Brain, Behavior, and Immunity 41 (2014) 191–199

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Greater inflammatory activity and blunted glucocorticoid signaling in monocytes of chronically stressed caregivers

Gregory E. Miller [a,*], Michael L.M. Murphy [a], Rosemary Cashman [b], Roy Ma [b], Jeffrey Ma [c,d,e], Jesusa M.G. Arevalo [c,d,e], Michael S. Kobor [f,g], Steve W. Cole [c,d,e]

[a] Department of Psychology and Institute for Policy Research, Northwestern University, United States
[b] British Columbia Cancer Agency, Vancouver Centre, Canada
[c] Division of Hematology-Oncology, UCLA School of Medicine, United States
[d] UCLA AIDS Institute, Molecular Biology Institute, Jonsson Comprehensive Cancer Center, UCLA School of Medicine, United States
[e] Norman Cousins Center at UCLA, United States
[f] Centre for Molecular Medicine and Therapeutics, Child and Family Research Institute, British Columbia Children's Hospital, Canada
[g] Human Early Learning Partnership, School of Population and Public Health, University of British Columbia, Canada

ARTICLE INFO

Article history:
Received 18 February 2014
Received in revised form 21 May 2014
Accepted 22 May 2014
Available online 2 June 2014

Keywords:
Stress
Inflammation
Depression
Cytokines
Glucocorticoids
Genomics

ABSTRACT

Chronic stress is associated with morbidity and mortality from numerous conditions, many of whose pathogenesis involves persistent inflammation. Here, we examine how chronic stress influences signaling pathways that regulate inflammation in monocytes. The sample consisted of 33 adults caring for a family member with glioblastoma and 47 controls whose lives were free of major stressors. The subjects were assessed four times over eight months. Relative to controls, caregivers' monocytes showed increased expression of genes bearing response elements for nuclear-factor kappa B, a key pro-inflammatory transcription factor. Simultaneously, caregivers showed reduced expression of genes with response elements for the glucocorticoid receptor, a transcription factor that conveys cortisol's anti-inflammatory signals to monocytes. Transcript origin analyses revealed that CD14+/CD16− cells, a population of immature monocytes, were the predominate source of inflammatory gene expression among caregivers. We considered hormonal, molecular, and functional explanations for caregivers' decreased glucocorticoid-mediated transcription. Across twelve days, the groups displayed similar diurnal cortisol profiles, suggesting that differential adrenocortical activity was not involved. Moreover, the groups' monocytes expressed similar amounts of glucocorticoid receptor protein, suggesting that differential receptor availability was not involved. In *ex vivo* studies, subjects' monocytes were stimulated with lipopolysaccharide, and caregivers showed greater production of the inflammatory cytokine interleukin-6 relative to controls. However, no group differences in functional glucocorticoid sensitivity were apparent; hydrocortisone was equally effective at inhibiting cytokine production in caregivers and controls. These findings may help shed light on the mechanisms through which caregiving increases vulnerability to inflammation-related diseases.

1. Introduction

Prospective studies show that chronic psychological stress undermines health. People who have persistent marital difficulties, lose their jobs and struggle to find work, or assume care for a terminally ill relative are prone to developing new health problems and worsening of existing ones (Christakis and Allison, 2006; Dupre et al., 2012; Ji et al., 2012; Matthews and Gump, 2002; Schulz and Beach, 1999; Schulz et al., 2003). The health consequences of chronic stress emerge in both mental and physical illnesses, with the most pronounced effects in depression, respiratory infections, HIV/AIDS, and cardiovascular disease (Cohen et al., 2007). Research shows that "nonresolving" inflammation plays a role in the pathogenesis and expression of all these conditions (Libby et al., 2011; Miller et al., 2009a; Nathan and Ding, 2010; Pace and Heim, 2011; Scrivo et al., 2011). Drawing on these insights, researchers have begun elucidating how chronic stress affects inflammation and its regulation by the immune, nervous, and endocrine systems (Irwin and Cole, 2011; Raison et al., 2006; Sternberg, 2006).

* Corresponding author. Address: Department of Psychology, Northwestern University, 102 Swift Hall, 2029 Sheridan Road, Evanston, IL 60208-2710, United States.
E-mail address: greg.miller@northwestern.edu (G.E. Miller).

http://dx.doi.org/10.1016/j.bbi.2014.05.016
0889-1591/

Recent gene expression profiling studies have revealed a "transcriptional fingerprint" of chronic stress in the monocytes of humans (Cole et al., 2011; Miller et al., 2008; O'Donovan et al., 2011), the immune cells that initiate and sustain many inflammatory responses. Bioinformatic analyses indicate this "conserved transcriptional response to adversity" is characterized by three predominate themes. First, chronic stress downregulates transcriptional activity mediated by interferon response factors. Second, chronic stress upregulates activity of pro-inflammatory transcription control pathways, especially those mediated by members of the nuclear factor-kappa B (NF-κB) family. Finally, chronic stress downregulates transcriptional activity mediated by the glucocorticoid receptor (GR), the apparatus that propagates cortisol signals to the genome of target cells. Cortisol has well-known anti-inflammatory properties, partly mediated through GR inhibition of NF-κB signaling (Beck et al., 2009; Busillo and Cidlowski, 2013). For this reason, researchers believe the transcriptional fingerprint reflects acquired glucocorticoid insensitivity. According to this hypothesis, chronic stress weakens the usual regulatory constraints on monocyte pro-inflammatory activity via diminution of cortisol-mediated signaling through GR (Cole et al., 2007; Miller et al., 2008, 2009b; Miller, 2008).

These findings have been substantiated in *ex vivo* functional studies, where monocytes are stimulated with bacterial products in the presence of cortisol, and production of pro-inflammatory cytokines is monitored. Under these conditions, chronically stressed individuals produce more inflammatory cytokines than controls, and their cells are less sensitive to inhibition by cortisol (Cohen et al., 2012; Miller et al., 2002; Rohleder et al., 2009; Rohleder, 2012). This stress-related diminution of cortisol sensitivity has implications for the pathophysiology of neuropsychiatric conditions like depression, chronic fatigue, and PTSD (Raison and Miller, 2003), as well as common diseases like upper respiratory infection (Cohen et al., 2012).

Despite this progress, little is known about the mechanism(s) through which chronic stress reduces glucocorticoid sensitivity and provokes inflammatory signaling. There are at least three plausible mechanistic scenarios. First, chronic stress might dampen the amount of cortisol signal that reaches the monocyte genome. Among people facing lengthy chronic stressors, the diurnal rhythm of cortisol release is often flattened, resulting in lower-than-normal output across the daily cycle (Fries et al., 2005; Lupien et al., 2009; Miller et al., 2007). Under these conditions, monocytes would have lower cortisol exposure, and thus less inhibition of inflammatory activity. Irrespective of cortisol, chronic stress could also downregulate monocyte GR expression, dampening these cells' ability to transduce glucocorticoids' anti-inflammatory signals (Pariante and Miller, 2001). In an earlier study, we found that chronic stress was unrelated to the quantity of GR mRNA expressed by monocytes (Miller et al., 2008). However, there is significant post-transcriptional regulation of GR message, such that only a portion is eventually translated into protein. Thus, to address this issue convincingly, studies of chronic stress and monocyte GR protein expression are needed.

