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ABSTRACT

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To identify molecular mechanisms by which early life social conditions might influence adult risk of disease in rhesus macaques (*Macaca mulatta*), we analyze changes in basal leukocyte gene expression profiles in 4-month-old animals reared under adverse social conditions. Compared to the basal condition of maternal rearing (MR), leukocytes from peer-reared (PR) animals and PR animals provided with an inanimate surrogate mother (surrogate/peer reared; SPR) show enhanced expression of genes involved in inflammation, cytokine signaling, and T lymphocyte activation, and suppression of genes involved in several innate antimicrobial defenses including Type I Interferon antiviral responses. Promoter-based bioinformatic analyses implicate increased activity of CREB and NF-κB transcription factors and decreased activity of Interferon Response Factors (IRFs) in structuring the observed differences in gene expression. Transcript origin analyses identify monocytes and CD4+ T lymphocytes as primary cellular mediators of transcriptional up-regulation and B lymphocytes as major sources of down-regulated genes. These findings show that adverse social conditions can become embedded within the basal transcriptome of primate immune cells within the first 4 months of life, and they implicate sympathetic nervous system-linked transcription control pathways as candidate mediators of those effects and potential targets for health-protective intervention.

JEL Classification: I12, J13

Keywords: immune system, gene expression, stress, social adversity, development, primates

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Exposure to adverse social environments during early life is associated with increased risk of disease in adulthood (1-5), but the biological mechanisms producing such effects remain poorly understood. One possible explanation suggests that neural and endocrine responses to adversity in childhood affect the development of health-relevant molecular systems (i.e., a "defensive programming" of the developing body) (4, 6-10), rendering the body more vulnerable to subsequent pathogen challenges in adulthood (11, 12). Given the transience of most neuroendocrine responses, however, it remains unclear how the extra-organismic social conditions that do "get into the body" during early life could "stay there" over decades to impact the risk of disease in adulthood (13).

One potential molecular mechanism that can create a persisting biological impact of early-life socio-environmental conditions involves the complex systems behavior of the gene transcriptional networks that govern cell growth, differentiation, and function (14, 15). Gene regulatory networks show dynamic landscapes in which the system's responses to external perturbations converge on a small number of stable "attractor" modes that can subsequently self-perpetuate (16). These self-perpetuating dynamics are sustained in part by the fact that the mRNA "output" of the system at one point in time (i.e., the genome-wide transcriptional profile) constitutes an "input" to the system at subsequent time points because translated mRNA shapes the cell's response to future environments (17). Mathematical models of human development that capture such recursive dynamics show that small exogenous influences early in life can significantly alter the course of subsequent life trajectories (18-20). What is not known is which specific genes might be sensitive to such early-life environments.

Several recent studies have linked adverse social conditions in early life with adult differences in gene expression in cells of the nervous and immune systems (8-10, 21, 22). Early

life social adversity has also been associated with adult cell differences in transcription-related epigenetic modifications such as DNA methylation (23-25). To determine whether these adult transcriptional alterations might potentially stem from a biological reprogramming of the developing immune system during early life, we analyze the genome-wide transcriptional profile of circulating leukocytes in infant rhesus macaques (*Macaca mulatta*) after 4 months of experimentally imposed social adversity (peer vs. maternal rearing) (26). To the extent that adverse social conditions become rapidly embedded into the gene regulatory regime of the developing immune system, we expect that: 1) surrogate/peer rearing (SPR) and peer rearing (PR) conditions increase the expression of genes involved in inflammation while decreasing expression of genes involved in Type I interferon-mediated innate antiviral responses (i.e., the "conserved transcriptional response to adversity" previously observed in adults (9, 27-32)), and, 2) these effects are structured by transcription control pathways linked to stress-responsive "social signal transduction" pathways such as the sympathetic nervous system (SNS) and hypothalamus-pituitary-adrenal (HPA)-axis (10, 13, 17, 32). These hypotheses are tested by coupling microarray-based assessment of the entire macaque transcriptome with recent advances in computational bioinformatics (33) and multiple hypothesis testing (34-36) to map large ensembles of differentially expressed genes into a small number of higher-order biological themes regarding their regulatory causes (e.g., transcription factor activity) (37), cellular contexts (e.g., originating leukocyte subtype) (30), and functional consequences (e.g., Gene Ontology functional annotations) (38).

