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HPA axis regulation and epigenetic programming of immune-related genes in chronically stressed and non-stressed mid-life women

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Abstract

Hypothalamic-pituitary-adrenal (HPA) axis dysregulation has been associated with altered immune function, but the underlying molecular mechanisms are unclear. Epigenetic processes, including DNA methylation, respond to the glucocorticoid end-products of the HPA axis (cortisol in humans) and could be involved in this neuroendocrine-immune crosstalk. Here we examined the extent to which variations in HPA axis regulation are associated with peripheral blood DNA (CpG) methylation changes in 57 chronically stressed caregivers and 67 control women. DNA methylation was determined with the Illumina 450k array for a panel of genes involved in HPA axis and immune function. HPA axis feedback was assessed with the low-dose dexamethasone suppression test (DST), measuring the extent to which cortisol secretion is suppressed by the synthetic glucocorticoid dexamethasone. After multiple testing correction in the entire cohort,

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Declarations of Interest

None.

higher post-DST cortisol, reflecting blunted HPA axis negative feedback, but not baseline waking cortisol, was associated with lower DNA methylation at eight *TNF* and two *FKBP5* CpG sites. Caregiver group status was associated with lower methylation at two *IL6* CpG sites. Since associations were most robust with *TNF* methylation (32% of the 450k-covered sites), we further examined functionality of this epigenetic signature in cultured peripheral blood mononuclear cells in 33 participants; intriguingly, lower *TNF* methylation resulted in higher ex vivo *TNF* mRNA following immune stimulation. Taken together, our findings link chronic stress and HPA axis regulation with epigenetic signatures at immune-related genes, thereby providing novel insights into how aberrant HPA axis function may contribute to heightened inflammation and disease risk.

Keywords

caregiving; dexamethasone suppression test; DNA methylation; FKBP5; HPA axis; interleukin 6; inflammation; TNF

1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is a highly conserved neuroendocrine axis in vertebrates that modulates multiple physiological processes, including stress responses and immune function (Chrousos and Gold, 1992; Horowitz and Zunszain, 2015; Rhen and Cidlowski, 2005). Cortisol, the primary glucocorticoid end product of the HPA axis in humans, and several pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF, increase in response to experimental stress (Marsland et al., 2017; Rohleder, 2014; Seddon et al., 2020). Notably, different timing and context of exposure to cortisol and other glucocorticoids have been reported to either promote or inhibit inflammatory signaling by binding to and activating the glucocorticoid receptor (GR) (Horowitz et al., 2020; Newton et al., 2017). Once activated, the GR functions as a ligand-dependent transcription factor that translocates to the nucleus and interacts with the DNA via glucocorticoid response elements (GREs). GR activation in the hypothalamus and pituitary inhibits HPA axis activity as part of a negative feedback loop, which is essential for homeostatic regulation of stress and immune responses (Cohen et al., 2012; Nikkheslat et al., 2015; Zannas and Chrousos, 2017).

To experimentally assess HPA axis feedback and homeostasis, studies in humans commonly employ the dexamethasone suppression test (DST). DST encompasses systemic administration of the synthetic glucocorticoid dexamethasone (DEX), which triggers the HPA axis negative feedback to decrease levels of circulating cortisol (Carroll et al., 1981). Decreased ability to suppress cortisol – that is, higher cortisol levels after oral DEX administration – reflects diminished HPA axis feedback regulation (Perrin et al., 2019). DST was first used in clinical settings to distinguish among etiologies of Cushing syndrome (Kennedy et al., 1984), but it was subsequently applied in fields as diverse as cardiology, immunology, and psychiatry (Di Dalmazi et al., 2019; Jokinen et al., 2008), highlighting its potential as an endophenotype for multiple disease entities. Among variations of the DST, the use of low oral DEX dose (0.25 mg) has been employed to allow exploration of subtler variations in HPA axis regulation (Direk et al., 2016; Gaffey et al., 2019).

Although previous research has examined possible mechanisms linking HPA axis and immune dysregulation (Barnes and Adcock, 2009), a paucity of studies have investigated the role of epigenetic mechanisms in this neuroimmune interplay (Jung et al., 2015; Misale et al., 2018; Zannas et al., 2019). One critical epigenetic mechanism is DNA methylation, which, with the advent of array technology, has become the most widely studied epigenetic mechanism in humans (Murphy and Mill, 2014). To our knowledge, two studies to date have explored the epigenetic correlates of DST (Kaminsky et al., 2015; Tyrka et al., 2016); hypermethylation at *NR3C1*, the gene encoding the GR, was associated with increased cortisol response to the DEX/CRH test, a modified version of the DST, whereas DNA methylation at *SKA2* gene, encoding a protein that binds to the GR, interacted with childhood trauma to predict post-DST cortisol. Exposure to stress has also been linked with differentially methylated regions at genes regulating HPA axis function, such as *NR3C1* (Palma-Gudiel et al., 2015) and *FKBP5* (Yehuda et al., 2016). Accordingly, stress-induced DNA methylation changes have been observed in close proximity to GREs (Klengel et al., 2013; Zannas et al., 2015) and have been associated with altered HPA axis regulation (Houtepen et al., 2018; Mulder et al., 2020). Furthermore, stress can influence DNA methylation at several genes with known roles in immune function (Janusek et al., 2017; McDade et al., 2019; Uddin et al., 2010; Zannas et al., 2019). While these studies have assessed cumulative lifetime stress, childhood adversity, and low socioeconomic status, no studies have examined the epigenetic correlates of caregiving burden, a chronic form of adult stress that has been associated with decreased quality of life and adverse health outcomes (Adelman et al., 2014).

