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ORIGINAL ARTICLE

Molecular signatures of peripheral blood mononuclear cells during chronic interferon-α treatment: relationship with depression and fatigue

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Background. Interferon-alpha (IFN-α) treatment for infectious disease and cancer causes high rates of depression and fatigue, and has been used to investigate the impact of inflammatory cytokines on brain and behavior. However, little is known about the transcriptional impact of chronic IFN-α on immune cells *in vivo* and its relationship to IFN-α-induced behavioral changes.

Method. Genome-wide transcriptional profiling was performed on peripheral blood mononuclear cells (PBMCs) from 21 patients with chronic hepatitis C virus (HCV) either awaiting IFN-α therapy ($n=10$) or at 12 weeks of IFN-α treatment ($n=11$).

Results. Significance analysis of microarray data identified 252 up-regulated and 116 down-regulated gene transcripts. Of the up-regulated genes, 2′-5′-oligoadenylate synthetase 2 (*OAS2*), a gene linked to chronic fatigue syndrome (CFS), was the only gene that was differentially expressed in patients with IFN-α-induced depression/fatigue, and correlated with depression and fatigue scores at 12 weeks ($r=0.80$, $p=0.003$ and $r=0.70$, $p=0.017$ respectively). Promoter-based bioinformatic analyses linked IFN-α-related transcriptional alterations to transcription factors involved in myeloid differentiation, IFN-α signaling, activator protein-1 (AP1) and cAMP responsive element binding protein/activation transcription factor (CREB/ATF) pathways, which were derived primarily from monocytes and plasmacytoid dendritic cells. IFN-α-treated patients with high depression/fatigue scores demonstrated up-regulation of genes bearing promoter motifs for transcription factors involved in myeloid differentiation, IFN-α and AP1 signaling, and reduced prevalence of motifs for CREB/ATF, which has been implicated in major depression.

Conclusions. Depression and fatigue during chronic IFN-α administration were associated with alterations in the expression (*OAS2*) and transcriptional control (CREB/ATF) of genes linked to behavioral disorders including CFS and major depression, further supporting an immune contribution to these diseases.

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Introduction

Inflammatory cytokines are thought to contribute to depression, fatigue and other neuropsychiatric disturbances in both medically ill and medically healthy individuals (Bower *et al*. 2002; Evans *et al*. 2005; Raison *et al*. 2006). For example, interferon-alpha (IFN-α), an inflammatory cytokine with antiviral and antiproliferative properties that is used to treat malignant melanoma and chronic hepatitis C virus (HCV) infection, produces clinically significant depression and/or fatigue in up to 80% of patients depending on the dose (Maddock *et al*. 2004; Raison *et al*. 2005; Lotrich 2009; Miller 2009). Therefore, patients undergoing treatment with IFN-α have been studied to explore immune and neurobiological pathways through which peripheral inflammatory cytokines can influence the brain and behavior.

Although the physiological consequences of IFN-α and their relationship to IFN-α-induced behavioral changes have been well studied, the molecular

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mechanisms involved have yet to be fully elucidated. Acutely, IFN-α binds to type-I IFN receptors expressed on immune cells to activate Janus kinase/signal transducers and activators of transcription (JAK/STAT) and tyrosine kinase signaling, which then increase the expression of cytokines and immunoregulatory genes (Darnell *et al*. 1994; Haque & Williams, 1998; Pfeffer *et al*. 1998). Following chronic exposure to IFN-α and to cytokines induced by IFN-α, it is likely that multiple inflammatory as well as regulatory genes and signaling pathways are recruited. However, a careful analysis of gene regulation within peripheral immune cells during chronic IFN-α administration in relation to behavior has not been reported. Identification of genes and gene-regulating transcription factors associated with chronic IFN-α may help to identify biomarkers or treatment targets for behavioral disorders associated with chronic inflammation, including major depression and chronic fatigue syndrome (CFS) (Yirmiya *et al*. 2000; Klimas & Koneru, 2007; Raison *et al*. 2009*b*).

This study sought to map functional genomic changes resulting from chronic IFN-α exposure in a genome-wide transcriptional survey of peripheral blood mononuclear cells (PBMCs) from HCV patients treated for 12 weeks with IFN-α plus ribavirin compared to HCV patients awaiting IFN-α/ribavirin therapy. Patterns of differentially regulated genes were investigated, and individual gene transcripts that correlated with IFN-α-induced depression and fatigue were assessed. Bioinformatics analysis of differentially expressed genes sought to identify specific transcription factors that might mediate such effects, and define specific leukocyte subsets within which those transcriptional alterations occurred. Of note, transcription factors can bind to closely related DNA promoter sequences, or transcription factor-binding motifs (TFBMs), in the promoter regions of multiple genes to drive gene expression. Therefore, examination of promoter sequences of large sets of differentially expressed genes can identify TFBMs selectively over-represented among those genes, thus providing information about transcriptional control pathways potentially mediating observed differences in gene expression (Cole *et al*. 2005). It was hypothesized that TFBMs related to IFN-α signaling would be over-represented in promoter regions of genes up-regulated in IFN-α-treated patients, and that the same TFBMs would be further over-represented in promoter regions of genes that were specifically associated with IFN-α-induced depression and fatigue. Administration of IFN-α is associated with increases in monocyte chemotactic protein-1 (MCP-1/CCL2) and other innate immune, monocyte-related cytokines [e.g. interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF)-α] (Taylor & Grossberg, 1998; Raison *et al*. 2009*a*), and therefore the signaling pathways related to monocyte/macrophage differentiation were also hypothesized to exhibit up-regulation. Finally, to examine TFBMs relevant to signaling pathways involved in depression and catecholamine control of immune function (Chen *et al*. 1999; Manji *et al*. 1999; Sanders & Straub, 2002; Blendy, 2006; Lutgendorf *et al*. 2009), cAMP responsive element binding protein/activation transcription factor (CREB/ATF) and the activator protein-1 (AP1)/c-Fos family of TFBMs were analyzed.

