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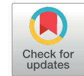
In vitro proinflammatory gene expression predicts *in vivo* telomere shortening: A preliminary study



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ABSTRACT

The chronic psychological stress of caregiving leads to higher risks for many diseases. One of the mechanisms through which caregiving is associated with disease risk is chronic inflammation. Chronic inflammation may accelerate cellular aging *via* telomere dysfunction and cell senescence, although this has not been examined in human cells from healthy people. We examined peripheral blood mononuclear cells (PBMCs) from 20 healthy mothers of children with autism (caregivers) and 19 mothers of neurotypical children (controls) in an *in vitro* culture system where PBMCs were stimulated with phytohaemagglutinin (PHA). We measured RNA expression levels of a panel of immune function genes before and after PHA stimulation, as well as telomere length from PBMCs collected from the participants at baseline and 15 months later. Caregivers and controls had similar gene expression profiles in unstimulated PBMCs, but after PHA stimulation, caregivers had increased RNA levels of the master inflammatory regulator NF-κB and its proinflammatory cytokine targets IL-1β, IL-6 and its receptor IL-6R as well as inflammatory chemokines IL-8, CXCL1 and CXCL2. Gene expression analysis suggested caregivers have increased Treg and Th17 T cell differentiation. Additionally, key signaling molecules involved in the upregulation of COX-2, a critical enzyme in the synthesis of the inflammatory mediator prostaglandin, were elevated. When both groups were examined together, higher expression levels of proinflammatory genes were associated with shorter telomere length in PBMCs from blood drawn 15 months later, independent of baseline telomere length. Taken together, these results suggest that chronic stress is associated with an exaggerated inflammatory response in PBMCs, which in turn is associated with shorter telomere length measured from PBMCs collected 15 months later. To our knowledge, this is the first human study that shows increased proinflammatory expression predicts future telomere shortening.

1. Introduction

Chronic psychological stress contributes to major diseases, including depression, cardiovascular disease, viral infection, and autoimmune diseases (Cohen et al., 2007). While detailed mechanisms of how chronic stress leads to increased risks for these various diseases are multiple, complex and still incomplete, a common underlying cause is chronic inflammation (Libby, 2007; Tracy, 2003). Clearly, acute and chronic stress have different effects. An elevated inflammatory response to acute stress leads to enhanced immunity, however, chronic stress is associated with impaired immune function with concomitant low-grade, unresolved inflammation, a critical contributor to pathogenesis of major diseases (Dhabhar, 2014; Nathan and Ding, 2010). Human disease processes are intricately tied with chronic stress-related processes that unfold over years and decades and may not be easily revealed through sampling of immune cells in a cross-sectional and static manner. *In vitro* studies in which immune cells are challenged with mitogens that mimic *in vivo* antigen stimulation may shed light on how inflammatory responses might interact with cell aging systems that unfold more slowly over time, such as telomere attrition.

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Telomeres are the protective end-complexes at the termini of eukaryotic chromosomes. Recent research points to a causal role for telomere attrition in major diseases and their risk factors (Blackburn et al., 2015). Chronic inflammation can potentially contribute to aging-related diseases through increased production of reactive oxygen species, which damage telomeres and lead to cellular senescence (Jurk et al., 2014). Senescent cells secrete proinflammatory proteins, thus reinforcing the inflammation in a positive feedback loop (Zhu et al., 2014). In humans, while various studies have shown an association between proinflammatory markers and shorter telomere length (Amsellem et al., 2011; Lopizzo et al., 2017; Masi et al., 2012; O'Donovan et al., 2011; Wolkowitz et al., 2010), the cross-sectional nature of these studies prevents conclusions about cause-effect relationship. To our knowledge, no study has reported whether inflammation predicts shorter telomere length over time in humans.