In this framework, we examined the extent to which HPA axis negative feedback regulation, assessed with the low-dose DST, as well as caregiving burden are associated with DNA methylation changes at genes involved in HPA axis and immune function. We addressed these questions in a cohort of women caregivers of children with autism spectrum disorders (ASD) and age-matched control mothers of neurotypical children. The functional relevance of the identified DNA methylation patterns was then examined using an *ex vivo* model of immune-stimulated peripheral blood mononuclear cells (PBMC) in a subset of study participants.

2. Materials and methods

2.1. Participants and sample collection

The current study is derived from a cohort of 183 women who were caregivers of either their children diagnosed with ASD (n = 92) or their neurotypical children (n = 91). Mothers of ASD children will be referred to as *caregivers* while mothers of neurotypical children will be referred to as *controls* hereinafter. Perceived stress was assessed in all participants with the Perceived Stress Scale (PSS) at all study timepoints (Cohen et al., 1983). Participants in the caregiver group had to report a PSS score ≥ 13 at enrollment to be included in the study. Further details on this cohort have been described elsewhere (Prather et al., 2018, 2015). This study was approved by the Institutional Review Board at the University of California, San Francisco, and written informed consent was obtained for each study participant prior to enrollment.

At the initial study visit to the laboratory, participants completed a battery of sociodemographic and psychological questionnaires and received instructions and kits to collect, store, and send saliva samples for cortisol determinations. Nine months after enrollment, a cohort subset consisting of the first 20 caregivers and the first 20 controls that were enrolled in the larger study provided a morning blood sample after overnight fasting for *ex vivo* immune stimulation analysis. PBMCs were isolated from fresh blood collected in EDTA tubes using Ficoll gradient, and collected cells were washed twice with Dulbecco's PBS, cryopreserved in freezing medium (90% FBS, 10% DMSO), and stored in liquid nitrogen. Blood DNA methylation was assessed at 18 and 24 months after enrollment in 57 caregivers and 67 controls. The study design is schematically depicted in Figure 1.

2.2. Cortisol measurements and dexamethasone suppression test (DST)

At study enrollment and for 3 consecutive days, saliva samples were collected at waking with Salivette tubes (Sarstedt, Germany) to obtain baseline and post-DST cortisol levels for each participant. Baseline cortisol was derived by averaging cortisol levels from the first two days. Subsequently, a single oral dose of 0.25 mg dexamethasone was self-administered on the second night, and post-DST cortisol was measured on the third day. We employed the low-dose version of the DST (0.25 mg), because it captures subtler variations in HPA axis suppression in healthy populations (Direk et al., 2016; Labad et al., 2018); in this setting, post-DST cortisol levels offer a continuous measure of the degree of HPA axis suppression (Gaffey et al., 2019). Salivary cortisol concentration was determined by competitive solid phase time-resolved fluorescence immunoassay, as described elsewhere (Dressendörfer et al., 1992). All samples were run in duplicates. Cortisol values were log-transformed to reduce skewness. Baseline and post-DST cortisol levels were available for 90 subjects out of the 124 assessed for DNA methylation. Cortisol values that were three standard deviations above or below the mean cortisol of the entire cohort were considered as outliers; one participant had cortisol values over this threshold and was discarded from further analyses. This resulted in a final total of 89 participants (39 caregivers and 50 controls).

2.3. *Ex vivo* immune stimulation

Cryopreserved PBMC were thawed at 37°C and washed with RPMI medium supplemented with 10% sterile heat-inactivated FBS, 1% L-glutamine, and 1% sterile penicillin-streptomycin, containing 2 μg/ml DNase I (Sigma Aldrich, St. Louis, MO). Cells used for stimulation were resuspended with 5 μg/ml of phytohaemagglutinin (PHA, Sigma Aldrich, St. Louis, MO) and 2 ng/ml of IL-2 (Thermo Fisher Scientific, Waltham, MA) for 5 days at 37°C with 5% CO2 in a cell culture incubator. Total RNA was extracted from both unstimulated and stimulated PBMC using RNeasy Plus Mini kits (QIAGEN, Hilden, Germany). Gene expression was measured by NanoString technology (Geiss et al., 2008) and analyzed by nCounter SPRINT Profiler according to manufacturer's instructions (Nanostring, Seattle, WA). Further details about immune stimulation and gene expression analysis are available elsewhere (Lin et al., 2018).

2.4. DNA methylation arrays

DNA methylation was assessed in 57 caregivers and 67 controls at both 18 and 24 months after enrollment. DNA was extracted from peripheral blood with the QIAamp DNA Blood

Midi Kit (QIAGEN). Extracted DNA was bisulfite converted using EZ-96 DNA Methylation-Gold™ Kit (Zymo Research). Genome-wide DNA methylation was measured with the Infinium HumanMethylation450 BeadChip (450K), which assays DNA methylation at 482,421 CpG sites (Bibikova et al., 2011). According to standard quality control procedures, the following probes were removed: i) CpG probes in cross-reactive regions and those containing single nucleotide polymorphisms with minor allele frequency above 1% within 10 bp as retrieved from (Chen et al., 2013); ii) CpG probes with less than 3 beads or with a detection p-value over 1% in at least 1% of the samples assayed. Following this standardized procedure, a total of 377,683 CpG were included in subsequent analyses. Raw signal intensities were normalized with subset quantile normalization available in the *minfi* package (Aryee et al., 2014). Normalized intensity values were then converted into beta values, which were used in all analyses. White blood cell type proportions were estimated from DNA methylation following the Houseman reference method (Houseman et al., 2012).

