3.579689
FK506-binding protein 1A (12 kDa)	FKBP1A	NM_000801	0	3.188564
Sequestosome 1	SQSTM1	NM_003900	0	2.963485
Caspase 4, apoptosis-related cysteine protease	CASP4	NM_001225	1.31E ≤ 13	2.827716
Death-associated protein 6	DAXX	NM_001350	0.000138	2.640925
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	NM_003118	0	2.625693
Regulator of G-protein signalling 4	RGS4	NM_005613	0	2.620301
Aconitase 2, mitochondrial	ACO2	NM_001098	0	2.485327
Heat shock 27-kDa protein 1	HSPB1	NM_001540	5.13E ≤ 09	2.367898

(Continued on next page)

Table 2C Genes up-regulated from ImmunoArray* *(Continued)*

	Abbreviation	*Accession no.*	P *value*	Z-*ratio*
Nuclear receptor subfamily 0, group B, member 2	NR0B2	AF044316	0	2.366236
BCL2-associated X protein	BAX	NM_004324	1.04E ≤ 11	2.295656
Vimentin	VIM	NM_003380	5.03E ≤ 06	2.269849
Cyclin-dependent kinase 6	CDK6	NM_001259	3.53E ≤ 09	2.235974
Tissue inhibitor of metalloproteinase 1 (erythroid-potentiating activity, collagenase inhibitor)	TIMP1	NM_003254	2.81E ≤ 05	2.193107
Integrin, beta 1 (fibronectin receptor, beta polypeptide)	ITGB1	XM_005799	0	2.163586
Interferon regulatory factor 2	IRF2	NM_002199	0	2.13388
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	PSMD2	NM_002808	0	2.120752
Interferon gamma receptor 1	IFNGR1	NM_000416	0.000554	2.031808
Wee1+ (*S. pombe*) homolog	WEE1	NM_003390	9.4E ≤ 13	1.949709
Gelsolin (amyloidosis, Finnish type)	GSN	NM_000177	0	1.92095
Transcription factor 7 (T-cell specific, HMG-box)	TCF7	NM_003202	1.07E ≤ 06	1.904207
ras homolog gene family, member E	ARHE	NM_005168	0.000144	1.885919
Protein kinase C, beta 1	PRKCB1	NM_002738	7.89E ≤ 08	1.842921
Transcription factor AP-4 (activating enhancer-binding protein 4)	TFAP4	NM_003223	0.00038	1.821991
GLUT4 enhancer factor	GEF	AF249267	0	1.777154
ras-related C3 botulinum toxin substrate 2 (rho family, small GTP-binding protein Rac2)	RAC2	NM_002872	1.47E ≤ 05	1.745914
Nuclear mitotic apparatus protein 1	NUMA1	NM_006185	6.77E ≤ 07	1.70841
Zinc finger protein 162	ZNF162	NM_004630	8E ≤ 08	1.597409
Adrenergic, beta-3-, receptor	ADRB3	NM_000025	4.14E ≤ 08	1.591778
Signal transducer and activator of transcription 1, 91 kDa	STAT1	NM_007315	0.000483	1.564258

*Expressed tagged sequences (ESTs) and duplicate genes have been deleted from the lists.

an importance for genes that are down-regulated in astrocytes following HIV infection or HIV protein interactions.

As expected, the most dramatic effects were observed on transducers involved in secondary signaling pathways. Many of these genes regulate the production of cytokines and chemokines that have shown to be modulated by HIV proteins and HIV infection. Similarly, it is likely that these transcription factors are also involved in modulation of adhesion

