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Biobehavioral Organization Shapes the Immune Epigenome in Infant Rhesus Macaques (*Macaca mulatta*)

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Abstract

How individuals respond to and cope with stress is linked with their health and well-being. It is presumed that early stress responsiveness helps shape the health of the developing organism, but the relationship between stress responsiveness and early immune function during development is not well-known. We hypothesized that stress responsiveness may shape epigenetic regulation of immune genes in infancy. We investigated whether aspects of behavioral responsiveness and hypothalamic-pituitary-adrenal stress-response were associated with epigenome-wide immune cell DNA methylation patterns in 154 infant rhesus monkeys (3–4 months old). Infants' behavioral and physiological responses were collected during a standardized biobehavioral assessment, which included temporary relocation and separation from their mother and social group. Genome-wide DNA methylation was quantified using restricted representation bisulfite sequencing (RRBS) from blood DNA collected 2-hours post-separation. Epigenome-wide analyses were conducted using simple regression, multiple regression controlling for immune cell counts, and permutation regression, all corrected for false discovery rate. Across the variables analyzed, there were 20,368 unique sites (in 9,040 genes) at which methylation was significantly associated with at least one behavioral responsiveness or cortisol measure across the three analyses. There were significant associations in 442 genes in the *Immune System Process* ontology category, and 94 genes in the *Inflammation mediated by chemokine and cytokine signaling* gene pathway. Out of 35 candidate genes that were selected for further investigation, there were 13 genes with at least one site at which methylation was significantly associated with behavioral responsiveness or cortisol, including two intron sites in the glucocorticoid receptor gene, at which methylation was negatively correlated with emotional behavior the day following the social separation (Day 2 Emotionality; $\beta = -.39$, $q < .001$) and cortisol response following a relocation stressor (Sample 1; $\beta = -.33$, $q < .001$). We conclude that biobehavioral stress responsiveness may correlate with the developing epigenome, and that DNA methylation of immune cells may be a mechanism by which patterns of stress response affect health and immune functioning.

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Keywords

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1 Introduction

It is widely recognized that severe and chronic stress can lead to poor health outcomes, including infectious disease, cardiovascular disease, respiratory disease, and diseases of inflammation (Caspi et al., 2006; Danese et al., 2008; Dong et al., 2004; Felitti et al., 1998). Early childhood is an especially sensitive period when the effects of stress can become "biologically embedded", leading to potentially long-lasting health consequences (Danese & McEwen, 2012; Dhabhar, 2014; Miller et al., 2011; Nusslock & Miller, 2016; Shonkoff et al., 2009). The biological embedding model of stress-disease (Miller et al., 2011) proposes that severe early-life stress has systemic epigenetic effects during development. These epigenetic modifications change how the brain, the hypothalamic-pituitary adrenal (HPA) axis, and the immune system respond to future and concurrent stressors, as well as how these systems interact. In this model, the effects of stress are relatively dose-dependent, and are compounded as stress exposure continues across time.

Biological stress embedding is also affected by differences in how individuals characteristically respond to and behave in stressful circumstances (i.e., behavioral responsiveness). For example, because children react differently from each other to stressors (see Carson & Bittner, 1994; Rothbart et al., 2000), children with more anxious or vigilant phenotypes may be especially sensitive to the biological embedding process (Strelau, 1995). Many other behavioral traits likely contribute to how individuals cope with stress, including behavioral inhibition, emotionality, reactivity, anxious temperament, aggression, impulsivity, physical activity / motor arousal, and extraversion / sociability (Carver & Connor-Smith, 2010; Compas et al., 2004; Goldsmith et al., 1987; Kagan et al., 2007). Consequently, by moderating the frequency, degree, and severity of the stress response (Strelau, 1995), behavioral responsiveness can affect the net stress effect on health (Miller et al., 2011). There is also evidence that behavioral responses can directly affect health, potentially independently of specific stressful experiences (see Capitanio, 2011). For example, some studies have linked anxious temperament with increased inflammatory profiles (Nelson et al., 2018; Tas & Caglar, 2019), and other studies suggest that affective response patterns like cyclothymia are related to hypertension (Körösi et al., 2019; László et al., 2016). Although it is not possible to completely rule out the effects of early life stressors in these correlational studies, animal studies using subjects with a controlled, uniform, and relatively-typical rearing history have shown that both nervous temperament (Capitanio et al., 2011) and behavioral inhibition (Chun et al., 2013; Michael et al., 2020) are associated with different aspects of immune system functioning. It is therefore possible that different biobehavioral response patterns can become biologically embedded, potentially through the same mechanisms as stressful experiences.

One of the primary epigenetic mechanisms underlying biological embedding is DNA methylation (Fagundes et al., 2013; Miller et al., 2011; Weaver et al., 2004), a process by which methyl groups bind to nucleotide base pairs to affect gene expression without altering gene sequence. DNA methylation is highly sensitive to individual experience in some parts of the genome, particularly during early development, and in some cases, experience-dependent changes can be relatively permanent and lead to long-lasting changes in gene expression (Bird, 2002; Szyf & Bick, 2013). Thus far, the biological embedding model has proved a useful framework for guiding investigations, and many studies have shown that stress can alter DNA methylation in genes and gene-networks related to inflammatory disease (for reviews, see Bick et al., 2012; Szyf & Bick, 2013; Vinkers et al., 2015), particularly in brain cells (Hostinar et al., 2018; Nusslock & Miller, 2016). There is still much to be learned about how stress responsiveness shapes the epigenome in the very cells that protect individuals from disease – the immune system. This path of investigation could provide a critical mechanistic link for the biological embedding hypothesis–for example, our previous work has shown that early epigenetic programming predicts health outcomes in adulthood (Kinnally, 2014).

There is some evidence that behavioral responses to stress are associated with different epigenetic signatures across various immune tissue and cell types, potentially in genes related to health. Studies using candidate gene approaches have shown that, across different tissue types, various behavioral domains correlate with methylation in candidate genes like the serotonin transporter gene (Kinnally et al., 2010; Kinnally et al., 2011; Montirosso et al., 2016) and the glucocorticoid receptor gene (Appleton et al., 2015; Conradt et al., 2015; Ostlund et al., 2016). Although few studies have investigated stress responsiveness and DNA methylation in older children or adults, one series of studies showed that, relative to the general population, individuals with a history of aggression and/or temperamental dispositions towards aggression exhibited differences in DNA methylation in several genes related to cytokine signaling and inflammation (Guillemin et al., 2014; Provencal et al., 2013) (however, see van Dongen et al., 2015). DNA methylation also correlates with HPA axis functioning. For example, variation in the cortisol response to stress has been linked with differential methylation of the glucocorticoid receptor gene in infants (Conradt et al., 2015; Oberlander et al., 2008), children (Stonawski et al., 2018), and adult women (Edelman et al., 2012), and with methylation of the serotonin transporter gene in young adolescents (Ouellet-Morin et al., 2013). Although these studies are correlational, these findings suggest bi-directional links between behavioral stress responses, HPA axis functioning, and DNA methylation in genes linked with inflammation and stress response.

To better understand how stress responses may shape the immune epigenome, the rhesus macaque (*Macaca mulatta*) offers a promising translational model for study. Rhesus monkeys share strong genetic homology (approximately 95%) with humans (Gibbs et al., 2007), show similar temperament domains exhibited by human infants and children (Kay et al., 2010; Suomi et al., 2011; Wood et al., 2020), and are a well-established model for investigating stress physiology, early life development, and health (Capitanio, 2017a; Harlow, 2008; Kinnally et al., 2019; Suomi, 2006). For example, one study found that infant rhesus monkeys rated high in nervous temperament showed dysregulation of cortisol-mediated leukocyte trafficking (Capitanio et al., 2011). In another study, Alisch

et al. (2014) showed that methylation determined from amygdala tissue was associated with a multi-dimensional measure of anxious temperament in juvenile rhesus monkeys at several genes, including *BCL11A* and *JAG1*, two genes linked with brain development. The use of infant rhesus monkeys to interrogate these relationships allows rigorous and controlled investigation during early life and allows investigators to detect potentially important associations during standardized and sensitive periods of development.

In this study, we used a genome-wide approach to assess whether infant rhesus monkeys' behavioral stress responses and parameters of the HPA response predict differential methylation profiles in mononuclear blood cells. We draw upon a comprehensive assessment of infant behavioral responsiveness and stress responsivity to assess associations between genome-wide methylation in immune cell DNA and eight measures of behavioral responsiveness (determined from two different assessments), as well as four different cortisol samples that span HPA activation and habituation to stress, response to dexamethasone suppression, and response to ACTH stimulation. To enhance the specificity of our findings, we considered the role of immune cell sub-populations, which differ in DNA methylation patterns, as well as sex. We hypothesized that infant rhesus monkeys' cortisol output and behavioral responses to stress would be specifically associated with differential methylation in immune related genes that are stress-responsive, such as inflammatory pathways, and identified differentially methylated regions (DMRs) across the genome that predicted each biobehavioral measure, using bioinformatics to implement rigorous correction for false discovery rate. To interrogate the functional significance of the genome-wide DMRs, we took three data analytic approaches to: 1.) identify which sites were classified as part of gene annotation sets specifically linked with immune function and inflammation, processes that are regulated by the tissue type in this study; 2.) explore other biological pathways that were statistically overrepresented among DMRs; and 3.) determine which DMRs were detected in candidate genes previously linked with stress response and health.

2 Methods

2.1 Subjects

Subjects were $n = 154$ infant rhesus monkeys (82 males), aged 3–4 months, housed at the California National Primate Research Center. Most subjects ($n = 143$) were housed in 0.2-hectare outdoor field pens with approximately 80–100 other monkeys, conditions that approximate naturalistic social groups and environmental conditions. A small subset of subjects ($n = 11$) was separated from their mother at birth and reared indoors with a peer group. Animals were selected for this study because they were genetically unrelated (average relatedness < 6%) and had undergone a biobehavioral assessment (see below). Microsatellite analysis was used to confirm the individual's place in the colony pedigree, i.e. the identity of their mothers, fathers, grandparents and great-grandparents (Kanthaswamy et al., 2006).

All procedures in this study conformed with the guidelines established in the National Institutes of Health guide for the care and use of Laboratory animals, and all procedures were approved by the Institutional Animal Care and Use Committee at UC Davis.

