

# Apolipoprotein E-Dependent Differences in Innate Immune Responses of Maturing Human Neuroepithelial Progenitor Cells Exposed to HIV-1

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**Abstract** HIV enters the brain early during infection and induces a chronic inflammatory state that can result in neurological abnormalities in a subset of infected individuals. To investigate the effects of HIV exposure on neurogenesis and neuronal survival in the brain, we have used a model system consisting of human neuroepithelial progenitor (NEP) cells that undergo directed differentiation into astrocytes and neurons *in vitro*. Changes in gene expression in NEP cultures as a result of HIV exposure were investigated using gene expression microarrays with the Illumina HT-12 V4\_0\_R1 platform array. Through this approach, we identified a group of genes specifically upregulated by exposure to virus that are strongly related to interferon induced responses and antigen presentation. When the data were stratified by their apolipoprotein genotype, this innate immune response was more robust in the apolipoprotein E3/E3 genotype cultures than in the apolipoprotein E3/E4 counterparts. Biological processes as defined by the gene ontology (GO) program were also differently affected upon virus exposure in cultures of the two genotypes, particularly those related to antigen presentation and

the actions of interferons. Differences occurred in both in numbers of genes affected and their significance in the GO processes in which they participate, with apoE3/E3 > apoE3/E4. These data suggest that maturing NEP cultures recognize HIV and respond to it by mounting an innate immune response with a vigor that is influenced by the apolipoprotein E genotype of the cells.

**Keywords** Human immunodeficiency virus · HIV · Neuron · Apolipoprotein E · Innate immune response

## Introduction

HIV invasion of the brain occurs early during infection and eventually may result in a neuroinflammatory state associated with neurocognitive and behavioral abnormalities known as HIV-associated neurocognitive disorders (HAND). Even though antiretroviral therapy has been able to ameliorate the severity of immunological and neurological symptoms, abnormalities in cognitive function still persist, affecting the long term clinical course of HIV infection.

The clinical consequences of HIV infection in the brain have been attributed to the collective effect of multiple factors. Infection of microglia will result in the release of a number of chemokines and cytokines, some of which are proinflammatory and capable of exerting an adverse effect on both astrocytes and neurons (Yao et al. 2010). Inflammatory processes are an important corollary of systemic HIV infection, and markers of chronic immune activation have been correlated with markers of disease progression (Hazenberget al. 2003). In addition, even though a small population of astrocytes is infected by HIV-1, this infection results in changes in their behavior and function (Nath and Geiger 1998; Wang et al. 2003). Some but not all of these effects are mirrored by exposure of astrocytes to viral proteins, namely gp120 and tat, both released from infected cells (Li et al. 2005). Since astrocytes have multiple

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roles in the maintenance of brain function and are critical for the support of neuronal growth and function, their dysfunction may have a profound effect on neurons (Wang et al. 2004).

When human neuroepithelial progenitor cells (NEP) are allowed to differentiate *in vitro* into a mixed population of astrocytes and neurons in the presence of HIV, cell fate is not affected. However, neurons “fail to thrive”, displaying lower total neurite lengths per cell (McCarthy et al. 2006). Expression of the neurofilament light protein (NF-L) is also moderately reduced upon HIV-1 exposure (McCarthy et al. 2006; Martinez et al. 2012). These effects have been observed with no apparent evidence of productive viral infection (McCarthy et al. 2006), and thus they are assumed to be the consequences solely of HIV-1 exposure. However, the moderate decline in NF-L expression associated with virus exposure was not observed in all cultures, but mainly in cells with the specific apolipoprotein genotype E3/34 (Martinez et al. 2012).

Apolipoprotein E (apoE) is a 34 Kd protein that is found in liver and brain, and is responsible for transfer of phospholipids and cholesterol to cells (Mahley 1988; Mahley and Rall 2000). In the brain, apoE is predominantly produced by astrocytes and is important for building of membranes and neuronal growth. Three different alleles exist in the human population,  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ , with a distribution of 8 %, 77 % and 15 % respectively (Mahley 1988). The alleles code for proteins with differences in two amino acids in positions 112 and 158. The variant isoforms are characterized by the presence or absence of cysteine residues; thus they have different spatial conformations. As a consequence, their characteristics with regards to phospholipid and cholesterol transport as well as with their binding to the apoE receptor are different; for review see Hauser et al. (Hauser et al. 2011). In humans, the presence of the  $\epsilon 4$  allele has been linked to increased risk of Alzheimer’s disease and other cognitive disorders (Corder et al. 1993; Poirier et al. 1993; Corder et al. 1994). In addition,  $\epsilon 4$  has been linked with the risk of acquisition of, or the severity of viral infections, such as hepatitis C, Herpes simplex, and HIV (Burt et al. 2008; Kuhlmann et al. 2010).

In seminal reports published in the past 8–10 years, the effects of human and simian immunodeficiency viruses were evaluated by measuring the changes in gene expression in brains of infected humans, macaques or mice (Roberts et al. 2003; Kim et al. 2004; Masliah et al. 2004; Stephens et al. 2006; Borjabad et al. 2011). These *in vivo* studies revealed a large list of genes whose expression is changed by HIV infection, some of them commonly reported. (For a review see: (Borjabad et al. 2010; Winkler et al. 2012)).

In order to better understand the observed effects of HIV on differentiating NEP *in vitro* in comparison with reported changes occurring in *in vivo* infections, we have employed the Illumina gene array chip to investigate changes in gene expression in maturing neuroepithelial progenitor cells exposed to HIV. We further compared the virus-associated

changes in gene expression between the two most common apoE genotypes.

## Materials and methods

### NEP proliferation and differentiation

Multipotent human NEP were derived from mechanically dissociated human fetal rostral CNS of 53–115 days gestation and cultured in suspension as proliferating cell clusters (“neurospheres”), prepared as previously reported in detail (McCarthy et al. 2006). Human fetal CNS specimens were obtained from the Birth Defects Laboratory of the Human Embryology program, University of Washington Medical School, Seattle, WA, through a tissue distribution program supported by the National Institutes of Health (NIH), and that is monitored by the Institutional Review Board of the University of Washington. The use of human fetal CNS tissue for this study was reviewed by the Institutional Review Board of the Miller School of Medicine, University of Miami. Cells derived from distinct fetal specimens were always cultured separately so that independent cultures represent distinct specimens. To initiate directed differentiation and start a synchronized differentiation timeline at  $t=0$ , neurospheres were adhered onto substrate (poly-D,L-ornithine plus fibronectin-(PON-FN)-coated glass or plastic) for 1 h, then incubated in differentiation medium for 21–22 days ( $t=21$  or  $22$ ). Differentiation medium contained DMEM/F12 supplemented with 1 % (w/v) bovine serum albumin (BSA), glutamine, N2 supplement, and 2.5 % (v/v) heat-inactivated fetal bovine serum (fbs) (Gibco Invitrogen, Carlsbad, CA). This differentiation medium generated a mixed population of astrocytes and neurons. Expression of neuronal marker  $\beta$ -III-tubulin and astrocyte marker glial fibrillary acidic protein (GFAP) increased within the first days of differentiation. Expression of post-mitotic neurofilament proteins (NF-L, NFM) and neuronal nuclear antigen Hu increased between 7 and 14 days of differentiation and were stable at 21–22 days. Previous studies (McCarthy et al. 2006) have indicated that microglia are absent from the neurosphere-derived cultures.

Preliminary real time, reverse transcription polymerase chain reaction (RT-PCR) studies were performed using mRNA harvested from differentiating NEP at different time points in the differentiation timeline (days 0, 1, 3, 7, 12, 16). The objective was to detect mRNA for neuronal and astrocyte genes and confirm that the time course of neural gene expression was similar for all cultures regardless of culture treatment or apolipoprotein E genotype. This analysis indicated that differentiation to 16 days was optimal for mRNA expression of neuronal and astrocyte genes (data not shown). There was consistency between mRNA and protein data, where the optimal time of mRNA expression was a few days prior to optimal protein expression. Separate time

course experiments of neuronal and astrocyte mRNA expression over time were performed in cultures corresponding to the three apoE genotypes included in this study, apoE3/E3, apoE3/E4 and apoE4/E4, with similar expression patterns over time (data not shown).