2.5. Statistical analysis

All statistical analyses were performed with R version 3.6.3 (R Development Core Team, 2011). Sociodemographic differences between caregivers and controls were tested by Student's t-test for continuous variables and Chi-square test (χ^2) for categorical variables. Suppression of cortisol levels by the DST was assessed with paired t-test. Based on prior evidence and to minimize multiple testing (Iwata et al., 2013; Marsland et al., 2017; Ronchetti et al., 2015; Tyrka et al., 2016; Zannas et al., 2019), we selected a panel of candidate genes with putative roles in the interplay between HPA axis and immune function: *IL1B*, *IL6*, *IL10*, *TNF*, *NLRP3*, *NR3C1*, *FKBP5,* and *GILZ*. This resulted in a total of 158 CpG sites (annotation in Supplementary Table 1). All associations were tested with linear mixed effects regression models implemented with the *lme4* package (Bates et al., 2015). Models examining cortisol and caregiving status included DNA methylation values at both timepoints as the dependent variable, baseline/post-DST cortisol or caregiving status as the independent variable, and subject ID as a random effects term. All models were further adjusted for blood cell type proportions (CD4 T cells, CD8 T cells, B cells, monocytes, NK cells, and granulocytes), array technical variables (slide, stripe, and median intensity of the methylated and unmethylated signals), age, race (White compared with other races), body mass index (BMI), and smoking history (ever-smoked compared with never-smoked), following iterative covariate pruning after PCA as implemented in the RaMWAS pipeline (Guintivano et al., 2020; Shabalin et al., 2018). Additionally, caregiving status and timepoint of DNA methylation assessment (18 or 24 months) were also included as covariates according to study design. Baseline cortisol concentration was included as an additional covariate in models assessing post-DST cortisol effect on DNA methylation as previously described (Direk et al., 2016). Gene-level false discovery rate (FDR) was used to correct for multiple testing, and adjusted q-values under 0.05 were considered statistically significant. Associations of the identified DST-associated DNA methylation patterns with proinflammatory gene mRNA expression were adjusted for unstimulated mRNA expression and DNA methylation timepoint and included subject ID as a random effects term.

2.6. Functional annotation of identified DNA methylation sites

Detailed functional annotation of the identified CpG sites covered by the 450k was performed using the WashU Epigenome Browser (https://epigenomegateway.wustl.edu/) adding the six main histone modification tracks (H3K4me3, H3K4me1, H3K9me1, H3K27me3, H3K27ac, and H3K36me3) as described elsewhere (Zhou et al., 2011). Genomic sequences obtained from NCBI reference sequence (GRCh37.p13 Primary Assembly) were bioinformatically screened for the presence of: (i) canonical glucocorticoid response elements (GREs) according to known chromatin immunoprecipitation (ChIP-exo) signals (Starick et al., 2015); and (ii) previously described "cryptic" potential GR binding sites present in NF-κB response elements (Hudson et al., 2018).

3. Results

3.1. Descriptive statistics

Clinicodemographic characteristics of the participants assessed for DNA methylation, stratified by caregiver status, are presented in Supplementary Table 2. Caregivers scored higher in the PSS when compared to controls at all timepoints (all p-values < 0.05). As expected, post-DST cortisol was significantly lower than baseline cortisol ($t = 11.4$, $p < 0.0001$). This decrease remained significant when separately analyzing caregivers ($t = 7.0$, $p < 0.0001$) and controls ($t = 9.2$, $p < 0.0001$). Cortisol measures were not significantly different between caregivers and controls at either baseline or post-DST (all p-values > 0.05). No significant differences in age, BMI, PSS, basal morning cortisol or post-DST cortisol were observed when comparing participants included in the DNA methylation analyses with participants who dropped out of the study.

3.2. Blunted HPA axis negative feedback is associated with lower *TNF* and *FKBP5* methylation

Among the 158 examined CpG sites, and after FDR correction for multiple testing, DST response was associated with DNA methylation at ten individual CpG sites (Supplementary Table 1); eight of these sites were located at the *TNF* gene and two at *FKBP5*. Strikingly, all DST-associated CpG sites exhibited negative associations with DST response, i.e. higher post-DST cortisol was associated with a hypomethylated pattern across the identified CpG sites. Given the widespread associations of post-DST cortisol with *TNF* methylation (involving 32% of the covered *TNF* CpG sites), we also examined average *TNF* methylation across all 25 *TNF*-annotated CpG sites as a simplified summary measure to use in subsequent analyses. This analysis revealed a robust negative association between post-DST cortisol and average *TNF* methylation ($\beta = -0.0060$, $SE = 0.0016$, $t = -3.81$, $p = 0.0003$; Figure 2). To provide a more intuitive effect size for the observed differences in DNA methylation, we further compared the upper and lower tertiles of DST response groups. Average *TNF* methylation was significantly different between upper and lower post-DST cortisol tertiles ($\beta = -0.0108$, $SE = 0.0046$, $t = -2.34$, $p = 0.0265$). Moreover, half of the individual DST-associated CpG sites (cg10717214, cg08553327, cg26729380 and cg17741993) exhibited significant methylation differences when comparing the upper and lower DST tertiles (effect sizes ranging from 1.75% to 2.55%, all p values < 0.05). Within-subject DNA methylation levels robustly correlated across the two timepoints at all

identified DST-associated CpG sites (all r > 0.4, all p-values < 0.0001). In striking contrast, no significant associations were found between baseline cortisol and any of the *TNF* CpG sites (all q-values > 0.18). Moreover, post-DST cortisol did not interact with caregiving status to influence any of the DST-associated CpG sites (all interaction q-values > 0.49).

Further examination of the *TNF* DNA methylation landscape, as covered by the 450K, revealed two clusters of CpG sites according to both location and DNA methylation levels (Figure 3). Average methylation levels of CpG sites included in Cluster 1 and 2 were thus calculated for further analysis. DST response was significantly associated with average methylation of both Cluster 1 ($\beta = -0.0088$, SE = 0.0025, $t = -3.54$, $p = 0.0008$) and Cluster 2 ($\beta = -0.0036$, SE = 0.0013, $t = -2.82$, $p = 0.0067$).