Second, there is considerable functional heterogeneity among monocytes (Auffray et al., 2009; Woollard and Geissmann, 2010). Via selective myelopoiesis, chronic stress could mobilize a subpopulation of monocytes with pro-inflammatory and cortisol-resistant tendencies. A recent mouse study found that repeated social defeat caused expansion and mobilization of Ly-6c^{high} cells into peripheral lymphoid tissues (Powell et al., 2013). Ly-6c^{high} cells are immature, pro-inflammatory monocytes, with a functional counterpart in humans identified as CD14+/CD16−. In humans, chronic stress could selectively populate lymphoid organs with these cells, creating an environment marked by glucocorticoid resistance and inflammatory signaling. This possibility has not yet been examined in studies of chronically stressed humans.

Finally, chronic stress could bring about functional alterations that diminish monocytes' capacity to transduce cortisol signals. For example, stress evokes post-translational modifications to proteins comprising GR (Pace et al., 2007). Some of these modifications can engender glucocorticoid resistance (Galliher-Beckley and Cidlowski, 2009). Nevertheless, transcriptional profiling studies published to date (Cole et al., 2011; Miller et al., 2008; O'Donovan et al., 2011) have not simultaneously reported genomic and functional outcomes. Thus, it remains unclear whether bioinformatic indications of glucocorticoid insensitivity among chronically stressed individuals are paralleled by functional indications, e.g., apparent in an *ex vivo* assay system.

In this article we present a multiwave study that builds upon previous research by considering these scenarios. It follows subjects as they grapple with a severe chronic stressor – caring for a family member with glioblastoma multiforme (GBM), an aggressive brain tumor. GBM treatment can be painful and disabling, and most patients die within a year of diagnosis. Even with promising new therapies, two-year survival rates are 27% (Stupp et al., 2005). As a consequence, GBM caregivers face numerous challenges, which can include watching a loved one deteriorate, anticipatory grieving of their death, and marked changes in family interpersonal dynamics. For some families, GBM treatment also poses a significant financial burden, which is compounded if the caregiver must quit work to assist with patient care. These challenges can have implications for health; a recent study showed that in the years following a spouse's cancer diagnosis, caregivers' rates of heart disease and ischemic stroke rose by 13% and 24%, respectively (Ji et al., 2012). Thus, GBM caregivers represent a scientifically and clinically relevant population in which to conduct behavioral immunology research. Here, we draw on them to clarify the linkages among chronic stress, glucocorticoid sensitivity, and inflammatory signaling, focusing specifically on the three mechanistic scenarios outlined above – reduced availability of cortisol or its receptor, selective expansion or mobilization of monocyte subsets, or functional alterations in glucocorticoid signal transduction.

2. Methods

2.1. Sample

Though our group has studied GBM caregivers previously (Miller et al., 2008; Rohleder et al., 2009), the data reported here are from an entirely new sample. There is no data overlap with our past studies. GBM caregivers were recruited from the CNS tumor clinics at the British Columbia Cancer Agency, Vancouver Centre. All caregivers who attended clinic prior to the onset of radiotherapy were approached about participation. Controls were recruited from the broader Vancouver community using advertisements in local media. They had to be without major stressors in their lives during the past year, including divorce, bereavement, unemployment, victimization, significant illness, hospitalization, or care giving responsibilities of their own. The project was approved by the Research Ethics Boards of the University of British Columbia and the British Columbia Cancer Agency, and all subjects gave written consent.

2.2. Assessments

Subjects completed four data-collection sequences. Each consisted of an in-person assessment and three days of ambulatory monitoring. GBM treatment typically involves surgical resection of the tumor, followed by radiotherapy and/or chemotherapy. Caregivers were enrolled after their family member had recovered

from surgery, but prior to radiotherapy. On average, caregivers began the study 3.8 months after surgery. They participated in follow-ups after completion of radiotherapy (roughly 2 months later) and after completion of chemotherapy (another 2 months later). The final sequence occurred four months after chemotherapy ended. Controls participated in four data-collection sequences along a similar timeline, beginning at study entry, and then 2, 4, and 8 months afterwards. At in-person assessments, subjects completed questionnaires and blood was drawn for assessment of inflammatory outcomes. During ambulatory monitoring, subjects collected saliva for cortisol measurement.

2.3. Psychological distress

Psychological distress was assessed with the Perceived Stress Scale (Cohen et al., 1983) and the Brief (ten-item) version of the Center for Epidemiologic Studies – Depression Scale (Radloff, 1977). Both instruments showed excellent psychometrics, with Cronbach's alpha's of .90, and .82, respectively.

2.4. Monocyte gene expression

To conduct whole transcriptome profiling of monocytes, 20-mL of blood was drawn by antecubital venipuncture into Vacutainer Cell Preparation Tubes (Becton–Dickinson). After isolation of mononuclear cells through density-gradient centrifugation, monocytes were captured via immuno-magnetic positive selection with antibodies against CD14 (using reagents and an autoMACS Separator from Miltenyi Biotec), resulting in >90% purity by flow cytometry. Total RNA was extracted using RNAlater/RNeasy kits from Qiagen, and its quantity and integrity were verified using NanoDrop ND100 and Agilent BioAnalyzer instruments. 100 ng of RNA was converted into biotinylated cRNA target and hybridized to Illumina Human HT-12 v4.0 beadchips, then scanned on an Illumina iScan instrument at the UCLA Neuroscience Genomics Core. The raw data are deposited in Gene Expression Omnibus (Accession No. GSE52319).

2.5. Patterns of cortisol output

During each sequence, diurnal cortisol was assessed as subjects went about 3 days of activities. Saliva was collected six times daily: at waking, and 1/2, 1, 4, 9, and 14 h later using Salivettes (Sarstedt). To monitor compliance, we lent subjects time/date stampers, and asked them to apply stamps to each Salivette upon collection. Salivettes with missing or incorrect time/date stamps were excluded from analyses. Cortisol was later measured via chemiluminescence (IBL-Hamburg) by the Laboratory for Biological Health Psychology at Brandeis University. This assay has a sensitivity of 0.43 nmol/L and intra- and inter-assay coefficients of variation <10%. Cortisol values were log-transformed, and used to create three indices: the cortisol awakening response, reflecting output over the first hour of the day, relative to a waking ground; total cortisol secretion, represented by an area-under-the-curve statistic; and the diurnal rhythm, estimated by regression of values onto time since waking. Values were averaged across collection days.