#### Determination of apoE genotype

ApoE genotyping was performed essentially as described previously (Hixson and Vernier 1990) and adapted for these NEP cultures (Martinez et al. 2012). Genotyping is based on amplification of apoE cellular DNA corresponding to aminoacids 112 through 158. Restriction mapping of the amplified DNA with specific enzymes allows the discrimination of the different isoforms. The method is described in detail in Martinez et al. 2012

#### NEP exposure to virus

Differentiating NEP were exposed in parallel cultures to either differentiation medium alone (“untreated”), HIV-1 supernatants (“virus-exposed”) and mock supernatants (“mock-exposed”). HIV supernatants were derived from peripheral blood mononuclear cells (PBMC) previously stimulated with Phytohemagglutinin (PHA) and IL-2 as described (McCarthy et al. 1998) and de novo infected with HIV. Virus-infected PBMC cultures were maintained from 7 to 14 days until virus production was above 100 ng/ml, and then HIV-containing supernatants were harvested, aliquoted, and frozen for future use. “Mock” supernatants were harvested from parallel PBMC cultures derived from the same donors, activated with PHA and IL-2 in an identical manner, and maintained in culture for the same length of time, but not infected with HIV. The mock supernatant was included to control for the potential effect that growth factors and cytokines released by activated PBMC might have on the gene expression of differentiating NEP. For HIV exposure, we have utilized the HIV-1 strain SF2, obtained from the NIH AIDS Reference Reagent Program. This virus strain recognizes both CXCR4 or CCR5 as co-receptor (Trkola et al. 1998). Virus stock was prepared in batches from infected PBMC supernatant that were aliquoted and frozen until use (McCarthy et al. 1998) Mock supernatants from mock-infected PBMC were similarly prepared and frozen at identical time periods of culture (McCarthy et al. 1998, 2006). Replicate NEP cultures were seeded for differentiation and appropriate PBMC supernatant aliquots were diluted into differentiation medium at  $t=0$ . HIV supernatants were diluted to a final p24 concentration of 11 ng/ml, and equal volumes of mock supernatants were diluted into differentiation medium of mock-exposed cultures. Cultures were incubated for varying number of days as indicated, with change of medium and replenishment with fresh

PBMC supernatant aliquots every 7 days, on days 7 and 14 of the differentiation timeline, followed by harvest for mRNA analysis.

#### Gene expression assayed by quantitative RT-PCR

NEP monolayers were washed 1 time with PBS, and lysed using the Illustra RNAspin Mini kit from GE Healthcare (Pittsburgh, PA) for RNA extraction. RNA concentrations, 260/280, and 260/230 absorbance ratios were determined using a Nanodrop from Thermo Scientific (Wilmington, DE). Samples were diluted to 5 ng/ $\mu$ l in nuclease free water. One-step RT-PCR reactions were carried out using a B-R 1-Step SYBR Green quantitative (qRT-PCR) kit from Quanta (Gaithersburg, MD) with 25 ng of RNA per 50  $\mu$ l reaction and 400 nM of oligonucleotide primers. All primers were made at Invitrogen (Grand Island, NY). The qRT-PCR samples were run in a Bio-Rad CFX-96 thermocycler with the following amplification conditions: 51 °C for 20 min for reverse transcription, an initial denaturing step at 95 °C for 10 min, then 40 cycles of 95 °C for 10 s and 57 °C for 30 s for annealing and elongation. Melting curves were performed with an initial denaturing step at 95 °C for 1 min and annealing step at 55 °C for 1 min, then increased from 65 °C to 95 °C at 0.5 °C increments for 5 s each step. Data was analyzed using the Bio-Rad CFX software (Bio-Rad, Hercules, CA).

Target gene expression was determined relative to the housekeeping control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplified from the same sample, chosen because GAPDH expression is not affected by exposure to mock or HIV supernatants. Calculations were done using the Bio-Rad CFX software which employs the ddCT method (Vandesompele et al. 2002). All primers used in our study were designed, verified, and optimized in-house using the Beacon Designer software suite and had amplification efficiencies >98 %. Fold change in gene expression (virus-exposed vs. mock-exposed, or mock-exposed vs. untreated) was calculated as a direct ratio of normalized gene expression values.

Oligonucleotide primers used in this study had the following sequences:

BST2 forward: 5'TCACTACATTAACCCATAAG3'  
reverse: 5'TTAAGACCTGGTTTTCTC3'  
GAPDH forward: 5'TGAGAAGTATGACAACAG3'  
reverse: 5'ATGAGTCCTTCCACGATA3'  
IFI6 forward: 5'GAACTTTGTCTATCACTCTC3'  
reverse: 5'AAGTGTTTTCTGGGTGAA3'  
IFI44 forward: 5'AATCTTGACTTGCTCAA3'  
reverse: 5'TCATCCAGTGAATCTTCG3'  
IFIT1 forward: 5'GGATAGTCTGGAGCAATT3'  
reverse: 5'TTCTAAATCAGGCATTCA3'

IFITM1 forward: 5'CCTCTTCTTGAAGTGGTG3' reverse: 5'CTAGACTTCACGGAGTAG3'  
 STAT1 forward: 5' CAGTAAAGTCAGAAATGTG3' reverse: 5'TTCATCTTGTAATCTTCCA3'  
 MX1 forward: 5' TGATACCATTAACTTGTGAC3' reverse: 5' GATGTCCACTTCGGAAAC3'  
 ISG15 forward: 5' TGGACAAATGCGACGAAC3' reverse: 5' GCCCTTGTATTCTCAC3'

#### RNA preparation and gene expression microarray generation

Total RNA was extracted from differentiated NEP-derived cells after 16 days of culture in differentiation medium. The RNA was extracted using the Illustra RNAspin Mini kit from GE Healthcare (Pittsburgh, PA). RNA analyses and microarray data generation were then performed by the technical staff of the Oncogenomics Core Laboratory, Miller School of Medicine, the University of Miami.

Total RNA was quantified with a Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington), and its quality was examined with a Bioanalyzer 2100 using the RNA 6000 Nano kit (Agilent, Santa Clara, CA). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions starting with 400 ng total RNA. Successful cRNA generation was checked using the Bioanalyzer 2100. Samples were added to the Beadchip after randomization using the randomized block design to reduce batch effects. Hybridization to the Sentrix Human-HT12 Expression BeadChip (Illumina, Inc., San Diego, CA), washing and scanning were performed according to the Illumina BeadStation 500 manual (revision C). The resulting microarray data was analyzed using Illumina GenomeStudio software.

#### Microarray analysis

Expression data from the HumanHT-12 V4\_0\_R1 platform array (Illumina) were loaded on GeneSpring® 12.5 GX (Agilent Technologies, Santa Clara, CA, USA). The raw intensities from the array were normalized using 'Quantile normalization' method and log (base 2) transformed. Data from different batches were processed separately and a batch bias correction was performed using *ComBat* algorithm (Johnson et al. 2007) on R/Bioconductor package (<http://www.R-project.org>). The batch corrected data was subjected to statistical analysis using paired Student's *T*-test (two-class comparisons) and one-tailed Analysis of Variance or ANOVA (multiclass comparisons). For ANOVA, probes were further tested using the Tukey post hoc analysis for determining significance. All significant and differentially expressed (DE) genes were subset using *P*-value  $\leq 0.05$  and

fold change  $\geq 1.5$  thresholds. These lists of DE genes were analyzed for enriched pathways using the MetaCore™ software on GeneGo from Thomson Reuters (New York, NY).

#### Results

##### Gene expression profiles of differentiating NEP exposed to HIV

For this study, tissues from 8 different brain specimens were analyzed. Four had the apoE3/E3 genotype, and had gestational ages between 54 and 108 days (mean=79.25). Three had the apoE3/E4 genotype, with gestational ages between 57 and 96 days (mean=70.33). One had the apoE4/E4 genotype, with a gestational age of 67 days. The NEP derived from these brain specimens were exposed to HIV-containing or mock supernatants throughout a 16 day differentiation timeline, at which time mRNA was harvested. During that timeline the population of cells transitioned from predominantly nestin-expressing NEP cells to a mixed population of astrocytes and neurons, including post-mitotic neurons (McCarthy et al. 2006).

Analysis of gene expression microarray results was performed as described in **Materials and methods**, comparing the three different treatments, untreated (differentiation medium alone), (mock-exposed), (mock supernatant added to differentiation medium), and HIV-exposed (HIV-containing supernatants added to differentiation medium). HIV-containing PBMC supernatants contain a mixture of cell-derived factors in addition to virus particles and viral proteins. Activated PBMC, stimulated with PHA and II-2, secrete growth factors, chemokines, cytokines, and cellular debris generated over time in culture. These factors contribute to a cell-generated inflammatory microenvironment in the background of virus production. To differentiate between the effects of virus-specific factors and the cell-specific components of the inflammatory microenvironment, gene expression was compared between virus and mock supernatant treatments, and also between mock supernatant treatment and no treatment (culture medium alone). Looking at the NEP-derived cell populations as a whole, regardless of apoE genotype, approximately 48,000 probes were tested, and the expression of 2,600 genes was differentially regulated among the three treatment groups (ANOVA,  $p \leq 0.05$ , no multiple test correction). When tukeyHSD post-hoc test was applied for pairwise comparisons, of these 2,600 genes, 990 genes were differentially regulated when comparing HIV-1-exposed to mock-exposed NEP-derived cultures; 974 genes were differentially regulated when comparing mock-exposed to untreated NEP-derived cultures; and 1,002 genes were differentially regulated when comparing HIV-1-exposed to untreated NEP-

derived cultures. Pairwise fold change was calculated for the 3 cell culture treatments, and the fold change values were then compared by paired *t*-test. This analysis found 32 genes that were significantly upregulated and 1 gene that was downregulated with a fold change of 1.5 or more when gene expression was compared between the HIV-exposed and mock-exposed cultures (Table 1, paired *t* test,  $p \leq 0.05$ ; no multiple testing correction). All of the upregulated genes were genes that can be induced by interferon, and one of them coded for the HIV restriction factor, tetherin, also known as BST2 (Table 1). Thirty four genes were significantly upregulated in mock-exposed NEP-derived cultures as compared to untreated NEP-derived cultures (Table 2). Of these 34 genes, 20 were associated with antigen presentation, and 7 genes including tetherin were also among the 32 genes that were relatively upregulated in HIV-1-exposed NEP-derived cultures as compared to mock-exposed cultures.