Examination of chromatin states of peripheral blood mononuclear primary cells using the Epigenome Browser showed that Cluster 1 colocalized with high mono- and tri-methylation at histone 3 lysine 4 (H3K4me1, H3K4me3, respectively) and low trimethylation at histone 3 lysine 36 (H3K36me3), while Cluster 2 colocalized with H3K36me3 peaks (see Figure 4). The whole region encompassing the *TNF* gene exhibits low levels of repressive marks such as histone 3 trimethylation at lysine 27 (H3K27me3). Based on these patterns, Cluster 1 is consistent with an enhancer/promoter localization and Cluster 2 with a gene body of an actively transcribed gene (Kimura, 2013). Notably, the DST-associated *TNF* CpG sites colocalized with either H3K4me3 or H3K9me3 peaks. Since unmethylated CpG-dense sequences interact with CpG-binding proteins to establish H3K4me3 domains (Thomson et al., 2010), DNA methylation at DST-associated CpG sites may impact the surrounding chromatin landscape. Furthermore, our search for predicted glucocorticoid binding sites in this region revealed the presence of a canonical GRE (chr6: 31,543,511–31,543,525, forward strand) and two NF-κB response elements with cryptic GR binding motifs (chr6: 31,544,443–31,544,433 and chr6:31,545,418–31,545,408; both in the reverse strand) (see Figure 4).

Detailed chromatin landscape characterization of the DST-associated *FKBP5* CpG sites (cg20813374 and cg00130530) has been described elsewhere (Zannas et al., 2019). Briefly, epigenetic modifications present in this region are consistent with a poised enhancer signature that may regulate *FKBP5* expression upon transcription factor binding.

Taken together, these data suggest that blunted HPA axis suppression in response to DEX is associated with DNA hypomethylation patterns at selected genes involved in immune function and HPA axis response, most notably *TNF*. Furthermore, our data highlight reduced HPA axis feedback, measured with the DST, as a more robust predictor of site-specific DNA methylation compared to baseline cortisol.

3.3. Caregiving status is associated with minor DNA methylation changes at inflammation-related genes

Among the 158 examined CpG sites and after FDR correction for multiple testing, caregivers exhibited lower methylation at only two CpG sites located within the *IL6* first exon (cg00087425; $\beta = -0.012$, SE = 0.004, $t = -3.14$, $p = 0.0023$, $q = 0.02$) and first intron (cg15703690; $\beta = -0.017$, SE = 0.006, $t = -2.92$, $p = 0.0044$, $q = 0.02$). Overall these

findings suggest that chronic stress (caregiving status) may predict a smaller number of distinct epigenetic changes as compared to metrics of HPA axis regulation.

3.4. DST-associated methylation influences *TNF* expression after *ex vivo* immune stimulation

To examine the functional relevance of the DST-associated DNA methylation changes, we measured *TNF* mRNA levels in PBMC derived from 33 study participants with DNA methylation data available (15 caregivers and 18 controls). To determine *TNF* mRNA responsivity in this context, PBMC were stimulated *ex vivo* with PHA, a generic and widely used immune stimulus that acutely promotes release of pro-inflammatory cytokines, including TNF (Janefjord et al., 2001; Kartika et al., 2020). Lower average *TNF* methylation was significantly associated with higher *TNF* mRNA expression after immune (PHA) stimulation ($\beta = -3.54 \times 10^{-5}$, SE = 1.22×10^{-5}, t = −3.16, p = 0.0036; Figure 5), but not with *TNF* mRNA expression in unstimulated PBMCs (p = 0.0941). These effects were not moderated by caregiving status (interaction p = 0.17). Analysis by clusters of CpG sites revealed that stimulated *TNF* mRNA expression was associated with methylation status of Cluster 2 ($\beta = -4.66 \times 10^{-5}$, SE = 1.43×10^{-5}, t = −3.26, p = 0.0028) but not Cluster 1 (p = 0.175). These data suggest that *TNF* hypomethylation associated with blunted HPA axis feedback may de-repress *TNF* transcriptional responses, predisposing to heightened peripheral inflammation following immune stimulation.

4. Discussion

The present findings suggest DNA methylation as a mechanistic link between varying degrees of HPA axis negative feedback regulation and immune function. Specifically, blunted HPA axis negative feedback, measured with the DST, was associated with lower methylation levels at eight *TNF* CpG sites and two *FKBP5* CpG sites. Furthermore, decreased methylation at the DST-associated *TNF* sites was associated with higher *TNF* expression after *ex vivo* immune stimulation, indicating functional relevance of the identified epigenetic signatures. In contrast, baseline cortisol was not linked with any DNA methylation changes and caregiving stress was only associated with lower methylation at two *IL6* CpG sites.

Previous studies support a complex interplay between HPA axis activity and the proinflammatory cytokine TNF. The DST-associated CpG sites identified herein are juxtaposed to a canonical GRE and two cryptic GR binding sites located within NF-κB response elements, indicating their potential involvement in epigenetic regulation of the molecular crosstalk between glucocorticoid and inflammatory signaling. Timing is a crucial factor in the bidirectional neuroimmune-neuroendocrine interplay (Horowitz and Zunszain, 2015), and since DNA methylation is a potentially dynamic epigenetic modification, there are two possible scenarios to explain our findings. Firstly, blunted HPA axis negative feedback could contribute to chronically increased circulating levels of cortisol that, in turn, lead to decreased *TNF* methylation and enhanced TNF signaling. Alternatively, higher cortisol levels after DST could be a consequence of preexisting TNF hyperreactivity. Although study design does not allow us to disentangle these different possibilities, our

novel findings point to the potential role of *TNF* methylation as a mechanistic link between HPA axis and immune function.