Method

Subjects

Twenty-one HCV-positive subjects (12 males, nine females) were enrolled. Subjects were serum positive for anti-HCV antibodies or HCV-RNA positive by reverse transcription–polymerase chain reaction (RT–PCR). Exclusion criteria included decompensated liver disease; liver disease from any cause other than HCV; infection with human immunodeficiency virus (HIV) (as reported by the subjects' treating physician); unstable cardiovascular, endocrinologic, hematologic, renal or neurologic disease (determined by physical examination and laboratory testing); history of schizophrenia or bipolar disorder or a diagnosis of major depression or substance abuse/dependence within 6 months of study entry, as determined by the Structured Clinical Interview for DSM-IV (SCID; First *et al*. 1997), and/or a score <28 on the Mini-Mental State Examination (MMSE; Folstein *et al*. 1975), indicating more than mild cognitive impairment. Patients were required to be off all antidepressant, antipsychotic or mood stabilizer medications for at least 4 months prior to blood sampling. Subjects were also required to discontinue other agents that might affect study results (i.e. narcotic analgesics, benzodiazepines and anti-inflammatory agents) at least 2 weeks prior to sample collection. In this study, the subjects reported on represent a subsample of subjects included in previous studies on effects of IFN-α on cognitive performance, neuroendocrine function and inflammatory responses (Majer *et al*. 2008; Raison *et al*. 2009*a*, 2010*a*, *b*; Felger *et al*. 2011). All subjects provided written informed consent, and study procedures were approved by the Emory University Institutional Review Board. α

Study design

Study participants were enrolled in a longitudinal study examining immune, neuroendocrine and neuropsychiatric variables at baseline and 12 weeks of either no treatment or treatment with IFN-α/ribavirin.

For purposes of this study, PBMCs were obtained at 12 weeks from HCV patients treated with IFN-α plus ribavirin ($n=11$) and untreated HCV patients awaiting IFN-α/ribavirin therapy (control subjects, $n=10$). All subjects who underwent IFN-α treatment received either pegylated IFN-α-2b (Pegintron, Schering Plough, USA; 1.5 μg/kg) ($n=5$) or pegylated IFN-α-2a (Pegasys, Roche-Genentech, USA; 180 mg) ($n=6$) administered subcutaneously and ribavirin (800–1400 mg/day). Participation in treatment *versus* control group was determined by patients and their physician based on scheduling constraints and personal preferences and was not based on standardized criteria or controlled by study protocol. Blood was collected at 10:00 hours into ethylenediaminetetraacetic acid (EDTA)-coated tubes through an indwelling catheter inserted at 08:00 hours. During blood sampling, subjects were asked to rest quietly for 30 min prior to blood withdrawal. PBMCs were isolated by centrifugation (400 *g*, 15 min at 22 °C) on lymphocyte separation medium (Mediatech, USA). PBMCs were stored in freezing serum [heat-inactivated fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO)] at −80 °C until RNA extraction. Urine drug screens were conducted at all visits to rule out substance abuse.

Behavioral assessments

Depression was evaluated using the Montgomery–Asberg Depression Rating Scale (MADRS; Montgomery & Asberg, 1979). The MADRS is a 10-item, clinician-administered scale that assesses severity of depressive symptoms. Fatigue was evaluated using the 20-item Multidimensional Fatigue Inventory (MFI-20; Smets *et al*. 1995). The MFI assesses five dimensions of fatigue, including general fatigue, physical fatigue, mental fatigue, reduced activity and reduced motivation. In addition to scores for each subscale, a total score was derived by summing the five-subscale scores (Wichers *et al*. 2005). Higher scores on the MADRS and MFI indicate greater symptom severity. The presence of IFN-α-induced depressive and fatigue symptoms after 12 weeks of treatment were defined as an MADRS score $\geqslant$15 and an MFI score $\geqslant$75 respectively. Of note, an MADRS score of 15 has been used as a cut-off for clinically significant depressive symptoms (Kearns *et al*. 1982; Potter *et al*. 2004; Felger *et al*. 2011), and a total MFI score $\geqslant$75 is similar to the mean score for patients diagnosed with CFS in a population-based study (Reeves *et al*. 2005).

Gene expression profiling

Total RNA was isolated from approximately 10 million PBMCs using RNeasy kits (Qiagen, USA) according to the manufacturer's instructions. RNA sample concentrations and the A260/280 ratio were determined using the MBA 2000 System (PerkinElmer, USA). Each sample was linearly amplified by the WT-Ovation RNA amplification system (NuGEN) and then submitted to the Emory Cancer Genomics Core for microarray analysis. After hybridization to Illumina Human HT-12 Expression BeadChips (Illumina, USA), which target over 47000 probes, BeadChips were scanned on the Illumina BeadArray Reader to determine probe fluorescence intensity. Raw probe intensities were normalized by the quantile normalization algorithm (Bolstad *et al*. 2003) using GenomeStudio software from Illumina, and data were deposited in NCBI Gene Expression Omnibus as series GSE31187.