Recent studies have shown that caregiving stress, a well-characterized model of chronic psychological stress, is associated with increased proinflammatory and decreased anti-inflammatory pathway activity (Cohen et al., 2012; Damjanovic et al., 2007; Miller et al., 2008, 2002; Miller et al., 2014; Rohleder et al., 2009). In addition to elevation of classical proinflammatory mediators such as IL-6, TNF-α, C-reactive protein (CRP) and IL-1β (Hansel et al., 2010; Kiecolt-Glaser et al., 2003; Miller et al., 2008; Rohleder et al., 2009), genome-wide gene expression and bioinformatics analysis showed elevated levels of the proinflammatory master transcriptional regulator nuclear factor-kappa B (NF-κB) family and its target genes, as well as concomitant decreased levels of target genes regulated by glucocorticoids (Creswell et al., 2012; Miller et al., 2014). Most of these prior studies either focused on monocytes, an important cell type in the innate immune system critical for cytokine production, or found that monocytes were the primary source of altered gene expression in unstimulated PBMC populations. Less is known about how psychological stress impacts the T and B cells of the adaptive immune system. Some studies described a lower CD4+/CD8+ T cell ratio in people under chronic stress while others reported an increased ratio of CD4+/CD8+ (Herkenham and Kigar, 2016). A shift in the CD4 + T cell repertoire, from Th1 to Th2 cytokine production, has also been reported (Glaser et al., 2001).

While all parents of young children are caregivers technically, parenting a child with an autism spectrum disorder (ASD) is a case of extreme caregiving. Parental caregivers of children with ASD experience tremendous psychological stress due to the prolonged duration of caregiving and the psychological and social challenges the child, and thus the family, faces (Tint and Weiss, 2016). Subsequently autism caregivers encounter health problems including depression, anxiety and physical strain, fatigue and compromised memory (Black et al., 2013; Lovell et al., 2014; Miller et al., 2008; Seltzer et al., 2010; Smith et al., 2010). However, to date, studies of parental caregiving for a child with ASD focus predominantly on the psychological effects, and investigations into the impact of this chronic stress on the immune system are very scarce.

To address this gap in the literature, we measured RNA expression levels of proinflammatory pathways in PBMCs with and without phytohaemagglutinin (PHA) stimulation from chronically stressed mothers of children with autism (caregivers) and low stress mothers of neurotypical children who served as a control group. We further investigated whether gene expression levels of proinflammatory pathways predicted telomere length measured 15 months later, controlling for baseline telomere length.

2. Methods

2.1. Description of the cohort and blood draw procedures

The current study is a sub-study of a larger cohort of 180 premenopausal mothers who were caring for their child with an autism spectrum disorder or a neurotypical child. Detailed information on the cohort was described earlier (Lin et al., 2016). The UCSF Institutional Review Board for Human Research approved the study and all participants provided written informed consent. Participants in this *in vitro* substudy included the first 20 caregiving and first 20 control participants who were enrolled in the larger study. Participants had their blood drawn in the morning after overnight fasting into EDTA Vacutanier® tubes (BD Bioscience, San Jose, CA, USA), as previously described (Lin et al., 2016). Participants did not have any symptoms of illness the day before and day of, and rested for 30 min before the blood draw to reduce any stimulation from the psychological and physical stress of arrival at the hospital. PBMCs from the 40 participants were prepared from fresh blood collected in EDTA tubes using Ficoll gradient. Cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), cryopreserved in freezing medium (90% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO)) and stored in liquid nitrogen until *in vitro* culturing. This allowed cells to be treated in batches, under identical conditions. An aliquot of PBMCs was also saved as cell pellets in −80 °C for telomere length analysis as described later.