2.2 Biobehavioral Assessment of Behavioral Responsiveness, Stress Physiology, and Immune Profile

All measures of behavioral responsiveness and physiology were obtained from a biobehavioral assessment, a standardized 25-hour battery of tests (see Capitanio, 2017b; Golub et al., 2006; Golub et al., 2009). From this assessment, the following metrics were used in the current study: behavioral responses to social separation and relocation determined from holding cage behavior observations, behavioral responses to an acute challenge determined from a human intruder test, cortisol determined from four blood samples, and immune cell counts from a hematology analysis (the distribution of these variables is shown in Supplementary Figure S1, and the intercorrelation between variables is shown in Supplementary Figure S2). Data from the biobehavioral assessment were collected in eight test-year cohorts (including 2005, and each year from 2009–2015). There were no cohort differences for any of the behavioral variables (ANOVA, $p > .17$) or immune cell counts (ANOVA, monocytes $p = .054$; all others $p > .44$). There were no cohort differences (ANOVA, $p > .30$) for any of the cortisol samples when analyzed separately by assay method (see below).

Holding cage behavior observations.—Infants were temporarily separated from their social group and relocated to a testing room for 25 hours. During this time, infants were observed for two five-minute periods, once at 15-minutes post-separation and relocation (the Day 1 assessment) and once at 22-hours post-separation and relocation (the Day 2 assessment). Behavioral data were live-scored by a trained observer, who sat approximately eight feet away from the holding cage and avoided direct eye contact with subjects (Capitanio et al., 2006). Exploratory and confirmatory factor analysis of the behavioral data (see Golub et al., 2009 for a full description) revealed the following factor scales: Activity (including the behaviors Locomotion, Exploration, Eating, Drinking, and Crouching) and Emotionality (included Cooing, Scratching, Threatening, and Lip-smacking). Based on these factors, holding cage scales for Day 1 Activity, Day 2 Activity, Day 1 Emotionality, and Day 2 Emotionality were created by summing the z-scored behaviors that loaded into each factor, and z-scoring within test-year cohort (see Golub et al., 2009).

Human intruder test.—At 5 hours post-separation and relocation (1400 hrs), subjects were tested in a human intruder paradigm modified for infant monkeys (see Capitanio et al., 2006; Kalin & Shelton, 1989). Prior to the human intruder testing, subjects were administered a blood draw (1100 hrs; see below) and two different behavioral tests (not considered here). For testing, subjects were relocated to an adjacent room and experienced four one-minute conditions in the following order: “Profile Far” (human intruder presents left-side profile to subject from 1 meter away), “Profile Near” (human intruder moves forwards to ~0.3 meters away and continues presenting left-side profile), “Stare Far” (human intruder steps back to 1 meter away and turns to makes direct eye contact), and “Stare Near” (human intruder steps forward to ~0.3 meters away and continues making eye contact with subject). Monkeys’ responses were video recorded and were later scored for stress response behaviors (including threats, activity, emotionality, and anxiety-like behaviors). Based on exploratory and confirmatory factor analyses performed on a larger population of previously tested infants (see Gottlieb & Capitanio, 2013), scales were created

based on the following four factors: Activity (locomotion, cage shaking, environment exploration), Aggression (threatening, barking, other vocalizations), Displacement (teeth grinding, yawning), and Emotionality (convulsive jerk, fear grimace, self-clasp, cooing). Following a similar approach used to create the holding cage scales (Golub et al., 2009), each human intruder scale was created by summing the average behavior frequency (across the four test conditions) of the z-scored behaviors that loaded into each factor, then z-scoring this sum within test-cohort year (Gottlieb & Capitanio, 2013).

Cortisol and HPA responsiveness.—Cortisol was determined from four blood samples obtained via femoral venipuncture during the 25-hour assessment period. All samples were obtained while subjects were awake and manually restrained, within five minutes of hand-capture from the holding cage. Sample 1 was taken two-hours after arrival in the test area (1100 hrs) and reflects infants' initial response to the separation and relocation. Sample 2 was taken at seven-hours post-separation and relocation (1600 hrs) and reflects infants' ability to adapt to the separation/relocation. Immediately after Sample 2 was taken, infants were injected intramuscularly with 500 μg/kg ml of dexamethasone (American Regent Laboratories, Inc., Shirley, NY). Sample 3 was taken at 23.5-hours post-separation and relocation (0830 hrs). Immediately after Sample 3 was taken, infants were injected with 2.5 IU ACTH (Organon, Inc., West Orange, NJ) i.m., and 30 minutes later Sample 4 was taken (24-hours post-separation and relocation, 0900 hrs). All samples were taken in un-heparinized syringes and immediately transferred to EDTA tubes. Samples were centrifuged at 4°C at 1277 RCF for 10 minutes, and plasma was pipetted into tubes and stored at −80°C until assay. Most samples (n = 128, those obtained before 2014) were assayed for cortisol using RIA (Diagnostic Products Corp., Los Angeles, CA), with the following coefficients of variations: inter-assay, 5.8%; intra-assay, 7.9%. Due to manufacturing changes in this kit, the remaining samples (n = 26, obtained during 2014–2015) were assayed using a competitive immunoassay (Siemens Healthcare Diagnostics, Tarrytown, NY). The immunoassay produced the following coefficients of variation: inter-assay, 5.0%, intra-assay 2.4%. We have previously shown in a validation study that both assays produce highly correlated cortisol estimates (r = 0.88) (see Vandeleest et al., 2019). We used these validation data to predict RIA cortisol values based on immunoassay cortisol values.

Hematology.—All hematological measures were determined from blood plasma obtained from blood Sample 1. To determine hematology, complete cell counting was performed on 0.5 ml plasma aliquots using an ABX Pentra 60C (Horiba Medical, Irvine, CA) with manual differential. From the available measures, the following white blood cell counts were considered in analyses: lymphocytes, monocytes, segmented neutrophils, and eosinophils.

2.3 Reduced Representation Bisulfite Sequencing

Methylation levels were determined from blood Sample 1 using reduced representation bisulfite sequencing (RRBS). After the sample was centrifuged and plasma was aliquoted (see above), the remaining blood sample was stored at −80° until RRBS was performed. RRBS libraries were generated using the Premium RRBS Kit from Diagenode (Liege, Belgium) according to the instructions of the manufacturer. In short 100 ng of each DNA sample were digested with the *MspI* restriction enzyme and ligated to barcoded adapters.

The ligation products were size-selected via magnetic bead purification and quantified by qPCR using a SybrGreen protocol and equal amounts of 8 samples were pooled. The pooled samples were bisulfite converted. The optimal PCR cycle number for the following enrichment PCR was again determined by qPCR of an aliquot of the bisulfite converted pool. The library pools were amplified with eleven PCR cycles. The fragment size distribution of resulting library pools was assessed via micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The library pools were quantified by qPCR with a Kapa Library Quantification kit (Kapa Biosystems/Roche, Basel Switzerland) and sequenced on an Illumina HiSeq 4000 (Illumina, San Diego, CA) run with single-end 90 bp reads. After adapter trimming and quality assessment, RRBS reads were mapped to the macaque genome using Bismarck (Chen et al., 2010). After removing unmapped sites and sites with less than 10x coverage, the RRBS yielded 9,548,543 individual sites with methylation data for at least one of the 154 samples (see Figure 1, Supplementary Tables S1A–C).

2.4 Genome Annotation

Sites yielding methylation data following RRBS were annotated for gene name, gene biotype, genomic function, and genomic position relative to transcription start sites (TSS) using the rhesus macaque Mmul_10 Ensembl (data base version 99.10) (http://uswest.ensembl.org/Macaca_mulatta/Info/Annotation). Based on the annotated gene biotypes, sites were classified into the following categories: protein coding genes (74% of annotated sites), non-coding RNAs (24% of annotated sites), pseudogenes (1.3% of annotated sites), or ribozymes (0.01% of annotated sites) (see Supplementary Table S1A).

Based on the annotated gene start and stop positions, sites were also classified as within the gene body, upstream from the gene body, or downstream from the gene body (see Supplementary Table S1B). Sites within the gene body were classified as being in one of the following genomic regions, according to the annotated genomic functions: *3 Prime UTR* (1.2% of all sites), *5 Prime UTR* (0.3% of all sites), *Exon* (2.2% of all sites), or *Intron* (47% of all sites). Because some genes were associated with multiple transcript variants, some sites were categorized as being in the *3 Prime UTR* of one transcript variant and in the *5 Prime UTR* of another transcript variant. In these few cases (67 sites, < .00001% of all sites), sites were classified as being in the *5 Prime UTR*. Sites located upstream of the gene body were classified as being in one of the following categories: *Promoter 200* (between 1 –200 bp upstream of the TSS; 0.2% of all sites), *Promoter 1500* (between 201–1,500 bp upstream of the TSS; 1.3% of all sites), *Promoter 10K* (between 1,501–10,000 bp upstream of the TSS; 5.9% of all sites), or *Intergenic – Upstream* (more than 10,000 bp upstream of TSS; 17.1% of all sites). Sites located downstream of the gene body were classified as being in one of the following categories: *Downstream 200* (between 1–200 bp downstream of gene body; 0.2% of all sites), *Downstream 1500* (between 201–1,500 bp downstream of gene body; 1.3% of all sites), *Downstream 10K* (between 1,501–10,000 bp downstream of gene body; 5.4% of all sites), or *Intergenic – Downstream* (more than 10,000 bp downstream of gene body; 17.8% of all sites) (see Supplementary Table S1B).

Based on the distribution of percent methylation at each site, sites were also classified according to predominant methylation state at a given site (see Supplementary Table S1C). Sites were classified as either predominantly Hypermethylated if more than 80% of subjects had 100% methylation at the site (43% of all sites), predominantly Hypomethylated if more than 80% of subjects had 0% methylation at the site (24% of all sites), or Intermediately methylated if neither criterion were met (33% of all sites) (see Supplementary Table S1C).

Using the rhesus macaque reference genome in the PANTHER data base (version 15.0, released 2020-02-14) (Mi et al., 2018; Mi et al., 2019), sites were also annotated for ontology terms related to biological process and gene pathways (Mi & Thomas, 2009).