#### Gene expression profiles according to apolipoprotein E genotype

Gene expression was next analyzed separately for the differentiated NEP-derived cultures, according to their apolipoprotein E genotype, apoE3/E3 or apoE3/E4. Maturing NEP-derived cultures with the apoE3/E3 genotype responded to HIV-1 exposure with a total of 2,105 genes differentially regulated among the three treatment groups (ANOVA,  $p \leq 0.05$ , no multiple test correction). When tukeyHSD post-hoc test was applied for pairwise comparisons, of the 2,105 genes, 724 were differentially regulated when comparing HIV-1-exposed to mock-exposed NEP-derived cultures, 832 were differentially regulated when comparing mock-exposed to untreated NEP-derived cultures, and 953 when comparing HIV-1-exposed to untreated NEP-derived cultures. Pairwise fold change was calculated for the 3 cell culture treatments, and the fold change values were then compared by paired *t*-test. This analysis found 17 genes that were upregulated 1.5 fold or higher when comparing HIV-1-exposed to mock-exposed NEP-derived cultures (Table 3), and 14 of these were interferon induced genes. Seven of these 17 genes were also significantly upregulated when mock-exposed NEP-derived cultures were compared to untreated NEP-derived cultures (Table 4). Thus 10 of these 17 genes were specifically expressed as a response to HIV exposure. Six of those 10 genes were interferon induced genes (Table 3). Maturing NEP-derived cultures with the apoE3/E3 genotype also responded to mock-infected supernatant exposure by upregulating 38 genes with a fold change of 1.5, when mock-exposed cultures were compared to untreated NEP-derived cultures (Table 4). The majority of these upregulated genes code for MHC class I and class II proteins.

Maturing NEP-derived cultures with the apoE3/E4 genotype differentially regulated 1,871 genes when comparing the

three treatments (ANOVA  $p \leq 0.05$ , no multiple test correction). When tukeyHSD post-hoc test was applied for pairwise comparisons, of the 1,871 genes, 790 genes were differentially regulated when comparing HIV-1-exposed to mock-exposed cultures, 782 genes were differentially regulated in mock-exposed versus untreated cultures, and 785 genes were differentially regulated in HIV-1-exposed versus untreated cultures. The total number of genes differentially regulated in apoE3/E4 cultures was 89 % of the total number of genes that were differentially regulated in the apoE3/E3 cultures, using one-way ANOVA to test for differences among the three treatment groups. When pairwise fold change values for gene expression by HIV-1-exposed versus mock-exposed apoE3/E4 cultures were analyzed by paired *t*-test, 13 genes were significantly upregulated with a fold change of 1.5 or more, compared to 17 genes in the apoE3/E3 cultures (Table 5, paired *t*-test  $p \leq 0.05$ ; no multiple testing correction). Ten genes can be upregulated by interferon, compared to 14 in the apoE3/E3 cultures. A lack of robust response was observed in mock-exposed NEP-derived apoE3/E4 cultures when compared to untreated NEP-derived apoE3/E4 cultures. Only 8 genes were upregulated with a fold change of 1.5 or more (Table 6) compared to 37 genes in the similarly-treated apoE3/E3 cultures (Table 4).

Inspection of the apoE4/E4 culture indicated that some genes are differentially regulated in response to virus or mock supernatant exposure, but the significance of this data cannot be assessed because only one sample of this rare phenotype was available for this study. However, the genes that are upregulated with a fold change of 1.5 or more are mainly related to antigen presentation (Table S1, supplementary material).

Genes affected by HIV-1 exposure were subjected to functional analysis using the MetaCore™ on GeneGo software from Thompson Reuters. The enrichment pathway analyses of genes differentially regulated among all 7 specimens (4 apoE3/E3 and 3 apoE3/E4 cultures) revealed that interferon related responses were significantly affected, independent of apolipoprotein E genotype, and constituting four of the top ten pathways differentially regulated, with a *p* value in the range of  $10^{-11}$  to  $10^{-4}$  (Table S2). The majority of the ten top gene ontology (GO) processes also involve genes associated with the actions of interferons, with *p* values in the range of  $10^{-37}$  to  $10^{-21}$  (Table S3), as well as antigen presentation processes, thus indicating a strong immune response to HIV was mounted by these NEP-derived cells. When the enrichment pathway analyses were stratified by apoE genotype, interferon related pathways and antigen presentation pathways were similarly affected in the apoE3/E3 and apoE3/E4 genotypes (Table S4 and S6). However, when Gene ontology processes were analyzed as a function of the apoE genotype, genes associated with the action of interferon and antigen processing

**Table 1** Genes differentially regulated in cells differentiated from NEP: fold change for virus vs. mock, *t*-test,  $p \leq 0.05$ 

Gene ID	Symbol	Name	Fold Change	<i>p</i> value
2537	IFI6	Interferon inducible protein 6	4.07	0.0000
			1.78	0.0006
4599	MX1	Interferon induced GTP binding protein	3.62	0.0003
9636	ISG15	Ubiquitin-like modifier	3.29	0.0001
684	BST2	Bone marrow stromal cell antigen 2- Tetherin	3.10	0.0002
			2.93	0.0004
10964	IFI44L	Interferon-induced protein 44-like	2.66	0.0008
3434	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	2.49	0.0002
8519	IFITM1	Interferon induced transmembrane protein 1	2.48	0.0004
55008	HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	2.24	0.0011
3437	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	2.20	0.0001
			1.78	0.0006
6772	STAT1	Signal transducer and activator of transcription 1	1.91	0.0001
			1.73	0.0002
			1.61	0.0010
10410	IFITM3	Interferon induced transmembrane protein 3	1.84	0.0005
4940	OAS3	2'-5'-oligoadenylate synthetase 3	1.76	0.0008
10561	IFI44	Interferon-induced protein 44	1.74	0.0004
4938	OAS1	2'-5'-oligoadenylate synthetase 1	1.73	0.0031
			1.54	0.0009
10581	IFITM2	Interferon induced transmembrane protein 2	1.73	0.0023
51191	HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	1.72	0.0004
94240	EPSTI1	Epithelial stromal interaction 1	1.71	0.0015
3429	IFI27	Interferon, alpha-inducible protein 27	1.70	0.0089
3106	HLA-B	Major histocompatibility complex, class I, B	1.65	0.0004
10379	IRF9	Interferon regulatory factor 9	1.61	0.0010
54625	PARP14	Poly (ADP-ribose) polymerase family, member 14	1.57	0.0006
3134	HLA-F	HLA class I histocompatibility antigen, alpha chain F	1.56	0.0026
85441	PRIC285	Peroxisomal proliferator-activated receptor alpha-interacting cofactor complex,285 kD subunit2	1.55	0.0032
3136	HLA-H	Major histocompatibility complex, class I, H Signal transducer and activator of transcription 1 (pseudogene)	1.54	0.0020
2633	GBP1	Guanylate binding protein 1, interferon-inducible	1.54	0.0088
			1.52	0.0003
5698	PSMB9	Proteasome subunit, beta type, 9	1.53	0.0007
83666	PARP9	poly (ADP-ribose) polymerase family, member 9	1.53	0.0019
10866	HCP5	HLA complex P5 (non-protein coding)	1.51	0.0006
51296	SLC15A3	Solute carrier family 15, member 3	1.51	0.0037
54809	SAMD9	Sterile alpha motif domain containing 9	1.51	0.0084
64761	PARP12	Poly (ADP-ribose) polymerase family, member 12	1.50	0.0005
3133	HLA-E	Major histocompatibility complex, class I, E	1.50	0.0057
642828	LOC642828	RPL6P10 ribosomal protein L6 pseudogene 10	-1.52	0.0033

Fold change (FC) is gene expression in differentiated, HIV-exposed NEP-derived cells compared to the gene expression in differentiated, mock-exposed NEP-derived cells. Differentially expressed genes with  $p$  value  $\leq 0.05$  were sorted by FC values, from the highest to the lowest. Listed are FC values of 1.5 or more. All cultures were included in this analysis, regardless of apoE genotype. Results for genes with more than one probe are listed individually

and presentation were prominently featured in the apoE3/E3 cultures, with  $p$  values ranging between  $10^{-18}$  and  $10^{-15}$