Results

Effects of Surrogate/Peer Rearing

Previous studies have identified substantial increases in adult health risk in macaques exposed to SPR conditions in early life (5). Our initial analyses compare leukocyte transcriptional profiles in peripheral blood mononuclear cells (PBMCs) from 4 month-old SPR animals (n = 4) relative to MR animals (n = 5). Genome-wide transcriptional profiling identified 249 transcripts showing ≥ 2-fold difference in average expression levels (85 up-regulated in SPR vs. MR; 164 down-regulated). Figure 1 displays these transcriptome differences. Supporting Information Table S1 lists specific up- and down-regulated genes.

Gene Ontology analyses (Supporting Information Table S2) characterize the genes up-regulated in PBMC from SPR animals as being involved in multiple biological processes mediating immune activation, including metabolic activation (e.g., oxidation/reduction and glycogen metabolism), cytokine signaling (e.g., Interleukin 27, Leukemia Inhibitory Factor, Ciliary Neurotrophic Factor, Notch, and STAT1/STAT3 signaling), and T cell proliferation. EntrezGene annotations for several up-regulated genes also indicate a common role in inflammation and tissue remodeling (e.g., Table S1: *IL8, MMP1*, *CCR3*, and *CCL2/MCP1*) (39). Down-regulated genes are characterized by involvement in innate immune response functions, such as antigen processing and presentation, anti-microbial defense responses (e.g., to bacteria and fungi), and liver development (Supporting Information Table S2). SPR down-regulated genes also include multiple transcripts identified in previous research as being involved in Type I interferon-mediated innate antiviral responses (e.g., Table S1: *GBP1*, *IFIT1*, *IFIT2*, *IFITM3*, and *IRF7*) (39-42). Consistent with these functional bioinformatic results implicating T cell activation and altered innate immune responses, Transcript Origin Analyses identify monocytes and CD4+ T lymphocytes as major cellular sources of SPR up-regulated genes and link down-regulated genes to B lymphocytes (Figure 1b).

To test the hypothesis that the observed differences in gene expression might be mediated by reciprocal up-regulation of pro-inflammatory NF-κB family transcription factors and down-regulation of IRF family transcription factors, we carry out TELiS bioinformatic analysis of transcription factor-binding motifs in the promoters of differentially expressed genes. As in previous studies of social adversity (9, 27-29, 31, 32, 43), NF-κB target motifs are significantly over-represented within the promoter sequences of up-regulated genes and IRF motifs are significantly enriched within the promoters sequences of down-regulated genes (Figure 1c). To determine whether such dynamics might be structured by social signal transduction pathways involving the SNS and HPA-axis (10, 13, 17, 32, 44), TELiS analyses also examine motifs associated with the CREB transcription factors involved in β-adrenergic signaling by SNS catecholamines, and glucocorticoid response elements (GREs) associated with HPA-axis signaling through the glucocorticoid receptor (GR). Results show significant enrichment of CREB sites within promoters of up-regulated genes, but no significant difference in GRE prevalence (Figure 1c).

Effects of Peer Rearing

To determine whether similar dynamics might emerge in PR animals that lacked access to an inanimate surrogate mother and spent more time in contact with peers, we also compare PBMC gene expression profiles in PR animals (n = 4) vs. MR animals (n = 5). Analyses identify 256 transcripts showing ≥ 2-fold difference in average expression (105 up-regulated in PR vs. MR; 151 down-regulated; Figure 2 and Supporting Information Table S3). Gene Ontology analyses again identify up-regulated genes as being involved in metabolic activation (ATP synthesis and electron transport chain, oxidation/reduction, glycogen metabolism), gene

translation, cytokine signaling (Interleukin 27, Leukemia Inhibitory Factor, Ciliary Neurotrophic Factor, Notch, and STAT1/STAT3 signaling), and T cell proliferation (Supporting Information Table S4). Down-regulated genes are again characterized by involvement in innate immune response functions including antigen processing and presentation, and anti-microbial responses to bacteria, fungi, and viruses. Prominent among down-regulated transcripts are multiple genes involved in Type I interferon-mediated innate antiviral responses (e.g., Table S3: *GBP1*, *IFI27*, *IFIT1*, *IFIT2*, *IFITM3*, *IRF7*, *MX1*, and *MX2*) (39-42). Gene Ontology annotations also link down-regulated genes to liver development and fat cell differentiation (Table S4). Transcript Origin Analyses identify monocytes and CD4+ T lymphocytes as cellular mediators of PR-up-regulated genes (Figure 2b) and B lymphocytes as contributors of down-regulated genes. TELiS promoter-based bioinformatics again implicate a reciprocal increase in activity of NF-κB and decrease in activity of IRF transcription factors in structuring the observed differences in immune response gene expression (Figure 2c). TELiS analyses also indicate increased CREB activity, but provide no evidence of decreased GR-mediated transcription (Figure 2c).