2.5 Data Analysis

All analyses were performed in R programming (R Core Team, 2019). First, a series of genome-wide analyses were performed to identify sites in the genome at which methylation was robustly associated with the stress response variables measured in the biobehavioral assessment (for a summary of the analyses performed, see Figure 1 and Supplementary Note 1). In all analyses, methylation was the dependent variable, and the independent variables analyzed included the four Holding Cage observation scales (Day 1 Emotionality, Day 1 Activity, Day 2 Emotionality, Day 2 Activity), the four Human Intruder scales (Activity, Emotionality, Displacement, and Aggression), and the four cortisol samples. Although not the primary variables of interest, to better understand how patterns of DNA methylation associated with different immune cell profiles, genome-wide analyses were also performed for the four immune cell counts (monocytes, lymphocytes, segmented neutrophils, and eosinophils). Given that sex differences have also been found in stress responsiveness and in health outcomes related to stress (see, for example, Burns et al., 2018; Gunnar et al., 2015; Kudielka & Kirschbaum, 2005), secondary analyses were also performed for sex. Although nursery rearing vs mother rearing in rhesus macaques has well-documented effects on stress responsiveness and immune-cell methylation profiles (Provençal et al., 2012), analyses for rearing status were not performed because the primary hypotheses in this study pertain to correlations between methylation and stress responsiveness.

Genome-wide analyses were performed using linear regression with false discovery rate (FDR) correction, and a series of sensitivity analyses were performed to a) control for immune cell subtype variation between subjects (using multiple regression) and b) to account for spurious associations driven by extreme methylation values (using permutation regression) (for a full description of these analyses and their justification, see Supplementary Note 1). Out of over 2.2 million sites that met inclusion criteria for analyses (see Figure 1 and Supplementary Table S2), this analytic approach yielded 20,368 unique sites (in 9,040 unique genes) that were significantly (FDR $q < .05$) associated with at least one of the twelve primary independent variables analyzed (i.e., the holding cage scales, the human intruder scales, and cortisol samples) (more information on these results is given in Supplementary Table S3A and Supplementary Notes 2–3; see Supplementary Table S3B for a summary of the genome-wide analyses of immune cell counts, and Supplementary Table S3C for a summary of the genome-wide analyses of sex). The sites that attained significance were relatively unique to each independent variable (see Supplementary Figure

S3) and included many positive and negative associations (see Supplementary Table S4). Few of the significant DMRs for sex overlapped with the significant DMRs from the other independent variables tested, suggesting that sex was not a major confound of the results (see Supplementary Tables S3C and S3D). For a full list and summary of all the sites that achieved genome-wide significance across all the analyses performed, see Supplementary File 1.

Interrogation of immune processes.—Using the biological process and gene pathway annotations obtained from the PANTHER data base, two annotation categories related to immune cell functioning were selected for post hoc exploration. These included *Immune System Process* (a biological process annotation) and *Inflammation mediated by chemokine and cytokine signaling pathway* (a gene pathway annotation; hereafter referred to as the *Inflammation pathway*). The *Immune System Process* annotation category was chosen because it contained a large subset of genes (1,376) related to general immune system functioning in the PANTHER data base. The *Inflammation pathway* was chosen because it contained a comprehensive list of genes (264) related to inflammation, a key process linking epigenetic stress programming and health (see, for example, Miller et al., 2011; Morales-Nebreda et al., 2019). There was relatively little overlap with the genes in the *Immune System Process* category (only 90 genes overlapped between the two annotation categories). Each group was explored separately by examining the sites that attained genome-wide significance across the simple regression, multiple regression controlling for immune cell counts, and the permutation regression, with particular focus given to sites located in promoter or other regulatory regions. For the *Inflammation pathway*, we also report summary statistics for sub-pathways of interest.

Pathway overrepresentation analyses.—In addition to assessing the two annotation groups described above, additional biological processes and gene pathways were selected for exploration by performing category overrepresentation analyses. For each independent variable, overrepresentation analyses were performed to assess whether the number of significant genes observed in each annotation category differed significantly from what would be expected given the base occurrence rate in a larger group. Overrepresentation analyses were performed relative to two different reference groups: the rhesus macaque reference genome available on the PANTHER data base and the subset of sites that met criteria for and underwent genome-wide analysis. This was done because preliminary assessments showed that using the PANTHER reference genome yielded liberal estimates of overrepresentation that were potentially driven by patterns of overrepresentation present in the larger subset of genes that met inclusion criteria for the analysis, and not necessarily by the genes that were significant in the analysis. Hence, by examining overrepresentation relative to the subset of sites that underwent genome-wide analysis, more robust and conservative estimates of overrepresentation were obtained (Mi et al., 2019). We focus our interpretation on pathways that were overrepresented in both comparisons.

Exploration of candidate genes.—Based on previous investigations of links among behavioral responsiveness, HPA function, and methylation in humans, the following rhesus macaque ortholog genes were explored, based on the availability of methylation data

returned from the sequencing: *SLC6A4* (Edelman et al., 2012; Montirosso et al., 2016), *NR3C1* (Edelman et al., 2012; Ostlund et al., 2016), *CXCL8*, *IL4*, *IL1A*, *NFAT5*, *NFKB1*, *STAT6* (Provencal et al., 2013), *EGR1* (also known as *NGFI-A*), *ESR1*, (Edelman et al., 2012), and *HSD11B2* (Appleton et al., 2015). We also explored beta-adrenergic receptors (*ADRB1*, *ADRB2*, and *ADRB3*). Although methylation of beta-adrenergic receptors has not previously been linked with behavioral responsiveness or cortisol, these genes were explored because beta-adrenergic receptors play an important role in modulating physiological stress responses (Elenkov et al., 2000; Stiles et al., 1984), and because polymorphisms in beta-adrenergic receptor genes have been linked with different adult personality dimensions (Numajiri et al., 2012). We also interrogated 22 other genes potentially associated with anxious temperament, to determine consistency with an epigenome-wide association study of rhesus monkey amygdala methylation (Alisch et al., 2014), including *BCL11A*, *JAG1*, *PIK3R1*, *KLHDC9*, *URM1*, *SHKBP1*, *ZNF521*, *GRIN1*, *PIP5K1B*, *ERC2*, *ABCB1*, *SCAMP3*, *SPTBN4*, *GDF11*, *ALDH7A1*, *FGD1*, *GRM5*, *CDH2*, *YIPF2*, *CLK2*, *TMEM121B*, and *KL*.

3 Results

A summary of the DMRs that attained significance across genome-wide analyses is given in Supplementary Tables S3A–C, Supplementary Notes 2–3, and Supplementary File 1.

3.1 Exploration of *Immune System Process* and *Inflammation* Pathway Genes

Of the 1,376 genes listed in the *Immune System Process* category of the PANTHER data base biological process category, 1,135 genes had at least one site with available methylation data for analysis in this study. Of these 1,135 genes, there were 442 unique genes (992 unique sites) that were significantly associated with at least one behavioral responsiveness or cortisol variable in the simple regression, multiple regression, and permutation regression (for the number of sites and genes across each independent variable, see Supplementary Tables S3E and S3F). There were 64 unique sites (in 45 *Immune System Process* genes) located in a functional region relevant for transcription (see Table 1 and Figure 2A for a summary of the sites in proximate promoters; for a full summary, see Supplementary File 1).

Of the 264 genes listed in the *Inflammation mediated by chemokine and cytokine signaling pathway* in the PANTHER reference genome, 181 genes had at least one site with available methylation data for analysis in this study. Of these 181 genes, there were 94 unique genes (262 unique sites) that were significantly associated with a behavioral responsiveness or cortisol variable in the simple regression, multiple regression, and permutation regression (for the number sites and genes across each independent variable, see Supplementary Tables S3G and S3H). The 94 genes that were robustly associated with at least one measure of behavioral responsiveness or cortisol were distributed widely across *Inflammation pathway* sub-pathways, including *Nuclear factor of activated T cells* (9 sites in 2 genes), *Chemokine* (3 sites in 3 genes), *Chemokine receptor* (4 sites in 3 genes), *Cytokine receptor* (3 sites in 2 genes), and *Interleukin 2* (6 sites in 1 gene) (see Supplementary File 2). There were 20 sites (in 16 *Inflammation pathway* genes) with at least one significant association in a region

functionally relevant for transcription (see Table 2 and Figure 2B for a summary of the sites most proximate to the gene body; for a full summary, see Supplementary File 1).

3.2 Exploratory PANTHER Overrepresentation Analyses

Across the sites that attained genome-wide significance in the behavioral responsiveness and cortisol analyses, there were 94 unique biological process categories that were significantly overrepresented (see Figure 3 for a condensed summary, and Supplementary File 3 for a full summary). Many of the overrepresented categories were related to cellular functioning, neuronal formation, and maturation (see Figure 3). The *Immune System Process* annotation category was not significantly overrepresented for any of the independent variables analyzed ($q > 0.98$; see Supplementary File 3).

There were six gene pathways that were significantly overrepresented among the sites that attained genome-wide significance (see Table 3). From these six pathways, the pathway most pertinent to stress physiology and inflammatory response was the *Angiotensin II-stimulated signaling through G proteins and beta-arrestin*. From this pathway, notable associations included a negative correlation between Day 1 Emotionality and methylation at an intergenic site proximate to the Angiotensin II Receptor Type 1 gene (*AGTR1*; $\beta = 0.46$, simple regression; see Figure 4), and a negative correlation between Day 1 Emotionality and methylation at an intron site in the Beta Arrestin 2 gene (*ARRB2*; $\beta = -0.31$, simple regression; see Figure 4). For a full summary of all the sites that attained significance in the six overrepresented pathways, see Supplementary Table S5. The *Inflammation mediated by chemokine and cytokine signaling pathway* was not significantly overrepresented across any of the behavioral responsiveness or cortisol variables when assessed relative to the genes meeting inclusion criteria for analysis ($q > .06$; see Supplementary File 4). However, we note that overrepresentation approached significance among the sites that were associated with Human Intruder Aggression (fold enrichment = 1.82, $q = .060$) and among the sites that were associated with Day 1 Emotionality (fold enrichment = 1.70, $q = .067$), and in both cases, the overrepresentation attained significance when assessed relative to the rhesus macaque reference genome ($q < .049$; see Supplementary File 4) rather than to the sites meeting inclusion criteria for analysis.

3.3 Exploration of Specific Candidate Genes

Across the 35 candidate genes that were explored, there were 13 unique genes (27 individual sites) at which methylation was significantly correlated with one of the behavioral responsiveness or cortisol variables across the simple regression, multiple regression, and permutation regression (see Supplementary Tables S6A and S6B). Notably, there was a significant correlation between Day 2 Emotionality and methylation of an intron site of the glucocorticoid receptor gene (*NR3C1*, $\beta = -0.39$ simple regression; see Figure 5). Methylation was also negatively correlated with Sample 1 Cortisol at a different intron site of the *NR3C1* gene ($\beta = -0.33$ simple regression; see Supplementary Table S6A).