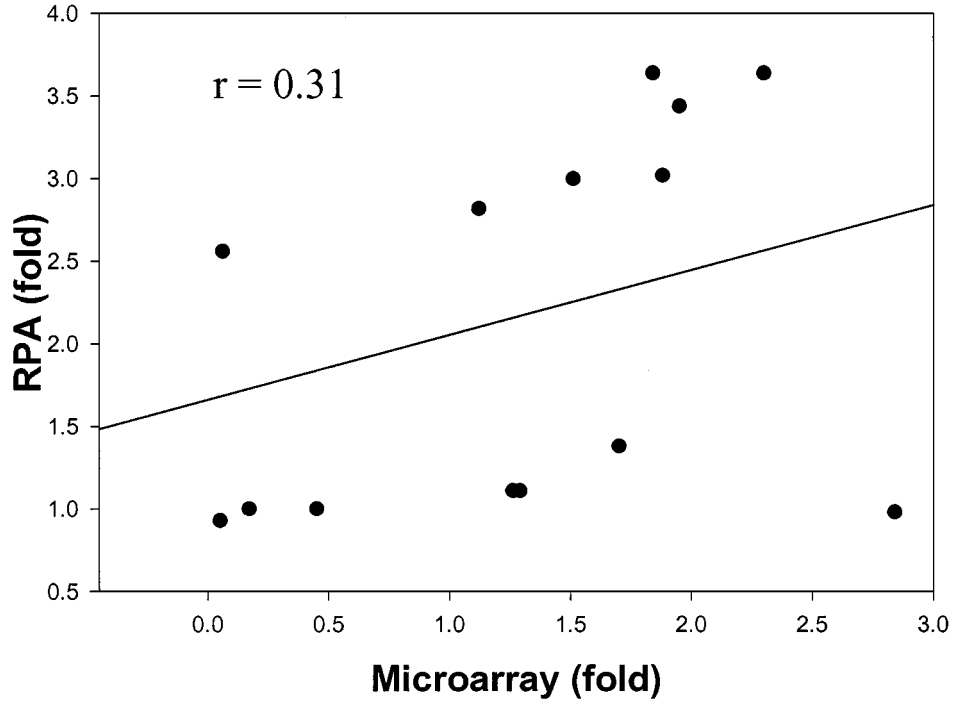

Figure 3 Correlation between microarray and RPA for cytokine and chemokine gene expression: Astrocytes were either infected with HIV or treated with gp120 and analyzed by microarray and RPA. Results are expressed as fold increases. Moderate correlation is seen between the two techniques.