Among genes involved in HPA axis function, reduced DST response was only associated with decreased DNA methylation at two closely juxtaposed CpG sites at *FKBP5*, a glucocorticoid-responsive gene that encodes a cochaperone of the GR complex that inhibits glucocorticoid signaling (Zannas et al., 2016). Notably, decreased DNA methylation at the same two *FKBP5* CpG sites was recently shown to contribute to NF-κB-driven peripheral inflammation (Zannas et al., 2019). Taken together, the prior and present findings indicate epigenetic regulation of selected stress- and immune-related genes, such as *FKBP5* and *TNF*, and its effects on NF-κB signaling as potential mediators of the crosstalk between HPA axis and immune function. In contrast, we did not find associations between DST response and other stress-related genes, including *NR3C1*, though such an association has been reported by a prior study employing the DEX/CRH test (Tyrka et al., 2016). Beyond the use of distinct tests of HPA axis regulation, disparate findings across studies may also be explained by the low overlap between assayed CpG sites due to the sparse CpG coverage by the arrays. Interestingly, however, one CpG site (cg15645634), was positively associated with DST response in the present study at a nominal p-value (Supplementary Table 1), a finding that is consistent with the prior study (Tyrka et al., 2016).

While caregivers did not differ in HPA axis feedback regulation as compared to controls, they exhibited lower *IL6* methylation. Notably, our previous work identified upregulated *IL6* mRNA in the caregiver group of the present cohort (Lin et al., 2018), and lower *IL6* promoter methylation has been associated with greater increases in salivary IL-6 levels following acute laboratory stress (Janusek et al., 2017), overall suggesting the involvement of this epigenetic signature in primed immune responses to stress.

TNF methylation was specifically associated with post-DST cortisol levels, whereas no associations were observed with baseline cortisol, highlighting the importance of measuring HPA axis regulation after experimental activation. Experimental tests of HPA axis regulation, such as the DST, may thus hold promise as a tool to mechanistically dissect the interplay between HPA axis and immune function in humans. While some studies have previously assessed the relationship between candidate gene methylation and HPA axis reactivity under exposure to psychosocial acute stress (Janusek et al., 2017; Ziegler et al., 2015), fewer studies have explored the epigenetic correlates of HPA axis suppression as a measure independent of stress exposure. The subjective measure of perceived stress (PSS) was not associated with morning salivary cortisol in the present sample. Salivary cortisol reflects momentary daily stressors rather than chronic exposure to stress; thus, cortisol measured in saliva has been used in the context of acute challenges such as the Trier Social Stress Test or the DST (Schlotz et al., 2019; Weckesser et al., 2013), and discordance between subjective measures of stress perception and biological metrics of stress response has been previously reported (Kerr et al., 2020; Metz et al., 2020). Notably, differences in *TNF* and *FKBP5* methylation were observed only in association with DST response rather than caregiver status, highlighting the specificity of the reported associations. In the same vein, *TNF* methylation was associated with *TNF* expression after immune stimulation but not at baseline, suggesting that this epigenetic pattern de-represses *TNF* transcription after

exposure to a challenge only, rather than increasing baseline *TNF* expression. These results are in line with previous work in human hippocampal progenitor cells exposed to glucocorticoids, which exhibited widespread long-lasting DNA methylation changes associated with enhanced transcriptional responses but only following subsequent glucocorticoid challenge (Provençal et al., 2019). While site-specific variation in chromatin structures may determine how DNA methylation patterns influence gene expression (Qi et al., 2019), further molecular studies are needed to disentangle the specificity of these associations across distinct genomic regions.

The present study has significant limitations but also notable strengths. Our approach is limited by the sparse coverage of the 450K array, which does not allow a fine-grained assessment of DNA methylation landscapes at the genomic regions of interest. Furthermore, the current study design cannot establish causality, which would require longitudinal studies assessing HPA axis regulation, DNA methylation, and systemic inflammation at repeated timepoints. The relationship between HPA axis suppression and DNA methylation is further obscured by the time lapse between both measures. Due to statistical power constraints, a genome-wide approach was not feasible in the current sample; however, our hypothesis-driven approach is less likely to yield false positives. Finally, there is a standing question of whether the observed DNA methylation differences are biologically meaningful, though the reported effect sizes are comparable to those of similar publications in the field (Chaix et al., 2020; Tyrka et al., 2016). Nevertheless, strengths include: (i) the association of DST response with 8 of the 25 examined *TNF* CpG sites and with selected disease-relevant *FKBP5* CpG sites; (ii) the consistent direction of these findings, showing that lower degrees of HPA axis feedback are associated with decreased DNA methylation at all identified sites; (iii) the stability of the observed DST-associated DNA methylation changes over time; and (iv) the functional relevance of the DST-associated epigenetic pattern of *TNF*, supported by our ex vivo study examining *TNF* mRNA expression after immune stimulation.

In conclusion, our findings identify DNA methylation as a novel link between HPA axis regulation and immune function. Intriguingly, lower *TNF* methylation has been previously observed with increasing age, an epigenetic pattern accompanied with increased *TNF* expression (Shinozaki et al., 2018), whereas higher *TNF* methylation has been associated with exposure to supportive parenting and with reported better health in young adults (Beach et al., 2017, 2015). Experimental manipulation of TNF methylation *in vitro* will help elucidate the functional relevance of this signature. As *TNF* and *IL6* mRNA levels have been recently suggested to discriminate between responders and non-responders to antidepressant treatment (Cattaneo et al., 2020), it would be intriguing to examine HPA axis suppression and DNA methylation in this population. Cell-type specific analysis will be crucial to identify which cell subpopulations drive the observed DNA methylation differences. Prior glucocorticoid exposure has been recently described to prime subsequent immune responses in hippocampal cells (Horowitz et al., 2020); thus, combined stimulation with glucocorticoid and immune stimuli may better reveal the temporal dynamics of the association between HPA axis regulation and *TNF* methylation. Future longitudinal and experimental studies will be necessary to dissect how HPA axis function contributes to epigenetic programming of immune function and the potential relevance of such programing for health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Blunted HPA axis negative feedback is associated with peripheral blood *TNF* and *FKBP5* hypomethylation
- Caregiving stress burden is associated with peripheral blood *IL6* hypomethylation
- *TNF* hypomethylation results in higher *TNF* expression upon *ex vivo* stimulation of immune cells

