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ORIGINAL RESEARCH

Acute Psychosocial Stress-Mediated Changes in the Expression and Methylation of Perforin in Chronic Fatigue Syndrome

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Abstract: Perforin (*PRF1*) is essential for immune surveillance and studies report decreased perforin in chronic fatigue syndrome (CFS), an illness potentially associated with stress and/or infection. We hypothesize that stress can influence regulation of *PRF1* expression, and that this regulation will differ between CFS and non-fatigued (NF) controls. We used the Trier Social Stress Test (TSST) as a standardized acute psychosocial stress, and evaluated its effect on *PRF1* expression and methylation in CFS (n = 34) compared with NF (n = 47) participants. During the TSST, natural killer (NK) cells increased significantly in both CFS ($P = <0.0001$) and NF subjects ($P = <0.0001$). Unlike previous reports, there was no significant difference in *PRF1* expression at baseline or during TSST between CFS and NF. However, whole blood *PRF1* expression increased 1.6 fold during the TSST in both CFS ($P = 0.0003$) and NF ($P = <0.0001$). Further, the peak response immediately following the TSST was lower in CFS compared with NF ($P = 0.04$). In addition, at 1.5 hours post TSST, *PRF1* expression was elevated in CFS compared with NF (whole blood, $P = 0.06$; PBMC, $P = 0.02$). Methylation of seven CpG sites in the methylation sensitive region of the *PRF1* promoter ranged from 38%-79% with no significant differences between CFS and NF. Although, the average baseline methylation of all seven CpG sites did not differ between CFS and NF groups, it showed a significant negative correlation with *PRF1* expression at all TSST time points in both CFS ($r = -0.56$, $P = <0.0001$) and NF ($r = -0.38$, $P = <0.0001$). Among participants with high average methylation (≥65%), *PRF1* expression was significantly lower in CFS than NF subjects immediately following TSST. These findings suggest methylation could be an important epigenetic determinant of inter-individual differences in *PRF1* expression and that the differences in *PRF1* expression and methylation between CFS and NF in the acute stress response require further investigation.

Keywords: pore forming cytotoxic proteins/genetics, transcription, genetic/immunology, DNA methylation, biological stress, fatigue syndrome, chronic/blood

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Introduction

Stress, whether physical or psychosocial, results in the activation of the hypothalamic pituitary adrenal (HPA) axis and the secretion of cortisol.[1] Studies of chronic fatigue syndrome (CFS) have identified a decreased cortisol awakening response[2] resulting from glucocorticoid-resistance, hypersensitivity to the negative feedback action of cortisol, or altered serotonergic activation of the hypothalamus.[3,4] In addition to these HPA axis findings, alterations in immune function have been documented in CFS. These include a shift from Th1- to Th2-type T-cell responses,[5,6] and reduced cytotoxic activity of both natural killer (NK) and CD8+ cytotoxic T-cells.[7–9] Stress can influence the immune system through its interactions with the HPA axis and the sympathetic nervous system (SNS).[10,11] Increased SNS activity has been indicated in CFS,[12–16] and could influence reduced cytotoxicity by suppressing Th1 and enhancing Th2 T-cell responses, resulting in reduced cytotoxic activity in humans and mice.[17–19] Perforin is a pore-forming protein that acts with granzymes and caspases to induce apoptosis in targeted cells,[20] and reduced perforin expression or function results in reduced cytotoxicity of NK and CD8+ T-cells. Since perforin is important for the cytolytic activity of both NK and cytotoxic CD8+ T-cells, stress regulation of the immune system may be in part mediated through perforin. Studies from three different groups identified reduced perforin in CFS subjects.[7–9] An 8-fold decrease in perforin (*PRF1*) messenger ribonucleic acid (mRNA) in peripheral blood mononuclear cells (PBMC) was identified by differential display Polymerase Chain Reaction (PCR).[9] Reduced function or expression of *PRF1* in CFS patients has been shown repeatedly;[7,8,21] generally, reduced intracellular perforin protein is correlated with *PRF1* mRNA down regulation, although one study found reduced cytotoxicity occurred with increased *PRF1* mRNA expression.[7] Studies indicate that methylation plays a role in the regulation of *PRF1* expression. Hypomethylation of the *PRF1* promoter, particularly in the 452 bp methylation-sensitive region (MSR), has been associated with increased expression of *PRF1*.[22] *In vitro* reporter gene assays showed that 70% of total *PRF1* mRNA expression could be reduced by methylation of the 7 CpG sites in the MSR.[23] In addition, overnight treatment of T-cells with a methylation inhibitor, 5-azacytidine, led to increased *PRF1* mRNA expression.[24]