RT-PCR

Total RNA was isolated as described above and reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). In brief, quantitative PCR was performed using Predesigned TaqMan Gene Expression Assay probes (Applied Biosystems) to target *MCP-1/CCL2* (Hs00234140_m1), *STAT1* (Hs00234829_m1), *STAT2* (Hs01013126_g1), protein kinase R (*PKR/EIF2AK2*) (Hs00169345_m1) and 2′-5′-oligoadenylate synthetase 2 (*OAS2*) (Hs00942643_m1) using TaqMan Universal PCR Master Mix (Applied Biosystems) and ABI 7900HT Sequence Detection System instrument and software (Applied Biosystems). All samples were run in duplicate. Expression levels were assessed in arbitrary units and normalized relative to housekeeping genes *18S* and *POL2RA*. Preliminary experiments confirmed that expression of housekeeping genes was not affected by 12 weeks of IFN-α (data not shown). Similar results were obtained using *18S* or *POL2RA*, and *18S* was used for the analysis presented here. Fold changes between control and IFN-α-treated patients were determined using the $\triangle\triangle$Ct method.

Microarray data analysis and bioinformatics

To derive a refined list of genes most affected by IFN-α, and to determine which of those genes were significantly different between subjects with low *versus* high MADRS ($\geqslant$15) and MFI ($\geqslant$75) scores at 12 weeks of IFN-α administration, the stringency of Significance Analysis of Microarray (SAM; Tusher *et al*. 2001) was used. SAM is bioinformatic strategy that uses a variety of statistical methods to identify genes that are differentially expressed between groups while correcting for multiple comparisons (see Supplementary Methods). To identify pathways affected by IFN-α compared to control and also genes

Table 1. *Characteristics of study participants*

Characteristic	Control ($n=10$)	IFN-α ($n=11$)	p value
Age (years), mean (S.D.)	47.8 (3.7)	48.5 (3.9)	0.69
Sex, males, *n* (%)	5 (50)	7 (63.6)	0.67
Race, *n* (%)			
Caucasian	4 (40)	3 (27.3)	0.49
Black	5 (50)	7 (63.6)	
Hispanic	0 (0)	1 (9.1)	
American Indian	1 (10)	0 (0)	
Education, *n* (%)			
Some high school	1 (10)	1 (9.1)	0.09
High school diploma	1 (10)	4 (36.4)	
Some college	2 (20)	5 (45.5)	
Standard college	5 (50)	0 (0)	
Post-graduate degree	1 (10)	1 (9.1)	
Past MD, *n* (%)	0 (0)	3 (27.3)	0.21
Past substance abuse, *n* (%)	6 (60)	7 (63.6)	1.00
Baseline MADRS, mean (S.D.)	4.1 (5.6)	4.7 (6.9)	0.82
Baseline MFI, mean (S.D.)	40.1 (13.9)	36.4 (8.4)	0.88
BMI, mean (S.D.) (kg/m^2)	29.6 (6.0)	30.0 (3.7)	0.46

IFN-α, Interferon-α; MD, major depression; MADRS, Montgomery–Asberg Depression Rating Scale; MFI, Multidimensional Fatigue Inventory; BMI, body mass index; S.D., standard deviation.

for confirmation by RT–PCR, Ingenuity Pathway Analysis (Ingenuity Systems, USA) was used. Ingenuity Pathway Analysis evaluates changes in gene expression as they relate to known pathways associated with a variety of cellular functions and disease states. To determine the TFBMs and immune cell types associated with patterns of differential gene expression, the Transcription Element Listening System (TELiS; www.telis.ucla.edu) (Cole *et al*. 2005) and transcript origin analysis (TOA; Cole *et al*. 2011) were used. TELiS searches promoters of differentially expressed genes to identify TFBMs that drive observed differences in gene expression. TOA examines specific patterns of gene expression that are associated with immune cell subtypes and generates a cell origin diagnosticity score, which provides information on which cell types contribute to changes in gene expression observed between groups. A comprehensive list of all genes with $>50\%$ expression difference (Cole *et al*. 2003; Miller *et al*. 2008; Bower *et al*. 2011) was entered into these analyses. Details are presented in the online Supplementary material.

Statistical analysis

Differences between groups were assessed using *t* tests or χ^2 or Fisher's tests (as appropriate) for categorical clinical variables. Differences in gene expression (fold change) between control and IFN-α-treated patients as measured by RT-PCR were assessed using Wilcoxon rank-sum tests. Pearson correlations were computed to evaluate associations between gene expression and MADRS/MFI scores. Where indicated, multivariate analyses (backward and forward multiple linear regression) were performed to assess the potential contribution of age, sex, type of IFN-α administered, body mass index (BMI), history of major depression or substance abuse. SPSS software (IBM, USA) was used for statistical analyses.

Results

Subject characteristics

As shown in Table 1, no significant differences between IFN-α/ribavirin-treated subjects and controls were observed for relevant clinical characteristics including age, race, gender, BMI and past history of major depression or substance abuse.

Differential gene expression after IFN-α administration

SAM revealed 252 up-regulated and 116 down-regulated gene transcripts out of 21244 detected probes (online Supplementary Table S1). Multiple probes representing a single gene were identified as significantly changed for some genes. Ingenuity Pathway Analysis demonstrated that 12 weeks of IFN-α administration significantly altered gene expression consistent with signaling involved in disease processes including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). As expected, it also revealed that many genes increased by IFN-α were related to antiviral and inflammatory responses (online Supplementary Table S2). From these pathways, select genes were chosen for validation by RT–PCR, including *OAS2*, *PKR/EIF2AK2*, *STAT1*, *STAT2* and *MCP-1/CCL2*. These genes were selected based on their role in IFN signaling (*STAT1*, *STAT2*), pattern recognition receptors in recognition of bacteria and viruses (*OAS2*, *PKR/EIF2AK2*), activation of interferon response factors (IRFs) by cytosolic pattern recognition receptors (*STAT1*, *STAT2*), dendritic cell maturation (*STAT1*, *STAT2*), the role of PKR in interferon induction and antiviral response (*PKR/EIF2AK2*, *STAT1*), and IL-17 signaling (*MCP-1/CCL2*), and for their association with IFN-α-induced behavioral alterations. Expression of these genes was significantly increased in samples from IFN-α-treated patients compared to controls at 12 weeks ($T=35.0$, df$=7,11$, $p<0.01$ for *OAS2*; $T=28.0$, df$=7,11$, $p<0.001$ for

EIF2AK2/PKR; $T=31.0$, df=7,11, $p<0.01$ for *STAT1*; $T=31.0$, df=7,11, $p<0.01$ for *STAT2*; and $T=31.0$, df=7,11, $p<0.01$ for *MCP-1/CCL2*) (Fig. 1).