(Table S5), while in the E3/E4 cultures, the top seven of the top ten GO processes differentially regulated were associated

**Table 2** Genes differentially regulated in cells differentiated from NEP: Fold change for mock vs. untreated, *t* test,  $p \leq 0.05$ 

Gene ID	Symbol	Name	FC	<i>p</i> value
3113	HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1	3.96	0.00
3122	HLA-DRA	HLA class II histocompatibility antigen, DR alpha chain	3.74	0.00
			2.99	0.00
730415	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	3.50	0.00
			1.90	0.02
3126	HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	3.34	0.00
972	CD74	HLA class II histocompatibility antigen gamma chain	3.19	0.00
			2.98	0.00
			1.77	0.01
3128	HLA-DRB6	Major histocompatibility complex, class II, DR beta 6	2.98	0.00
3108	HLA-DMA	Major histocompatibility complex, class II, DM alpha	2.66	0.00
649143	LOC649143	Similar to HLA class II histocompatibility antigen, DRB1-9 beta chain precursor	2.65	0.01
10866	HCP5	HLA Complex P5	2.60	0.00
3117	HLA-DQA1	HLA class II histocompatibility antigen, DQ alpha 1 chain-like	2.60	0.02
			2.52	0.02
3109	HLA-DMB	Major histocompatibility complex, class II, DM beta	2.58	0.00
2633	GBP1	IFN induced glutamate binding protein 1 (HLA class II histocompatibility antigen gamma chain)	2.50	0.00
			2.03	0.00
3106	HLA-B	Major histocompatibility complex, class I, B	2.30	0.00
3136	HLA-H	Major histocompatibility complex, class I, H	2.02	0.00
			1.58	0.00
5698	PSMB9	Proteasome subunit beta type-9	1.99	0.00
3134	HLA-F	HLA class I histocompatibility antigen, alpha chain F	1.85	0.00
			1.58	0.00
100133583	LOC100133583	HLA class II histocompatibility antigen, DQ beta 1 chain-like	1.83	0.01
3115	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	1.77	0.03
3111	HLA-DOA	Major histocompatibility complex, class II, DO alpha	1.74	0.01
586	BCAT1	Branched chain amino-acid transaminase	1.70	0.00
5696	PSMB8	Proteasome subunit beta type-8	1.66	0.00
			1.61	0.00
12	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1.64	0.04
3133	HLA-E	HLA class I histocompatibility antigen, alpha chain E	1.62	0.00
6890	TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	1.61	0.00
3105	HLA-A	Major histocompatibility complex, class I, A	1.59	0.00
81035	COLEC12	Collectin sub-family member 12	1.58	0.03
4837	NNMT	Nicotinamide N-methyltransferase	1.58	0.00
146802	SLC47A2	Solute carrier family 47, member 2	1.56	0.01
4057	LTF	Lactotransferrin	1.54	0.05
6281	S100A10	S100 calcium binding protein A10	1.53	0.01
10457	GPNMB	Glycoprotein (transmembrane) nmb	1.53	0.00
2634	GBP2	Guanylate binding protein 2, interferon-inducible	1.52	0.01
684	BST2	Bone marrow stromal cell antigen 2	1.52	0.00
			1.51	0.00
5920	RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	1.51	0.00

Fold change (FC) is gene expression in differentiated, mock-exposed NEP-derived cells compared to the gene expression in differentiated, untreated NEP-derived cells. Differentially expressed genes with  $p$  value  $\leq 0.05$  were sorted by FC values, from the highest to the lowest. Listed are FC values of 1.5 or more. All cultures were included in this analysis, regardless of apoE genotype. Results for genes with more than one probe are listed individually

**Table 3** Genes differentially regulated in cells differentiated from apoE3/E3 NEP: fold change for virus vs. mock, *t* test,  $p \leq 0.05$ 

Gene ID	Symbol	Name	FC	<i>p</i> value
4599	MX1	Interferon induced GTP binding protein	2.67	0.030
684	BST2	Bone marrow stromal cell antigen 2- (Tetherin)	2.53	0.007
			2.18	
8519	IFITM1	Interferon-induced transmembrane protein 1 (cd225)	2.45	0.009
3437	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.90	0.021
3429	IFI27	Interferon, alpha-inducible protein 27	1.79	0.010
6772	STAT1	Signal transducer and activator of transcription 1, 91 kDa	1.71	0.010
			1.51	
3136	HLA-H	Major histocompatibility complex, class I, H	1.69	0.011
			1.52	
3106	HLA-B	HLA-B major histocompatibility complex, class I, B	1.62	0.004
4940	OAS3	2'-5'-oligoadenylate synthetase 3, 100 kDa	1.62	0.036
10581	IFITM2	Interferon induced transmembrane protein 2 (1-8D)	1.60	0.015
5698	PSMB9	Proteasome subunit beta type-9	1.59	0.003
10866	HCP5	HLA Complex P5	1.59	0.009
3134	HLA-F	major histocompatibility complex, class I, F	1.59	0.009
10410	IFITM3	Interferon induced transmembrane protein 3	1.56	0.019
2634	GBP2	Guanylate binding protein 2, interferon-inducible	1.54	0.023
2537	IFI6	Interferon alpha-inducible protein 6	1.52	0.044
51296	SLC15A3	Solute carrier family 15, member 3	1.50	0.031

Fold change (FC) is gene expression in differentiated, HIV-exposed NEP-derived cells compared to the gene expression in differentiated, mock-exposed NEP-derived cells. Differentially expressed genes with *p* value  $\leq 0.05$  were sorted by FC values, from the highest to the lowest. Listed are FC values of 1.5 or more. Only cultures with the apoE3/E3 genotype were included in this analysis. Results for genes with more than one probe are listed individually

with tolerance induction and not with the actions of interferons (Table S7).

Genes that were differentially affected by HIV exposure according to apoE genotype were also subjected to functional analysis using the MetaCore™ on GeneGo software from Thompson Reuters. This generated a set of enrichment pathways and GO processes that directly compare the two apoE genotypes (Fig. 1). There were notable differences between the responses of the two genotypes, most prominent in the analysis of GO processes. These differences related to antigen presentation and interferon related responses, both in the number of genes that are differentially regulated as well as the significance of the *p* values (Fig. 1). Among the top 10 enrichment pathways, immune response antiviral actions of interferons were more significant with the apoE3/E3 cultures. The top 8 GO processes that were differentially regulated during HIV exposure were processes that involve antigen presentation and interferon-related responses. In all of these GO processes, there was a dramatic difference between the 2 genotypes, both in the number of genes and the significance of their participation in the GO process. Among all the GO processes that were identified, the response of the apoE3/E3 cultures was more significant than that of the apoE3/E4 cultures.

#### Confirmatory RT-PCR of differentially regulated genes

Quantitative RT-PCR was used to independently confirm changes in gene expression determined by microarray analysis of differentiating NEP exposed to HIV-1 or mock

supernatants. Eight representative genes were selected among genes showing changes in expression in either the apoE3/E3 or the apoE3/E4 cultures. These genes were BST2, STAT1, MX1, ISG15, IFI44, IFI6, IFIT1 and IFITM1. Their expression was independently evaluated by quantitative RT-PCR using 5 of the 7 specimens in this study, 3 having the apoE3/E4 genotype and two having the apoE3/E3 genotype. Gene expression regardless of apoE genotype was then compared between the two methods (Fig. 2). Results obtained by the two methods were comparable, though FC values were in general higher when derived from the quantitative RT-PCR data than from the microarray data. This may be due to the fact that FC values from RT-PCR data used direct ratios of normalized gene expression values, while the FC values from the microarray data used ratios of log (base 2) transformations of gene expression values.