Stress regulation of perforin has not been directly explored. We hypothesize that stress can influence regulation of *PRF1* expression, and that this regulation will differ between CFS and non-fatigued (NF) controls. We used the Trier Social Stress Test (TSST), an established and validated method to study the stress response by activating the sympathetic nervous system and the HPA axis,[25] as a standardized acute psychosocial stress, and evaluated its effect on *PRF1* expression and methylation in CFS (n = 34) compared with NF (n = 47) participants.

Materials and Methods

Subjects

The Centers for Disease Control and Prevention (CDC) Human Subjects committee approved the study protocol, which met the ethical standards of the Helsinki Declaration, and all subjects gave written informed consent. This study included the 81 subjects (34 CFS and 47 NF) from a population-based follow-up study of CFS in Georgia, USA who had completed the TSST as part of a three-day study in the Emory General Clinical Research Center. CFS cases met the 1994 international research definition of CFS as evaluated by standardized questionnaires, including the Multidimensional Fatigue Inventory, the SF-36® Health Survey, and the CDC Symptom Inventory.[26] There were no statistical differences between CFS and NF groups in the mean age (CFS, 45 ± 9 years; NF, 46 ± 9 years; $P = 0.75$), sex (CFS, 82% female; NF, 77% female; $P = 0.53$), race (CFS, 76% Caucasians; NF, 85% Caucasians; $P = 0.23$) or body mass index (CFS, 28 ± 5; NF, 26 ± 5; $P = 0.075$).

Trier social stress test

The TSST was performed to assess stress-induced regulation of neuroendocrine, autonomic and immune responses to challenge. The TSST was consistently started at the same time of day to ensure a similar diurnal response between subjects. The test consists of a preparatory and anticipation phase (beginning at 1:15 pm) and a subsequent 10-minute public speaking and 10-minute mental arithmetic task in front of three trained staff members (TSST panel, 1:30 pm to 1:50 pm). An indwelling catheter was placed at 7:30 am for blood draws. Blood was collected in Tempus™ tubes (Applied Biosystems, CA) for microarray analysis at 8:00 am and at 1:00 pm baseline, as well as immediately prior to the TSST panel at 1:30 pm, immediately

following the TSST panel at 1:50 pm, and at subsequent fifteen minute intervals until 3:05 pm. Blood was also collected in 8 mL Cell Preparation Tubes (CPT™) for PBMC isolation (BD Biosciences, CA) at 10:00 am (3.5 hours prior to TSST) and at 3:05 pm (1.5 hours post TSST). Cell counts were determined in blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes corresponding to all Tempus™ tube blood collection times except 8:00 am.

Blood processing and DNA/RNA extraction

Blood drawn into CPT™ was processed within 1.5–5 hours to isolate PBMCs according to the manufacturer's instructions. PBMCs were frozen in RPMI 1640 media (Invitrogen, CA) at 5×10^6 cells/mL, and stored in liquid nitrogen until use. Deoxyribonucleic acid (DNA) and RNA were isolated from aliquots of the stored PBMC. PBMC RNA was isolated using Trizol (Sigma Aldrich, MO) and PBMC DNA was extracted using the Roche DNA Isolation Kit for Mammalian Blood (Roche Applied Science, IN) following the manufacturer's protocol.

Tempus™ tubes were frozen at –20 °C until extraction (<one month). Whole blood RNA was extracted from Tempus™ tube blood using the 5 PRIME Perfect Pure RNA Cultured Cell Kit (Fisher Scientific, PA). For all samples, RNA quality and quantity were assessed using Agilent 2100 Bioanalyzer RNA Nano Chips (Agilent Technologies, CA) and a Nanodrop 1000 spectrophotometer (Thermo Scientific, DE).