Genes associated with depressive symptoms and fatigue

Of the 11 IFN-α-treated subjects, four demonstrated both clinically significant depressive symptoms (MADRS score ⩾15) and fatigue (total MFI ⩾75) at 12 weeks of IFN-α administration. Three of these subjects also met symptom criteria for major depression according to DSM-IV. To identify genes that were increased in subjects with high MADRS and MFI scores, gene expression profiles of these individuals were compared to IFN-α-treated subjects with low depression (MADRS <15) and fatigue (MFI <75). Examination of the 252 up-regulated and 116 down-regulated genes by SAM identified only two genes that were differentially expressed in high MADRS/MFI subjects: *OAS2* (ILMN_2248970) was significantly up-regulated, and *FCER1A* (ILMN_1688423) was significantly down-regulated. Of interest, *OAS2* expression values significantly correlated with 12-week MADRS and MFI scores in IFN-α-treated subjects ($r=0.80$, df=9, $p=0.003$ and $r=0.70$, df=9, $p=0.017$ respectively) (Fig. 2*a*, *b*). Significant correlations with *OAS2* expression were also observed for mental fatigue (MF) ($r=0.77$, df=9, $p=0.006$) and reduced motivation (RM) ($r=0.76$, df=9, $p=0.007$) (Fig. 1*c*, *d*). Similar results were found with *OAS2* as measured by RT–PCR (data not shown). Multivariate analyses including age, sex, race, BMI, type of IFN-α and past history of depression and substance abuse as covariates revealed a final model including only *OAS2* for MADRS ($F_{1,9}=14.25$, $p<0.01$), MFI ($F_{1,9}=5.95$, $p<0.05$), MF ($F_{1,9}=11.75$, $p<0.01$) and RM ($F_{1,9}=14.68$, $p<0.01$) in separate analyses. To evaluate the contribution of baseline depression and IFN-α-induced fatigue (week 12) to the association between *OAS2* expression and high MADRS scores at week 12, partial correlations were used. The analyses revealed that the relationship between *OAS2* and week-12 MADRS scores persisted when controlling for baseline MADRS scores ($r=0.80$, df=8, $p=0.005$), and also for week-12 MFI scores ($r=0.63$, df=8, $p=0.05$). Decreased *FCER1A* was negatively correlated with MADRS scores at 12 weeks ($r=-0.74$, df=9, $p=0.009$), but this relationship was not significant when entered into multivariate analyses including clinical covariates.

Transcription control pathways

To examine transcriptional control pathways that might mediate observed differences in gene expression, a two-sample variant of TELiS bioinformatics analysis was used to assess the prevalence of TFBMs involved in IFN-α signaling, myeloid differentiation (myeloid zinc finger protein, MZF), CREB/ATF and AP1 signaling pathways in promoter regions of differentially expressed genes (see Supplementary Table S3 for gene lists analyzed). The prevalence of TFBMs of interest is summarized in Table 2 as the mean ratio (fold difference), standard error and *p* value. We observed highly consistent results between the two databases that the TELiS system queries, TRANFAC and JASPAR, for the TFBMs investigated.

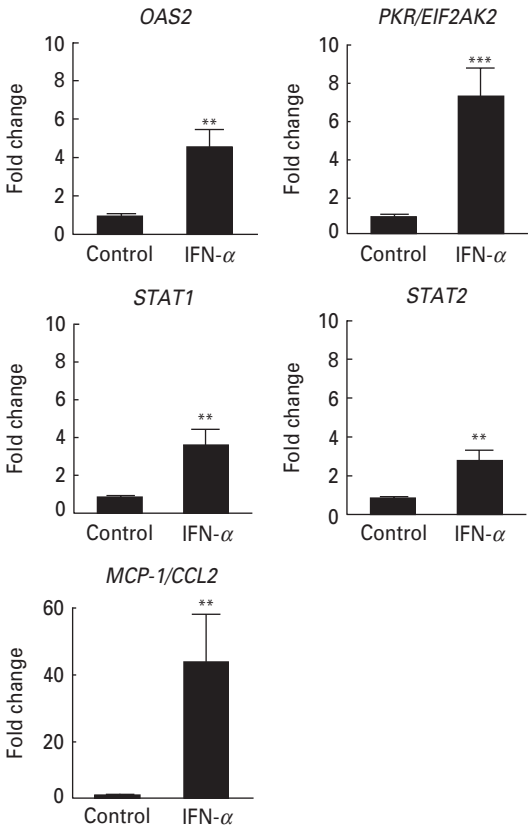

Fig. 1. Verification of gene expression for 2′-5′-oligoadenylate synthetase 2 (*OAS2*), protein kinase R (*PKR/EIF2AK2*), signal transducers and activators of transcription (*STAT*)*1*, *STAT2* and monocyte chemotactic protein-1 (*MCP-1/CCL2*). To verify altered mRNA expression in the context of interferon (IFN)-α administration, changes in gene expression were compared in hepatitis C virus (HCV) control subjects and HCV patients treated with IFN-α/ribavirin for 12 weeks. Expression of IFN-α/antiviral genes, including *OAS2*, *PKR/EIF2AK2*, *STAT1* and *STAT2*, and the inflammatory transcript MCP-1/CCL2 were measured by reverse transcription polymerase chain reaction (RT-PCR). Expression of these genes was significantly increased after 12 weeks of IFN-α administration ($n=11$) compared to control ($n=7$). Data are summarized as mean ± standard error (S.E.). ** $p<0.01$, *** $p<0.001$.