The gene expression data clearly reflected the significance of innate immune gene expression by these neural cells after exposure to HIV during their differentiation from NEP. Accordingly, we examined the expression of selected innate immune genes by quantitative RT-PCR at earlier time points in the differentiation timeline, to determine how soon after virus exposure these genes are expressed. NEP cultures from two brain specimens were used. One was an apoE3/E4 culture that had been analyzed at day 16 by gene expression microarray. The other was an independent apoE3/E3 culture. Five innate immune response genes were tested: BST2, IFIT1, ISG15, MX1 and STAT1. All genes reached maximal



**Table 4** Genes differentially regulated in cells differentiated from apoE3/E3 NEP: fold change for mock vs untreated, *t* test,  $p \leq 0.05$ 

Gene ID	Symbol	Name	FC Mock vs. Unt	<i>p</i> value
3113	HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1	4.97	0.048
3126	HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	4.30	0.027
730415	LOC730415	Major histocompatibility complex, class II, DR beta 1	4.08	0.047
3109	HLA-DMB	Major histocompatibility complex, class II, DM beta	3.13	0.032
3108	HLA-DMA	Major histocompatibility complex, class II, DM alpha	3.02	0.031
3106	HLA-B	Major histocompatibility complex, class I, B	2.64	0.001
10866	HCP5	HLA Complex P5	2.60	0.003
2633	GBP1	IFN induced glutamate binding protein 1 (HLA class II histocompatibility antigen gamma chain)	2.50	0.014
			2.06	0.024
3115	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	2.32	0.047
100133583	LOC100133583	HLA class II histocompatibility antigen, DQ beta 1 chain-like	2.18	0.049
3134	HLA-F	HLA class I histocompatibility antigen, alpha chain F	2.12	0.003
			1.62	0.006
3136	HLA-H	Major histocompatibility complex, class I, H (pseudogene)	2.11	0.000
			1.68	0.000
5698	PSMB9	Proteasome subunit beta type-9	2.08	0.003
3111	HLA-DOA	Major histocompatibility complex, class II, DO alpha	1.97	0.049
972	CD74	HLA class II histocompatibility antigen gamma chain	1.87	0.044
5920	RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	1.81	0.003
3133	HLA-E	Major histocompatibility complex, class I, E	1.80	0.003
4837	NNMT	Nicotinamide N-methyltransferase	1.80	0.022
5696	PSMB8	Proteasome subunit beta type-8	1.79	0.003
			1.74	0.008
			1.6	0.006
2634	GBP2	Guanylate binding protein 2, interferon-inducible	1.76	0.011
3105	HLA-A	Major histocompatibility complex, class I, A	1.75	0.004
6890	TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	1.73	0.022
100129681	LOC100129681	Bone marrow stromal cell antigen 2	1.71	0.015
11118	BTN3A2	Butyrophilin, subfamily 3, member A2	1.64	0.001
497190	LOC497190	C-type lectin domain family 18, member B	1.64	0.038
1052	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	1.63	0.019
54435	HCG4	HLA complex group 4 (non-protein coding)	1.61	0.000
586	BCAT1	Branched chain amino-acid transaminase 1, cytosolic	1.58	0.037
684	BST2	Bone marrow stromal cell antigen 2- (Tetherin)	1.58	0.046
3428	IFI16	Interferon, gamma-inducible protein 16	1.58	0.029
5630	PRPH	Peripherin	1.58	0.015
7045	TGFBI	Transforming growth factor, beta 1	1.55	0.046
6648	SOD2	Superoxide dismutase 2, mitochondrial	1.55	0.024
715	C1R	Complement component 1, r subcomponent	1.53	0.002
10410	IFITM3	Interferon induced transmembrane protein 3	1.51	0.012
11118	BTN3A2	Butyrophilin, subfamily 3, member A2	1.51	0.011
834	CASP1	Caspase 1, apoptosis-related cysteine peptidase	1.51	0.026
3135	HLA-G	Major histocompatibility complex, class I, G	1.51	0.004

Fold change (FC) is gene expression in differentiated, mock-exposed NEP-derived cells compared to the gene expression in differentiated, untreated NEP-derived cells. Differentially expressed genes with  $p$  value  $\leq 0.05$  were sorted by FC values, from the highest to the lowest. Listed are FC values of 1.5 or more. Only cultures with the apoE3/E3 genotype were included in this analysis. Results for genes with more than one probe are listed individually

**Table 5** Genes differentially regulated in cells differentiated from apoE3/E4 NEP: fold change for virus vs. mock, *t* test,  $p \leq 0.05$ 

Gene ID	Symbol	Name	Fold Change	<i>p</i> value
2537	IFI6	Interferon alpha-inducible protein 6	3.74	0.01843
			1.62	0.041401
9636	ISG15	Ubiquitin-like modifier	2.91	0.039843
3434	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	2.53	0.028373
3437	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	2.30	0.037042
			1.57	0.010078
4938	OAS1	2'-5'-oligoadenylate synthetase 3	2.10	0.045357
6772	STAT1	Signal transducer and activator of transcription 1	2.06	0.031311
			1.81	0.027569
10561	IFI44	Interferon-induced protein 44	1.98	0.031434
10379	IRF9	Interferon regulatory factor 9	1.66	0.009863
3959	LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	1.60	0.032152
5920	RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	1.58	0.027926
55601	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	1.53	0.034285
642989	LOC642989	Similar to 40S ribosomal protein S25	-1.51	0.042072
3202	HOXA5	Homeobox A5	-1.54	0.024202

Fold change (FC) is gene expression in differentiated, HIV-exposed NEP-derived cells compared to the gene expression in differentiated, mock-exposed NEP-derived cells. Differentially expressed genes with  $p$  value  $\leq 0.05$  were sorted by FC values, from the highest to the lowest. Listed are FC values of 1.5 or more. Only cultures with the apoE3/E4 genotype were included in this analysis. Results for genes with more than one probe are listed individually

expression by day 1 (Fig. 3), with subsequent levels declining as compared to day 1, but remaining constant over time after that. This pattern occurred in cultures of both genotypes (full time course data not shown). However, differences in gene expression between the virus-exposed apoE3/E3 and apoE3/E4 cultures were noticeable at this early time point (Fig. 3). This difference in gene expression also occurred, for some of the genes, after exposure to mock supernatants, but was less dramatic than the difference in expression after virus exposure.

## Discussion

In this study we have used maturing human fetal brain neuroepithelial progenitor cells (NEP) to model the effects of HIV exposure on neurogenesis and neuronal survival in the CNS in vivo. In this culture model, exposure to virus occurs during a 3 week differentiation timeline (McCarthy et al. 2006). By the third week of the timeline, the population of cells has transitioned from predominantly nestin-expressing NEP cells to a mixed population of astrocytes

**Table 6** Genes differentially regulated in cells differentiated from apoE3/E4 NEP: fold change for mock vs. untreated, *t* test,  $p \leq 0.05$ 

Gene ID	Symbol	Name	FC	<i>p</i> value
3128	HLA-DRB6	Major histocompatibility complex, class II, DR beta 6	2.34	0.0310
2633	GBP1	IFN induced glutamate binding protein 1 (HLA class II histocompatibility antigen gamma chain)	1.84	0.0383
586	BCAT1	Branched chain amino-acid transaminase 1, cytosolic	1.82	0.0120
642073	LOC642073	Similar to HLA class II histocompatibility antigen, DRB1-1 beta chain precursor	1.55	0.0205
3134	HLA-F		1.53	0.0043
140771	SMCR5	Smith-Magenis syndrome chromosome region, candidate 5 (non-protein coding)	-1.51	0.0500
202243	CCDC125	Coiled-coil domain containing 125	-1.58	0.0137
100130445	LOC100130445	Uncharacterized	-1.58	0.0196

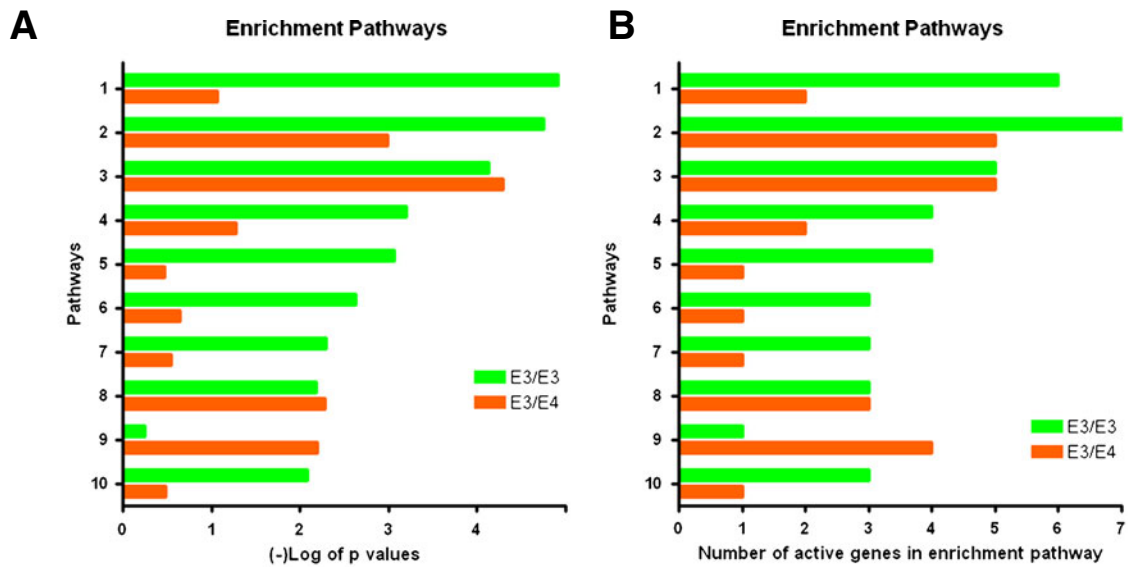
Fold change (FC) is gene expression in differentiated, mock-exposed NEP-derived cells compared to the gene expression in differentiated, untreated NEP-derived cells. Differentially expressed genes with  $p$  value  $\leq 0.05$  were sorted by FC values, from the highest to the lowest. Listed are FC values of 1.5 or more. Only cultures with the apoE3/E4 genotype were included in this analysis. Results for genes with more than one probe are listed individually

and neurons, including post-mitotic neurons. Since RNA for gene expression analysis was harvested at day 16 of the timeline, gene expression data reflect the output from astrocytes and neurons more than from progenitor cells. However, since the cell population at day 16 was differentiated from NEP exposed to HIV during the timeline, gene expression data also reflect the impact of HIV exposure on both neurogenesis and astrogenesis in the cultures. Neurogenesis in the adult human is spatially localized in the brain, largely to the hippocampus, whereas neurogenesis during early human fetal development is more pervasive, laying down the architecture of the brain. Differences in neurogenesis put some limitation on interpretation of data generated with cells derived from fetal brain. However, though there may be functional differences between fetal and adult NEP, they have demonstrated similar developmental potential (i.e. markers of neurons, astrocytes, oligodendrocytes) and similar gene expression patterns when studied *in vitro* (Palm et al. 2000).