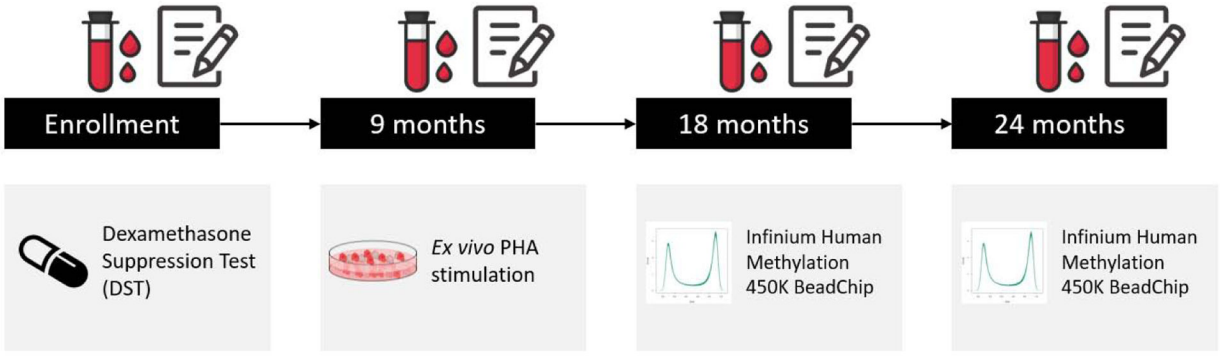

Figure 1. Data collection timeline.

Dexamethasone suppression test was performed at study enrollment. Blood samples were additionally collected in subsequent visits 9, 18, and 24 months after enrollment. Peripheral blood mononuclear cells were isolated from the 9-month samples for ex vivo stimulation analyses in the first 20 controls and the first 20 caregivers. DNA was extracted from peripheral whole blood collected at the 18- and 24-month timepoints for DNA methylation assessment in 124 participants. Perceived stress was assessed in all participants with the Perceived Stress Scale at all study timepoints. Abbreviations: PHA, phytohaemagglutinin.

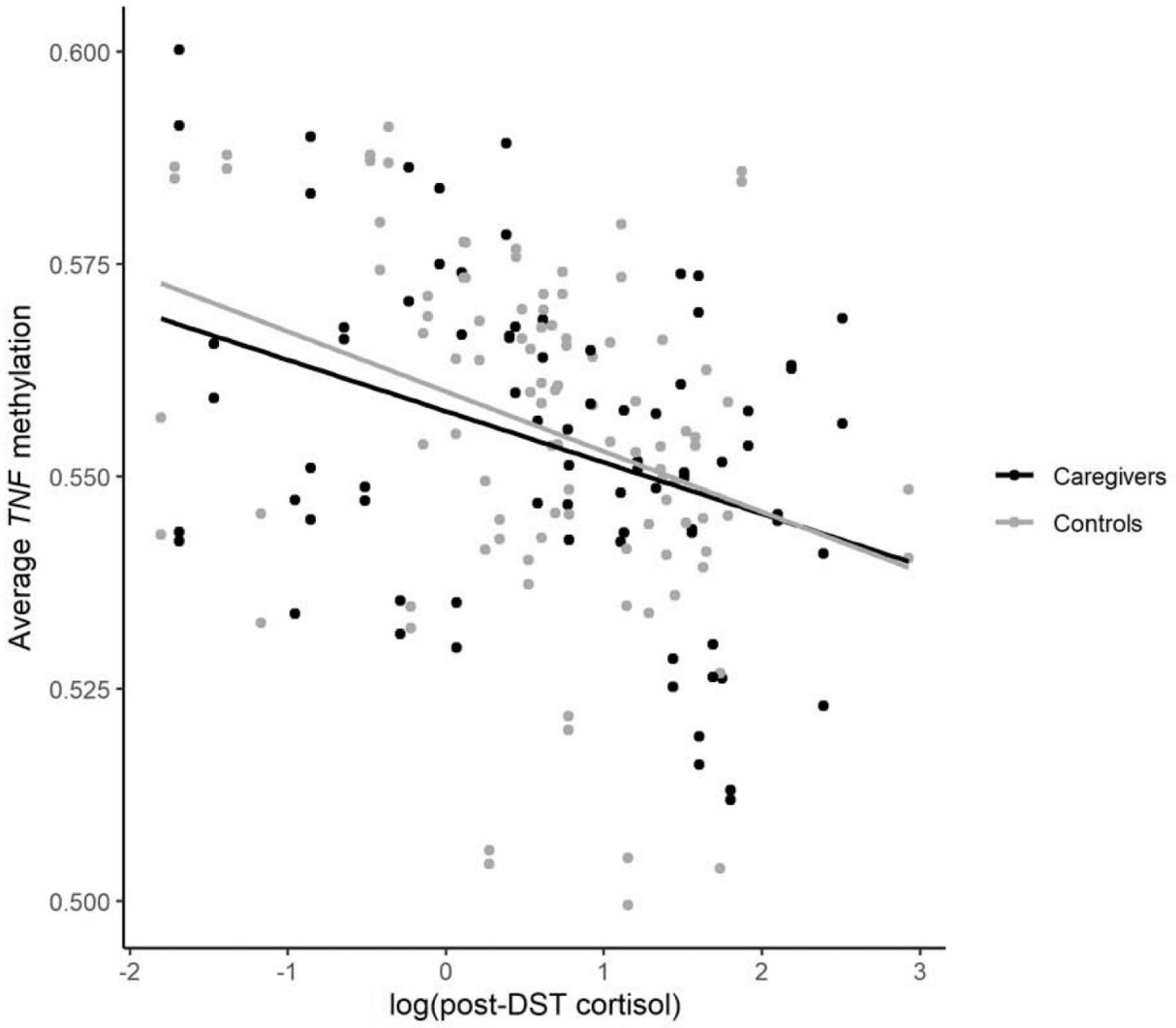

Figure 2. DST response is negatively associated with average *TNF* methylation (β = −0.0060, SE = 0.0016, t = −3.81, p = 0.0003).