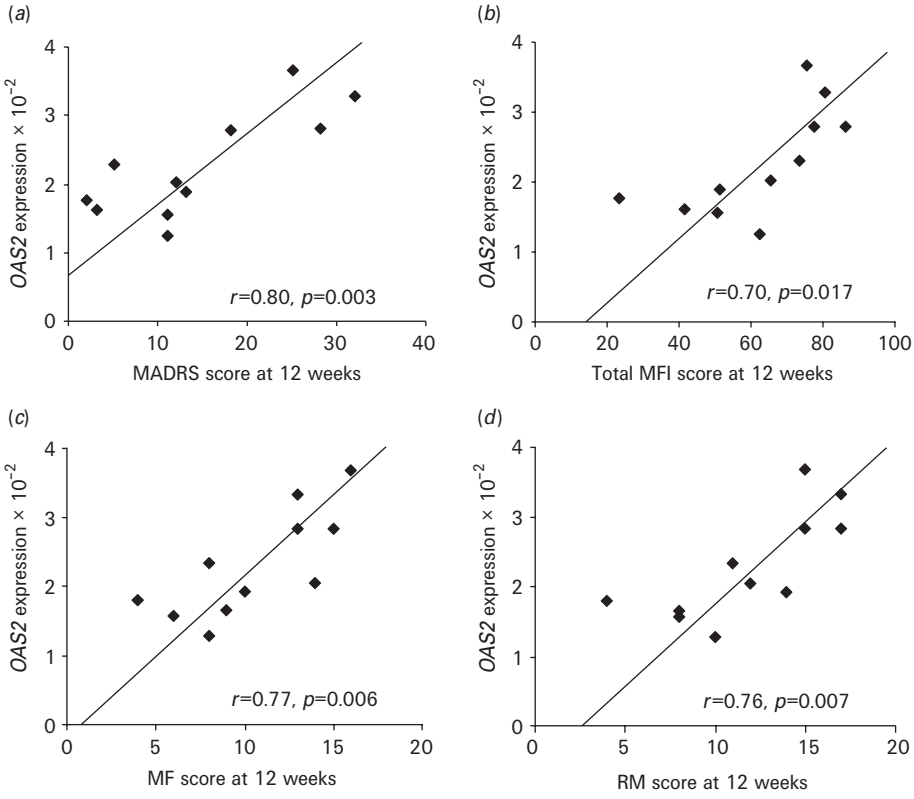

Fig. 2. Expression levels of the 2′-5′-oligoadenylate synthetase 2 (*OAS2*) gene correlated with Montgomery–Asberg Depression Rating Scale (MADRS) and Multidimensional Fatigue Inventory (MFI) scores at 12 weeks of interferon (IFN)-α administration. *OAS2* expression was positively correlated with (*a*) MADRS and (*b*) total MFI scores at 12 weeks of IFN-α administration. Assessment of subsets of the MFI score that were related to *OAS2* expression revealed significant positive correlations for (*c*) mental fatigue (MF) and (*d*) reduced motivation (RM).

As anticipated, IFN-α administration was associated with an increase in genes regulated by interferon-sensitive response element V$ISRE_01 and IRFs (V$IRF1_01, V$IRF2_01, Jaspar Irf-1, and Jaspar Irf-2). V$ISRE_01 and V$STAT1_01 were over-represented in genes of high compared to low MADRS/MFI subjects. Genes with TFBMs associated with myeloid differentiation (V$MZF1_02 and Jaspar MZF_5-13) were increased in subjects treated with IFN-α compared to control. When comparing high and low MADRS/MFI groups, there was a significant increase in V$MZF1_01, Jaspar MZF_1-4 and Jaspar MZF_5-13 TFBM prevalence. Enrichment of CREB/ATF binding sites, V$CREB_01, V$CREB_Q2 and Jaspar CREB, was revealed in IFN-α compared to control subjects, yet a marked decrease in the prevalence of multiple CREB/ATF binding sites (V$CREB_02, V$CREB_Q2, V$CREB_Q4, V$ATF_01 and Jaspar CREB) was observed in differentially expressed genes from high compared to low MADRS/MFI subjects. The prevalence of AP1-related TFBMs was also found to be significantly increased in promoters of IFN-α-related genes compared to control (V$AP1_C, V$AP1_Q2, V$AP1_Q4, V$AP1_Q6, V$AP1FJ_Q2 and Jaspar c-Fos), and also in genes up-regulated in high *versus* low MADRS/MFI subjects (V$AP1_C, V$AP1_Q4 and Jaspar c-Fos).

TOA

To investigate the cellular origin of differentially regulated transcripts, we conducted TOA (Cole *et al*. 2011) for differentially expressed genes in IFN-α compared to control, and high ($n=4$) *versus* low ($n=7$) MADRS/MFI subjects (Table 3). Transcripts that were up-regulated by IFN-α were derived primarily from monocytes and plasmacytoid dendritic cells. Whereas transcripts expressed by $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and natural killer (NK) cells appeared at approximately the same rate among IFN-α-increased transcripts as they did across a random sample of all human genes, transcripts originating from monocytes and B cells were significantly more prevalent in genes down-regulated by IFN-α. Of note, cell origin analysis revealed a significant presence of monocyte and plasmacytoid dendritic cell-related genes in the down-regulated transcripts from high *versus* low MADRS/MFI subjects. Conversely, the