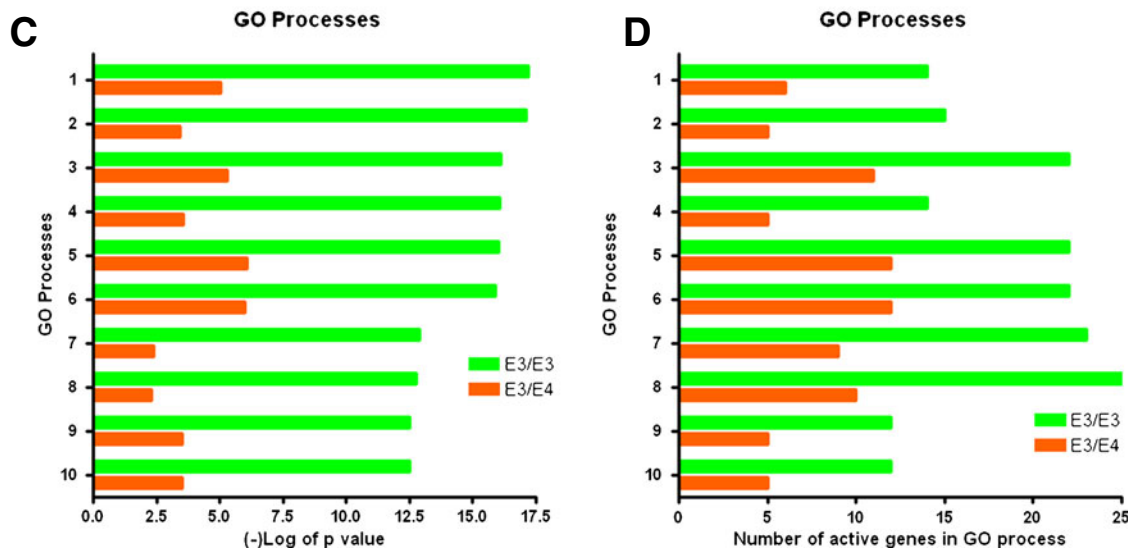
We utilized functional genomics to elucidate the processes and pathways affected when astrocytes and neurons are exposed to the virus during differentiation from NEP, and we also analyzed changes in gene expression as a function of the apoE genotype of the NEP. Our first observations were that differentiating NEP-derived cultures exposed to HIV-1 upregulated a number of genes as compared to the mock exposed cultures, and the majority of these are related to interferon induced responses and antigen presentation. This indicates that these human NEP-derived cells identify the virus and respond to it. We have also observed that mock exposed cultures upregulated genes related to antigen presentation as compared to untreated cultures. This mock effect may be due to the fact that these “mock” supernatants, derived from PHA and IL2 stimulated PBMC, contain potential allogeneic antigens from cell debris and an array of chemokines, growth factors, and interleukins (Martinez et al. 2012). Therefore the background neuroinflammatory environment represented by mock treatment of differentiating NEP results in an upregulation of adaptive-type immune response genes. Virus containing supernatants augment the transcription of antigen presenting genes, suggesting an added effect of virus exposure. Interferon induced responses were not significantly upregulated in cultures exposed to mock supernatants, indicating that the innate immune response to virus exposure is specific. It is of interest to note that fold changes in gene expression reported in other studies using a variety of neural cell sources, including simian and human brain tissue, as well as human fetal brain derived astrocytes (Roberts et al. 2003; Kim et al. 2004; Masliah et al. 2004; Stephens et al. 2006; Borjabad et al. 2011) were noted in both directions, i.e. increases and decreases in expression of certain genes, though in this system, most fold change values were 1.5 or less (HIV as compared to mock, and mock as compared to untreated). In addition, other interferon induced genes specifically related to anti-HIV responses were upregulated in these cultures. They include four genes that function as HIV-1

restriction factors: 1) BST2, also known as tetherin (FC=3.101  $p=0.0002$ , Table 1), which plays an important role in preventing budding from infected cells and is counteracted by the viral protein vpu (Neil et al. 2008; Perez-Caballero et al. 2009), 2) TRIM22 (FC=1.39,  $p=0.00002$ , data not shown) (Barr et al. 2008), which down-regulates transcription from the HIV-1 promoter region, 3) ISG15, which interferes with virus production during the budding process (Okumura et al. 2006; Pincetic et al. 2010), and 4) the IFTM proteins, that restrict HIV infection at different stages including entry (Lu et al. 2011).

In a review summary by Winkler et al. (Winkler et al. 2012), five genes were listed as commonly upregulated in tissues from all these studies. These include B2M, IFI44, IFIT3, MX1 and STAT1. All of these were significantly upregulated ( $p\leq 0.05$ ) in the NEP cultures exposed to HIV-1 as compared to mock exposed cultures, regardless of apoE genotype. Three of these genes, IFIT3, MX1 and STAT1 showed a fold change of 1.5 or more. In a study comparing autopsy brain samples from 9 individuals with HIV encephalitis against samples from 8 individuals with HIV infection but without encephalitis, Masliah et al. reported that 59 genes were preferentially upregulated in encephalitis brain tissues (Masliah et al. 2004). Among those 59 genes, 8 were interferon induced genes; 7 of these genes were upregulated in differentiating NEP-derived cultures exposed to HIV (Table 1), with remarkably similar fold change values, even though two different gene chips were used for determining gene expression in each study (Affymetrix in Masliah et al. vs. Illumina HT in our study). Table 7 presents a direct comparison of 3 published gene expression analyses of *in vivo* gene responses to HIV with the gene expression patterns from *in vitro* responses summarized in this study. This comparison emphasizes the great degree of similarity in the genes that are upregulated by HIV (Table 7). The greatest agreement was found between our study and that published by Borjabad et al. (Borjabad et al. 2011), which examined gene expression in brain tissue of HIV-infected individuals not treated with antiretroviral drugs as compared with expression in uninfected control brain. Of the 32 genes upregulated *in vitro* in differentiating NEP regardless of the apoE genotype (Table 1), 24 (75 %) are upregulated *in vivo* in HIV-infected compared to uninfected brain (Table 7). Comparing a second study from human tissue (Masliah et al. 2004), with the *in vitro* cell cultures, a smaller number of genes are commonly upregulated (8 of 32). But this study (Masliah et al.) compares gene expression between infected tissue of infected individuals with and without encephalitis and not between infected and uninfected. In a third *in vivo* study (Roberts et al. 2003), using tissues from SIV-infected macaques, 13/32 genes are commonly upregulated (Table 7), half of which are the genes which have the highest FC values in our study (Table 1). Seven of the 32 genes upregulated in differentiating NEP were not found in the three studies reviewed, but two of these are non protein coding genes.



Gene Pathway Name:	Total Number of Genes in Pathway:
1. Cell cycle_Spindle assembly and chromosome separation	33
2. Immune response_Antiviral actions of Interferons	52
3. Immune response_Antigen presentation by MHC class I	28
4. Cell cycle_Initiation of mitosis	25
5. Transcription_Role of Akt in hypoxia induced HIF1 activation	27
6. DNA damage_Role of SUMO in p53 regulation	17
7. LRRK2 and immune function in Parkinson's disease	22
8. Immune response_IFN alpha/beta signaling pathway	24
9. Immune response_Function of MEF2 in T lymphocytes	50
10. DNA damage_ATM / ATR regulation of G2 / M checkpoint	26



GO Process Name:	Total Number of Genes in Process:
1. antigen processing and presentation of exogenous Peptide antigen via MHC class I, TAP-independent	22
2. antigen processing and presentation of endogenous antigen	27
3. type I interferon-mediated signaling pathway	85
4. antigen processing and presentation of endogenous peptide antigen	25
5. cellular response to type I interferon	86
6. response to type I interferon	87
7. cellular response to interferon-gamma	131
8. response to interferon-gamma	160
9. negative regulation of natural killer cell mediated immunity	26
10. negative regulation of natural killer cell mediated cytotoxicity	26

◀ **Fig. 1** Functional analysis of genes that were differentially affected by HIV exposure (HIV-exposed vs. mock-exposed) according to apoE genotype, comparing apoE3/E3 to apoE3/E4. Functional analysis used the MetaCore™ on GeneGo software from Thompson Reuters. Panels A and B depict the top ten enrichment pathways, while panels C and D depict the top ten GO processes that were differentially regulated in maturing NEP exposed to HIV vs. mock-exposed maturing NEP. *Bar graphs* depict the  $-\log$  of  $p$  values for each pathway (a) or process (c), as well as the number of genes differentially expressed in the pathway (b) or process (d). Below the figures, the top ten enrichment pathways or GO processes are listed by description, as well as the total number of genes that are involved in that enrichment pathway or GO process

However, other genes upregulated *in vivo*, both in human and macaque tissues, related to transcription regulation, cell cycle, cell adhesion and signaling were not differentially activated in the NEP-derived cell culture system, suggesting the existence of some systemic effects of HIV may not be fully captured *in vitro*. In addition, a significant difference between our *in vitro* cell culture system and autopsy studies is the lack of productive infection of microglia (McCarthy et al. 2006). In this context of virus exposure of neuroepithelial derived cells, a more limited set of genes might be affected.