Comparison of Peer and Surrogate/Peer Rearing

The qualitative similarity in effects of SPR and PR conditions is underscored by the fact that relatively few genes show ≥ 2-fold difference in average expression in direct comparison of these two groups (n = 48 up-regulated and 31 down-regulated, or < 1/3 the number of differences observed in comparisons of each group with MR gene expression profiles; Supporting Information Table S5). Both SPR and PR groups show similar patterns of transcriptional differentiation from MR animals, with 49% (42/85) of SPR-up-regulated transcripts also up-regulated by PR, and 48% (79/164) of SPR down-regulated transcripts also down-regulated by

PR (both exceeding the < 0.1% overlap expected by chance; $p < .0001$). Gene Ontology analyses also identify few differences in the functional characteristics of SPR and PR PBMC transcriptomes (Supporting Information Table S6). Among the few functional differences that are identified is a comparative up-regulation of genes involved in antigen presentation (including proteolysis and antigen processing) in SPR animals relative to PR animals. No Gene Ontology annotations are identified as significantly up-regulated in PBMC from PR animals relative to SPR animals.

Discussion

The results of this study show that adverse social conditions can become embedded in the basal transcriptome of primate immune cells within the first 4 months of life. Compared to PBMC from maternally reared (MR) rhesus macaques, those from peer-reared animals (both SPR and PR) show enhanced expression of genes involved in inflammation and T lymphocyte activation, and reduced expression of genes involved in Type I Interferon-mediated innate antiviral responses and other pathogen-specific innate anti-microbial responses. This pattern of enhanced inflammatory gene expression and inhibited antiviral gene expression parallels the "conserved transcriptional response to adversity" (CTRA) observed in previous correlational studies of humans confronting adverse life circumstances (9, 27-32, 43). The experimental manipulation of early life social conditions in this study demonstrates that social adversity plays a causal role in activating CTRA dynamics, and can do so during the earliest stages of post-natal immune system development. To the extent that such environmentally-mediated transcriptome remodeling persists to affect immune responses to pathogens encountered later in life (e.g., inhibiting immune responses to viral infections (45, 46) or amplifying allergic inflammation (43)), the present findings provide a molecular framework for understanding the long-observed

epidemiologic association between social adversity and reduced host resistance to disease (47-50), as well as more recently recognized effects of early life social conditions on adult immune function (10, 12, 13, 51) and disease risk (1-5).

These data provide additional insights into the specific immune cell subtypes that are most sensitive to socio-environmental regulation, and the neural and endocrine pathways that may mediate such effects. Transcript Origin Analyses link SPR/PR-induced transcriptional up-regulation to monocytes and CD4+ T lymphocytes, and transcriptional down-regulation to B lymphocytes. These findings parallel previous primate studies documenting altered CD4/CD8 T lymphocyte ratios as a function of social vs. non-social housing conditions (52, 53) and defining leukocyte subset alterations as a key mechanism of social influences on the aggregate leukocyte transcriptome (54). These findings are also consistent with previous studies indicating monocyte-derived gene activation in humans confronting adversity (30, 31). Based on the known functions of these specific cell subtypes (30), SPR/PR animals might be expected to show reduced antibody responses (mediated by B cells, e.g., in response to vaccines or infections), and increased chronic inflammation (initiated by monocytes and perpetuated by CD4+ T lymphocytes, e.g., in responses to injury or infection). Additional research will be required to directly assess these specific immune system functional alterations, but these results are broadly consistent with the increased disease risk observed in SPR/PR animals (5).