Of the 22 genes in which Alisch et al. (2014) found robust associations between methylation and anxious behavioral responsiveness in adolescent rhesus monkeys, we found that nine of these genes had at least one site that was significantly associated with one of the behavioral

responsiveness or cortisol variables across the simple regression, multiple regression, and permutation regression (in total, there were 19 sites across the 9 genes). Of these sites, one that was particularly relevant to peripheral blood cells was the B-cell lymphoma/leukemia 11A gene (*BCL11A*), in which methylation was negatively correlated with Day 2 Emotionality ($\beta = -0.36$, simple regression. The other eight genes included *ABCB1*, *ERC2*, *GRIN1*, *GRM5*, *JAG1*, *KL*, *PIP5K1B*, and *ZNF521* (see Supplementary Table S6A). Under our criteria for genome-wide significance, none of the other genes identified by Alisch et al. (2014) were significantly associated with behavioral responsiveness or cortisol, including: *PIK3R1*, *KLHDC9*, *URM1*, *SHKBP1*, *SCAMP3*, *GDF11*, *ALDH7A1*, *FGD1*, *CDH2*, *YIPF2*, *TMEM121B*, and *CLK2*.

Of the three beta-adrenergic genes that were investigated (*ADRB1*, *ADRB2*, *ADRB3*), only the *ADRB3* gene contained a site at which methylation was significantly associated with behavioral responsiveness or cortisol. The site that attained genome-wide significance was an intergenic site, at which methylation was negatively correlated with both Sample 3 post-dexamethasone Cortisol ($\beta = 0.67$, simple regression) and Sample 4 post-ACTH injection cortisol ($\beta = 0.69$, simple regression; see Supplementary Table S6A).

Of the six genes that Provencal et al. (2013) found to be associated with aggressive temperament in adult humans, we found that two of these genes contained at least one site at which methylation was significantly associated with behavioral responsiveness (in total there were 4 sites across the 2 genes; see Supplementary Table S6A). Two of these sites included an intergenic site most proximate to the interleukin 1A (*IL1A*) gene, at which methylation was negatively correlated ($\beta = -0.31$, simple regression; see Figure 5) with Human Intruder Aggression, and an intron site in the signal transducer and activator of transcription 6 (*STAT6*) gene, at which methylation was positively correlated with Human Intruder Aggression ($\beta = 0.53$, simple regression; see Figure 5). The four other genes identified by Provencal et al. (2013) were not significantly associated with behavioral responsiveness or cortisol, including: *CXCL8*, *IL4*, *NFAT5*, or *NFKB1*.

None of the other candidate genes explored (*SLC6A4*, *ESR1*, *ERG1*, and *HSD11B2*) attained genome-wide significance under the simple regression, multiple regression, and permutation regression (a summary of the analyses for each available site in these and other candidate genes is available in Supplementary File 1).

3.4 Analysis of Immune Cell Counts

The epigenome-wide analysis revealed 2,327 unique sites (in 1,962 genes) that attained significance across the four immune cell count variables analyzed (see Supplementary Table S3B); most of these sites were for eosinophils (2,306 sites in 1,949 genes). In the case of eosinophils, there were 104 significant sites (in 91 genes) in the *Immune System Process* annotation category (see Supplementary Table S3F), as well as 23 sites (in 20 genes) in the *Inflammation pathway* (see Supplementary Tables S3H). Although these annotation categories were not significantly overrepresented, there were three other biological processes (but no gene pathways) that were overrepresented for eosinophils, including *Biological Regulation* (727 genes), *Regulation of Cellular Process* (659 genes), and *Regulation of Biological Process* (688 genes) (fold enrichment ranged from 1.12 – 1.13, $q < .026$; see

Supplementary File 3). Among the candidate genes that were explored, eosinophils were associated with methylation at two sites in the *TMEM121B* gene (see Supplementary Table S6B). We note that when the behavioral responsiveness and cortisol analyses were repeated using multiple regression to control for immune cell counts, in some cases one or more of the immune cell variables were significant covariates (see Supplementary File 1). However, in most cases the sites that were associated with the immune cell counts were relatively independent of the sites that were associated with any of the behavioral responsiveness or cortisol variables (see Supplementary Figure S3, panels E1 and E2).

4 Discussion

How one responds to a stressor affects their long-term health trajectories: usually the more intense and negative the stress response, the poorer the health outcomes (for reviews, see Danese & McEwen, 2012; Dhabhar, 2014; Miller et al., 2011). Here we show that, very early in life, how an infant monkey responds to a brief stressor is associated with variation in the immune epigenome. Out of the approximately 2.2 million sites in the genome that were assessed, we found 20,368 unique differentially methylated regions (DMRs) (in 9,040 genes) at which immune cell methylation was associated with at least one biobehavioral measure. We found significant genome-wide associations between DNA methylation and all four cortisol samples measured, and six of the eight (6/8) behavioral responsiveness measures assessed (see Supplementary Table S3A). The DMRs were associated with a broad range of biological processes, including maturation, cellular differentiation, and intracellular signaling and communication (see Figure 3 and Supplementary Files 3–4). Approximately 6% of the DMRs were in genes directly involved with immunity, and others (1.3%) were primarily involved with cell-level signaling processes involving β-adrenergic receptors, angiotensin II, glutamate, and GABA (See Supplementary Table S5 and Supplementary File 3), which may be relevant to coordinating the stress-immune axis. We found 992 unique sites (in 442 unique genes) in the *Immune System Process* annotation category at which methylation was significantly associated with at least one behavioral responsiveness or cortisol variable analyzed, as well as 262 unique sites (in 94 unique genes) in the *Inflammation mediated by chemokine and cytokine signaling pathway* (see Supplementary Table S3E and S3G). Exploratory overrepresentation analyses revealed six gene pathways and 94 biological function categories that were statistically overrepresented (see Figure 3 and Table 3, as well as Supplementary Files 3–4), including several pathways related to inflammation and immune cell functioning. We also explored 35 candidate genes based on prior studies linking DNA methylation with biobehavioral measures and found 27 unique DMRs (in 13 genes) that were associated with at least one measure of behavioral responsiveness or cortisol, including two sites in the glucocorticoid receptor gene (*NR3C1*; see Figure 5 and Supplementary Table S6A). Congruent with systems models of biological stress embedding (see, for example, Fagundes et al., 2013; Hostinar et al., 2018; Miller et al., 2011), our results suggest that individual stress response patterns may re-program epigenetic regulation of the immune system.

Our investigation was based on the premise that DNA methylation likely regulates *how* immune cells function, and may also be re-programmed by responses to ongoing challenges (see Morales-Nebreda et al., 2019; Suarez-Alvarez et al., 2012). For example, infection

triggers active demethylation at distal gene enhancers, particularly at sites associated with nuclear factor-κB and interferon (Pacis et al., 2015), primary regulators of inflammation. Altered immune cell methylation profiles are also associated with specific auto-immune disorders and immune-system cancers (Morales-Nebreda et al., 2019; Suarez-Alvarez et al., 2012), suggesting that some changes in DNA methylation may be specifically involved with disease. Over-representation analyses of the entire dataset revealed many affected pathways (see Figure 3 and Supplementary Files 3–4). We focused on the *Immune System Process* and *Inflammation Mediated by Chemokine and Cytokine Signaling pathway*s to specifically identify how biobehavioral organization shapes immune function in immune cells. Although neither category were significantly overrepresented when assessed relative to the genes that met analysis inclusion criteria, we note that for the *Inflammation pathway*, there was a trend towards significant overrepresentation in the Human Intruder Aggression and Day 1 Emotionality analyses (and in these cases overrepresentation was significant when assessed relative to the less-stringent reference group; see Supplementary File 4). Across the measures considered in this study, biobehavioral stress responses predicted methylation in 992 sites in 442 distinct genes annotated in the PANTHER *Immune System Process* category and in 262 sites in 94 genes in the *Inflammation Mediated by Chemokine and Cytokine Signaling pathway* (see Supplementary Tables S3E and S3G). In total there were 1,189 sites (in 507 genes) between the two annotation categories, indicating little overlap between the two categories. Within the *Inflammation pathway*, these DMRs were associated with many different sub-processes, including chemotaxis, T cell and B cell signaling, cytokine and chemokine receptor signaling, and regulation of inflammatory transcription factors (see Supplementary File 2). Between the two annotation categories, there were many DMRs located in upstream- or downstream-regulatory regions or in the 5 prime or 3 prime UTR (184 sites in 147 genes). Pending replication of our findings, bio-behaviorally linked DMRs in these categories are likely to be immunologically meaningful. It is notable that, in general, emotion-related (rather than motor- or exploration-related) behaviors were more strongly associated with immune cell DNA methylation (see Supplementary Table S3A). Given that stress-responses involve many limbic brain regions that interact with both the sympathetic nervous system and the HPA axis, this finding may suggest that emotional responses to stress exert a top-down influence on the epigenetic organization of immune cells.

Exploratory analyses of all the DMRs revealed 94 biological processes (see Figure 3 and Supplementary File 3) and six gene pathways (see Table 3) that were statistically over-represented among the sites that met criteria for genome-wide significance (i.e., associations that remained significant even when controlling for immune cell subtype counts per sample and when using a permutation approach). Although the overrepresented categories contained genes that may not be highly expressed in immune cells, there were several that are potentially relevant to immune functioning. One pathway was the *Angiotensin II-stimulated signaling through G proteins and beta-arrestin pathway*, which was linked with Day 1 Emotionality. Beyond its role in regulating blood pressure and volume, this process also contributes to inflammatory response (for a comprehensive reviews, see Fan, 2014; Suzuki et al., 2003). Among the genes in this pathway that were associated with Day 1 Emotionality were angiotensin type II 1a receptor (*AGTR1*), the beta arrestin 1 (*ARRB1*) and beta arrestin 2 (*ARRB2*) genes, and two g-protein genes (*GNB4* and

GNG12) (see Supplementary Table S5 and Figure 4). Other overrepresented pathways of interest included the *Beta1-* and *Beta2-andrenergic receptor pathways*, processes that are linked with regulating stress physiology and initiating inflammation (Elenkov et al., 2000; Farmer & Pugin, 2000; Kolmus et al., 2015). However, an important caveat is that neither the beta1- nor the beta2-adrenergic receptor genes (*ADRB1* and *ADRB2*) attained genome-wide significance in any of the analyses performed (see Supplementary File 1), meaning that the overrepresentation of these pathways was driven by the other elements of the signaling pathway (see Supplementary Table S5). Three other gene pathways that were significantly overrepresented across different behavioral responsiveness analyses, including the *Metabotropic glutamate receptor group III pathway*, the *Ionotropic glutamate receptor pathway*, and the *GABA-B receptor II signaling* pathway (see Table 3). Beyond their traditional roles in the central nervous system, there is evidence that both glutamate (for reviews, see Boldyrev et al., 2005; Ganor & Levite, 2014) and GABA (for reviews, see Barragan et al., 2015; Jin et al., 2013) can affect immune cell functioning and signaling. Significant genes from these pathways included the kainate receptor genes *GRIK1* and *GRIK2*, the NMDA receptor genes *GRIN2B* and *GRIN3A*, and the GABA-B receptor gene *GABBR2* (see Supplementary Table S5). These and the other overrepresented pathways merit further investigation, and are possible candidates linking behavioral responsiveness and methylation with different health outcomes.