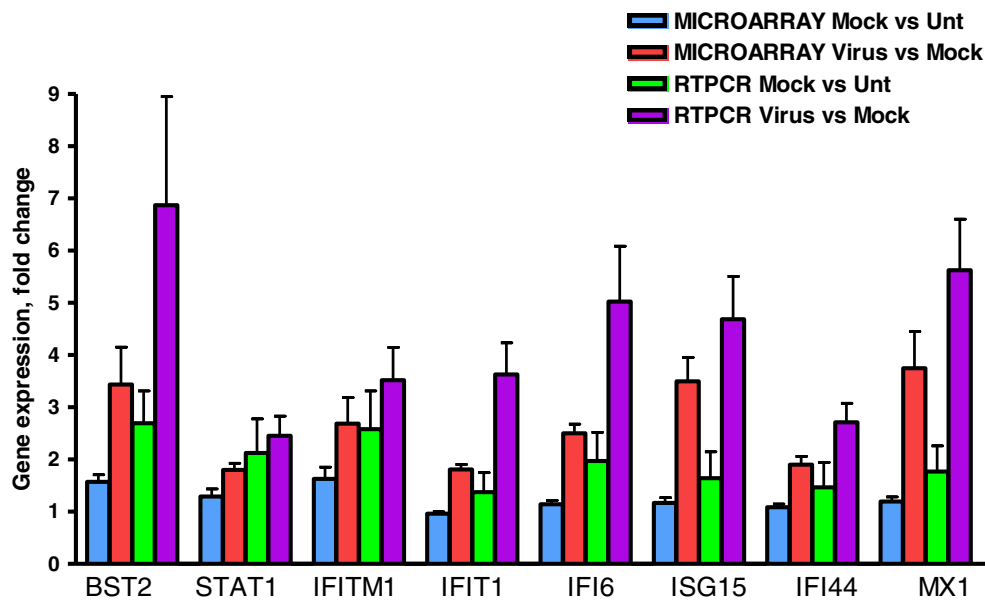
Results from experimental infections of astrocytes *in vitro*, with either HIV strain NL4-3 (Galey et al. 2003), or with a VSV-G pseudotyped NL43 (Kim et al. 2004), revealed a number of genes that were differentially expressed between infected and non-infected astrocytes. In these three studies, gene expression was evaluated using rapid subtraction hybridization (Su et al. 2002), using neuro and immuno arrays with approximately 1,100 test oligonucleotides in each (Galey et al. 2003), or Affymetrix gene chip microarrays (Kim et al. 2004). The identity of the genes differentially regulated between infected and uninfected astrocytes were different in the three studies. This could be due to differences in infection (HIV vs. VSV-G pseudotyped), the times of incubation, and the methods for evaluating gene expression. Among these three studies, that of Kim et al. had the larger number of genes studied and the one with data most similar to ours, noting an elevation in interferon related genes, and no elevation in chemokines, their receptors, or cytokine transcripts. Other genes overrepresented in Kim's study, related to cell cycle regulation and cell proliferation were not observed in our studies, probably because the levels of infection are very limited if at all compared to the enhanced infection obtained with a VSV-G pseudotyped virus. Thus, it is likely that the vigorous expression of interferon related genes in our studies originated with the astrocytes more than the neurons. Wang et al. in a review of related studies that included the three described above, attributes the discrepancies also to the heterogeneity of the astrocyte population, reporting the extensive variability found among batches of astrocytes in their ability to take up glutamate and respond to HIV or gp120 (Wang et al. 2003).

It is widely accepted that a great heterogeneity is found among astrocytes *in vivo* and *in vitro* (Davies et al. 2000; Bachoo et al. 2004). Only a small population of astrocytes is

infected *in vivo*, and can be infected *in vitro* (Dewhurst et al. 1987; Tornatore et al. 1991; Nath et al. 1995). The results described herein have identified at least one important potential variable responsible for differences in astrocyte response to HIV exposure namely, the apoE genotype. While apoE3/E3 cultures respond strongly to HIV exposure by upregulating a number of genes related to antiviral defense, cultures containing the  $\epsilon 4$  allele are less robust. Interestingly, this was also true for the comparison of mock treatment versus exposure to medium alone, suggesting basic differences between these two genotypes in their response to antigenic stimuli or to virus.

Maximal gene expression of a representative group of interferon response genes at day one after virus exposure confirmed the differences in gene expression between apoE3/E3 and apoE3/E4 cultures, and indicates that a prompt response to HIV occurs quickly, as the NEP are beginning to differentiate into astrocytes and neurons. This could point to some important capabilities of progenitor cells in mounting an innate immune response. Data from our laboratory indicate that the transition from NEP markers (e.g. intermediate filament nestin) to neuronal and astrocyte differentiation markers occurs by day 5 in differentiation medium (McCarthy et al. 2006).

The influence of apolipoprotein E alleles has been implicated in the risk of developing a number of neurocognitive disorders, among which, Alzheimer's disease is the most prominent one (Corder et al. 1993; Poirier et al. 1993; Corder et al. 1994). ApoE isoforms have varied effects on the aggregation, deposition, and clearance of amyloid  $\beta$  proteins in the brain (Carter 2005); however, the mechanisms for these effects are incompletely understood. ApoE is critical for the turnover of cholesterol and phospholipids in membranes and essential for neurite elaboration and extension. The seemingly insignificant change of two amino acids between the apoE3 and the apoE4 isoforms has profound consequences in the folding and conformation of the proteins, in their binding to cholesterol and phospholipids, and in their binding to receptor and consequent internalization. This leads to different consequences in basic cellular processes and pathways in which these isoforms participate, reviewed by Hauser et al. (Hauser et al. 2011). During HIV infection, viral gp120 may compete with apolipoprotein E binding at the cell surface; a recent *in vitro* study showed that addition of apoE4 increases HIV entry and apoE3 decreases it (Burt et al. 2008). In addition, cell-surface proteoglycans such as heparan sulfate proteoglycan (HSPG) may bind both apolipoprotein E (Chen et al. 2011) and stabilize the binding of HIV (Roderiquez et al. 1995; Mondor et al. 1998), facilitating competitive inhibition of each other's binding to the cell surface. Thus HIV-1 could act at the cell surface to interfere differentially with internalization of apoE3.

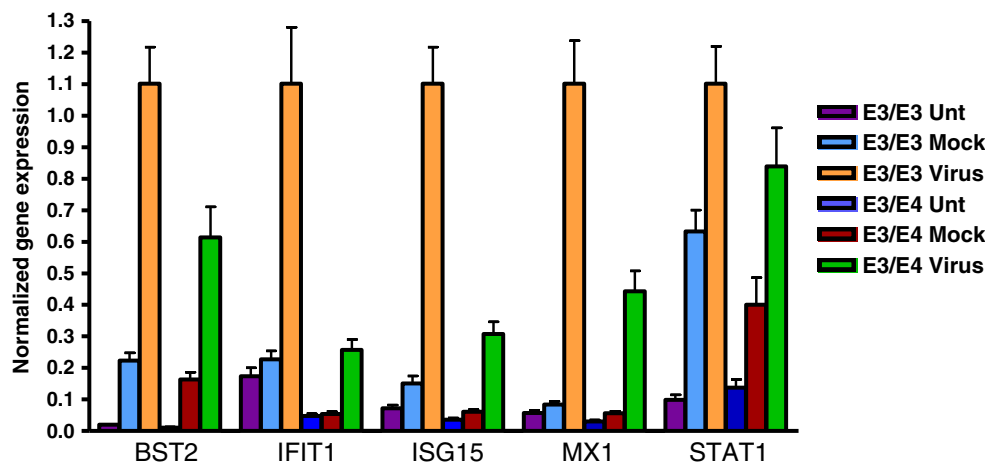


**Fig. 2** Validation of changes in gene expression in the cells differentiated from NEP regardless of apoE genotype. Fold change (FC) values for gene expression were determined for 8 representative genes that were significantly expressed by the HIV-exposed or mock-exposed cultures identified in Table 1. Fold change of gene expression (virus vs. mock, mock vs. untreated) was independently evaluated by quantitative RT-PCR and gene expression microarray. Quantitative RT-PCR

used cultures from 5 of the 7 specimens in this study. Gene expression microarray data (Illumina HumanHT-12 V4\_0\_R1 platform array) from these same specimens was used to generate the comparison FC values depicted in the *bar graphs*. Panel A depicts mean FC values  $\pm$  standard error for the gene expression by apoE3/E3 cultures; panel B depicts mean FC values  $\pm$  standard error for gene expression by the apoE3/E4 cultures