EDTA tubes were submitted to Quest Diagnostics (Atlanta, GA) on the day of collection for determination of Complete Blood Count with differential and flow cytometric determination of T, B, and NK cell counts and percentages.

Microarray procedure

Microarray analysis was carried out as previously described,[27] using whole blood RNA. One microgram of RNA was labeled using the Exon WT Sense Target Labeling Assay (Affymetrix, CA) and after hybridization to the Affymetrix Human Exon 1.0 ST array, chips were scanned using the Affymetrix GeneChip Scanner 3000. Array analysis was performed using Affymetrix® Expression Console™ (v 1.1) at the transcript level using core-level probe sets. For this analysis, only *PRF1* expression was used from this microarray data set.

Quantitative reverse transcription PCR (qRT-PCR)

PBMC RNA (500 ng) was DNase I treated in a 10 µL volume using the MessageClean® Kit (GenHunter, TN) and then reverse transcribed in the same tubes using 20 µL reactions with Superscript™ III (Invitrogen, CA) and a combination of oligo(dT) and random hexanucleotide primers (2.5 µM each). LightCycler PCR (20 µL) was performed using the SybrGreen 480 Master Mix (Roche Applied Sciences) that contained 2 µL of 1:20 dilution of complementary DNA (cDNA) and 0.5 µM of each primer. Thermal cycling conditions were as follows: 1 cycle of 94 °C for 5 minutes (min), 50 cycles of 94 °C 15 seconds (s), 62 °C 15 s, and 72 °C 15 s. All reactions were carried out in duplicate with previously described *peptidylprolyl isomerase B* (*PPIB*),[28] and *PRF1* primers (forward 5′AGG AGC TGG GCA GAA GGA CAA GA 3′, reverse 5′ CAC CAT AGA GGG CTC AAG GGA AGG 3′, product 88 bp).[9] PCR efficiencies of *PRF1* and *PPIB* reactions were 1.96 and 1.97 respectively. Relative quantitation was done using the $2^{-\Delta\Delta CT}$ method using the equation 2^-((sample *PRF1* Ct – *PPIB* Ct)-(calibrator *PRF1* Ct – *PPIB* Ct)) where the calibrator was a 1:100 dilution of HeLa cell cDNA (prepared as above for PBMC cDNA), included in each plate.

Quantitative methylation by bisulfite-pyrosequencing

PBMC DNA (200 ng/reaction) was bisulfite treated using the Epitect Bisulfite Kit (Qiagen, CA) according to the manufacturer's instructions. Bisulfite-pyrosequencing was conducted as previously described to examine methylation levels at seven CpG sites in the MSR of the *PRF1* promoter (Fig. 1; sites –876, –776, –744, –720, –691, –670 and –650 base pairs upstream of the transcription start site).[29] Three amplicons, D, E and F, and a total of five sequencing primers were used to cover all seven CpG sites. We used a touchdown PCR that consisted of one cycle of 94 °C for 5 min for the initial denaturation step followed by 5 cycles each of denaturation at 94 °C for 30 s. This process involved varying annealing temperatures for 30 s, and an extension at 72 °C for 30 s. Annealing temperatures for the touchdown portion were as follows: 65 °C for 5 cycles, 62 °C for 5 cycles, 59 °C for 5 cycles, 56 °C for 5 cycles and 52 °C for 5 cycles. And a further 25 cycles of the following: 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. PCR was terminated after a final cycle at 72 °C