Our candidate gene analysis allowed us to compare our results with those of previous studies, and to determine the generalizability of our results. Thirteen out of thirty-five (13/35) candidate genes that were assessed showed a robust association between methylation and at least one of the behavioral responsiveness or cortisol measures considered in this study (see Figure 5 and Supplementary Table S6A). In two out of six (2/6) candidate inflammatory genes previously linked with temperament in humans (Provencal et al., 2013), we found significant associations between methylation and behavioral responsiveness. We found a negative correlation between Human Intruder Aggression and methylation of an intergenic site upstream from the Interleukin-1 alpha (*IL1A*) gene ($\beta = -0.31$), and a positive correlation between Human Intruder Aggression and methylation of an intron site in the signal transducer and activator of transcription 6 (*STAT6*) gene (see Figure 5 and Supplementary Table S6A). The direction of these results is relatively consistent with the findings of Provencal et al. (2013), who showed that in monocytes, methylation of the *IL1A* gene was generally higher in men with a history of aggression when compared to controls, whereas methylation of the *STAT6* gene was generally lower in men with high aggression compared to controls (however, these associations were not always in the same direction when T cells were investigated). Together with this study, our results suggest that an aggressive behavioral disposition may contribute to methylation signatures underlying inflammatory profiles. However, it is important to note that these results were not replicated in two other epigenome-wide investigations of methylation and aggressive temperament (Guillemin et al., 2014; van Dongen et al., 2015). Further, for both *IL1A* and *STAT6*, there were additional associations between methylation and other behavioral responsiveness measures (Human Intruder Displacement and Emotionality, respectively) at different sites in the genes, suggesting that other high-arousal behavioral response patterns may contribute to inflammatory profiles.

Another key candidate gene we explored was the glucocorticoid receptor gene (*NR3C1*). We found a negative correlation between Sample 1 cortisol (taken two hours post-separation and relocation) and methylation of an intron site in the *NR3C1* gene, analogous to previous studies (Conradt et al., 2015; Conradt et al., 2016; Edelman et al., 2012; Oberlander et al., 2008). The negative association found in this investigation was consistent with human studies of women (Edelman et al., 2012) and infants (Conradt et al., 2016), but was in the opposite direction of the positive associations reported in other human studies of infants (Conradt et al., 2015; Lester et al., 2018; Oberlander et al., 2008) and children (Stonawski et al., 2018). We also linked high intronic *NR3C1* methylation with low Day 2 Emotionality in the Holding Cage observations (at a different site than the one linked with Sample 1 cortisol; see Supplementary Table S6A and Figure 5). This finding was consistent with a study showing a negative correlation between *NR3C1* methylation and infant socio-emotional functioning (Folger et al., 2019), but was in the opposite direction compared to other studies showing positive correlations between the severity of infants' stress responses and methylation in promoters of the *NR3C1* gene (Appleton et al., 2015; Conradt et al., 2015; Conradt et al., 2013; Ostlund et al., 2016; Sheinkopf et al., 2016; Stroud et al., 2016). However, in these cases the directional discrepancies may be due to artifact, different age groups studied, different functional locations assessed, or the different cell types investigated in the different studies (immune cells in this study, vs buccal cells or placenta in other studies). Nonetheless, our findings add to a growing body of literature implicating methylation of the *NR3C1* gene as an important factor underlying biological stress embedding (for a review, see Palma-Gudiel et al., 2015), and further research is needed to explore how behavioral responsiveness traits like emotionality and fearfulness affect *NR3C1* regulation.

We also compared our results to a study that examined the relationship of amygdala DNA methylation and a composite measure of anxious temperament in juvenile rhesus monkeys. This study identified 22 genes with functional associations between anxious temperament and gene methylation (Alisch et al., 2014). We found that nine of those 22 genes (9/22) had at least one robust association between methylation and one of the behavioral responsiveness or cortisol variables analyzed in our study, including the following: BAF Chromatin Remodeling Complex Subunit BCL11A (*BCL11A*), ATP Binding Cassette Subfamily B Member 1 (*ABCB1*), ELKS/RAB6-Interacting/CAST Family Member 2 (*ERC2*), Glutamate Ionotropic Receptor NMDA Type Subunit 1 (*GRIN1*), Glutamate Metabotropic Receptor 5 (*GRM5*), Jagged Canonical Notch Ligand 1 (*JAG1*), Klotho (*KL*), Phosphatidylinositol-4-Phosphate 5-Kinase Type 1 Beta (*PIP5K1B*), and Zinc Finger Protein 521 (*ZNF521*) (see Supplementary Table S6A). The degree of overlap between the current study and Alisch et al. (2014) suggests that there might be some parallel epigenetic programming in brain and blood. This possibility is supported by the overrepresentation of many biological pathways related to central nervous system development and regulation in our immune cell gene ontology analysis, even though these genes play a negligible role in immune function (see Figure 3 and Supplementary File 3). This is consistent with a seminal primate epigenomics study that found over 200 differentially methylated gene promoters in T cells and over 1,300 differentially methylated gene promoters in the prefrontal cortex when mother-reared and nursery-reared infants were compared, with significant enrichment of gene ontology terms

related to immune functioning in both cell types (Provençal et al., 2012). Notably, of the top 50 DMRs in T cells and brain tissue in this prior study (Provençal et al., 2012), eleven of the T cell DMRs (11/50) and nine of the brain cell DMRs (9/50) emerged as significant in our study. This may support the idea that some immune cell programming reflects or mirrors neural re-programming (see Davies et al., 2012). This would also help explain why, even though we examined immune cell DNA, only some of the significant DMRs were involved with normative immune cell processes, while others were more likely to affect expression in other tissues (see Figure 3). We conclude that while some DMRs may be tissue specific, DMRs observed across multiple tissues may arise from more global physiological processes that are distributed throughout the body.

Our study considered a potential role for sex differences. None of our behavioral measures differed based on sex. Consistent with previous studies, there was a sex difference in plasma cortisol, such that females exhibited higher concentrations across sampling conditions (Capitanio et al., 2005). While we observed a significant number of DMRs based on infant sex (3,330 sites in 476 genes; see Supplementary Table S3C), across the eight behavioral variables, four cortisol samples, and four immune cell counts assessed, there were few DMRs that overlapped with the DMRs from the analysis of sex differences (most variables had zero overlapping DMRs, and at most there were 26 overlapping DMRs, equivalent to < .01% of the DMRs) (see Supplementary Table S3D). Hence, it is not likely that sex differences confounded the primary results of this study. Our findings add to a growing body of literature showing that sex differences exist in epigenome-wide methylation. Consistent with several genome-wide investigations in humans (see, for example, Gong et al., 2018; Inoshita et al., 2015; Liu et al., 2010; Yousefi et al., 2015), most of the DMRs for sex in this study were located on the X chromosome (3,275 sites, approximately 98%). As might be expected, females exhibited higher methylation than males at most of these sites (2,252 sites, approximately 69%), likely due to the role of DNA methylation in X-inactivation (Mohandas et al., 1981). These data may be informative for future studies of genome-wide sex differences in methylation.

Overall, our results support the view that individual differences in biobehavioral organization influence epigenetic regulation of the immune system (Miller et al., 2011). It is also possible that some methylation patterns change biobehavioral organization, via immune-brain communication (see Nusslock & Miller, 2016), or that immune cell methylation profiles correlate with neural DNA methylation patterns that guide stress response. Understanding these potentially cumulative forces in epigenome-behavior links will be a key next step in this research. Regardless of origin, these epigenetic changes linked to biobehavioral organization likely impact individual immunity, and possibly explain the associations between intense emotional and physiological stress responses and adverse health outcomes (see, for example, Michael et al., 2020; Nelson et al., 2018; Tang et al., 2019). A major advantage of our approach is that we examined associations between biobehavioral traits and DNA methylation by cell subtype (see Supplementary Table S3B and Supplementary File 1), including eosinophils, monocytes, lymphocytes, and segmented neutrophils. These profiles likely contribute to immune cell production and/or differentiation (see Supplementary Figure S1), as well as cell-specific roles in creating and recruiting the necessary immune cell types to respond to injury or pathogens (Deaton et al., 2011;

Fitzpatrick & Wilson, 2003; Schuyler et al., 2016). We found 2,327 unique sites (in 1,962 unique genes) at which methylation was associated with at least one of these cell counts. However, these cell specific DMRs did not confound our results, as our models controlled for cell subtype concentration, and there were relatively few overlapping DMRs between the immune cell count analyses and the primary behavioral and cortisol analyses (see Supplementary Figure S3). Our approach may be useful in guiding future studies with DNA from whole-blood samples when cell subtype concentrations are known.

To our knowledge, this study is the largest genome-wide study of immune cell DNA methylation in rhesus macaques thus far. A primary strength of our analytic strategy was that we used a genome-wide approach that maximized the amount of data analyzed, and enhanced reliability by identifying DMRs that survived three types of analysis: simple regression, multiple regression controlling for immune cell counts, and permutation regression (see Supplementary Note 1). We do not yet know if the biobehavioral-epigenome associations found in this study are stable across development or how they might affect long-term health and immune functioning, although our previous work suggests that early epigenetic programming impacts health into adulthood (Kinnally, 2014). A disadvantage of our study is that, while a small number (n = 11) of our subjects were differentially reared, our study was not powered to estimate the role of early stress on epigenetic and behavioral development. We expect that it does, as early life experiences can impact behavioral stress responses (Suomi, 2006), immune cell functioning (Cole et al., 2012), and DNA methylation (Provençal et al., 2012). Future studies should investigate how variability in biobehavioral stress responsiveness interacts with early stressful experiences to impact health.