Even though the ApoE specific differences in neural cell responses to HIV presented herein is novel, the influence of apoE genotypes on innate immune responses has been documented in several studies. It has been shown that microglia from targeted replacement mice (TR) expressing apoE2/E2, apoE3/E3 and apoE4/E4 differ in their response to in vivo

activation to lipopolysaccharide (LPS), an activator of the innate immune response. ApoE4/E4 mice display increased secretion of proinflammatory cytokines TNF $\alpha$  and IL-6 and subsequent induction of nitric oxide synthase 2 (NOS2) (Vitek et al. 2009). Primary microglia cultures from apoE2, apoE3 and apoE4 targeted mice stimulated with



**Fig. 3** Quantitative RT-PCR determination of 5 selected innate immune response genes expressed by differentiating NEP after 1 day in differentiation medium ( $t=1$ ). All three culture treatments are depicted: untreated (“unt”), mock-exposed (“mock”), and HIV(SF2)-exposed (“virus”). NEP cultures from two brain specimens were used. One was an apoE3/E4 culture that had been analyzed at day 16 by gene expression microarray. The other was an independent apoE3/E3

culture. *Bar graphs* show mean normalized gene expression  $\pm$  standard error from triplicate experiments. Expression values for each gene were normalized for GAPDH as described in *Materials and methods*. The difference in gene expression is already evident in apoE3/E3 versus apoE3/E4 NEP 1 day after starting the differentiation and exposure with the three different treatments

**Table 7** Comparison of genes differentially upregulated in brain tissues of infected macaques and humans with genes upregulated in differentiating NEP exposed to HIV

Gene ID	Symbol	Name	Roberts et al.	Maslah et al.	Borjabad et al.
2537	IFI6	Interferon inducible protein 6	U	NF	U
4599	MX1	Interferon induced GTP binding protein	U	U	U
9636	ISG15	Ubiquitin-like modifier	U	U	U
684	BST2	Bone marrow stromal cell antigen 2- Tetherin	U	U	U
10964	IFI44L	Interferon-induced protein 44-like	U	NF	U
3434	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	U	U	U
8519	IFITM1	Interferon induced transmembrane protein 1	U	U	U
55008	HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	NF	NF	U
3437	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	NF	NF	U
6772	STAT1	Signal transducer and activator of transcription 1	U	U	U
10410	IFITM3	Interferon induced transmembrane protein 3	U	NF	U
4940	OAS3	2'-5'-oligoadenylate synthetase 3	NF	NF	NF
10561	IFI44	Interferon-induced protein 44	U	U	U
4938	OAS1	2'-5'-oligoadenylate synthetase 1	NF	NF	U
10581	IFITM2	Interferon induced transmembrane protein 2	NF	NF	NF
51191	HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	NF	NF	U
94240	EPSTI1	Epithelial stromal interaction 1	NF	NF	U
3429	IFI27	Interferon, alpha-inducible protein 27	NF	U	NF
3106	HLA-B	Major histocompatibility complex, class I, B	NF	NF	U
10379	IRF9	Interferon regulatory factor 9	NF	NF	NF
54625	PARP14	Poly (ADP-ribose) polymerase family, member 14	NF	NF	U
3134	HLA-F	HLA class I histocompatibility antigen, alpha chain F	U	U	U
85441	PRIC285	Peroxisomal proliferator-activated receptor alpha-interacting cofactor complex, 285 kD subunit2	NF	NF	NF
3136	HLA-H	Major histocompatibility complex, class I, H Signal transducer and activator of transcription 1 (pseudogene)	NF	NF	NF
2633	GBP1	Guanylate binding protein 1, interferon-inducible	NF	NF	U
5698	PSMB9	Proteasome subunit, beta type, 9	U	NF	U
83666	PARP9	poly (ADP-ribose) polymerase family, member 9	NF	NF	U
10866	HCP5	HLA complex P5 (non-protein coding)	NF	NF	NF
51296	SLC15A3	Solute carrier family 15, member 3	NF	NF	NF
54809	SAMD9	Sterile alpha motif domain containing 9	NF	NF	U
64761	PARP12	Poly (ADP-ribose) polymerase family, member 12	NF	NF	U
3133	HLA-E	Major histocompatibility complex, class I, E	U	NF	U

Changes in gene expression in differentiating NEP exposed to HIV as compared to mock were compared to three studies that used genome wide gene expression profiles of brain tissues obtained from HIV infected humans and macaques. Roberts et al. (Roberts et al. 2003) reported genes differentially regulated in the frontal lobe of simian immunodeficiency virus associated encephalitis as compared to uninfected controls. Maslah et al. studied genes differentially regulated in HIV infected individuals with encephalitis as compared to HIV infected individuals without encephalitis (Maslah et al. 2004), and Borjabad et al. reported on genes differentially regulated in brain tissues of untreated patients with HIV-associated neurocognitive disorders as compared to brain tissues from uninfected controls (Borjabad et al. 2011). For this comparison, genes significantly upregulated with FC of 1.5 or above in differentiating NEP exposed to HIV, listed in Table 1, were queried for differential upregulation in the above three studies

*U* upregulated in the study, *NF* not found or not upregulated in the study

LPS display an isoform dependent innate immune response that is highest with apoE4 (Maezawa et al. 2006b). In mixed primary cultures containing neurons, this was accompanied by higher p38MAPK-dependent damage to neurons. However, primary astrocytes from apoE TR mice stimulated with LPS

respond in an opposite, but also apoE isoform dependent manner, secreting TNF $\alpha$ , IL-6 and IL-1 $\beta$  that was higher in apoE2 cultures, and lowest in apoE4 cultures, without toxicity to neurons (Maezawa et al. 2006a). Stimulation of innate immunity in TR apoE mice with LPS resulted in loss of

dendrite length in hippocampal neurons after 24 h that was similar for mice expressing all three apoE isoforms. However, recovery of dendrite length over the subsequent 48 h was apoE isoform dependent, with the greatest recovery found in neurons of TR apoE2 mice vs. TR apoE3 mice. TR apoE4 mice did not display any observable dendrite regeneration.

Collectively, these published data together with the data from this study suggest that innate immune activation in the CNS shows differences in response that are apoE isoform -dependent. This response can be beneficial or not depending on multiple factors that include the cells that are part of the response and the chronic nature of the stimulus, among others. In this context, the response to HIV exposure of human apoE3/E4 NEP-derived cultures is not protective of neurons. Previous studies from our laboratory indicate that exposing differentiating NEP cultures of the apoE3/E4 genotype to HIV results in neuronal “failure to thrive,” as measured by lower neurite length of differentiating neurons (McCarthy et al. 2006). This in vitro cell culture model presents certain advantages in designing studies of the mechanisms underlying these apoE isoform -related differences in response to HIV exposure. In preliminary developmental studies, differentiating NEP were treated with specific siRNA to suppress apoE expression. When apoE production is suppressed, differentiating, HIV-1-exposed NEP cultures of the apoE3/E3 genotype show decreased expression of neurofilament proteins. By using siRNA to suppress endogenous apoE production, and adding exogenous recombinant apoE isoform proteins, it is possible to modify the apoE phenotype of differentiating NEP cultures, facilitating the study of how apoE3 versus apoE4 isoforms affect neurofilament expression, which itself is a reflection of neurogenesis (unpublished observations).

This study demonstrates that human NEP, when expanded and then differentiated in vitro, show virus-specific gene expression responses when passively exposed to HIV-1 virus, even in the absence of productively infected microglia. These responses are detected in cultures of progenitor cells early in the in vitro differentiation timeline, as well as in cultures of astrocytes and neurons differentiated from NEP later in the differentiation timeline. These in vitro gene expression patterns, particularly with respect to innate immune responses, are also found in in vivo HIV brain infection (Roberts et al. 2003; Masliah et al. 2004). Moreover, the in vitro gene expression patterns suggest that apolipoprotein E is a host factor that could potentially explain the variability among HIV-infected individuals with regards to their neuroimmune response, the severity of their neurological symptoms, and their neurological disease course. Thus apolipoprotein E could become an important factor to help stratify patients and guide treatment of HAND and related neurological disorders in patients living with chronic HIV-1 infection.

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**Conflicts of interest** The authors declare that they have no conflict of interest.

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