Also consistent with previous observations are results from promoter-based bioinformatic analyses implicating increased activity of pro-inflammatory NF-κB transcription factors and decreased activity of IRF family transcription factors in structuring the observed gene expression differences (32). These analyses also implicate CREB family transcription factors as potential molecular mediators of PR/SPR effects on the basal leukocyte transcriptome. CREB factors play

a central role in mediating the transcriptional effects of SNS activation via β-adrenergic receptors (55), and β-adrenergic signaling can also activate NF-κB (56), up-regulate transcription of pro-inflammatory cytokine genes (57), and inhibit IRF transcription factors and Type I interferon gene expression (46, 58) (i.e., the same pro-inflammatory / anti-antiviral transcriptional shift observed here and in other adversity studies (32)). A potential increase in SNS-induced β-adrenergic signaling would parsimoniously account for many of the transcriptional dynamics observed here and provide a specific social signal transduction pathway by which early life social adversity alters basal leukocyte gene expression profiles and immune cell function (51, 52, 59, 60). Parallel bioinformatic analyses provide little support for the hypothesis that reduced glucocorticoid-mediated transcription plays a major role in structuring the effects observed here (as has previously been observed in studies of long-term adversity in adults (9, 27, 28, 54)). It is possible that the effects of early life adversity do not involve changes in cortisol signaling or glucocorticoid receptor (GR) function as previously observed (12). However, it is also possible that GR-related functional alterations may have been present, but remain undetected due to the limited statistical power available in this study. Alternatively, the SNS/β-adrenergic/CREB-related transcriptional dynamics observed here at 4 months of age may constitute the initial phase of a long-term regulatory trajectory that subsequently induces GR desensitization and propagates systemic inflammation into adulthood. Ongoing longitudinal analyses of gene expression dynamics in the cohort studied in this paper will help clarify the possibilities. If analyses continue to indicate a role for SNS/β-adrenergic/CREB signaling in structuring the CTRA gene expression dynamics observed here, pharmacologic inhibition of that pathway (e.g., with β-adrenergic antagonists (55-58)) might represent one potential strategy for mitigating the transcriptional dynamics and health risks associated with social adversity (13, 32).

Gene products that act recursively on the transcriptome (e.g., transcription factors and chromatin regulators) also show marked changes in expression in response to early life social adversity. Recursive dynamics can help to explain how transient periods of environmental adversity in early life propagate over time to influence adult health many years later. To the extent that transient environmental perturbations alter the expression of genes that control the basal dynamic equilibrium of the leukocyte transcriptome and/or alter the expression of molecules that mediate social signal transduction, early life social conditions may establish a long-lasting propensity to respond to challenges (either socio-environmental or microbial) that becomes manifest in health vulnerability only when the organism is challenged later in life (10-12). Quantitative modeling of such recursive developmental systems shows that corrective interventions are likely to be far more effective when deployed early in life than when delivered later in adulthood (16, 18-20, 61). The present study's observation that adverse social conditions can alter basal gene expression profiles in circulating immune cells within the first 4 months implies an opportunity to remediate the biological impact of adverse environments shortly after they initially manifest, and well before these gene regulatory regimes consolidate to drive the emergence of frank disease (e.g., late-life "diseases of aging" such as cardiovascular, neurodegenerative, or neoplastic disease) (62).

The scope of the present findings is limited in several important respects. First, this study does not include any direct measure of immune system functional activity (e.g., response to a pathogenic challenge (11, 12)), so the health significance of the present results remains to be determined in future studies. However, recent analyses have shown that macaques exposed to same adverse conditions in early life show significantly elevated physical and mental health problems in adulthood (5). The present study also focuses on circulating leukocytes, and

implications for the more disease-relevant cells in peripheral tissues and lymphoid organs will require additional studies. Both of these limitations may be addressed to some extent by the fact that the Type I interferon inhibition observed here parallels that observed in previous studies of social stress effects on macaque interferon responses to viral infection in lymph nodes (46). This study is also limited in its focus on a one-time analysis of immune cell gene expression after 4 months of adverse social conditions in a small sample of animals. Replication of these findings in larger study samples with extended follow-up will be important to gauge the generalizability of these findings. Despite the fact that this small sample size available limits statistical power, the present analyses were nevertheless able to detect the pro-inflammatory and anti-antiviral transcriptional dynamics that have previously been observed in larger studies of social adversity in adult humans and macaques (54). Future longitudinal analyses will be required to determine how rapidly such transcriptional alterations emerge, how long they persist, and whether or how quickly they might reverse following the cessation of environmental adversity. Previous analyses of the macaque SPR/PR paradigm have documented an enduring impact on other biobehavioral phenotypes (63), raising the possibility that the transcriptional alterations observed here may persist as well (e.g., due to self-perpetuating transcriptional dynamics (15-17)). However, until more information is available regarding the longitudinal trajectory of the transcriptome dynamics documented here and their impact on cellular function and liability to disease, these findings should be considered the first steps toward addressing the question of molecular pathways by which early life social conditions affect subsequent health trajectories. Bioinformatic indications that SNS/β-adrenergic/CREB activation may mediate the observed effects are consistent with previous experimental results (46, 56, 57), but future pharmacologic inhibition studies will also be required to decisively confirm the role of SNS signaling in the