Table 2D Genes down-regulated from ImmunoArray*

	Abbreviation	*Accession no.*	P *value*	Z-*ratio*
gp120				
Integrin, beta 1 (fibronectin receptor, beta polypeptide)	ITGB1	XM_005799	0	≤5.50259
Cysteine-rich, angiogenic inducer, 61	CYR61	NM_001554	8.61E ≤ 13	≤3.96071
Fibronectin 1	FN1	M10905	0	≤3.95357
TNF receptor–associated factor 3	TRAF3	NM_003300	0	≤3.75092
Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	PIP5K1C	AB011161	4.65E ≤ 10	≤3.68745
Fibroblast growth factor receptor 4	FGFR4	NM_002011	1.6E ≤ 12	≤3.6507
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 1	DDX1	NM_004939	0	≤3.58625
Ubiquitin-like 1 (sentrin)	UBL1	NM_003352	0	≤3.5057
fms-related tyrosine kinase 1 (vascular endothelial growth factor/permeability)	FLT1	NM_002019	0	≤3.47192
Chromobox homolog 3 (Drosophila HP1 gamma)	CBX3	NM_007276	0	≤3.30449
Opioid-binding protein/cell adhesion molecule≤like	OPCML	NM_002545	0	≤3.12111
Caspase 2, apoptosis-related cysteine protease	CASP2	NM_001224	0	≤3.10145
Survival of motor neuron 1, telomeric	SMN1	NM_000344	2.03E ≤ 11	≤2.95659
Leptin (murine obesity homolog)	LEP	NM_000230	3.63E ≤ 08	≤2.95305
Upstream transcription factor 2, c-fos interacting	USF2	NM_003367	0	≤2.92168
Gamma-glutamyl carboxylase	GGCX	NM_000821	0	≤2.89008
Ribosomal protein S6	RPS6	NM_001010	0	≤2.71536
Peroxisome proliferative activated receptor, gamma	PPARG	NM_005037	3.45E ≤ 13	≤2.63342
Flotillin 1	FLOT1	NM_005803	1.02E ≤ 05	≤2.57852
Ubiquinol-cytochrome *c* reductase, Rieske iron-sulfur polypeptide 1	UQCRFSL1	XM_009131	2.79E ≤ 06	≤2.49688
Transcription factor 17	TCF17	NM_005649	1.68E ≤ 12	≤2.485
Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	ITGA2	NM_002203	2.22E ≤ 09	≤2.48375
Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	ITGAV	NM_002210	0	≤2.39817
Ephrin-B2	EFNB2	NM_004093	0	≤2.29757
Tissue inhibitor of metalloproteinase 1	TIMP1	NM_003254	4.49E ≤ 11	≤2.26668
Cholinergic receptor, nicotinic, alpha polypeptide 4	CHRNA4	NM_000744	1.92E≤13	≤2.20186
Fibroblast growth factor receptor 3	FGFR3	NM_000142	4E ≤ 06	≤2.18977
Interleukin-10 receptor, beta	IL10RB	NM_000628	8.16E ≤ 06	≤2.18864
Nuclear mitotic apparatus protein 1	NUMA1	NM_006185	0	≤2.17912
Nuclear transcription factor Y, beta	NFYB	NM_006166	0	≤2.14087
v-myc avian myelocytomatosis viral oncogene homolog	MYC	J00120	3.01E ≤ 09	≤2.06761
Platelet-derived growth factor receptor, alpha polypeptide	PDGFRA	NM_006206	4.24E ≤ 05	≤2.02787
UDP-glucose pyrophosphorylase 2	UGP2	NM_006759	1.3E ≤ 05	≤1.95701
Core promoter element–binding protein	COPEB	NM_001300	1.25E ≤ 05	≤1.92985
Caspase 7, apoptosis-related cysteine protease	CASP7	NM_001227	0	≤1.91028
Zinc finger protein 220	ZNF220	NM_006766	5.7E ≤ 10	≤1.88573
Protein phosphatase 3, catalytic subunit, gamma (calcineurin A gamma)	PPP3CC	NM_005605	0.000809	≤1.8813
Growth factor receptor-bound protein 2	GRB2	NM_002086	0	≤1.82986
General transcription factor IIE, polypeptide 2 (beta subunit, 34 kDa)	GTF2E2	NM_002095	0	≤1.82259
SHC (Src homology 2 domain-containing) transforming protein 1	SHC1	NM_003029	1.87E ≤ 11	≤1.81799
BCL2-related protein A1	BCL2A1	NM_004049	0.000523	≤1.78786
Formyl peptide receptor 1	FPR1	NM_002029	0	≤1.78778
Transcription factor 6-like 1 (mitochondrial transcription factor 1-like)	TCF6L1	NM_003201	0.000256	≤1.74374
BCL2-associated X protein	BAX	NM_004324	0	≤1.73046
Basic transcription factor 3	BTF3	NM_001207	7.74E ≤ 06	≤1.70669
Pyrimidinergic receptor P2Y, G-protein coupled, 6	P2RY6	NM_004154	5.18E ≤ 10	≤1.68012
Glycosylphosphatidylinositol specific phospholipase D1	GPLD1	NM_001503	0	≤1.68003
2,4-dienoyl CoA reductase 1, mitochondrial	DECR	NM_001359	0	≤1.66628
Macrophage migration inhibitory factor	MIF	NM_002415	0.000628	≤1.59046
Heat shock 70-kDa protein 1A	HSPA1A	NM_005345	1.58E ≤ 12	≤1.56857
Prostatic binding protein	PBP	NM_002567	0	≤1.55179
Transcription factor 12 (helix-loop-helix transcription factors 4)	TCF12	NM_003205	6.66E ≤ 14	≤1.54991
N-acylaminoacyl-peptide hydrolase	APEH	NM_001640	0	≤1.51841
GATA-binding protein 4	GATA4	NM_002052	7.78E ≤ 11	≤1.51504
Mitogen-activated protein kinase 11	MAPK11	NM_002751	5.6E≤06	≤1.50653
HIV				
Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	PIP5K1C	AB011161	0	≤5.96738
Ephrin-B2	EFNB2	NM_004093	0	≤4.94714
Ubiquitin specific protease 8	USP8	NM_005154	2.22E ≤ 16	≤4.26141
Growth factor receptor–bound protein 2	GRB2	NM_002086	1.22E≤08	≤3.55023
Methionine aminopeptidase; eIF-2-associated p67	MNPEP	NM_006838	3.03E≤12	≤3.49615
Ribosomal protein L7a	RPL7A	NM_000972	0	≤3.35416
Ribosomal protein L5	RPL5	NM_000969	0	≤3.35217
Thyroid hormone receptor interactor 13	TRIP13	NM_004237	8.13E ≤ 10	≤3.26518
Ribosomal protein S25	RPS25	NM_001028	0.00032	≤3.07487
KIAA0535 gene product	KIAA0535	NM_014682	0.000439	≤2.94605

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Table 2D Genes down-regulated from ImmunoArray* *(Continued)*