Response to the dexamethasone suppresion test (DST) was defined as log-transformed cortisol concentration in saliva (measured in nmol/L) at awakening after dexamethasone administration the night before. Higher post-DST cortisol levels reflect blunted HPA axis negative feedback. *TNF* methylation here corresponds to average DNA methylation of all *TNF*-annotated CpG sites covered by the 450K array. This analysis was conducted on 89 participants, 39 caregivers and 50 controls.

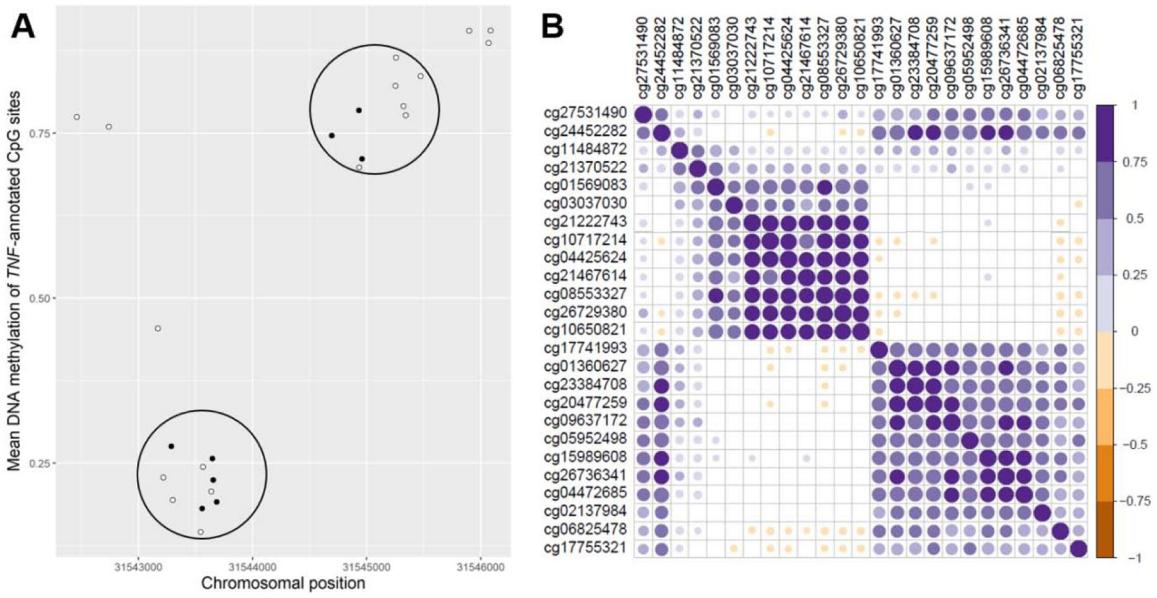

Figure 3. DNA methylation landscape of the *TNF* gene.

(A) Mean DNA methylation levels (across all subjects) for each of the *TNF*-annotated CpG sites covered by the 450K array arranged by chromosomal position. The genomic annotation for the CpG sites is detailed in Supplementary Table 1. Each CpG site is depicted as a black dot. Filled dots depict CpG sites significantly associated with response to the dexamethasone suppression test (DST). Blank dots depict CpG sites not associated with DST response. As illustrated, the DST-associated sites fall within one of two of adjacent clusters of CpG sites with similar methylation patterns (enclosed in black circles). The first cluster (Cluster 1) is located at the promoter region/first exon of the *TNF* gene and encompasses 10 adjacent CpG sites with low-intermediate levels of DNA methylation (range from 15 to 30%). The second cluster (Cluster 2) is located within the *TNF* gene body and includes 9 CpG sites with intermediate-high DNA methylation levels (range from 68 to 86%). *(B)* Correlation plot depicting pairwise correlations between mean DNA methylation levels of the *TNF*-annotated CpG sites. Circle size and color tone correspond to the magnitude of the correlation coefficient. Only significant correlations are depicted. Purple and orange are used to depict positive and negative correlations, respectively.

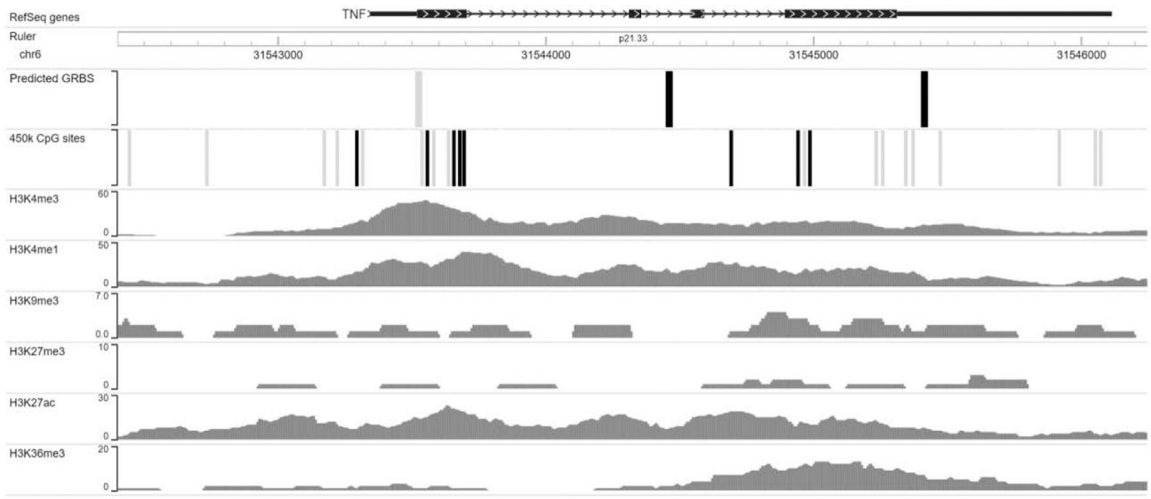

Figure 4. Functional annotation of the examined CpG sites at the *TNF* locus.