2.6. Functional glucocorticoid sensitivity and glucocorticoid receptor expression

As a functional indicator of glucocorticoid sensitivity, we performed a standard *ex vivo* assay wherein cytokine production was monitored in leukocytes co-incubated with lipopolysaccharide and hydrocortisone (Cohen et al., 2012; Rohleder et al., 2009). The assay was adapted for a flow cytometric platform using a protocol that BD Biosciences developed and validated for assessing monocyte cytokine responses to lipopolysaccharide stimulation (BD Biosciences, 1999). After blood was drawn into Sodium Heparin Vacutainers, it was diluted 9:1 with saline, and 1.6 mL aliquots were dispensed into six-well plates. To each well (except the unstimulated control), we added 200 μL of lipopolysaccharide (from Sigma; final concentration of 50 ng/mL), 200 μL of saline-diluted hydrocortisone at varying concentrations (from Sigma; final concentrations of 0, 10^{-7}, 10^{-6}, or 10^{-5} mol/L), and 2 μL of Brefeldin A (BD Biosciences). After a 6-h incubation, red blood cells were removed with Pharmlyse (BD Biosciences) and re-suspended. To minimize nonspecific binding to Fc receptors, we then incubated cells for 15 min with 10% normal human serum. After washing, cells were incubated with monoclonal antibodies against CD14 (10% APC-conjugated CD14, BD BioSciences) for 20 min. Following another wash, cells were permeabilized (Cytofix/Cytoperm Plus; BD Biosciences), then stained with monoclonal antibodies against interleukin-6 (4% PE-conjugated IL-6 Ab, BD BioSciences). After a 20-min incubation, the samples were washed and data were acquired on a FACSCaliber (BD Biosciences). Data were analyzed with FlowJo (Tree Star Inc). Quadrants were set using data from concurrently run negative control samples (unstained cells and unstimulated cells), along with compensation beads stained with appropriate antibodies.

We also used flow cytometry to quantify monocyte expression of GR protein. These assays followed a similar protocol, except they were done with unstimulated cells, and used monoclonal antibodies against GR (10% FITC-conjugated GR Ab, from AbD Serotec).

2.7. C-reactive protein

To index low-grade inflammation, we measured C-reactive protein (CRP) in serum that had been harvested from blood drawn into Serum Separator Tubes (BD). CRP was assayed with a high-sensitivity, chemiluminescent technique on an IMMULITE 2000 (Diagnostic Products Corporation). The assay had an average inter-assay coefficient of variation of 2.2% and a detection threshold of .20 mg/L.

2.8. Potential confounders

Using questionnaires and measurements (Miller et al., 1999, 2008; Paffenbarger et al., 1993), we solicited information on plausible demographic (age, gender, ethnicity, educational attainment) and biobehavioral (daily cigarette use, weekly alcoholic drinks and physical activity, and waist circumferences) confounders, and treated them as covariates in statistical analyses. We also ascertained medical history via self-report, using items from the Medical Condition Survey of the National Health and Nutrition Examination Study Centers for Disease Control (2009).

2.9. Statistical analyses

Transcriptional activity was analyzed with methods employed and validated in our previous research (Cole et al., 2003, 2005, 2011; Fredrickson et al., 2013; Miller et al., 2008). Briefly, raw data were quantile-normalized and log-2 transformed. To identify differentially expressed genes, we conducted Linear Mixed Models that included demographic and biobehavioral confounders. Differentially expressed genes were defined as those showing $\geqslant$1.25-fold difference between groups and a transcript-level false discovery rate $\leqslant$5%, based on Benjamini and Hochberg (Cole et al., 2003). Analyses were performed in R using the lme4 and fdrtool packages. To identify transcription control pathways underlying differential gene expression, we used a 2-sample variant of the Transcription Element Listening System (TELiS; Cole et al., 2005). TELiS quantifies the prevalence of transcription factor-binding

motifs (TFBMs) within promoters of differentially expressed genes. We present aggregate prevalence indices, pooled across 9 technical specifications involving variations of promoter length (−300 bp, −600 bp, −1000 to +200 bp) and match stringency (MatSim .80, .90, .95). Results are based on paired *t*-tests with bootstrapped standard errors.

Generalized Estimating Equations (GEE) were used to analyze remaining outcomes (Hanley et al., 2003). All models included caregiving status, visit number, and confounders, and employed unstructured covariance matrices to account for dependencies created by repeated assessments.

Not all subjects completed the study. Among caregivers, attrition was principally due to bereavement, treatment complications, or patient deterioration. At baseline, the caregiver sample consisted of 33 individuals. Follow-up assessments were done with 27, 21, and 18 caregivers at Visits 2, 3, and 4, respectively. The parallel numbers for controls were 47, 41, 37, and 35. We modeled all available data in statistical analyses, taking advantage of the procedures' robustness to missing values. However, given the declining size of the sample across follow-up, the dataset was not suited for modeling changes over time. Thus, the findings reflect aggregated group differences, collapsed across assessments.

3. Results

Table 1 displays the sample characteristics. Demographically, the groups were similar on age, ethnic/racial background, and educational attainment (p's > .24), but caregivers were marginally more likely to be female (p = .07). Behaviorally, caregivers reported fewer weekly minutes of exercise than controls (p = .02), but the groups were similar on cigarette and alcohol use and waist circumference (p's > .64). Despite these similarities, many statisticians argue that covariates should be chosen *a priori*, and not on the basis of a univariate prescreening (Babyak, 2004). Thus, in the analyses below, we adjust for demographic and biobehavioral confounders.

3.1. Psychological distress

Fig. 1 shows that caregivers' perceived stress levels were almost double those of controls (17.3 ± 1.0 vs. 9.3 ± 0.7; Wald χ^2 = 42.2; p = .0001), indicating they found life significantly more overwhelming and uncontrollable. Similarly, caregivers' levels of depressive symptoms, as reported on the CES-D, were more than twice that of controls (9.9 ± 0.7 vs. 4.8 ± 0.4; Wald χ^2 = 37.4; p = .0001). Over the study, 14 caregivers (42.4%) reported a Brief CES-D score ⩾ 10, the cutoff used to identify patients at high-risk for a major depressive episode. By contrast, only 3 controls had scores that crossed this threshold (6.4%).

3.2. Monocyte gene expression

Covariate-adjusted linear-mixed models identified 461 transcripts differentially expressed by ⩾1.25-fold (listed in Table S1).

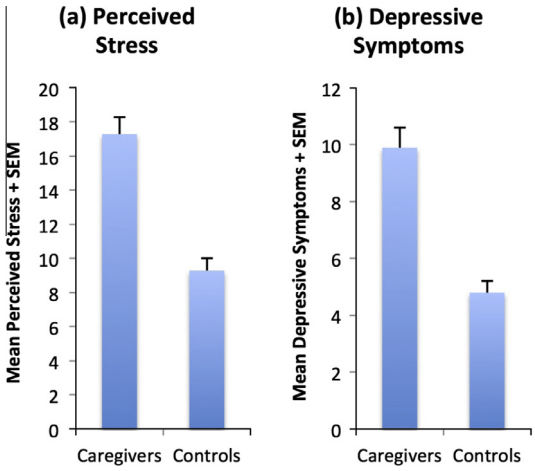

Fig. 1. Psychological sequelae of caregiving. Self-reports of perceived stress and depressive symptoms were collected from 33 adults caring for a family member with glioblastoma, and 47 control subjects without major stressors in their lives. Averaged across assessments, caregivers' levels of (a) perceived stress (p = .0001) and (b) depressive symptoms (p = .0001) were nearly twice that of controls.