Table 2. *Transcriptional activity of IFN-α, myeloid differentiation, CREB/ATF and AP1 signaling pathways as assessed by TELiS bioinformatics analysis of the prevalence of response elements in the promoters of genes differentially expressed by IFN-α compared to control, and in patients with high versus low depression and fatigue scores*

Transcription factor-binding motif (TFBM)	IFN-α *versus* Control			High *versus* Low MADRS/MFI		
	Ratio[a]	S.E.	*p* value[b]	Ratio[a]	S.E.	*p* value[b]
IFN-α signaling						
V$ISRE_01	3.73	1.4	**0.022**	1.53	1.1	**0.021**
V$IRF1_01	2.68	1.4	**0.023**	−1.16	1.1	0.320
V$IRF2_01	3.15	1.3	**0.010**	1.48	1.1	0.069
V$STAT_01	0.78	1.4	0.529	1.60	1.1	**0.011**
Jaspar Irf-1	2.88	1.3	**0.003**	1.10	1.1	0.429
Jaspar Irf-2	2.47	1.1	**0.000**	1.34	1.2	0.250
Myeloid differentiation						
V$MZF1_01	1.00	1.0	0.790	1.44	1.1	**0.012**
V$MZF1_02	1.47	1.1	**0.014**	1.42	1.2	0.096
Jaspar MZF_1-4	1.00	1.0	0.753	1.39	1.1	**0.026**
Jaspar MZF_5-13	1.14	1.1	**0.032**	1.40	1.1	**0.003**
CREB/ATF						
V$CREB_01	1.23	1.1	**0.007**	−1.10	1.1	0.399
V$CREB_02	1.00	1.1	0.915	−1.47	1.1	**0.000**
V$CREB_Q2	1.25	1.1	**0.013**	−2.01	1.3	**0.029**
V$CREB_Q4	1.09	1.1	0.183	−1.60	1.1	**0.002**
V$ATF_01	1.23	1.1	0.070	−1.72	1.1	**0.004**
Jaspar CREB	1.07	1.0	**0.032**	−1.48	1.0	**0.000**
AP1						
V$AP1_C	1.57	1.1	**0.002**	2.02	1.2	**0.010**
V$AP1_Q2	1.42	1.1	**0.001**	−1.06	1.1	0.532
V$AP1_Q4	1.56	1.1	**0.004**	1.73	1.2	**0.019**
V$AP1_Q6	1.46	1.1	**0.007**	1.32	1.2	0.146
V$AP1FJ_Q2	1.28	1.0	**0.000**	−1.03	1.1	0.668
Jaspar c-Fos	1.44	1.1	**0.001**	2.25	1.3	**0.007**

IFN-α, Interferon-α; CREB/ATF, cAMP responsive element binding protein/activation transcription factor; AP1, activator protein-1; TELiS, Transcription Element Listening System; MADRS, Montgomery–Asberg Depression Rating Scale; MFI, Multidimensional Fatigue Inventory.

Bold values indicate statistical significance at $p < 0.05$.

[a] Data represent the ratio (fold difference) and standard error (S.E.) of response element prevalence in promoter regions of genes differentially expressed in subjects at 12 weeks of IFN-α compared to controls, and in IFN-α-treated patients with high *versus* low MADRS and MFI scores.

[b] *p* values from single-sample *t* tests.

up-regulated genes of high MADRS/MFI subjects were characteristic of $CD8^+$ T lymphocytes, and NK cell-related genes were significantly present in both up- and down-regulated genes for these subjects. Similar to that of IFN-α compared to control, the down-regulated gene profile of high MADRS/MFI subjects was significantly represented by transcripts of B-cell origin.

Discussion

A systematic analysis of gene transcripts regulated by chronic IFN-α exposure using genome-wide profiling as it relates to IFN-α-induced depression and fatigue was performed. As expected, genes up-regulated at 12 weeks of IFN-α administration were predominantly related to IFN-α/antiviral and inflammatory signaling pathways. However, there was a striking specificity of transcriptional alterations associated with the onset of IFN-α-induced depression and fatigue. *OAS2* mRNA emerged as the primary transcript associated with the advent of depression and fatigue symptoms within the IFN-α-treated group. Subsequent bioinformatic analyses suggested that cytokine-induced depression and fatigue were also linked to a relative reduction in activity of the CREB/ATF signaling pathway,

Table 3. *Transcript origin analysis (TOA) of IFN-α-induced transcriptional alterations in isolated leukocyte subsets compared to control, and in patients with high versus low depression and fatigue scores*

	IFN-α *versus* Control			High *versus* Low MADRS/MFI		
Cell type	Mean TOA score	Difference from genome (±S.E.)[a]	*p* value	Mean TOA score	Difference from genome (±S.E.)[a]	*p* value
(*a*) Up-regulated gene transcripts						
Monocytes	1.44	1.23±0.18	**0.000**	0.5	0.29±0.27	0.136
Plasmacytoid dendritic cells	1.10	0.71±0.26	**0.003**	−0.03	−0.42± −0.37	0.868
NK cells	0.53	−0.40±0.33	0.885	4.47	3.54±0.48	**0.000**
$CD4^+$ T cells	0.10	−0.31±0.10	0.998	0.41	0.00±0.15	0.492
$CD8^+$ T cells	−0.04	−0.25±0.08	0.998	0.44	0.23±0.12	**0.030**
$CD19^+$ B cells	−1.01	−0.13±0.18	0.755	−0.97	−0.08±0.27	0.621
(*b*) Down-regulated gene transcripts						
Monocytes	0.67	0.46±0.21	**0.014**	1.55	1.34±0.27	**0.000**
Plasmacytoid dendritic cells	0.71	0.32±0.29	0.133	1.08	0.69±0.37	**0.034**
NK cells	0.25	−0.67±0.37	0.964	5.32	4.40±0.48	**0.000**
$CD4^+$ T cells	−0.02	−0.42±0.12	1.000	−0.22	−0.62±0.15	1.000
$CD8^+$ T cells	−0.08	−0.29±0.10	0.998	−0.39	−0.59±0.12	1.000
$CD19^+$ B cells	0.46	1.34±0.21	**0.000**	−0.22	0.66±0.27	**0.008**

IFN-α, Interferon-α; MADRS, Montgomery–Asberg Depression Rating Scale; MFI, Multidimensional Fatigue Inventory; NK, natural killer.