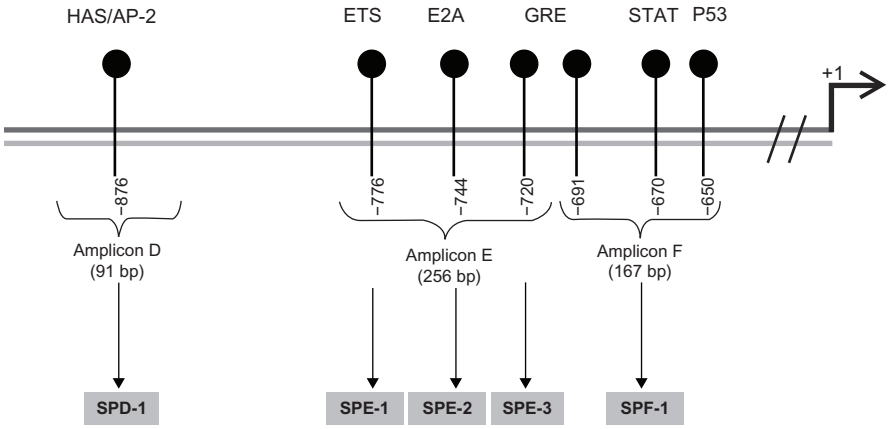

Figure 1. CpG-specific sites in the methylation-sensitive region of perforin promoter (+1 indicates transcription start site).
Notes: The pyrosequencing assay consists of three amplicons (D–F) and five sequencing primers (SP) to cover seven CpG sites indicated by lollipops. Putative transcription factors binding to the CpG sites are listed above the lollipops.

for 7 min. The PyroGold Kit was used in conjunction with the PSQ 96MA instrument (Qiagen), and each pyrosequencing reaction used 20 µL of PCR product. All reactions were carried out in duplicate.

Bioinformatic analysis of the *PRF1* MSR

Bioinformatic analysis of transcription factor binding sites (TFBS) in the MSR was carried out using the Genomatix Matinspector database (Genomatix Software GmbH (http://www.genomatix.de).[30] The identified TFBS are as follows: HIF-1 ancillary sequence family, previously identified as AP-2 (HAS/AP-2),[31] E-Twenty Six family (ETS), E2A basic helix loop helix family (E2A), Glucocorticoid Response Element (GRE), Signal Transduction and Activator of Transcription (STAT),[32] and p53 transcription factor family (P53; Fig. 1).

Statistical analysis

Statistical analysis was carried out using either SAS version 9.3 or SAS enterprise guide version 4.3. Normality was tested using the D'Agostino and Pearson Omnibus Normality Test, and parametric tests including two-tailed *t*-tests and Pearson correlation coefficients were used for analysis.

Results

Impact of TSST on blood cell counts and *PRF1* expression

As shown in Figure 2A, there was a significant increase in the overall percentage of NK cells in both CFS ($P < 0.0001$) and NF ($P < 0.0001$) subjects during the TSST, with no significant difference between groups. Similar to NK cells, other cell type percentages (neutrophils, T-cells, and B-cells) also increased in response to TSST and did not differ with respect to disease status (data not shown).

Microarray analysis of whole blood (Fig. 2B) showed a significant increase in *PRF1* expression between the introduction/preparation phase and immediately prior to the TSST oral presentation (1:00 pm–1:30 pm) of about 1.6-fold in both NF ($P < 0.0001$) and CFS ($P = 0.0003$). *PRF1* expression continued to increase in NF subjects during the TSST oral presentation (1:30–1:50 pm) whereas expression decreased slightly in CFS subjects, resulting in a significant difference between NF and CFS subjects ($P = 0.04$) at 1:50 pm. Over the 45 minutes following the TSST, *PRF1* expression declined and reached baseline levels in both groups (sampling time 2:35 pm). *PRF1* expression in NF subjects continued to decrease, but increased in CFS subjects, resulting in 1.3-fold higher expression in CFS than NF at this time, $P = 0.06$ (Fig. 2B). *PRF1* expression at 10:00 am and 3:05 pm in PBMCs determined by qRT-PCR (Fig. 2C) were generally consistent with whole blood microarray results. *PRF1* expression in PBMCs did not differ between CFS and NF at 10:00 am, but at 3:05 pm, *PRF1* expression was higher in CFS than NF (1.4 fold, $P = 0.02$).

Impact of TSST on *PRF1* methylation

Site-specific CpG methylation in the MSR at 10 am ranged from 38% to 79% and increased at all 7 CpG sites by 2.1% to 4% after the TSST (at 3:05 pm) for the