5 Conclusion

Congruent with many studies showing links between temperament, epigenetics, and health, we identified 20,368 unique sites in the rhesus macaque epigenome that were associated with behavioral or physiological responses to stressful situations. The results of this study suggest that stress responsiveness is associated with epigenetic regulation of immune system function and inflammatory response. This study adds to other epigenome-wide investigations of methylation-temperament associations in humans (Guillemin et al., 2014; van Dongen et al., 2015) and rhesus monkeys (Alisch et al., 2014), and is the first epigenome-wide investigation, to our knowledge, to explore the association of immune epigenomics with multi-domain measures of stress response and temperament in infants. We speculate that, like stressful events (Danese & McEwen, 2012; Miller et al., 2011), individual differences in early-life stress responsivity can have a programming effect on the immune epigenome, potentially affecting later health outcomes. Continued research is needed to explore how these epigenetic signatures emerge across development, and their role in individual differences in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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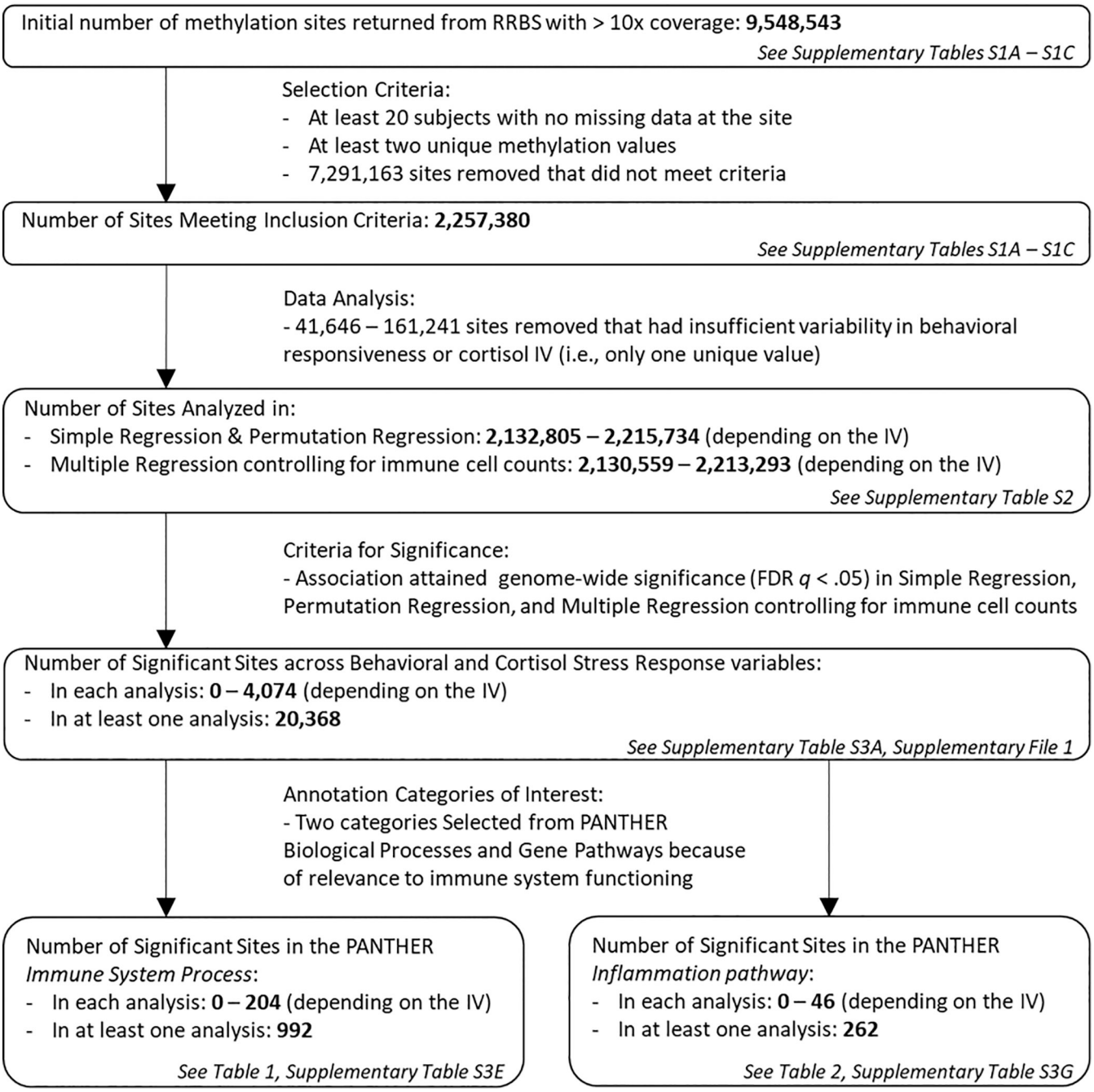

Figure 1.

Summary of Data Analysis.

The flow chart summarizes the amount of methylation data at each stage of data collection and analysis for the primary independent variables (i.e., the holding cage observation scales, the human intruder scales, and the cortisol samples).

Abbreviations: *IV* indicates independent variable.

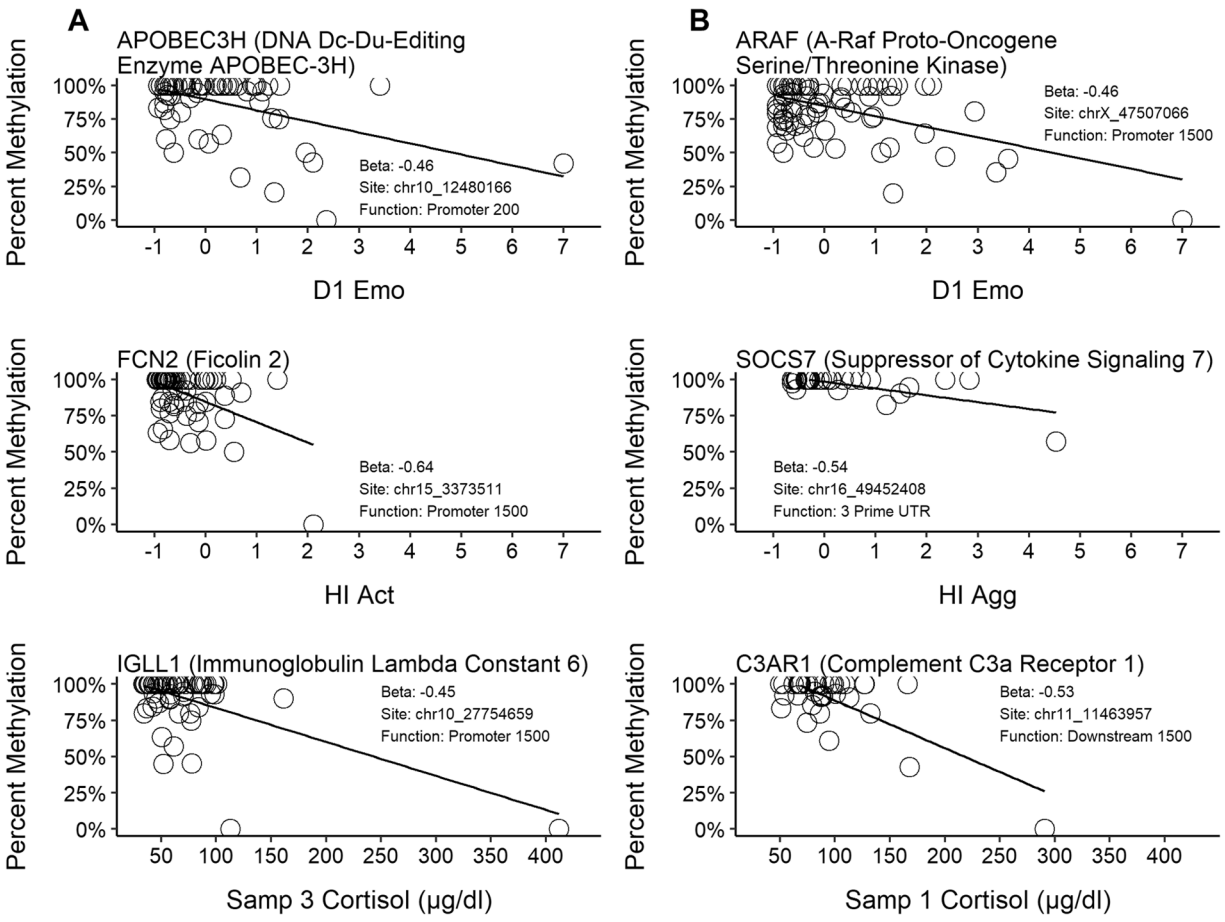

Figure 2.

Selected Associations of Interest from Immune-related Annotation Categories.

The figure shows the raw data for three selected sites in the *Immune System Process* biological process annotation category (Panel A, left) and the *Inflammation Mediated by Chemokine and Cytokine Signaling pathway* (Panel B, right) in genomic regions that are functionally relevant for transcription. The beta values indicate the beta weight from the simple regression for the association between the independent and dependent variables shown. For an expanded summary of these analyses, see Table 1 and Table 2.

Abbreviations: *D1 Emo* indicates Day 1 Emotionality; *HI Act* indicates Human Intruder Activity; *HI Agg* indicates Human Intruder Aggression; *Samp 3* indicates Sample 3 Cortisol (23.5 hours post-separation, 16.5 hours post-dexamethasone); *Samp 1* indicates Sample 1 Cortisol (2 hours post-separation).