2.2. Cell culture

Cryopreserved PBMCs were thawed at 37 °C and washed with R10 (RPMI-1640 medium supplemented with 10% sterile heat-inactivated FBS, 1% L-glutamine, 1% sterile penicillin-streptomycin) containing 2 μg/ml DNase I (Sigma Aldrich, St. Louis, MO). Live cells were counted by Trypan Blue exclusion method. Samples used in subsequent analyses had greater than 93% viability. To examine unstimulated cells, aliquots of cells were saved for flow cytometry analysis for phenotyping and gene expression analysis. The remaining cells, used for stimulation, were resuspended in R10 at 1 million live cells/ml with 5 μg/ml of PHA (Sigma Aldrich, St. Louis, MO) and 2 ng/ml of IL-2 (Thermo Fisher Scientific, Waltham, MA) and cultured in 6-well tissue culture dishes (Thermo Fisher Scientific, Waltham, MA) for 5 days at 37 °C with 5% CO2 in a cell culture incubator. Live cells were counted after 5 days and aliquots of cells were saved for immunophenotyping by flow cytometry and gene expression analysis. We chose 5 days for stimulation based on our pilot work that showed PBMCs continue to grow for up to 7 days after PHA stimulation. The 5-days growth period allowed us to obtain sufficient cells for all the measurements. The cell culturing experiments were done in batches of 8 participants, 4 caregivers and 4 controls. All the experimental procedures described here and below were conducted by investigators without knowledge of caregiver status or other demographic data.

2.3. Flow cytometry analysis

Flow cytometry analysis was performed on both the unstimulated and stimulated PBMCs. 500,000 cells were stained at 4 °C for 15 min with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA) and fluorescent-conjugated monoclonal antibodies: V450-conjugated anti-CD3 (clone UCHT1), Allophycocyanin (APC)-conjugated anti-CD8 (clone SK1), APC-H7-conjugated anti-CD14 (clone MφP9), Peridinin Chlorophyll Protein Complex-Cy™5.5 (PerCp-Cy5.5)-conjugated anti-CD19 (clone HIB19), Phycoerythrin (PE)-conjugated anti-CD28 (clone L293), Fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (clone2D1) (all from BD Biosciences, San Jose, CA) and PE-Texas Red-conjugated anti-CD4 (clone S3.5) (Thermo Fisher Scientific, Waltham, MA). Stained cells were analyzed with a BD Biosciences LSR II cell analyzer. Data was analyzed with FlowJo software (version 10.1). Cells were classified as the following: monocytes (CD45+CD3-CD14+), CD4 + T cells (CD45+CD3+CD14-CD4+), CD8+CD28+ T cells (CD45+CD3+CD14-CD4-CD8+CD28+), CD8+CD28- T cells (CD45+CD3+CD14-CD4-CD8+CD28-) and B cells (CD45+CD14-CD3-CD19+).

2.4. Gene expression analysis

Total RNA was extracted from PBMCs (unstimulated from cryopreservation and stimulated) using RNeasy Plus Mini kits (QIAGEN, Hilden, Germany). The expression levels of a panel of 54 immune pathway genes were measured by NanoString technology and analyzed by nCounter SPRINT™ Profiler according to the manufacturer's instructions (Nanostring, Seattle, WA). In NanoString technology, each gene of interest is recognized by two sequence-specific probes, one of which is coupled to biotin for affinity purification, and the other is labeled with a unique barcode. The barcoding design allows measurement of up to several hundred targets in one hybridization reaction. The hybridized reaction products are immobilized on a sample cartridge by electrophoresis and the number of hybridized molecules for each gene is counted by imaging the cartridge (Geiss et al., 2008). This technology uses RNA without the need to convert to cDNA or amplification, this eliminating possible bias during these processes. Six housekeeping genes were included in each sample and the geometric mean of mRNA expression copies for these 6 genes was used to normalize the mRNA expression reads of each gene in each sample. The normalized data was used for all analyses.

2.5. Telomere length measurement

As described earlier, PBMCs were purified by Ficoll gradient and stored as dry pellets at −80 °C for batch DNA purification. The remaining PBMCs were cryopreserved and stored in liquid nitrogen for the cell culture experiments described above. Total genomic DNA was purified using the QIAamp® DNA Mini kit (QIAGEN, Hilden, Germany; Cat# 51104) in batches. DNA was stored at −80 °C for batch telomere length measurement. Telomere length was measured in PBMCs at baseline, the same visit for which the other measurements were performed, as well PBMCs collected 15 months later. Baseline and 15-month DNA samples from the same participant were assayed in the same batch.