333 were relatively over-expressed by caregivers, including genes that encode classic pro-inflammatory mediators such as interleukin-1β, interleukin-6, COX-2, intercellular adhesion molecule 1, and macrophage inflammatory protein 2α. Also over-expressed were multiple transcription factors involved in monocyte/macrophage activation, including RELA, a member of the NF-κB family, and EGR1, FOSL2, and MAFF/MAFFG. The other 128 transcripts were relatively under-expressed by caregivers', and they included receptors for the chemokines monocyte chemotactic protein-1 (CCR2) and Fractalkine (CX3CR1). To examine whether caregivers showed the "conserved transcriptional response to adversity" observed in past studies, we used an *a priori*-defined contrast (Fredrickson et al., 2013) to compare expression of 19 pro-inflammatory, 31 interferon, and 3 immunoglobulin-related transcripts. The overall profile was significantly greater in caregivers vs. controls (difference: 7.9%; SE = 1.5%; p < .0001). This difference was driven mainly by upregulated pro-inflammatory genes (difference: 16.1%; SE = 2.3%, p < .0001), and to a lesser extent downregulated interferon- and antibody-related genes (difference: −3.4%; SE = 1.5%; p = .031).

TELiS was used to identify transcription control pathways underlying these disparities. Caregivers' monocytes displayed significant upregulation of transcripts bearing response elements for NF-κB, the chief pro-inflammatory transcription factor. There was a 1.65-fold greater prevalence of NF-κB/Rel TFBMs in the promoters of genes over-expressed by caregivers, relative to those over-expressed by controls (TRANSFAC V$NFKAPPAB_01 motif: SE = 0.16; p = .0017; Fig. 2). At the same time, caregivers' exhibited significant downregulation of transcripts with response elements for GR. There was a 0.68-fold lower prevalence of GR TFBMs in genes over-expressed by caregivers, relative to those over-expressed by controls (V$GR_Q6 motif; SE = 0.11; p = .04; Fig. 2).

Table 1
Demographic, lifestyle, and biomedical characteristics.

	Caregivers (n = 33) Mean ± SEM or %	Controls (n = 47) Mean ± SEM or %
Age at entry, years	54.2 ± 2.8	50.1 ± 1.9
Gender, % male/female	36.4/63.6	38.3/61.7
Ethnicity, % caucasian	81.8	63.8
Education, % four-year university degree	81.8	75.0
Cigarette smoking, % daily smokers	6.1	10.6
Exercise, minutes weekly	134.8 ± 28.6	239.0 ± 22.6
Alcohol consumption, drinks weekly	1.9 ± 0.3	2.1 ± 0.4
Waist circumference, cm	90.6 ± 2.9	88.0 ± 1.8

Exploratory analyses highlighted two additional signaling pathways associated with chronic stress. First, caregivers exhibited significant upregulation of genes containing response elements for Early Growth Response Protein (EGR-1; 1.85-fold greater promoter V$EGR1_01 prevalence; SE = 0.25; $p = .04$; Fig. 2). These results are consistent with the upregulation of EGR-1 itself among caregivers. Second, caregivers displayed significant upregulation of genes bearing CREB/ATF response elements (1.75-fold greater V$CREB_04 prevalence; SE = 0.23; $p = .007$; Fig. 2). Among other activities, this family of transcription factors propagates signals sympathetic nervous system signals to the monocyte genome.

To identify the cellular sources of these disparities, we performed Transcript Origin Analyses (Cole et al., 2011), using reference profiles of CD14+/CD16+ vs. CD14+/CD16− monocytes (GSE18565) (Ingersoll et al., 2010). The results suggested that caregivers' upregulated genes were predominately expressed by CD14+/16− cells, a population of immature, highly inflammatory monocytes (Diagnosticity Score = 1.05; SE = 0.17; $p < .001$; Fig. 3). To clarify whether these cells were simply more prevalent among caregivers, we performed a Transcriptome Representation Analysis (Powell et al., 2013). The results indicated that caregivers and controls expressed similar quantities of transcripts specifically diagnostic of CD14+/16− cells (Ingersoll et al., 2010) prevalence ratio = 1.01; $p = .25$). Together, these findings suggest that caregivers' upregulated genes resulted from increased per-cell transcriptional activity of CD14+/16− cells, rather than a higher prevalence of these cells in circulation. In analyses of downregulated genes, Transcript Origin Analyses did not identify either CD14+/16− or CD14+/16+ cells as mediators.