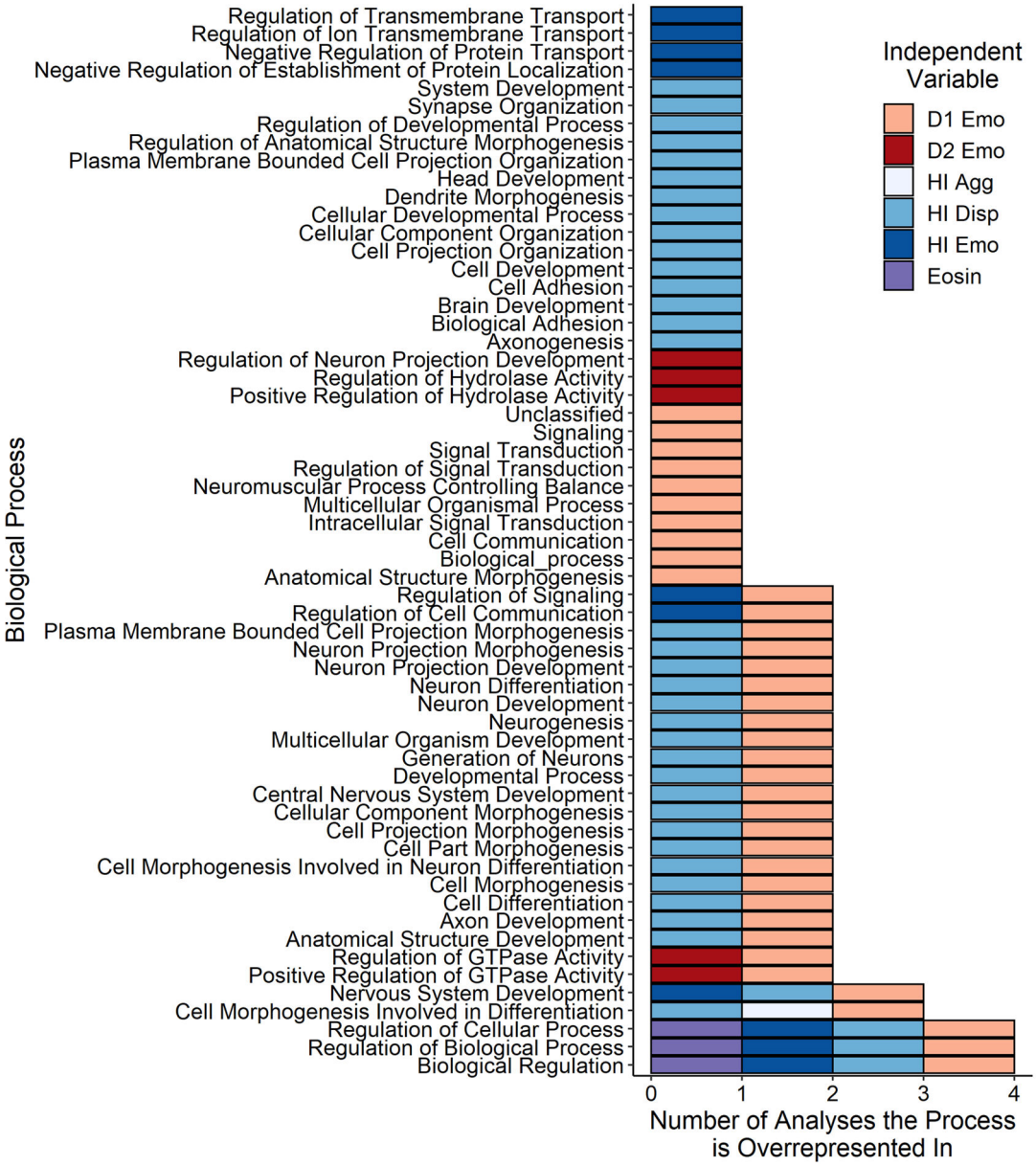

Figure 3.

Summary of Significantly Overrepresented PANTHER Biological Processes.

The figure shows the PANTHER biological pathways that were significantly overrepresented across the independent variables analyzed (FDR $q < .05$). For a full summary of these analyses, see Supplementary File 3.

Abbreviations: *D1 Emo* and *D2 Emo* indicate Day 1 and Day 2 Emotionality; *HI Agg* indicates Human Intruder Aggression; *HI Disp* indicates Human Intruder Displacement; *HI Emo* indicates Human Intruder Emotionality; *Eosin* indicates Eosinophil cell counts.

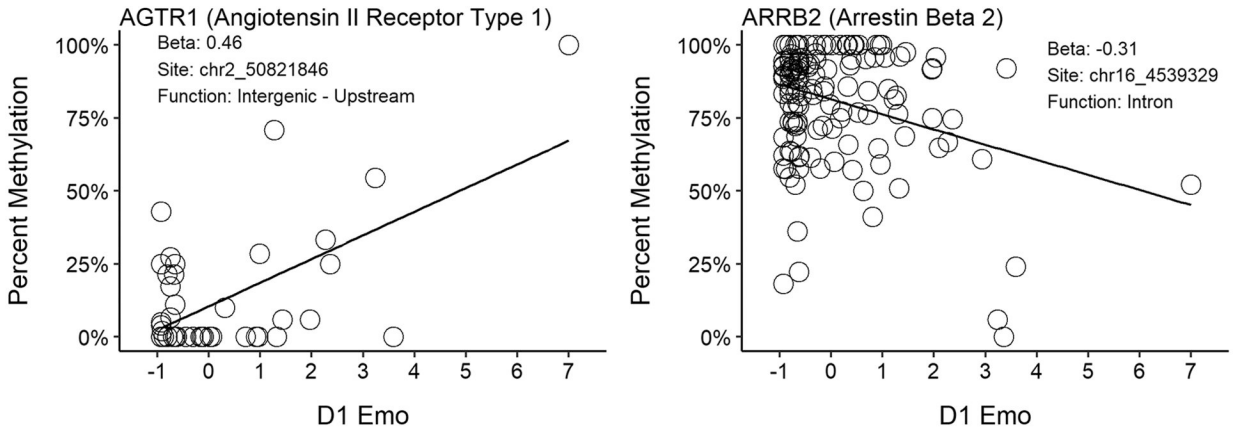

Figure 4.

Raw Data of Associations Between Day 1 Emotionality and Methylation of Two Selected Sites in the *Angiotensin II-stimulated signaling through G proteins and beta-arrestin* pathway.

The figure shows the raw data for two selected sites in the *Angiotensin II-stimulated signaling through G proteins and beta-arrestin* in which methylation was associated with Day 1 Emotionality. The beta values indicate the beta weight from the simple regression for the association between the independent and dependent variables shown. For an expanded summary of these analyses, see Supplementary Table S5.

Abbreviations: *D1 Emo* indicates Day 1 Emotionality.

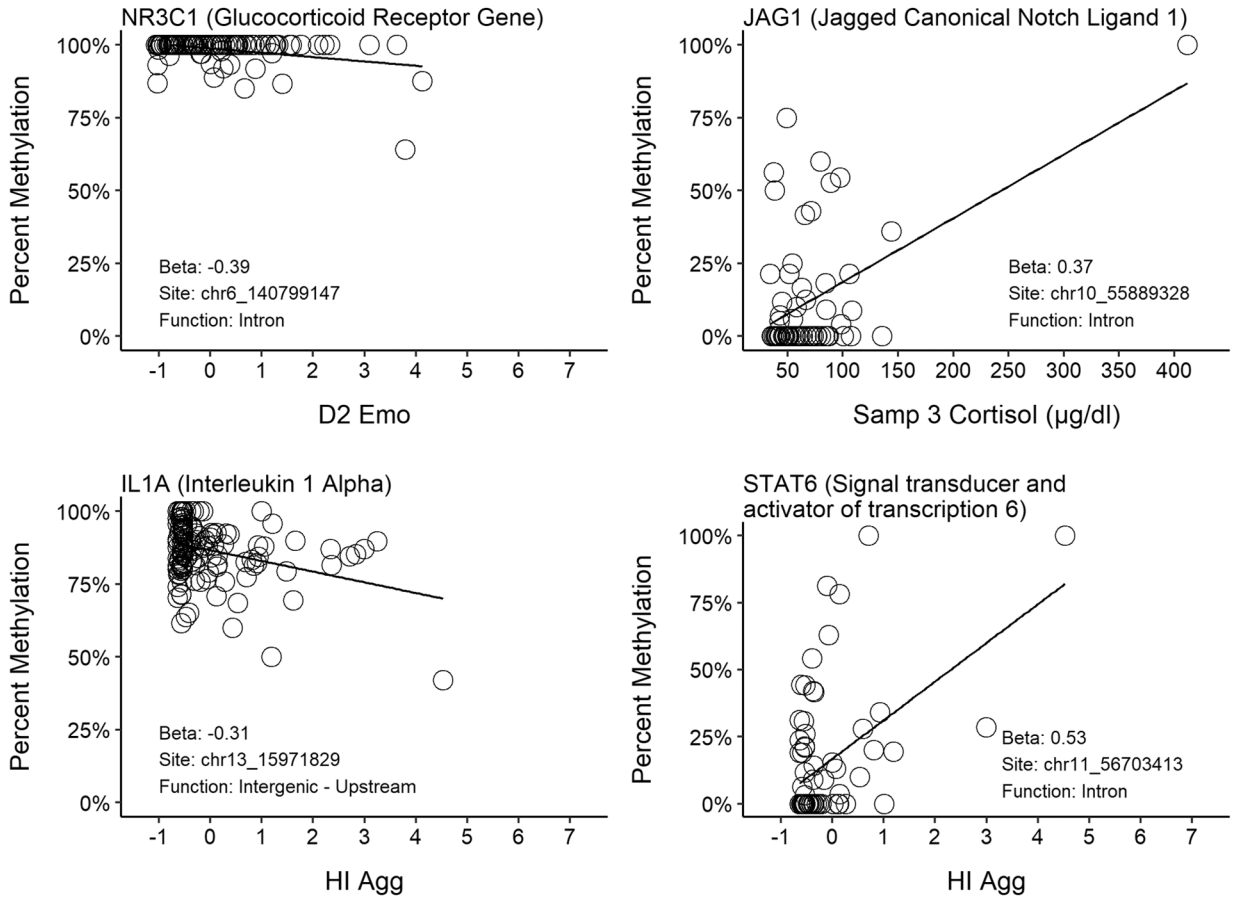

Figure 5.

Raw Data for Selected Candidate Genes of Interest.

The figure shows the raw data for four selected candidate genes in which methylation was significantly correlated with behavioral responsiveness or cortisol. The beta values indicate the beta weight from the simple regression for the association between the independent and dependent variables shown. For a full summary of these and other candidate gene analyses, see Supplementary Tables S6A and S6B.

Abbreviations. *D2 Emo* indicates Day 2 Emotionality; *Samp 3* indicates Sample 3 Cortisol (23.5 hours post-separation, 16.5 hours post-dexamethasone); *HI Agg* indicates Human Intruder Aggression.

Table 1.

Selected Associations of Interest between Methylation and Behavioral Responsiveness and Methylation and Cortisol in the *Immune System Process* Annotation Category.