Positive TOA diagnosticity scores indicate that differentially expressed genes originate predominately from the analyzed cell type. Negative values are uninformative, implying that transcripts originate from other cell types or from the analyzed cell type and also other cell types.

Bold values indicate statistical significance at $p < 0.05$.

[a] Scores are presented as the difference from the genome mean and standard error (S.E.) of the difference.

and a substantial transcriptional shift toward genes originating from NK and $CD8^+$ T lymphocytes. These depression and fatigue-related transcriptional alterations were distinctive relative to the more general pattern of transcriptional (TFBMs) changes associated with IFN-α treatment compared to controls, which involved a shift to predominately myeloid lineage monocytes and plasmacytoid dendritic cells, and activation of IRF and AP1 family transcription factors accompanied by a comparative increase in CREB/ATF activity. Thus, the present study identifies a distinctive pattern of leukocyte transcriptional alterations associated with the onset of clinically significant depression and fatigue during IFN-α administration that could serve as biomarkers and/or targets for diagnosis or intervention to reduce behavioral symptoms associated with chronic exposure to an inflammatory stimulus, as during medical illness or chronic stress.

Consistent with a role for IFN-α signaling in development of behavioral symptoms, functional clustering of genes using Ingenuity Pathways Analysis indicated patterns of differential gene expression consistent with SLE and RA, both of which have well-documented behavioral changes including depression and fatigue (Kozora *et al*. 2006; Kojima *et al*. 2009). This analysis further classified genes related to IFN-α/antiviral and inflammatory responses, and increased mRNA expression of common and behaviorally relevant IFN-α-induced genes (*STAT1, STAT2, PKR/EIF2AK2* and *OAS2*) and the inflammatory gene *MCP-1/CCL2* (confirmed by RT–PCR). *STAT1* and *PKR* are activated in the brain following peripheral IFN-α administration, and are thought to be related to IFN-α-induced behavioral alterations in rodents (Wang *et al*. 2008, 2009). Furthermore, *OAS2* and *PKR* are activated by IFN-α and have been associated with behavioral disturbances in patients with CFS (Suhadolnik *et al*. 1994*b*; Vojdani *et al*. 1997; Vojdani & Lapp, 1999). Interestingly, we have previously reported increased MCP-1, an inflammatory chemokine released by monocytes, in the periphery and cerebrospinal fluid of IFN-α-treated patients (Raison *et al*. 2009*a*), and this mRNA transcript was expressed at high levels in IFN-α-treated patients.

Of the genes identified with SAM as most affected by IFN-α, *OAS2* was the only gene significantly increased in subjects that evinced high MADRS and MFI scores. Interestingly, *OAS2* mRNA expression, as measured by both microarray and RT–PCR, was

correlated with depression and fatigue scores in IFN-α-treated subjects at 12 weeks. Analysis of the MFI subscales indicated a significant association of *OAS2* expression with subscales related to mental fatigue and reduced motivation. This relationship between *OAS2* and IFN-α-induced fatigue is of particular relevance in the context of CFS, in which *OAS2* activity has consistently been found to be elevated and is thought to indicate increased IFN-α-mediated antiviral activity in these subjects (Suhadolnik *et al*. 1994*a*, *b*; Vojdani & Lapp, 1999; Nijs & De Meirleir, 2005). Although previous studies have reported increased *OAS2* activity following IFN-α administration (Merritt *et al*. 1985; Okuno *et al*. 1991), this is the first study to directly link increased *OAS2* expression with depression and fatigue in IFN-α-treated subjects. Further support for these findings were apparent when considering transcriptional control of differentially expressed genes. Not surprisingly, IFN-α signaling was increased in IFN-α patients compared to controls. However, IFN-α-related TFBMs were also over-represented in genes of high *versus* low MADRS/MFI subjects, indicating that these innate antiviral signaling pathways are, in fact, related to depression and fatigue. The relationship between IFN-α signaling and peripheral *OAS2* activity in IFN-α-induced depression and fatigue supports the hypothesis that chronic exposure to antiviral and/or inflammatory cytokines such as IFN-α may contribute to the pathophysiology of diseases associated with chronic inflammation and/or viral infection, including CFS (Suhadolnik *et al*. 1994*b*; Vojdani & Lapp, 1999). In terms of inflammation, *OAS2* is involved in innate immune responses to viral infection. Although the role of *OAS2* in the brain has not been characterized in relation to behavioral alterations, *OAS* family transcripts are activated in rodent brains in response to viral infections (Sandberg *et al*. 1994; Saha & Rangarajan, 2003) and to overexpression of IFN-α (Akwa *et al*. 1998). Whether activation of this enzyme in the brain is sufficient to induce behavioral change remains to be determined. Nevertheless, increased peripheral *OAS2* observed in CFS, and in this study during IFN-α-induced depression/fatigue, indicates that *OAS2* or downstream pathways activated by *OAS2* may precipitate behavioral alterations associated with innate immune activation.