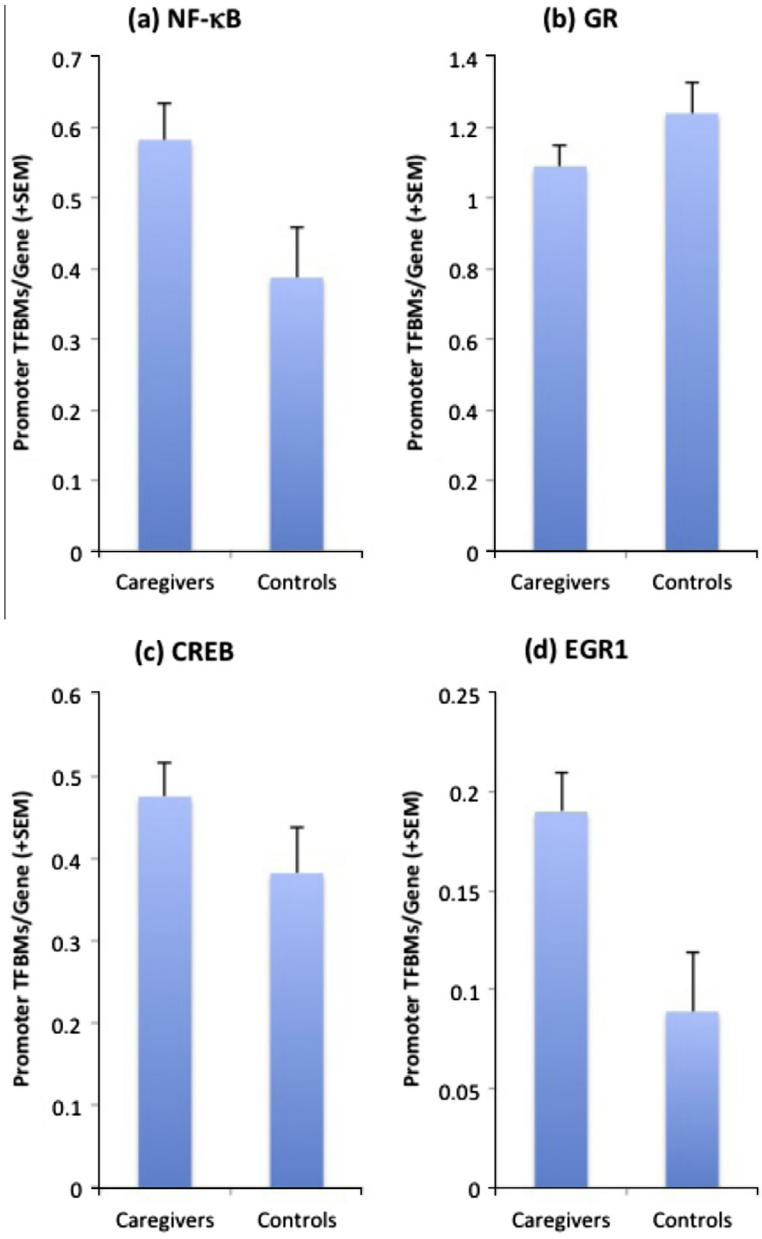

Fig. 2. Monocyte transcriptional activity. Genome-wide expression profiling was performed on immunomagnetically isolated CD14+ monocytes techniques. TELiS bioinformatics analyses quantified response element prevalence in promoters of differentially expressed genes; the results were averaged across assessments. Relative to controls, monocytes of caregivers showed (a) increased expression of genes bearing response elements for the pro-inflammatory transcription factor nuclear factor kappa B ($p = .0017$), and (b) decreased expression of genes bearing response elements for the glucocorticoid receptor ($p = .04$). Caregivers' monocytes also showed upregulation of genes with response elements for (c) Cyclic AMP Response Element Binding Protein ($p = .007$) and (d) Early Growth Response Protein 1 ($p = .04$).

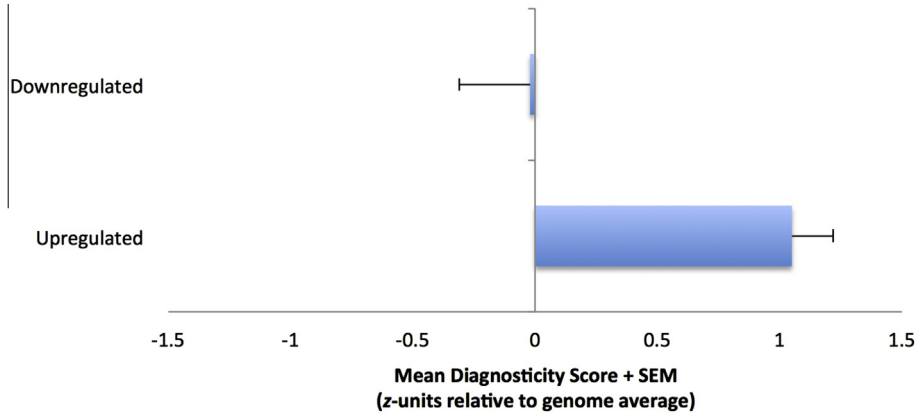

Fig. 3. Cellular sources of transcriptional disparity. Transcript origin analyses were performed to identify the cellular origins of differentially expressed genes. Data from Ingersoll et al., 2010 were used to calculate diagnosticity scores, indicating whether transcripts are expressed predominately by a specific cell type. Averaged across assessments, results indicated that upregulated genes in caregivers were predominately expressed by CD14+/16− cells, a population of immature, inflammatory monocytes ($p < .001$). Down-regulated genes were not selectively associated with either subset.

3.3. Cortisol output and GR expression

To evaluate cortisol's role in these processes, we had subjects collect saliva for three days following each in-person assessment. Covariate-adjusted GEE's indicated that caregivers and controls had similar diurnal cortisol profiles. Specifically, there were no group differences in cortisol awakening response, diurnal rhythm, or total daily output (Wald χ^2 values from 0.1 to 0.5, p values range .25–.81; Fig. 4). Regardless of cortisol, stress could alter monocyte GR expression. On flow cytometry, nearly all monocytes stained positive for GR protein (average 96.9%). We thus focused analyses on mean fluorescence intensity, an indicator of GR abundance. However, covariate-adjusted GEE's suggested that GR was expressed at similar levels by monocytes of caregivers and controls (Wald $\chi^2 = 0.7$, $p = .41$; Fig. 4). Moreover, on microarray, no group differences in GR mRNA were seen ($p = .48$; data not shown).

3.4. Functional indications of glucocorticoid sensitivity

To evaluate the functional hypothesis, we cultured leukocytes with LPS and cortisol, and quantified monocyte IL-6 production by flow cytometry. In no-hydrocortisone wells the vast majority of monocytes stained positive for IL-6 (72.8% across runs), so to maximize variance we focused on mean fluorescence intensity, indicative of IL-6 abundance. Covariate-adjusted GEE's revealed significant group differences (Fig. 5). Relative to controls, caregivers' monocytes expressed more IL-6 following LPS stimulation, and this disparity persisted when cells were co-incubated with hydrocortisone (for LPS + saline: Wald $\chi^2 = 6.4$, $p = .01$; for LPS + hydrocortisone 10^{-7} mol/L: Wald $\chi^2 = 4.0$, $p = .04$; for LPS + hydrocortisone 10^{-6} mol/L: Wald $\chi^2 = 12.9$, $p = .001$; for LPS + hydrocortisone 10^{-5} mol/L: Wald $\chi^2 = 4.7$, $p = .03$). When MFI values from all the wells were aggregated – using an area-under-the-curve statistic to form a composite indicator – GEE yielded the same pattern of results, with greater overall IL-6 expression by caregivers (Wald $\chi^2 = 6.0$, $p = .01$). However, further analyses suggested these findings were secondary to disparities in LPS responsivity. Specifically, when values from the hydrocortisone wells were normalized for IL-6 production in the LPS-only well, caregiving-related differences were no longer significant (p's > .32).

3.5. Low-grade inflammation

We measured CRP in serum to index low-grade inflammation. However, in covariate-adjusted GEE's, no group differences in CRP were apparent (Wald $\chi^2 = 0.3$, p's > .62; Caregivers: 1.63 ± .48 mg/L; Controls: 1.86 ± .27 mg/L).

3.6. Role of pre-existing conditions

Most of our subjects were middle-aged or older, and some of them reported a history of serious illnesses. These conditions

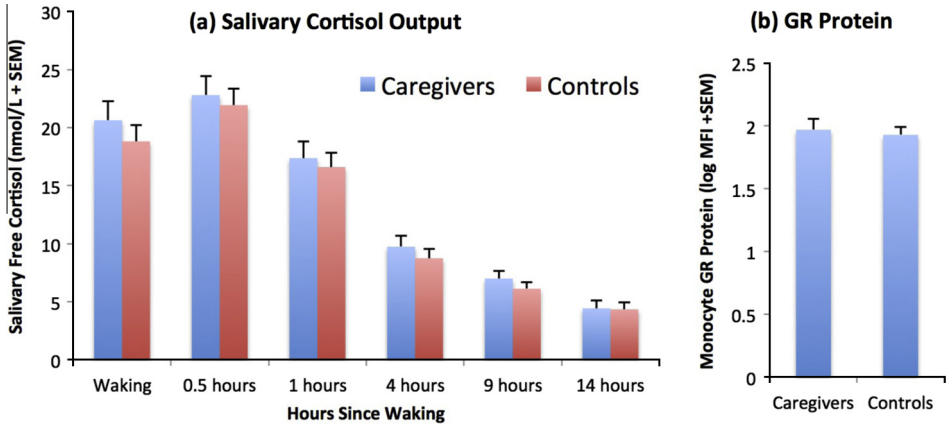

Fig. 4. Diurnal cortisol output and glucocorticoid receptor expression. (a) Salivary cortisol was assessed at six points over the diurnal cycle, on a total of twelve days across the study. Averaged across assessments, caregivers and controls displayed similar patterns of diurnal cortisol output (p's > .25). (b) Flow cytometry was used to quantify glucocorticoid receptor protein expression. Averaged across assessments, the groups' monocytes had similar GR abundance ($p = .41$).