Gene	Name	Chr	Base Pair	Function	IV	Beta (Simple Reg.)	N	UV	N 0	N 100	Range
APOBEC3H	DNA dC-dU-editing Enzyme APOBEC-3H	10	12480166	Promoter 200	D1 Emo	−0.46	77	23	1	51	100
CARD11	Caspase Recruitment Domain Family Member 11	3	36166894	Promoter 1500	HI Agg	−0.47	85	13	0	73	25
CCL13	C-C Motif Chemokine Ligand 13	16	30717375	Promoter 1500	D2 Emo	−0.66	45	3	0	41	70
CCL13	C-C Motif Chemokine Ligand 13	16	30717375	Promoter 1500	Samp 3	−0.89	45	3	0	41	70
DOCK1	Dedicator of Cytokinesis 1	9	127400246	Promoter 1500	Samp 1	−0.40	67	18	2	45	100
DOCK1	Dedicator of Cytokinesis 1	9	127400246	Promoter 1500	Samp 3	−0.42	64	16	2	44	100
FCN2	Ficolin 2	15	3373511	Promoter 1500	HI Act	−0.64	71	22	1	45	100
IGLL1	Immunoglobulin Lambda Like Polypeptide 1	10	27754659	Promoter 1500	Samp 3	−0.45	70	20	2	44	100
MRC1	Mannose Receptor C-Type 1	9	18958109	Promoter 1500	Samp 4	−0.28	125	33	0	90	52.78
MTHFD1	Methylenetetrahydrofolate Dehydrogenase 1	7	126443033	Promoter 1500	D2 Emo	−0.61	41	16	1	25	100
PAFAH1B1	Platelet Activating Factor Acetylhydrolase 1b Regulatory Subunit 1	16	2446534	Promoter 1500	HI Act	0.98	47	4	44	0	25
PAFAH1B1	Platelet Activating Factor Acetylhydrolase 1b Regulatory Subunit 1	16	2446534	Promoter 1500	HI Emo	1.13	47	4	44	0	25
PAFAH1B1	Platelet Activating Factor Acetylhydrolase 1b Regulatory Subunit 1	16	2446586	Promoter 1500	D1 Emo	0.38	144	16	128	0	36.36
PGLYRP2	Peptidoglycan Recognition Protein 2	19	15079828	Promoter 1500	D2 Emo	0.56	43	6	38	0	30.43
PGLYRP2	Peptidoglycan Recognition Protein 2	19	15079864	Promoter 1500	D2 Emo	0.52	42	5	38	0	17.39
PTPRZ1	Protein Tyrosine Phosphatase Receptor Type Z1	3	148280553	Promoter 1500	D1 Emo	0.44	64	13	51	0	50
SIX4	SIX Homeobox 4	7	122817666	Promoter 200	HI Disp	−0.99	33	12	0	22	27.27
TOLLIP	Toll Interacting Protein	14	1349051	Promoter 200	D1 Emo	−0.48	75	12	1	63	100
UMODL1	Uromodulin Like 1	3	4566361	Promoter 1500	D1 Emo	0.53	74	4	71	0	18.75
VIM	Vimentin	9	18374107	Promoter 1500	Samp 3	0.41	107	37	59	1	100

The table shows a summary of sites in the *Immune System Process* annotation category (from PANTHER data base) that were significantly associated with behavioral responsiveness or cortisol in the simple linear regression, the multiple regression controlling for immune cell counts, and the permutation regression. For brevity, only sites that were in proximate promoter regions (Promoter 200, Promoter 1500) are shown (for a full summary see Supplementary File 1).

Abbreviations. *Chr* indicates chromosome; *IV* indicates independent variable analyzed; *Reg.* indicates regression; *UV* indicates number of unique methylation values; *UTR* indicates untranslated region; *Promoter 200* indicates the site was within 200 base pairs of the transcription start site; *Promoter 1500* indicates the site was within 1,500 base pairs of the transcription start site; *D1Emo* and *D2 Emo* indicate Day 1 and Day 2 Emotionality; *HI Act*, *HI Agg*, *HI Disp*, and *HI Emo* indicate Human Intruder Activity, Aggression, Displacement, and Emotionality; *Samp 1*, *Samp 3*, and *Samp 4* indicates Sample 1 Cortisol (2 hours post-separation), Sample 3 Cortisol (23.5 hours post-separation, 16.5 hours post-dexamethasone), and Sample 4 Cortisol (24 hours post-separation,.5 hours post-ACTH).

Table 2.

Selected Associations of Interest between Methylation and Behavioral Responsiveness and Methylation and Cortisol in the *Inflammation Mediated by Chemokine and Cytokine Signaling pathway*.

Gene	Name	Chr	Base Pair	Function	IV	Beta (Simple Reg.)	N	UV	N 0	N 100	Range
ARAF	Serine/threonine-protein kinase A-Raf	X	47507066	Promoter 1500	D1 Emo	−0.46	86	42	1	35	100
C3AR1	Complement C3a Receptor 1	11	11463957	Downstream 1500	Samp 1	−0.53	42	14	1	25	100
CCL13	C-C motif chemokine	16	30717375	Promoter 1500	D2 Emo	−0.66	45	3	0	41	70
CCL13	C-C motif chemokine	16	30717375	Promoter 1500	Samp 3	−0.89	45	3	0	41	70
GRAP	GRB2-related adapter protein	16	18621419	Promoter 1500	D1 Emo	0.36	110	35	68	0	79.31
GRAP	GRB2-related adapter protein	16	18621419	Promoter 1500	Samp 4	0.34	105	33	65	0	79.31
ITPR1	Inositol 1,4,5-trisphosphate receptor type 1 isoform 3	2	135800641	5 Prime UTR	D1 Emo	−1.04	40	5	0	36	46.15
PLCB3	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase	14	10292476	5 Prime UTR	Samp 3	0.52	38	5	34	0	42.86
SOCS7	Suppressor of Cytokine Signaling 7	16	49452408	3 Prime UTR	HI Agg	−0.54	40	8	0	33	42.86

The table shows a summary of sites in the *Inflammation Mediated by Chemokine and Cytokine Signaling pathway* (from PANTHER data base) that were significantly associated with behavioral responsiveness or cortisol in the simple linear regression, the multiple regression controlling for immune cell counts, and the permutation regression. For brevity, only sites that were in the 5 prime UTR, the 3 prime UTR, or in a proximate promoter or downstream region (Promoter 200, Promoter 1500, Downstream 200, Downstream 1500) are shown (for a full summary see Supplementary File 1).

Abbreviations. *Chr* indicates chromosome; *IV* indicates independent variable analyzed; *Reg.* indicates regression; *UV* indicates number of unique methylation values; *UTR* indicates untranslated region; *Promoter 1500* indicates the site was within 1,500 base pairs of the transcription start site; *Promoter 10K* indicates the site was within 10,000 base pairs of the transcription start site; *Downstream 10K* indicates the site was within 10,000 base pairs of the end of the gene body; *D1 Emo* and *D2 Emo* indicate Day 1 and Day 2 Emotionality; *HI Agg* indicates Human Intruder Aggression; *Samp 1*, *Samp 3*, and *Samp 4* indicates Sample 1 Cortisol (2 hours post-separation), Sample 3 Cortisol (23.5 hours post-separation, 16.5 hours post-dexamethasone), and Sample 4 Cortisol (24 hours post-separation, .5 hours post-ACTH).

Table 3.

Summary of the Six Significantly Overrepresented PANTHER Gene Ontology Pathways for Sites Significantly Associated with Behavioral Responsiveness or Cortisol.

				Overrepresentation Analysis Relative to Analyzed Data (Relative to Reference Genome)				Total Genes Across all Pathways	
Pathway	**ID**	**IV**	**Number of Sig. Genes in Pathway**	**Total in Pathway**	**Expected in Pathway**	**Fold Enrichment**	**FDR *q***	**Attained Sig.**	**In Analyzed Data (In Reference Genome)**
Ionotropic glutamate receptor pathway	P00037	HI Act	14	43 (49)	2.9 (2.1)	4.79 (6.68)	<.001 (<.001)	901	13,257 (21,073)
GABA-B receptor II signaling	P05731	HI Act	9	30 (40)	2.0 (1.7)	4.41 (5.26)	.022 (.004)	901	13,257 (21,073)
Angiotensin II-stimulated signaling through G proteins and beta-arrestin	P05911	D1 Emo	10	29 (40)	2.8 (2.5)	3.53 (4.07)	.038 (.006)	1,293	13,257 (21,073)
Beta1 adrenergic receptor signaling pathway	P04377	HI Agg	9	32 (47)	2.8 (2.6)	3.21 (3.47)	.044 (0.02)	1,163	13,257 (21,073)
Beta2 adrenergic receptor signaling pathway	P04378	HI Agg	9	32 (46)	2.8 (2.5)	3.21 (3.55)	.049 (.02)	1,163	13,257 (21,073)
Metabotropic glutamate receptor group III pathway	P00039	HI Agg	12	53 (69)	4.7 (3.8)	2.58 (3.15)	.049 (.016)	1,163	13,257 (21,073)

The table shows a summary of the overrepresentation analyses performed on the sites that attained genome-wide significance in the simple regression, multiple regression controlling for immune cell counts, and the permutation regression for each independent variable analyzed. Only the pathways that were significantly overrepresented relative to the rhesus macaque reference genome and to the subset of sites that were analyzed are shown (see Supplementary File 4 for a list of all pathways, including genes in Unclassified pathways, which are omitted from this table). The *Pathway* column indicates the PANTHER gene pathway that was overrepresented, and the *IV* column indicates the pathway was significantly overrepresented among the sites significantly associated with the specified independent variable (see Supplementary Table S3A). The *Number of Sig. Genes in Pathway* column shows the number of unique genes with at least one site at which methylation was significantly correlated with the independent variable indicated. The *Total in Pathway* column indicates how many genes in the pathway were present in the data that were analyzed (or in the rhesus macaque reference genome, in parentheses). The *Expected in Pathway* column indicates the number of genes in the pathway that would be expected to be significant hits based on chance, and is calculated by dividing the *Total in Pathway* by the *Total Genes Across All Pathways in Analyzed Data* (or *In Reference Genome*, in parentheses), then multiplying this ratio by the *Total Genes Across All Pathways* that *Attained Sig.* (for example, in the first row, (43 / 13,257) * 901 = 2.9). The *Fold Enrichment* indicates the degree to which the number of observed significant hits in the pathway exceeds the number of genes that would be expected, and was calculated by dividing the *Number of Sig. Genes in Pathway* by the *Expected in Pathway* (for example, in the first row, 14 / 2.9 = 4.79). Note, not all of the sites that attained significance were annotated in a PANTHER gene pathway, and the numbers presented in the *Attained Sig.* column correspond to the number of significant sites that were annotated in a gene pathway; for this reason, the numbers in this column are lower than the numbers presented in Supplementary Table S3A. The *FDR q* column shows the *p* value of the binomial proportion test for overrepresentation, corrected for False Discovery Rate.

Abbreviations. *IV* indicates independent variable; *Sig.* indicates significant; *D1 Emo* indicates Day 1 Emotionality; *HI Act* and *HI Agg* indicate Human Intruder Activity and Human Intruder Aggression.