All chromatin modification tracks correspond to "Peripheral Blood Mononuclear Primary cells" from the publicly available Roadmap Epigenome data. The upper track "Predicted GRBS" displays glucocorticoid receptor binding sites identified by means of logos available in JASPAR (MA0105.3, including a cryptic GRBS as described in Hudson et al., 2018) and Starick et. al. (2015); cryptic sites are shown as black bars while canonical GRE are shown as light grey bars. The track "450k CpG sites" displays the 25 *TNF*-annotated CpG sites covered by the 450K array according to the genomic coordinates reported in Supplementary Table 1; CpG sites significantly associated with response to the dexamethasone suppression test DST are shown as black bars, whereas non-significant CpG sites are shown as light grey bars. Genomic coordinates (chr6:31,542,401–31,546,250) correspond to human genome assembly 19 (hg19).

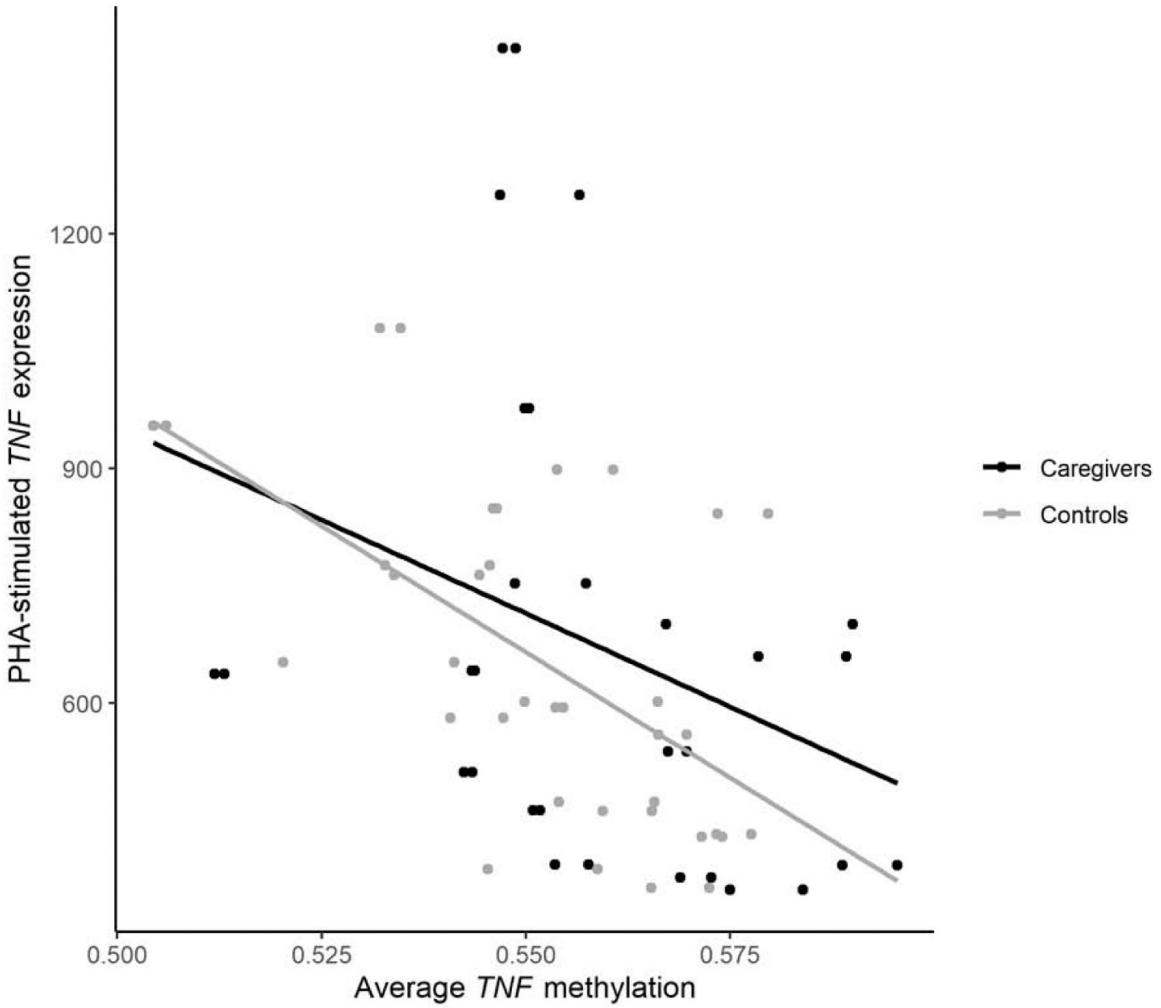

Figure 5. Average *TNF* methylation is negatively associated with *TNF* expression after PHA stimulation.

Peripheral blood mononuclear cells from a subset of 33 participants (15 caregivers and 18 controls) were cultured ex vivo to assess *TNF* expression in response to immune stimulation with phytohaemagglutinin. *TNF* methylation here corresponds to average DNA methylation of all 25 *TNF*-annotated CpG sites covered by the 450K array. No interaction was found between caregiving status and *TNF* expression ($p = 0.17$).