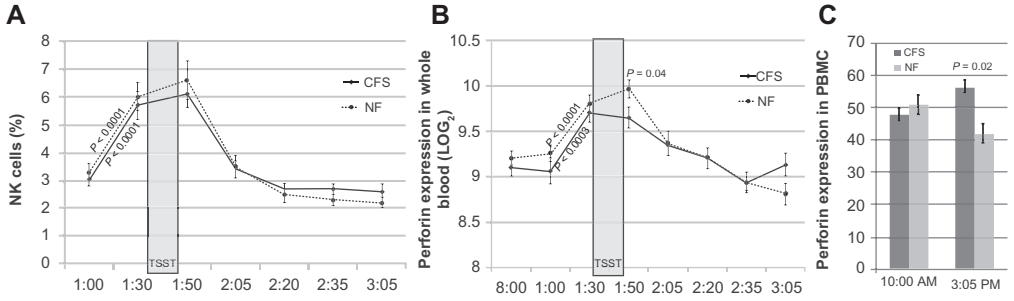

Figure 2. NK cells and *PRF1* expression in response to TSST stratified by illness. Percentage of NK cells (**A**) and *PRF1* expression (average ± SEM) in whole blood microarray analyses (**B**) in samples at each time point during the afternoon of the TSST; CFS (solid line) and NF (dotted line). Gray vertical bar designates time of speech and math challenge in TSST. *P*-values along time-course lines in A and B indicate change between baseline and start of TSST speech. In B, *P*-values above time points indicate significant difference between CFS and NF at 1:50 pm. (**C**) Relative expression of *PRF1* in PBMC by qRT-PCR in CFS and NF subjects at 10:00 am and 3:05 pm. *P*-value above data indicates significant difference between CFS and NF at 3:05 pm.

study population as a whole ($P < 0.0001$ to $P = 0.01$, Fig. 3A). However, in CFS subjects (Fig. 3B) the increase in methylation levels after TSST were significant at only two CpG sites (−776 and −744) whereas in NF controls (Fig. 3C) the increase was significant at four CpG sites (−876, −744, −691, and −670). There were no significant overall differences between CFS and NF in terms of site-specific methylation or average methylation of all 7 CpG sites before or after the TSST.

Impact of *PRF1* methylation on its expression

To examine the impact of *PRF1* methylation on *PRF1* expression, we examined correlations between average methylation of the 7 CpG sites and gene expression for each participant and time point in whole blood and PBMCs. Average baseline methylation (10:00 am) was negatively correlated with *PRF1* expression in whole blood at all 8 time points (Table 1) in both CFS and NF, statistically significant for all except one NF time point (2:35 pm). Average methylation at 3:05 pm was also negatively correlated with whole blood *PRF1* expression at 3:05 pm (Table 1) in both NF and CFS subjects, although correlation was not significant in CFS. Similar negative correlations between *PRF1* methylation and *PRF1* expression in PBMCs were observed in both NF and CFS subjects (Table 1).

We used linear regression to quantify the relationship between *PRF1* average methylation at 10 am