present paradigm. It is also important to note that the present findings can only be interpreted in the context of immune function, and the CNS neurobiological mechanisms of the present effects represent an important topic for future research (10, 21, 64, 65).

A major strength of this study is its ability to clearly map causal effects of early life social conditions on immune cell gene expression profiles in the context of a randomized experiment. Future analysis of larger samples and longitudinal trajectories from this paradigm hold great promise for clarifying the specific environmental conditions that mediate and moderate social genomic dynamics. For example, we observed particularly strong expression of interferon-related genes in MR animals reared in outdoor field cages (although laboratory-reared MR animals also showed elevations relative to SPR/PR animals). Such findings suggest that the physical environment may interact with the social environment to shape transcriptional adaptation. A larger sample of field-reared MR animals would be required for any definitive conclusion. The potential health significance of field versus laboratory MR conditions also remains to be defined because previous epidemiologic analysis of this paradigm have excluded field-reared animals (5). Thus, ongoing longitudinal analyses of the rhesus macaque peer rearing paradigm will provide valuable information with which to dissect the physical, behavioral, neural, and molecular pathways through which early life social conditions affect adult health and well-being (5).

Methods

Social conditions

Newborn rhesus macaques were randomized to maternal rearing (MR), peer rearing (PR), or surrogate/peer rearing (SPR), as previously described in (26). MR infants were housed in social groups approximating natural conditions (i.e., 8-10 adult females including the infant's mother, 2 adult males, and other similar-aged infants), with 2 of the 5 MR animals raised in 5 acre outdoor field cages and 3 reared in 14.6 m^2 indoor/outdoor laboratory enclosures (26). (Note that this distribution differs from the sample recently analyzed by Conti et al.(5), which did not include any MR animals reared in field cage environments.) Infants assigned to PR and SPR conditions were removed from their mothers between birth and 2 days of age and taken to a neonatal nursery, where they were housed individually with an inanimate surrogate mother for 37 days, after which they either entered a permanent group of 4 age-matched peers (PR) or continued individual housing with a surrogate mother supplemented by 2 hour/weekday play sessions in groups of 4 age-matched peers (SPR) (26).[*]

At four months of age, peripheral blood mononuclear leukocytes (i.e., monocytes, dendritic cells, B lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, and NK cells) were isolated by standard Ficoll density gradient centrifugation of venipuncture blood samples obtained under resting conditions from a consecutive series of 14 healthy animals (8 females and 6 males) in the course of routine veterinary health examinations. Additional procedural details are provided in Supporting Information S7 - Detailed Methods. All procedures were carried out at the National Institutes of Health Animal Center in Poolesville, MD and approved by the Institutional Animal Care and Use Committee.

[*] The PR monkeys also had a surrogate mother in the cage until they were 4 months old.

Gene expression and transcriptional bioinformatics

Genome-wide transcriptional profiling and bioinformatic analyses were carried out essentially as described in previous studies (27, 30, 57) using Affymetrix Rhesus Genome Arrays. Detailed methods are provided in Supporting Information S7, and data are deposited as Gene Expression Omnibus series GSE35850. Briefly, raw expression values for 52,024 probes assessing ~47,000 distinct macaque mRNA transcripts (including 7,185 distinct named macaque genes) were quantile normalized and $\log_2$-transformed to identify genes showing ≥ 2-fold differences in average expression levels across groups. Functional characteristics of differentially expressed genes were identified through NCBI EntrezGene annotations (66) and Gene Ontology (GO) annotations in the Affymetrix Rhesus Genome Array annotation file (i.e., testing both for significant differences in average levels of gene expression across rearing conditions using small-sample permutation-based inference and correcting for multiple hypotheses testing using the stepdown algorithm of Romano and Wolf as developed and applied in (34), and for over-representation of annotations in differentially expressed gene lists relative to the sampling frame of all genes present on the microarray, as outlined in Supporting Information S7 – Detailed Methods). Activity of specific transcriptional control pathways was assessed by TELiS bioinformatics analysis of transcription factor-binding motifs (TFBMs) in the promoters of differentially expressed genes (www.telis.ucla.edu) (37, 57). Promoter sequences derived from the *Macaca mulatta* genome sequence were analyzed for TFBMs corresponding to NF-κB (TRANSFAC V$NFKAPPAB65_01 motif), IRFs (V$IRF1_01), CREB (V$CREB_01), and the glucocorticoid receptor (V$GR_Q6), using PromoterScan and PromoterStats algorithms as previously described (37). Differential prevalence was quantified by the ratio of TFBM frequency in promoters of genes up-regulated in one group vs. another, and summarized by the