	Abbreviation	*Accession no.*	P *value*	Z-*ratio*
Proteasome (prosome, macropain) subunit, beta type, 6	PSMB6	D29012	0	≤2.81729
Gamma-glutamyl carboxylase	GGCX	NM_000821	0	≤2.76876
Ribosomal protein S6	RPS6	NM_001010	9.06E ≤ 06	≤2.69543
Transcription elongation factor A (SII), 1	TCEA1	NM_006756	2.22E ≤ 16	≤2.66119
Selectin L (lymphocyte adhesion molecule 1)	SELL	NM_000655	5.54E ≤ 13	≤2.60374
CD53 antigen	CD53	NM_000560	7.91E ≤ 07	≤2.52785
Forkhead box J1	FOXJ1	NM_001454	1.7E ≤ 06	≤2.50935
Insulin-like growth factor 1 receptor	IGF1R	NM_000875	0	≤2.33489
Formyl peptide receptor 1	FPR1	NM_002029	6.44E ≤ 13	≤2.29446
PTK2 protein tyrosine kinase 2	PTK2	NM_005607	0	≤2.26282
Phospholipase C, beta 4	PLCB4	NM_000933	3.38E ≤ 05	≤2.25637
Small inducible cytokine A2 (monocyte chemotactic protein 1)	SCYA2	NM_002982	2.94E ≤ 10	≤2.20671
Opioid receptor, kappa 1	OPRK1	NM_000912	2.4E ≤ 10	≤2.20498
Major histocompatibility complex, class II, DN alpha	HLA-DNA	NM_002119	9.76E ≤ 07	≤2.17854
Tyrosine kinase(immunoglobulin and epidermal growth factor homology)	TIE	NM_005424	0	≤2.13453
Angiotensin receptor 1	AGTR1	NM_000685	0.00025	≤2.13159
Nuclear factor (erythroid-derived 2)-like 1	NFE2L1	NM_003204	0.000171	≤2.12037
Apolipoprotein D	APOD	NM_001647	7.14E ≤ 05	≤2.08651
Colony-stimulating factor 1 receptor	CSF1R	NM_005211	0.000167	≤2.03586
Histamine receptor H1	HRH1	NM_000861	2.29E ≤ 07	≤1.97787
Midkine (neurite growth-promoting factor 2)	MDK	NM_002391	4.88E ≤ 15	≤1.97282
Transcription elongation factor A (SII), 2	TCEA2	NM_003195	0	≤1.9664
KIAA1046 protein	KIAA1046	NM_014928	6.88E ≤ 15	≤1.95447
Eukaryotic translation initiation factor 2B, subunit 1 (alpha, 26 kDa)	EIF2B1	NM_001414	1.9E≤05	≤1.94494
Interleukin 1, beta	IL1B	NM_000576	4.69E ≤ 05	≤1.94059
Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	NM_003807	0.000124	≤1.91659
Cytochrome *c*-1	CYC1	NM_001916	2.22E ≤ 16	≤1.8793
MAD (mothers against decapentaplegic, *Drosophila*) homolog 3	MADH3	NM_005902	3.13E ≤ 13	≤1.86847
Granulin	GRN	NM_002087	1.24E ≤ 08	≤1.73984
Breakpoint cluster region	BCR	NM_004327	8.06E ≤ 05	≤1.68178
T cell receptor alpha locus	TCRA	M12959	2.48E ≤ 07	≤1.67123
Protein tyrosine phosphatase, receptor type, O	PTPRO	NM_002848	2.22E ≤ 06	≤1.67012
MAD (mothers against decapentaplegic, *Drosophila*) homolog 6	MADH6	NM_005585	1.36E ≤ 11	≤1.66749
Major histocompatibility complex, class I, A	HLA≤A	NM_002116	4.04E ≤ 07	≤1.64658
Retinoic acid receptor, gamma	RARG	NM_000966	0.000165	≤1.62596
Nuclear receptor subfamily 3, group C, member 1	NR3C1	NM_000176	0.000791	≤1.60687
Stromal cell–derived factor 1 (SCYB12)	SDF1	NM_000609	1.04E ≤ 12	≤1.60199
MutS (*E. coli*) homolog 2 (colon cancer, nonpolyposis type 1)	MSH2	NM_000251	3.38E ≤ 08	≤1.58744
Mitogen-activated protein kinase kinase kinase kinase 1	MAP4K1	NM_007181	1.55E ≤ 15	≤1.57077
Transforming growth factor, beta 1	TGFB1	NM_000660	7.5E ≤ 13	≤1.56571

*Expressed tagged sequences (ESTs) and duplicate genes have been deleted from the lists.

molecules, structural proteins, and major histocompatibility complex (MHC) antigens.

Only two genes were up-regulated by both treatments. Stat1, or signal transducer and activator of transcription 1, is a potent intermediary in cell activation. It acts as the transcriptional activator for such ligands as interferon alpha, interferon gamma, epidermal growth factor, platelet-derived growth factor, and IL-6. It has been demonstrated by others that Stat1 is activated through phosphoryaltion in astrocytes following treatment with gp120 (Shrikant *et al*, 1996). The induction of Stat1 and its activation might be part of the astrocyte's antiviral response, given the involvement of Stat1 in interferon signaling. Indeed, others have found antiretroviral involvement for Stat1 in other cell types (Vidal *et al*, 2001; Chang *et al*, 2002; Sarol *et al*, 2002). The other gene up-regulated by both treatments was the the alpha M integrin, also known as CD11b. This integrin is important in the complement cascade, as well as the adherence of neurophils and monocytes to endothelium. Given the proximity and relative abundance of astrocytes to the blood-brain barrier, the later activity might be quite important. It has also been thought that CD11b might be able to act as an alternative coreceptor for CCR5-trophic HIV (Bouhlal *et al*, 2001).