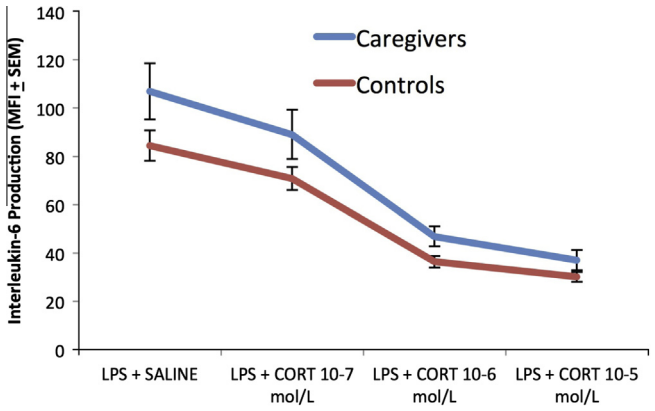

Fig. 5. Functional glucocorticoid sensitivity. Whole blood was cultured for 6 h with the bacterial product lipoploysaccharide (50 ng/mL) and varying dosages of hydrocortisone. Production of the inflammatory cytokine interleukin-6 was measured by flow cytometric analysis of mean fluorescence intensity in CD14+ cells, and averaged across assessments. Relative to control subjects, the caregivers' monocytes produced higher levels of IL-6 when stimulated with LPS, and this disparity persisted when the cells were co-incubated with hydrocortisone at doses from 10^{-5} to 10^{-7} mol/L (*p*'s from .01– to .04).

included cancer (5 caregivers, 4 controls), myocardial infarction (2 caregivers, 0 controls), and diabetes (0 caregivers, 1 control). Some also were taking anti-depressant medications, or had in the past year (7 caregivers, 6 controls). To determine what impact these subjects had on results, we re-conducted analyses after removing them from the dataset. The findings were identical, or in some instances, even stronger than reported above.

4. Discussion

Chronic stress is associated with excess morbidity and mortality from numerous diseases (Cohen et al., 2007). Recent genomic studies have identified a plausible inflammatory mechanism for these effects, whereby chronic stress upregulates pro-inflammatory transcriptional activity, and simultaneously downregulates anti-inflammatory signaling through the GR. Here we replicate these findings, documenting that caregiving is associated with a transcriptional profile characterized by higher NF-κB signaling and lower GR signaling. These disparities were independent of lifestyle variables (tobacco and alcoholic use, physical activity, and abdominal obesity) as well as pre-existing disease.

Extending previous research, we conducted analyses to define the cellular and molecular mechanisms of these differences, and explore their functional correlates. Bioinformatic analyses of cellular origins revealed CD14+/16− cells as the principle source of upregulated inflammatory genes among caregivers. Follow-up analyses suggested the groups possessed similar numbers of these cells. Together, these results imply that caregiving heightens expression of selected pro-inflammatory transcripts by CD14+/16− cells, rather than increasing the prevalence of these cells in circulation. This scenario converges with evidence from a mouse model of chronic social threat (Powell et al., 2013) in implicating immature monocytes in stress-evoked upregulation of inflammatory gene expression.

Transcript Origin Analyses did not identify any distinct cellular origins for caregivers' downregulated genes. Because these downregulated genes' promoters contained a relative enrichment of response elements for the GR, we considered whether chronic stress might have reduced the amount of cortisol signal delivered to the monocyte genome by altering levels of ligand and/or receptor. However, across a dozen days of monitoring, caregivers and controls displayed similar patterns of salivary cortisol output. The groups also expressed similar quantities of monocyte GR protein on flow cytometry. Together, these findings suggest the diminution of cortisol-mediated transcription among caregivers was not a consequence of the bioavailability of cortisol or its receptor. These findings build on previous research (Miller et al., 2008), whose focus has been limited to GR mRNA. The patterns here also converge with the results of glucocorticoid insensitivity studies in depressed patients. There, disruptions in negative feedback to the HPA axis arise because of functional modifications that compromise GR signal transduction, as opposed to downregulated receptor expression (Pariante and Miller, 2001). An alternative possibility, which should be explored in future research, is that chronic stress affects subcellular trafficking of GR. Indeed, caregiving may have interfered with GR's ability to translocate to the nucleus, where it exerts many of its anti-inflammatory actions. Our flow cytometric approach could not differentiate between GR in cytoplasmic vs. nuclear compartments.

Using *ex vivo* studies, we found that caregivers produced more IL-6 following LPS stimulation than controls. These findings substantiate the bioinformatic results of increased NF-κB activity in monocytes. When hydrocortisone was added to these cultures to model glucocorticoid sensitivity, caregivers continued to produce larger quantities of IL-6. However, further analyses suggested these disparities were secondary to caregivers' greater LPS responsivity. Interpretation of these findings is complicated, particularly in light of the bioinformatic evidence of glucocorticoid insensitivity among caregivers. Several factors could account for the disparate findings. The functional studies performed here were *ex vivo*, involved just a single microbial stimulus (LPS), utilized supra-physiologic doses of hydrocortisone, and focused on a single gene product outcome (IL-6). By contrast, the bioinformatic findings reflect *in vivo* GR-mediated transcription, which is regulated by multiple inflammatory stimuli and by physiologic variations in cortisol, and which involved many hundreds of gene products. Moreover, the transcriptional and functional indicators are likely to capture somewhat different pathways of glucocorticoid action. By quantifying expression of GR-mediated transcription, TELiS focuses principally on cortisol's genomic actions – that is, the extent to which it has switched on target genes. In the *ex vivo* assay hyrdrocortisone acts genomically as well, but its anti-inflammatory effects also depend on protein–protein interactions, wherein GR interferes with the activity of other transcription factors, like NF-κB. Moreover, the hydrocortisone in these assays could be acting, in part, through the mineralocorticoid receptor, which can inhibit monocyte cytokine responses to LPS (Sauer et al., 1996). Future research is needed to clarify the similarities and differences between these approaches to quantifying glucocorticoid sensitivity. Moreover, additional research is needed to understand the mechanisms through which caregiving becomes associated with lower GR-mediated transcription. As noted, several routes of action are plausible, including stress-related changes in mitogen-activated protein kinase activity, which can induce post-translational modifications to residues that comprise GR (Pace et al., 2007). Some of these post-translational modifications, especially phosphorylation of serine and threonine residues, dampen glucocorticoid sensitivity (Galliher-Beckley and Cidlowski, 2009). Acting through epigenetic pathways like methylation, chronic stress might also dampen the expression of genes that are necessary for glucocorticoid signal transduction, e.g., chaperones like FKBP5 (Klengel et al., 2013).