geometric mean ratio computed over 9 parametric variations of promoter length (-300 bp relative to transcription start site, -600, and -1000 to +200) and TFBM detection stringency (mat_sim = .85, .90, .95). Geometric mean ratios were tested for statistical significance using a single-sample *t* statistic with bootstrap-derived standard errors (37). Transcript Origin Analyses were conducted as previously described (30) to identify specific leukocyte subsets contributing to the observed PBMC transcriptome alterations.

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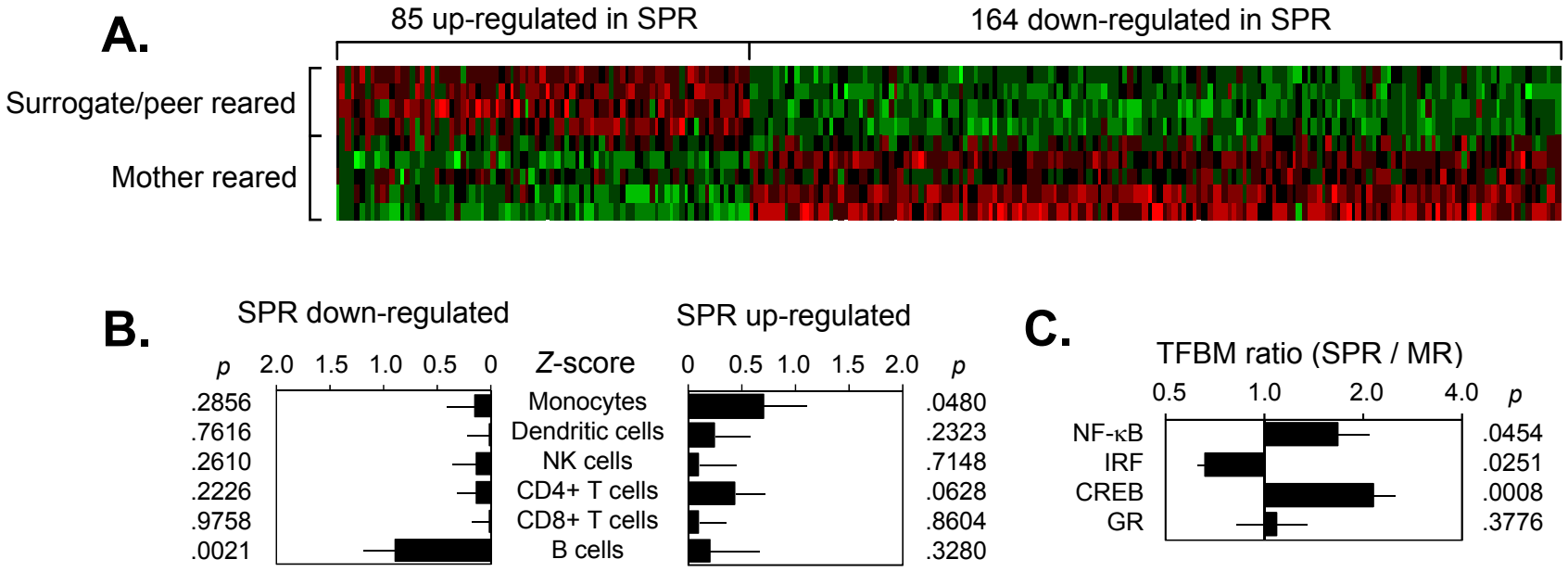
Figure 1