Conversely, 15 genes were found to be down-regulated by both treatments. FPR1 activation leads to a down-regulation of chemokine receptors CCR5

Table 3A Genes up-regulated by gp120 and HIV

	gp120	*HIV*
Integrin, alpha M (ITGAM, NM_000632)	2.32	1.74
Signal transducer and activator of transcription 1 (STAT1, NM_007315)	1.87	1.56

Table 3B Genes down-regulated by gp120 and HIV

	gp120	*HIV*
A kinase (PRKA) anchor protein 12 (AKAP12, AA478542)	≤3.00	≤3.21
Activated leukocyte cell adhesion molecule (ALCAM, R13558)	≤2.39	≤1.64
Basic transcription factor 3 (BTF3, NM_001207)	≤1.71	≤2.40
Collagen, type III, alpha 1 (COL3A1, T98612)	≤2.91	≤2.67
Cytochrome *c*-1 (CYC1, AA447774)	≤3.24	≤1.87
Ephrin-B2 (EFNB2, NM_004093)	≤2.30	≤4.95
Formyl peptide receptor 1 (FPR1, NM_002029)	≤1.79	≤2.29
Gamma-glutamyl carboxylase (GGCX, NM_000821)	≤2.89	≤2.77
Glial fibrillary acidic protein (GFAP, AA069414)	≤3.09	≤6.73
Growth factor receptor-bound protein 2 (GRB2, NM_002086)	≤1.83	≤3.55
Membrane metalloendopeptidase (enkephalinase) (MME, R98851)	≤2.93	≤2.00
Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma (PIP5K1C, AB011161)	≤3.69	≤5.97
Protein phosphatase 1, catalytic subunit alpha (PPP1CA, AA443982)	≤3.20	≤2.46
Ribosomal protein S6 (RPS6, NM_001010)	≤2.72	≤2.70
Solute carrier family 9 (Na/H exchanger) isoform 3 regulatory factor 1 (SLC9A3R1, AA425299)	≤1.50	≤2.83

Table 3C Genes activated by gp120 but suppressed by HIV infection

	gp120	*HIV*
Protease, serine, 2 (trypsin 2) (PRSS2, AA284528)	2.13	≤2.79
Ribosomal protein S25 (RPS25, NM_001028)	1.91	≤3.07

Table 3D Genes suppressed by gp120 but activated by HIV infection

	gp120	*HIV*
BCL2-associated X protein (BAX, NM_004324)	≤1.73	2.29
Cyclic nucleotide–gated channel, cGMP-gated 2 (H82535)	≤5.26	4.45
Human beta-1D integrin, cytoplasmic domain (W67174)	≤5.19	2.23
Insulin-like growth factor–binding protein 3 (IGFBP3, AA598601)	≤3.10	4.66
Integrin, beta 1 (fibronectin receptor) (ITGB1, AA409975)	≤5.50	2.16
Nuclear mitotic apparatus protein 1 (NUMA1, NM_006185)	≤2.18	1.71
Opioid-binding protein (OPCML, NM_002545)	≤3.12	3.67
Tissue inhibitor of metalloproteinase 1 (TIMP1, NM_003254)	≤2.27	2.19

and CXCR4 and inhibition of HIV entry. Previous studies have shown that peptides derived from gp120 can cause formyl peptide receptor (FPR) activation (Le *et al*, 1999; Shen *et al*, 2000). It has been suggested that compounds that activate FPR may be of therapeutic benefit in HIV infection. GRB2, which is associated with apoptosis, was found to be down-regulated in a similar manner. This may partially explain the lack of apoptosis among infected astrocytes. BTF3 was also down-regulated by both treatments, which could have wide-ranging effects on transcription. Although the direct role of enkephalinase has also not been investigated in the pathogenesis of HIV infection, its activation would likely lead to increased degradation of endogenous opiates, which have been shown to play an important role in HIV neuropathogenesis (Gekker *et al*, 2001; Gurwell *et al*, 2001; Peterson *et al*, 1998). In contrast to much of the published literature, which has focused on activation of transcription factors, our observations show that just as many transcription factors are down-regulated as are up-regulated with HIV proteins or infection, suggesting a complex balance of gene regulation during HIV infection. ALCAM down-regulation by HIV and gp120 might be an attempt to avoid immune surveillance. It is interesting to note that ALCAM has also been insinuated in neuronal interactions and development (Sato *et al*, 2002; Mothe and Brown 2000). Down-regulation of cytochrome *c* would potentially cause disruptions in the electron transport chain and a decrease in the ability of the astrocytes to activate caspase-9.