When considering these results, readers should keep in mind several caveats. First, the study's design precludes us from making causal inferences. By assessing subjects on multiple occasions and adjusting for demographic and behavioral confounders, we have ruled out many plausible alternative explanations. But without a truly experimental design, we cannot be certain that caregiving

acted causally. Second, attrition rates over follow-up were high, particularly for caregivers whose family members' health deteriorated. Because these dropouts were non-random, they limit the generalizability of findings at later timepoints. As a result of this problem, and the associated loss of statistical power, we could not meaningfully map trajectories of inflammatory signaling and glucocorticoid sensitivity over time, or how they differed by caregiving status. (We ran exploratory analyses of this nature among subjects who completed the entire study, and did not observe strong time-related trends. But as a result of attrition, we do not have great confidence in these findings).

With that said, those caregivers lost to follow-up are likely to have been facing greater psychosocial challenges than completers (e.g., deterioration of the family member with GBM, a lack of social and coping resources, psychiatric problems of their own). If so, our sample might be biased towards caregivers who are relatively less distressed, and as a result would be expected to show less pronounced changes in inflammatory biology vis a vis controls. Thus, the present study could be under-estimating the true magnitude of disparities associated with caregiving. This scenario could explain why differences we observed in transcriptional activity and LPS responsivity were not paralleled by indications of low-grade inflammation (via CRP) among the caregivers. Consistent with this possibility, caregivers enrolled in this project were less likely to score above the CES-D's clinical cutoff than caregivers in our pilot study (that is, scores $\geqslant$10 at 45% vs. 63% of assessments; see Miller et al., 2008). They also had lower CRP than caregivers in the pilot (average values of 1.6 vs. 2.9 mg/L). Together, these results suggest that our subjects had relatively favorable caregiving experiences and/or had developed resilience to this stressor.

Third, there is likely to be much variability in the experiences that caregivers have as their family member grapples with GBM (e.g., more vs. less complicated courses) and the resources they bring to this difficult situation (e.g., social support, health problems of their own, other stressors, assistance with caregiving). Our sample is too small for moderator analyses exploring how these factors relate to glucocorticoid sensitivity and inflammatory signaling. But addressing these questions should be a high priority in future research. Fourth, the fold-change threshold we used to identify differentially expressed genes (1.25) was modest (consistent with standard threshold used in other studies of psychological/social influences on immune cell gene expression). However, it bears noting that the RNA source here was quiescent cells from healthy adults, and that transcriptional patterns were paralleled by functional disparities in IL-6 production. Lastly, Transcript Origin Analysis is an indirect method for identifying transcripts' cellular origins. Future research must substantiate our conclusions about CD14+/16− cells with more direct methods, e.g., flow cytometric enumeration of monocyte subsets.

Despite these limitations, the study provides fresh insights about the changes in monocyte behavior that accompany chronic stress. Conceptually, the study's findings advance understanding of the cells responsible for stress-related upregulation of inflammatory gene expression. They also provide clues about how stress dampens glucocorticoid-mediated transcription, and rule out the possibility of changes in ligand and receptor availability. Clinically, the study's findings highlight mechanisms that might contribute to caregivers' vulnerability to mental and physical health problems (Ji et al., 2012; Schulz and Beach, 1999; Schulz et al., 2003). Based on mounting evidence from mechanistic studies, future research might attempt to forestall or ameliorate these problems via agents that boost efficiency of monocyte GR signaling and/or attenuate these cells' pro-inflammatory activity (Haroon et al., 2012).

Funding and disclosures

This research was supported by grants from the Canadian Institutes of Health Research (#89736), the Brain and Behavior Research Foundation, and the National Institutes of Health (P30 AG017265). None of the authors has a conflict of interest to declare related to this project, financial or otherwise.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2014.05.016.

References

Auffray, C., Sieweke, M.H., Geissmann, F., 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu. Rev. Immunol. 27, 669–692.
Babyak, M.A., 2004. What you see may not be what you get: a brief, nontechnical introduction to overfitting in regression-type models. Psychosom. Med. 66, 411–421.
Biosciences, B.D., 1999. Application Note: Detecting Intracellular Cytokines in Activated Monocytes. Becton, Dickinson, and Company, Franklin Lakes, NJ.
Beck, I.M., Vanden Berghe, W., Vermeulen, L., Yamamoto, K.R., Haegeman, G., De Bosscher, K., 2009. Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. Endocr. Rev. 30, 830–882.
Busillo, J.M., Cidlowski, J.A., 2013. The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. Trends Endocrinol. Metab. 24, 109–119.
Centers for Disease Control, 2009. National Health and Nutrition Examination Survey Questionnaire. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Hyattsville, MD.
Christakis, N.A., Allison, P.D., 2006. Mortality after the hospitalization of a spouse. N. Engl. J. Med. 354, 719–730.
Cohen, S., Janicki-Deverts, D., Doyle, W.J., Miller, G.E., Frank, E., Rabin, B.S., Turner, R.B., 2012. Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. Proc. Natl. Acad. Sci. USA 109, 5995–5999.
Cohen, S., Janicki-Deverts, D.L., Miller, G.E., 2007. Psychological stress and disease. J. Am. Med. Assoc. 298, 1685–1687.
Cohen, S., Kamarck, T.W., Mermelstein, R., 1983. A global measure of perceived stress. J. Health Soc. Behav. 24, 385–396.
Cole, S.W., Hawkley, L.C., Arevalo, J.M., Sung, C.S., Rose, R.M., Cacioppo, J.T., 2007. Social regulation of gene expression: inflammation and the human transcriptional response to loneliness. Genome Biol. 8, r89.
Cole, S.W., Galic, Z., Zack, J.A., 2003. Controlling false-negative errors in microarray differential expression analysis: a PRIM approach. Bioinformatics 19, 1808–1816.
Cole, S.W., Hawkley, L.C., Arevalo, J.M., Cacioppo, J.T., 2011. Transcript origin analysis identifies antigen-presenting cells as primary targets of socially regulated gene expression in leukocytes. Proc. Natl. Acad. Sci. USA 108, 3080–3085.
Cole, S.W., Yan, W., Galic, Z., Arevalo, J., Zack, J.A., 2005. Expression-based monitoring of transcription factor activity: the TELiS database. Bioinformatics 21, 803–810.
Dupre, M.E., George, L.K., Liu, G., Peterson, E.D., 2012. The cumulative effect of unemployment on risks for acute myocardial infarction. Arch. Intern. Med. 172, 1731–1737.
Fredrickson, B.L., Grewen, K.M., Coffey, K.A., Algoe, S.B., Firestine, A.M., Arevalo, J.M., Ma, J., Cole, S.W., 2013. A functional genomic perspective on human well-being. Proc. Natl. Acad. Sci. USA 110, 13684–13689.
Fries, E., Hesse, J., Hellhammer, J., Hellhammer, D.H., 2005. A new view on hypocortisolism. Psychoneuroendocrinology 30, 1010–1016.
Galliher-Beckley, A.J., Cidlowski, J.A., 2009. Emerging roles of glucocorticoid receptor phosphorylation in modulating glucocorticoid hormone action in health and disease. IUBMB Life 61, 979–986.
Hanley, J.A., Negassa, A., Edwardes, M.D., Forrester, J.E., 2003. Statistical analysis of correlated data using generalized estimating equations: an orientation. Am. J. Epidemiol. 157, 364–375.
Haroon, E., Raison, C.L., Miller, A.H., 2012. Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior. Neuropsychopharmacology 37, 137–162.
Ingersoll, M.A., Spanbroek, R., Lottaz, C., Gautier, E.L., Frankenberger, M., Hoffmann, R., Lang, R., Haniffa, M., Collin, M., Tacke, F., Habenicht, A.J., Ziegler-Heitbrock, L., Randolph, G.J., 2010. Comparison of gene expression profiles between human and mouse monocyte subsets. Blood 115, e10–9.
Irwin, M.R., Cole, S.W., 2011. Reciprocal regulation of the neural and innate immune systems. Nat. Rev. Immunol. 11, 625–632.
Ji, J., Zoller, B., Sundquist, K., Sundquist, J., 2012. Increased risks of coronary heart disease and stroke among spousal caregivers of cancer patients. Circulation 125, 1742–1747.

Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J.C., Pariante, C.M., Pace, T.W., Mercer, K.B., Mayberg, H.S., Bradley, B., Nemeroff, C.B., Holsboer, F., Heim, C.M., Ressler, K.J., Rein, T., Binder, E.B., 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat. Neurosci. 16, 33–41.

Libby, P., Ridker, P.M., Hansson, G.K., 2011. Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317–325.

Lupien, S.J., McEwen, B.S., Gunnar, M.R., Heim, C., 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci. 10, 434–445.

Matthews, K., Gump, B.B., 2002. Chronic work stress and marital dissolution increase risk of posttrial mortality in men from the Multiple Risk Factor Intervention Trial. Arch. Intern. Med. 162, 309–315.

Miller, A.H., 2008. Inflammation versus glucocorticoids as purveyors of pathology during stress: have we reached the tipping point? Biol. Psychiatry 64, 263–265.

Miller, A.H., Maletic, V., Raison, C.L., 2009a. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol. Psychiatry 65, 732–741.

Miller, G.E., Chen, E., Sze, J., Marin, T., Arevalo, J.M.G., Doll, R., Ma, R., Cole, S.W., 2008. A genomic fingerprint of chronic stress in humans: blunted glucocorticoid and increased NF-κB signaling. Biol. Psychiatry 64, 266–272.

Miller, G.E., Chen, E., Fok, A., Walker, H., Lim, A., Nicholls, E.P., Cole, S.W., Kobor, M.S., 2009b. Low early-life social class leaves a biological residue manifest by decreased glucocorticoid and increased pro-inflammatory signaling. Proc. Natl. Acad. Sci. USA 106, 14716–14721.

Miller, G.E., Chen, E., Zhou, E.S., 2007. If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychol. Bull. 133, 25–45.

Miller, G.E., Cohen, S., Herbert, T.B., 1999. Pathways linking major depression and immunity in ambulatory female patients. Psychosom. Med. 61, 850–860.

Miller, G.E., Cohen, S., Ritchey, A.K., 2002. Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid resistance model. Health Psychol. 21, 531–541.

Nathan, C., Ding, A., 2010. Nonresolving inflammation. Cell 140, 871–882.

O'Donovan, A., Sun, B., Cole, S., Rempel, H., Lenoci, M., Pulliam, L., Neylan, T., 2011. Transcriptional control of monocyte gene expression in post-traumatic stress disorder. Dis. Markers 30, 123–132.

Pace, T.W., Heim, C.M., 2011. A short review on the psychoneuroimmunology of posttraumatic stress disorder: from risk factors to medical comorbidities. Brain Behav. Immun. 25, 6–13.

Pace, T.W., Hu, F., Miller, A.H., 2007. Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. Brain Behav. Immun. 21, 9–19.

Paffenbarger, R.S., Blair, S.N., Lee, I., Hyde, R.T., 1993. Measurement of physical activity to assess health effects in a free-living population. Med. Sci. Sports Exerc. 25, 60–70.

Pariante, C.M., Miller, A.H., 2001. Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. Biol. Psychiatry 49, 391–404.

Powell, N.D., Sloan, E.K., Bailey, M.T., Arevalo, J.M.G., Miller, G.E., Chen, E., Kobor, M.S., Reader, B.F., Sheridan, J.F., Cole, S.W., 2013. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via beta-adrenergic induction of myelopoiesis. Proc. Natl. Acad. Sci. USA 110, 16574–16579.

Radloff, L.S., 1977. The CES-D scale: a self-report depression scale for research in the general population. J. Appl. Psychol. Meas. 1, 385–401.

Raison, C.L., Capuron, L., Miller, A.H., 2006. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol. 27, 24–31.

Raison, C.L., Miller, A.H., 2003. When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. Am. J. Psychiatry 160, 1554–1565.

Rohleder, N., 2012. Acute and chronic stress induced changes in sensitivity of peripheral inflammatory pathways to the signals of multiple stress systems – 2011 Curt Richter Award Winner. Psychoneuroendocrinology 37, 307–316.

Rohleder, N., Marin, T.J., Ma, R., Miller, G.E., 2009. Biologic cost of caring for a cancer patient: dysregulation of pro- and anti-inflammatory signaling pathways. J. Clin. Oncol. 27, 2909–2915.

Sauer, J., Castren, M., Hopfner, U., Holsboer, F., Stalla, G.K., Arzt, E., 1996. Inhibition of lipopolysaccharide-induced monocyte interleukin-1 receptor antagonist synthesis by cortisol: involvement of the mineralocorticoid receptor. J. Clin. Endocrinol. Metab. 81, 73–79.

Schulz, R., Mendelsohn, A.B., Haley, W.E., Mahoney, D., Allen, R.S., Zhang, S., Thompson, L., Belle, S.H., 2003. End-of-life care and the effects of bereavement on family caregivers of persons with dementia. N. Engl. J. Med. 349, 1936–1942.

Schulz, R., Beach, S.R., 1999. Caregiving as a risk factor for mortality. J. Am. Med. Assoc. 282, 2215–2219.

Scrivo, R., Vasile, M., Bartosiewicz, I., Valesini, G., 2011. Inflammation as "common soil" of the multifactorial diseases. Autoimmun. Rev. 10, 369–374.

Sternberg, E.M., 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat. Rev. Immunol. 6, 318–328.

Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., Belanger, K., Brandes, A.A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R.C., Ludwin, S.K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J.G., Eisenhauer, E., Mirimanoff, R.O., 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N. Engl. J. Med. 352, 987–996.

Woollard, K.J., Geissmann, F., 2010. Monocytes in atherosclerosis: subsets and functions. Nat. Rev. Cardiol. 7, 77–86.