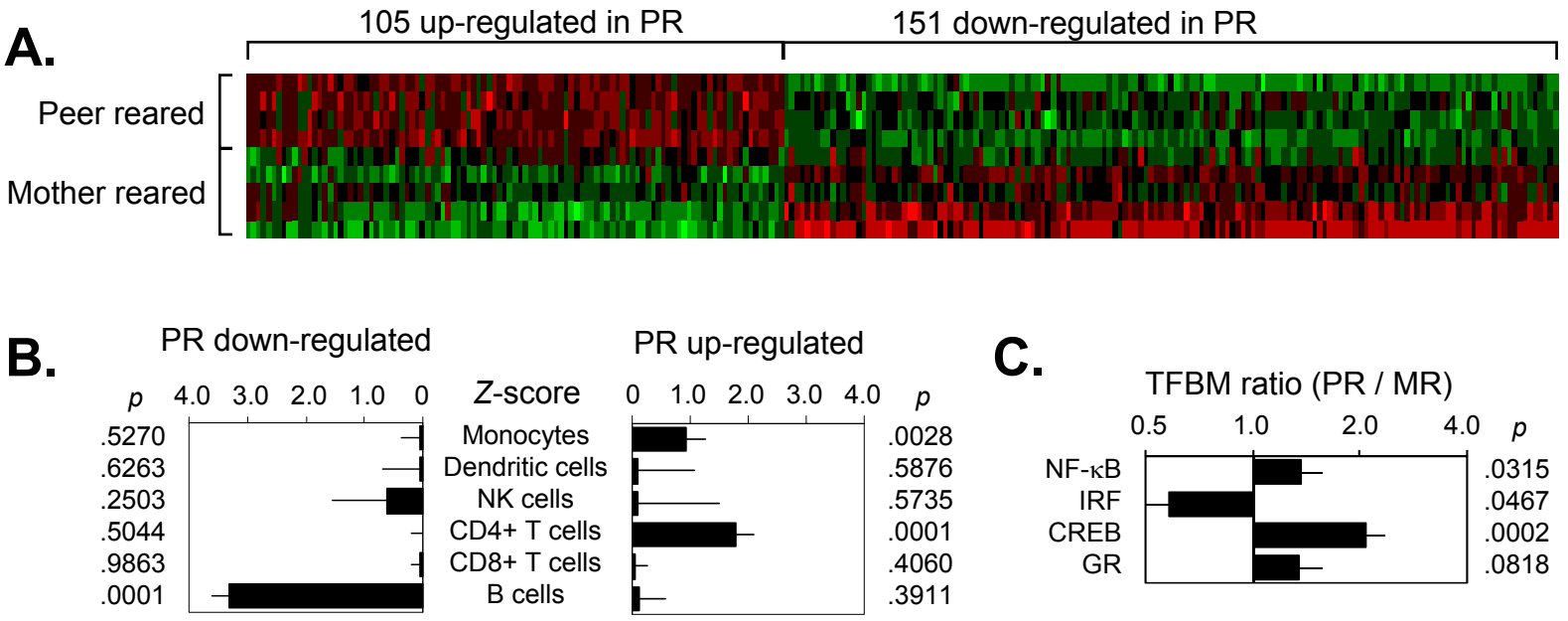
Differential gene expression in leukocytes from mother-reared vs. surrogate/peer-reared macaques



Notes: A. Heat plot representation of gene expression values for 249 transcripts showing ≥ 2-fold difference in average expression between peripheral blood mononuclear cells (PBMC) from surrogate/peer-reared (SPR) vs. maternally reared (MR) rhesus macaques.
Rows = animals, columns = gene transcripts; red = up-regulated gene expression, green = down-regulated gene expression.
B. Transcript Origin Analyses assession cellular origins of differentially expressed genes within specific PBMC subsets.
C. Relative prevalence of binding motifs for NF-κB, IRF, CREB, and GR transcription factors within promoters of genes up-regulated in PBMC from SPR vs. MR animals.

Figure 2

Differential gene expression in leukocytes from mother-reared vs. peer-reared macaques



Notes: A. Heat plot representation of gene expression values for 256 transcripts showing ≥ 2-fold difference in average expression between PBMC from peer-reared (PR) vs. maternally reared (MR) rhesus macaques.
B. Transcript Origin Analyses assessing cellular origins of differentially expressed genes within specific PBMC subsets.
C. Relative prevalence of binding motifs for NF-κB, IRF, CREB, and GR transcription factors within promoters of genes up-regulated in PBMC from PR vs. MR animals.