Interestingly, a reciprocal effect was noted with HIV infection versus extracellular treatment with gp120. HIV infection with intracellular expression of HIV proteins may promote apoptosis by increasing transcription of BAX, an effect similar to that seen in lymphocytes (Petit *et al*, 2002), whereas extracellular treatment with gp120 causes down-regulation of BAX. This is in keeping with previous observations that gp120 does not cause cell death in astrocytes. However, they may induce apoptosis in other cell types where they up-regulate BAX (Castedo *et al*, 2001; Park *et al*, 2001). This implies a differential signaling cascade initiated at the cell membrane. Our observations on the effect of HIV infection on insulin-like growth factor (Corry and Tuck, 2001; Mynarcik *et al*, 2000) and tumor necrosis factor (TNF) receptor are consistent with previous studies (Chiao *et al*, 2001; Ryan *et al*, 2001). OPCML is a dual role protein that not only acts as a glycosyl-phosphatidylinositol (GPI)-anchored cell adhesion molecule, but also is an opioid receptor with selectivity for mu ligands. Furthermore, TIMP1 decreases with gp120 treatment might lead to an increase in many active matrix metalloproteinase family members.

Our laboratory has extensive experience in studying the effect of viral proteins on cytokine and chemokine production in glial cells, therefore we compared the results for these genes obtained from

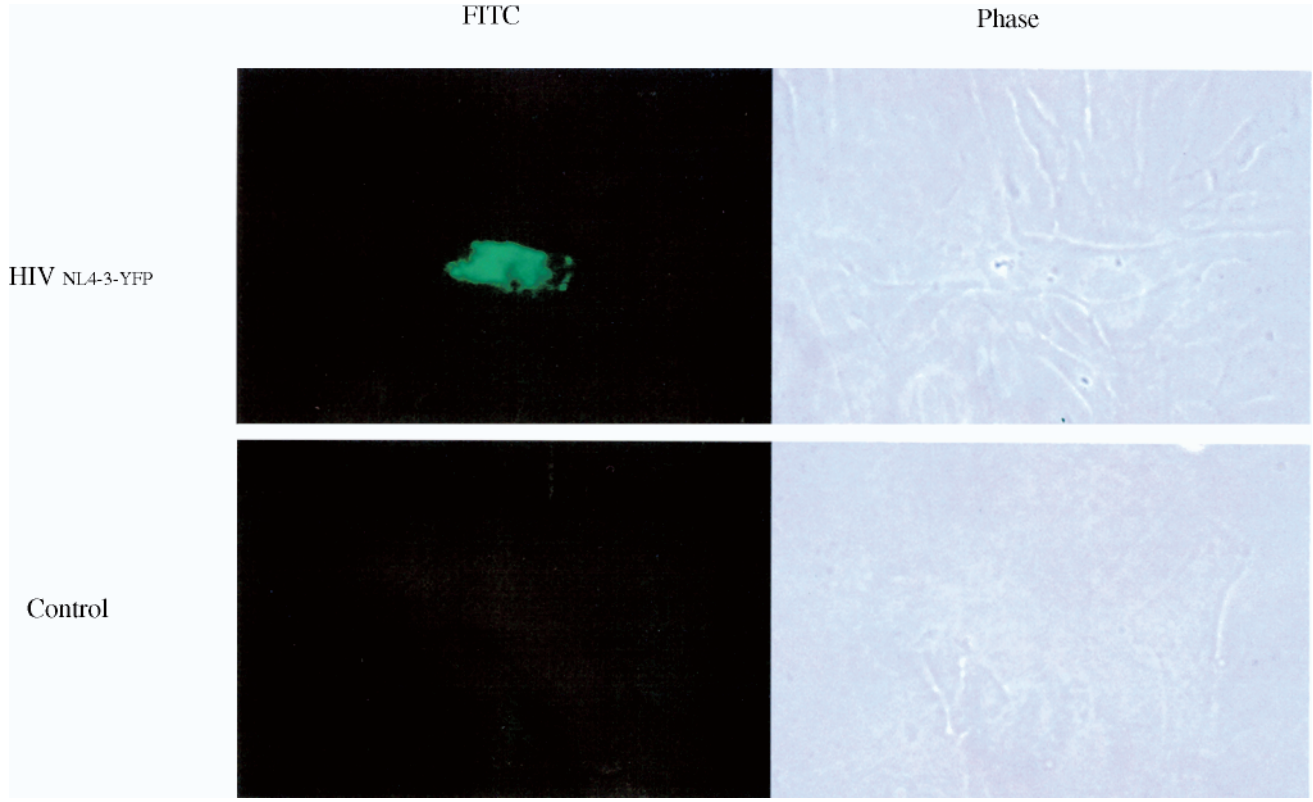

Figure 4 Images of primary human astrocytes infected by $HIV_{NL4\text{-}3\text{-}YFP}$ or uninfected controls. The left column shows fields visualized using a FITC filter. Note the fluorescent astrocytes in the $HIV_{NL4\text{-}3\text{-}YFP}$ row. The right column shows the same field with normal light. 40≤ magnification.