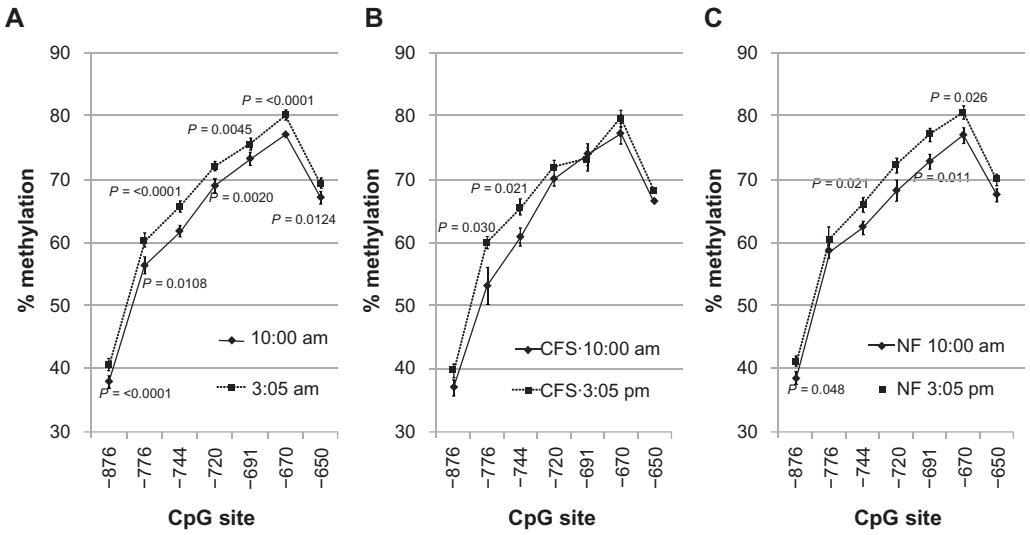

Figure 3. Methylation levels at each CpG site in *PRF1* promoter before (AM) and after (PM) TSST. Percentage methylation was measured in DNA isolated from PBMCs collected at pre- (AM = 10:00 am) and post- (PM = 3:05 pm) TSST. (**A**) All subjects, (**B**) CFS subjects, and (**C**) NF subjects.
Notes: *P*-values indicate significant differences between AM and PM times. Methylation level is expressed as percentage ± SEM. CpG sites are numbered with reference to transcription start site as +1.

Table 1. Impact of *PRF1* methylation on its expression in whole blood and PBMCs with reference to TSST administration.

***PRF1* methylation**	**Time of *PRF1* expression**	**NF subjects**		**CFS subjects**	
		r-value[c]	***P*-value**	**r-value[c]**	***P*-value**
Average methylation 10:00 am (Baseline[a])	8:00 am	–0.64707	<0.0001	–0.57851	0.0008
	10:00 am[b]	–0.60079	<0.0001	–0.70634	<0.0001
	1:00 pm	–0.44063	0.0056	–0.63819	0.0002
	1:30 pm	–0.38157	0.0165	–0.55888	0.0016
	1:50 pm	–0.37414	0.0268	–0.67297	<0.0001
	2:05 pm	–0.37259	0.0195	–0.66001	0.0001
	2:20 pm	–0.55224	0.0002	–0.62388	0.0005
	2:35 pm	–0.18219	0.2876	–0.51622	0.0049
	3:05 pm	–0.5073	0.0004	–0.53012	0.0077
Average methylation 3:05 pm	3:05 pm	–0.66987	<0.0001	–0.2441	0.239
	3:05 pm[b]	–0.52646	0.0014	–0.3000	0.1544

Notes: [a]Baseline methylation defined as the average methylation of 7 CpG sites in the MSR at 10:00 am; [b]indicates *PRF1* expression measured by qRT-PCR; [c]indicates Pearson correlation coefficients.

and whole blood *PRF1* expression at all time-points (Fig. 4). In CFS subjects, this analysis predicted a 3% increase in *PRF1* expression (95% confidence interval 2.5% to 3.7%) with every 1% decrease in *PRF1* methylation ($y = -0.044x + 12$; the antilog_2 of 0.044 = 1.03). A similar inverse relationship was found with NF subjects, however 31% and 14% of the variance in *PRF1* expression was explained by *PRF1* promoter methylation in CFS ($R^2 = 0.3133$) and NF ($R^2 = 0.1425$) respectively.

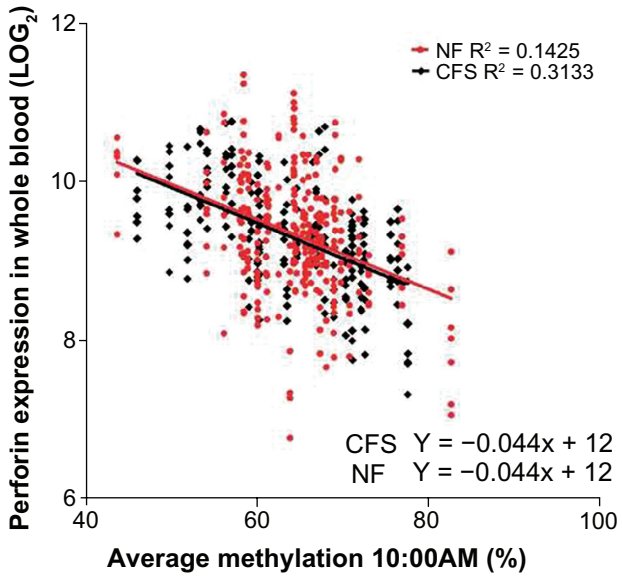

Figure 4. Correlation of average methylation in *PRF1* promoter and *PRF1* expression.
Notes: Scatter plot showing *PRF1* expression in whole blood at all time-points on Y-axis and average methylation of 7 CpG sites measured at 10:00 am on X-axis in PBMCs, stratified by illness (NF = red, CFS = black). Slope of regression line is the same for NF and CFS, –0.044, indicating a negative correlation between methylation and expression. R^2 indicates the amount of variance in expression that can be explained by the average methylation of the MSR.