Interestingly, transcriptional differences related to IFN-α-induced depression and fatigue were found in CREB/ATF and AP1/c-Fos signaling pathways. Although genes related to CREB transcriptional control were increased in IFN-α-treated subjects compared to controls, it was not surprising that patients with high MADRS/MFI scores displayed a marked decrease in genes controlled by CREB signaling. Unlike CREB, increased AP1/c-Fos transcriptional activity was apparent in genes differentially expressed by both IFN-α compared to control, and also high compared to low MADRS/MFI scores. In relation to decreased CREB signaling and IFN-α-induced depression/fatigue, CREB activity has been implicated in major depression and increases in PBMCs following response to antidepressant treatment (Koch *et al*. 2002; Lai *et al*. 2003; Yamada *et al*. 2003; Blendy 2006). Furthermore, CREB activity in PBMCs is under control of catecholamines, which may be reduced in both the peripheral and central nervous system during IFN-α-induced depression and fatigue (Trask *et al*. 2000; Miller, 2009). Moreover, alterations in CREB and AP1/c-Fos activity in specific brain regions are related to exposure to chronic stress and the development of depressive and anxiety-like behaviors in rodents (Morinobu *et al*. 1995; Wallace *et al*. 2004; Kuipers *et al*. 2006). In the brain, these transcription factors are thought to act as gene expression regulators that produce long-term modifications at the neuronal level. Transcriptional activity of chronic IFN-α in the periphery may mirror regulation of gene expression in brain, and represent transcriptional modifications that potentially underlie chronic cytokine-induced behavioral alterations. In relation to cytokine-induced depression, AP1/c-Fos is a downstream target of MAPK/ERK signaling pathways (Kyriakis & Avruch, 2001; Zarubin & Han, 2005), and activation (phosphorylation) of p38-MAPK following the initial injection of IFN-α is associated with depression and fatigue during IFN-α treatment (Felger *et al*. 2011). Therefore, over-representation of AP1-regulated genes associated with IFN-α-induced depressed and fatigue may reflect overactivity of MAPK/ERK signaling pathways at 12 weeks of IFN-α administration.

Monocyte and plasmacytoid dendritic cell-related genes were significantly represented in the genes up-regulated by IFN-α as determined by TOA. Further support for IFN-α-mediated increases in monocyte signaling is the finding that *MCP-1/CCL2* was the inflammatory transcript demonstrating the greatest fold increase by IFN-α, and that genes differentially regulated by IFN-α demonstrated significant transcriptional control by MZF TFBMs related to monocyte/macrophage differentiation (Krishnaraju *et al*. 1995; Moeenrezakhanlou *et al*. 2008). Regarding plasmacytoid dendritic cells, these cells are a rich source of type-I IFNs, and are prominently activated in autoimmune disorders including SLE and Sjögren's syndrome (Ronnblom *et al*. 2003; Gottenberg *et al*. 2006; Ng & Bowman, 2010). Another interesting preliminary finding from subjects with high MADRS/MFI was that up-regulated genes were derived primarily from cytotoxic $CD8^+$ T cells and NK cells,

and monocyte-related genes were more prevalent in the down-regulated genes. Nevertheless, a significant over-representation of genes under MZF transcriptional control was observed, indicating that increased myeloid-related signaling persisted in a background of cytotoxic lymphocyte transcriptional activity. Of note, NK cells contributed significantly to both up- and down-regulated genes associated with high MADRS/MFI, suggesting a shift in transcriptional activity within this cell type. Interestingly, alterations in NK cell activity have been frequently described in individuals with major depression (Irwin *et al*. 1990; Evans *et al*. 1992; Pariante & Miller, 1995) and CFS (Klimas *et al*. 1990; Nijs & De Meirleir, 2005). Future examination of PBMCs by flow cytometry after 12 weeks of IFN-α administration will determine whether transcriptional alterations in cytotoxic and monocytic cells translate into differences in cell number or activation state in patients with IFN-α-induced depression/fatigue.

In terms of limitations of this study, foremost is the small sample size. Thus, findings from genes differentially expressed in high compared to low MADRS/MFI subjects should be interpreted with caution. Furthermore, this study used a cross-sectional design that did not permit identification of gene expression at baseline, which might predict development of IFN-α-induced depression/fatigue. It should also be noted that all IFN-α-treated patients were concomitantly treated with ribavirin, and it is possible that some transcriptional dynamics observed were induced by ribavirin. However, an *in vitro* study comparing transcriptional activity of ribavirin to that of pegylated IFN-α in PBMCs found that ribavirin induced differential expression of a negligible number of genes compared to IFN-α alone (Taylor *et al*. 2004). Additionally, the present study was not a randomized experiment in which patients were experimentally allocated to treatment and control groups. Although there does not seem to be any significant demographic or disease history-related condition confounded with IFN-α/ribavirin therapy in this sample, it remains possible that some other extraneous feature may differ between groups and potentially contribute to gene expression differences. However, the fact that IFN-α response genes dominated transcriptional signatures suggests that any confounding effects of ribavirin or other unmeasured variables probably contributed relatively little to the observed results. Furthermore, IFN-α mono-therapy for malignant melanoma has been associated with profound induction of depression and fatigue (Musselman *et al*. 2001; Raison *et al*. 2005). Thus, the transcriptional activity associated with depression and fatigue in this study can most probably be attributed to specific effects of IFN-α.

In sum, this study affords a comprehensive examination of molecular alterations induced by chronic IFN-α exposure in a small sample of patients with HCV, and provides important clues and a theoretical framework for future studies examining changes in transcriptional activity related to cytokine-induced depression and fatigue.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/psm).

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Declaration of Interest

C. L. Raison serves as a consultant for Pamlab LLC and Biolex Therapeutics. A. H. Miller has served as a consultant for Abbott Laboratories, AstraZeneca, GlaxoSmithKline, Lundbeck Research USA, F. Hoffmann-La Roche Ltd, Schering-Plough Research Institute and Wyeth/Pfizer Inc. and has received research support from Centocor Inc., GlaxoSmithKline and Schering-Plough Research Institute.

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