microarray analysis with that of RPA. We found that there was a moderate positive correlation for those cytokine and chemokine ($r = .31$). Hence, microarray technique seems to provide reliable results for those genes that show moderate or strong responses to stimuli. Further studies are needed to confirm our observations with protein analysis for the genes discussed above. In conclusion, a multitude of transcriptional abnormalities occur in astrocytes in the setting of HIV infection. As many genes are down-regulated as are up-regulated, often HIV infection of astrocytes and exposure of uninfected astrocytes to HIV proteins have reciprocal effects. Our studies support an important role for astrocytes in HIV neuropathogenesis.

Methods

Tissue culture

Human fetal astrocyte cultures were prepared from human fetal brain specimens of 12 to 17 weeks, gestation (Furer *et al*, 1993). The astrocytes were cultured in 75-cm^2 flasks at 37≤C in Dulbecco's modified Eagle medium (DMEM; GibcoBRL) with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and 1% antibiotic-antimycotic solution (penicillin G sodium, streptomycin sulfate, and amphotericin B in 0.85% saline; GibcoBRL).

Astrocyte treatments

Astrocyte cultures were either treated with gp120 or infected with HIV. Gp120 from HIV_{SF2} was produced recombinantly in Chinese hamster ovary cells and obtained as a gift from Chiron Corporation. The protein was purified to >99% purity.

Prior to treatment with gp120, the culture medium was removed from the flasks and replaced with serum-free DMEM. The cells were incubated at 37≤C for 6 h with gp120 (250 pM) and then processed as described below. These concentrations were chosen from previous dose and time course studies monitoring neurotoxicity and intracellular calcium changes in neural or glial cell cultures (Holden *et al*, 1999; Nath *et al*, 2000a). Another set of astrocytes were infected with $HIV_{NL4\text{-}3\text{-}YFP}$ (multiplicity of infection [MOI] 0.2) for 1 h in serum-free DMEM and then maintained in DMEM + 10%FBS at 37≤C for 3 days, at which time there is maximal HIV production (Tornatore *et al*, 1991). This virus has a YFP insert in the *nef* gene. The percentage of infected astrocytes were monitored by counting the number of fluorescent cells at 3 days post infection.

cDNA microarray

Astrocytes were lysed using RNAwiz (Ambion) and RNA extracted according to manufacturers protocols. Five micrograms of RNA was diluted in 14 μl

of diethyl pyrocarbonate (DEPC)-treated H_2O. First-strand cDNA synthesis was performed, incorporating in [μ-^{33}P]-dCTP. RNA was incubated at 65≤C with 5 μl 0.5 M EDTA and 10 μl 0.1 N NaOH, and then 25 μl of 1 M Tris-HCl was added. cDNA was then purified on a Biospin P-30 spin column (Bio-Rad). The membrane arrays were washed with 45 ml of 2≤ saline-sodium citrate (SSC) buffer (BioSource 357-000, 20≤ SSC), and incubated in prehybridization buffer (4 ml of Microhyb, 10 μl human Cot1 DNA, 10 μl polyA at 8 mg/ml) at 42≤C for 4 h in a rotating oven. The probes were heat denatured at 95≤C for 5 min and added to the tubes containing the microarray membranes and prehybridization solution and incubated overnight at 42≤C. The membranes were washed twice at 50≤C for 15 min each in 2≤ SSC and 0.1% sodium dodecyl sulfate (SDS), and analyzed using a Storm phosphoimager (Molecular Dynamics). Two types of microarray membranes were used, neuro-based and immuno-based, each of which have 1153 genes (*http://www.grc.nia.nih.gov/branches/rrb/dna/dna.htm*). Each sample was analyzed in duplicate.

Microarray quantitation

Raw intensity data obtained from the phosphoimager was subjected to a log10 transformation to reduce the variance due to extreme values. The mean and standard deviation of the log10 scores for each sample was calculated. These values were entered into the *Z*-score normalization formula:

$$\text{Observed gene } Z\text{-score} = (\text{observed gene log10 intensity} \leq \text{mean of all genes on microarray log10 intensity})/(\text{standard deviation of all genes on microarray log10 intensity})$$

Gene expression differences between two arrays were calculated as follows:

$$Z\text{-score difference gene 1} = [(z_{S1a} + z_{S1b})/2] \leq [(z_{C1a} + z_{C1b})/2]$$

where S1 and C1 equal experimental gene 1 and control gene 1, respectively, and a, b represent individual *Z*-scores obtained from two measurements of the gene. Two arrays conducted in duplicate were used and the *Z*-score differences averaged. *Z*-ratios were calculated as follows:

$$Z\text{-score difference gene 1/standard deviation of the } Z\text{-differences distribution}$$