We used the median split of the average methylation level of the 7 CpG sites at 10:00 am to categorize subjects into high (≥65%) and low (<65%) methylation. Whole blood *PRF1* expression was significantly higher in the low methylation group at all TSST time-points (*P*-value from 0.0002 to 0.05; Fig. 5A). When the high or low methylation group was stratified by illness, a significant difference was noted between CFS and NF only at the 1:50 pm time-point (immediately following TSST). The CFS high-methylation group had a significantly lower *PRF1* expression than the NF high-methylation group ($P = 0.005$, Fig. 5B).

Discussion

This study demonstrates that acute psychosocial stress impacts *PRF1* gene expression, and that *PRF1* methylation contributes to individual differences in *PRF1* expression. *PRF1* expression increased approximately 1.6-fold over baseline in response to the TSST and this level of change falls within the relative expression change in perforin seen in other paradigms.[33–35] However, the peak *PRF1* expression in response to TSST was reduced in CFS compared to NF subjects (Fig. 2B, $P = 0.04$). NK cells increased in response to the TSST in both CFS and NF, and since *PRF1* expression is mostly restricted to NK cells,[36] it is likely that the increased expression of *PRF1* as a response to the TSST could be related to NK cell numbers. Since we

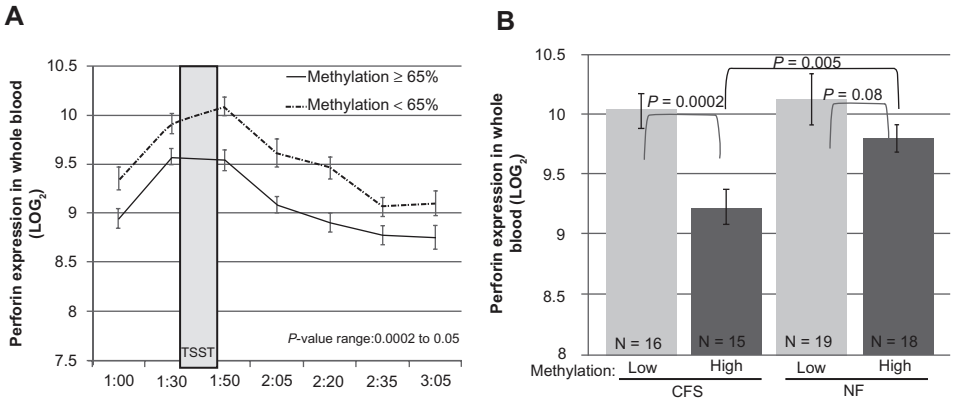

Figure 5. Differential expression of *PRF1* based on low versus high methylation status of its promoter in the MSR. Subjects were grouped into low or high methylation status based on the median split (65%) of the average 10:00 am methylation of 7 CpG sites in the MSR in all subjects. (**A**) *PRF1* expression in whole blood (average ± SEM) in subjects grouped into low (dotted line) versus high (solid line) methylation at all TSST time-points. Gray vertical bar designates time of speech and math challenge in TSST. Subjects with low methylation had significantly higher expression than subjects with high methylation at all TSST time-points ($P = 0.0002–0.05$). (**B**) Histograms show the whole blood *PRF1* expression immediately following TSST (1:50 pm) in CFS and NF subjects categorized into low and high methylation.
Notes: *P*-values above grey brackets indicate significant differences in *PRF1* expression between low versus high methylation subjects within CFS and NF. *P*-value above the black bracket indicates significant difference in *PRF1* expression between CFS and NF subjects with high methylation.

did not delineate the developmental heterogeneity in NK cells, we cannot distinguish whether all NK cells or only a proportion of mature NK cells contributed to *PRF1* expression following TSST. Further analyses using enriched NK cells are needed to evaluate the increase in *PRF1* expression in terms of transcript copies/cell, and its relationship to NK cell heterogeneity in response to acute stress.[37–39]