These ratios were used to compare treatment groups versus untreated controls. *Z*-ratios greater than 1.5 s.d. from the untreated control group were considered significant. *Z*-score ratios were utilized as the statistical method employed for analysis of the genes, as described above. This method is more conservative than the traditional fold-induction calculations. This allows for greater comparability between arrays and experiments. Four replicates were used to calculate the *Z*-ratios. Genes that were altered by 1.5 s.d. or more were then filtered by their *P* value following a two-tailed *t* test, with a *P* value of .001 or less considered significant. Therefore, results reported herein are altered by at least 1.5 s.d. and have a *P* value of .001 or less.

Probe synthesis

Multiprobe RPA templates and kits were obtained from PharMingen. One microliter of template with 10 μl [μ-^{32}P]-UTP, 1 μl GACU pool, 2 μl dithiothreitol (100 mM), 4 μl 5≤ transcription buffer, 1 μl RNAsin (40 U/μl), and 1 μl T7 RNA polymerase (20 U/μl) and incubated for 1 h at 37≤C. The reaction was terminated with 2 μl DNAse (1 U/μl) and incubated for 30 min at 37≤C. Twenty-six microliter 20 mM ethylenediaminetetraacetic acid (EDTA, 20 mM), 25 μl water-saturated phenol, 25 μl chloroform:isoamyl alcohol (50:1), and 2 μl yeast tRNA (2 mg/ml) were added to the samples, vortexed, and centrifuged for 5 min at room temperature. The aqueous phase was mixed with 50 μl chloroform:isoamyl alcohol (50:1), vortexed into an emulsion, and centrifuged for 2 min at room temperature. The aqueous phase was removed and mixed with 50 μl of 4 M ammonium acetate and 250 μl ice-cold 100% ethanol, incubated at ≤70≤C for 30 min., and centrifuged for 15 min at 4≤C. The pellet was washed in ice-cold 90% ethanol, centrifuged for 5 min at 4≤C, and the pellet air-dried. The probe was resuspended in 50 μl hybridization buffer (80% formamide, 1 mM EDTA, 400 mM NaCl, 40 mM piperazine-*N*,*N*′-bis(ethanesulfonic acid), pH 6.7). The radioactivity was quantitated in duplicate for each probe from a 1-μl sample in a scintillation counter.

Ribonuclease protection assay

Following RNA isolation from cell treatment groups as described above, the RNA was diluted to 5 μg/μl in 50 μl H_2O. Fifty microliters of 4 M ammonium acetate (NH_4 ≤$CHCO_3$) and 250 μl of ice-cold ethanol were added and incubated for 1 h at ≤70≤C and then centrifuged for 15 min at 4≤C. The pellet rinsed with ice-cold 90% ethanol, air-dried, and resuspended in 8 μl of hybridization buffer. Two microliters of the probe, diluted to 3 ≤ 10^5 cpm/μl, was added, layered with mineral oil, warmed to 90≤C, and cooled to 56≤C for a 12-h incubation, and then to 37≤C for 15 min. One hundred microliters of the RNase cocktail (2.5 ml RNase buffer [10 mM Tris, 300 mM NaCl, 5 mM EDTA], 6 μl RNase A + T1[A: 80 ng/μl; T1: 250 U/μl]) was mixed with the samples under the mineral oil and incubated for 45 min at 30≤C. The RNase digest was removed and treated with 18 μl of the proteinase K cocktail (390 μl proteinase K buffer [4% SDS], 30 μl proteinase K [10 mg/ml], 30 μl yeast tRNA [2 mg/ml]).

The mixture was incubated for 15 min at 37°C, mixed with 65 μl water-saturated phenol and 65 μl chloroform:isoamyl alcohol (50:1), and centrifuged at room temperature for 5 min. One hundred twenty microliters of the aqueous phase was mixed with 120 μl 4 M ammonium acetate and 650 μl ice-cold 100% ethanol, incubated for 1 h at $\leq$70°C and centrifuged for 15 min at 4°C. The pellet was washed with ice-cold 90% ethanol, centrifuged for 5 min at 4°C, and the pellet air-dried. The pellets were resuspended in 5 μl loading buffer, heated for 3 min at 90°C, and immediately placed on ice. The samples were resolved on a 6% polyacrylamide gel and analyzed by a Storm phosphoimager (Molecular Dynamics) using ImageQuant 5.0 (Molecular Dynamics). Each data point was standardized to L32 RNA, expressed as fold increases compared to control, and reported as mean values from three experiments done in triplicates.

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