We found support for methylation as a mechanism for regulation of *PRF1* expression, as there was a significant negative correlation between the average methylation of all 7 CpG sites in the *PRF1* promoter and its expression. We observed this relationship at all TSST time points, when methylation levels were scored on a continuous scale or categorized into low or high status. Based on this, it is possible that the lack of a significant difference in average methylation of all 7 CpG sites between CFS and NF might contribute to the lack of difference in *PRF1* expression between CFS and NF in this study. In general, our results suggest methylation could be an important epigenetic determinant of inter-individual differences in *PRF1* expression and thus the average methylation of all 7 CpG sites may account for the reported discrepancies (increase, decrease or no change) in *PRF1* expression among different CFS studies.[7–9] These results further imply the importance of measuring both *PRF1* methylation and expression in understanding mechanisms of *PRF1* expression in immune surveillance. Although there was no difference in methylation levels between CFS and NF, these groups showed some differences in the relationship between methylation and expression, immediately following TSST (1:50 pm) and at the last time point following TSST (3:05 pm). It appears that at 1:50 pm following TSST, a subset of CFS subjects with high baseline methylation (10:00 am methylation) in the MSR contributed to the blunted response in the *PRF1* expression in comparison to NF subjects (compare Figs. 2B and 5B). At the last time point following TSST (3:05 pm) when *PRF1* expression significantly increased in CFS (both in whole blood and PBMCs), its negative relationship with methylation at its closest time (3:05 pm) became non-significant. A mechanistic explanation appears complex for these disease-specific differences observed in this study. We focused on the relationship between *PRF1* promoter methylation and mRNA, but many other mechanisms may be involved to regulate the expression of *PRF1*, including a GRE located between −720 and −691 (Fig. 1) and the IL-2 responsive regulation of upstream enhancers in the *PRF1* promoter.[40,41] There are also mechanisms involving post-transcriptional, post-translational and circadian regulation of *PRF1* that could contribute to its differential expression or activity.[32,42–46]

There are several limitations to this study. This study is limited in sample size, although it is comparable with earlier studies using the TSST chal-

lenge paradigm.[25] Study participants were screened for medication and no participant was on immunosuppressors. However, the impact of medications on HPA axis responsivity, if any, is unknown. Although statistically significant, the observed differences (2.6%–6.8%) in CpG site-specific methylation in the MSR between the pre- (10:00 am) and post (3:05 pm) TSST samples were small, possibly confounded due to the mixture of cell types present in PBMC. The methylation profile of the *PRF1* MSR in this study (81 subjects) differed compared to an earlier study (5 subjects),[29] possibly due to a substantial difference between these studies in the number of samples. One of the important questions, however, is whether the small percentage changes in methylation observed in this study are likely to be an important biological mechanism for regulating *PRF1* expression. Some recent studies suggest that small changes in DNA methylation (2%–10%) can indeed translate to larger changes (1.5- to 32-fold) in gene expression.[47–49] While these reported estimates of the impact of DNA methylation on expression vary considerably depending on the gene, tissue, environment and statistical analysis, our estimate of a 3% increase in *PRF1* expression with every 1% decrease in methylation agrees with the estimate of impact of *FXN* methylation on its expression.[49] These results, although limited to cross sectional studies, support the view that subtle epigenetic changes can influence gene expression in response to environment.

Conclusion

In conclusion, we have documented an increase in *PRF1* expression that parallels an increase in NK cells in response to acute psychosocial stress where patients with CFS had a blunted response compared to NF controls. Blunted expression by CFS may be related to high baseline *PRF1* promoter methylation that was found to be an important epigenetic determinant of inter-individual differences in *PRF1* expression. Further studies are needed to confirm these results and to evaluate explanations for the observed dynamics of *PRF1* expression. It will also be interesting to investigate the signal transduction events resulting in peripheral influx of NK cells, as well as *PRF1* expression and its functional role in the context of acute stress, and to identify molecular mechanisms that may be shared between stress and infection.

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Author Contributions

VRF, MSR and ERU conceived and designed the experiments. VRF and JM conducted the experiments. VRF and MSR analyzed the data. VRF wrote the first draft of the manuscript. VRF, MSR, TAW and ERU contributed to manuscript writing and revision. All authors reviewed and approved the final manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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