Average relative telomere length was measured by quantitative PCR using a method adapted from the original published method by Cawthon (Cawthon, 2002; Lin et al., 2010) and expressed as the ratio of telomere abundance *vs*. a single copy gene (human β-globin) abundance (T/S ratios). Detailed descriptions of the telomere length assay used in the current study were published previously (Lin et al., 2016). The average coefficient of variation (CV) of this study was 2.3% (± 1.8%) (Lin et al., 2016). Lab personnel who performed the telomere length measurement were blind to the group status of each sample and all other demographic data.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 7.0a). D'Agostino-Pearson test was performed to test if the data sets (raw data and BMI-adjusted residuals) were normally distributed. In cases where data were normally distributed, we employed independent t-tests; however, a Welch correction was used when the SDs of the two groups were not equivalent. Mann-Whitney tests were carried out on data that were not normally distributed. Pearson product-moment correlations were used to explore associations between gene expression levels and telomere length. This study is an exploratory study that aimed to detect patterns in a range of gene expression profiles. This was done in a small initial sample size, and thus without adjusting for multiple comparisons. Results will be considered preliminary and necessary to replicate.

Multiple comparisons using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with false discovery rate Q = 5% as recommended by Graphpad Prism were also performed to compare the gene expression levels of caregiver *vs*. control mothers and results are presented in Supplemental Table S3.

Table 1
Participant demographics from thecurrent study (N = 39).

Characteristic	Caregivers (N = 20) Mean (SD) / %	Controls (N = 19) Mean (SD) / %
Age	43.2 (5.6)	42.7 (4.6)
BMI (kg/m^2)	26.6 (5.3)	23.5 (3.5)
Education, years	16.6 (2.2)	17.7 (2.4)
Household Income ($1,000)	134.1 (57.5)	168.4 (32.1)
Race/ ethnicity (%)		
Caucasian	80%	84%
Black	–	–
Hispanic or Latina	20%	11%
Asian, Pacific Islander, or Native American	–	5%

3. Results

3.1. Caregivers have higher perceived stress scores

As a sub-study of the larger cohort of 180 mothers that investigates the consequences of caregiving on cellular aging, we included the first 20 caregiving and first 20 control participants to examine the RNA expression levels of a panel of *a priori* selected 54 genes based on their key roles in proinflammatory immune function and regulation. These genes were chosen as they are key players of immune function and inflammation and some of them have been reported to be altered in chronic stress (Damjanovic et al., 2007; Glaser et al., 2001; Miller et al., 2008, 2014). One sample from a control participant had low cell viability after thawing from cryopreservation, and was therefore excluded from data analysis, resulting in a final sample of 20 caregivers and 19 controls. Table 1 describes the demographic characteristic of the participants in the current study. The sample was mostly white (82%), with a mean age of 43.0 (range: 31.2–51.6), mean BMI of 25.1 (range: 18.5–34.5) and the majority had completed college. Caregivers and controls did not differ significantly in educational level, nor in age [controls: mean (SD) = 42.7 (4.6), caregivers: mean (SD) = 43.2 (5.6), df = 37, t = -0.29, p = 0.771]. Age was not used as a covariate when comparing group differences (Table 2). However, caregivers had higher BMI values [controls: mean (SD) = 23.5 (3.5), caregivers: mean (SD) = 26.6 (5.3), df = 37, t = -2.10, p = 0.043] and BMI was examined in secondary analyses as a covariate (Table 2). As expected, caregivers had higher scores on the Perceived Stress Scale (Cohen et al., 1983) compared to controls (controls: mean (SD) = 14.05 (5.85), caregivers: mean (SD) = 20.83 (5.61), df = 36, t = 3.648, $p < 0.001$). In this sample, the caregiver duration ranges from 1.9 to 13.9 years (from diagnosis to baseline visit), with an average of 6.0 years (SD = 2.9 years).

3.2. PBMCs from caregivers have higher levels of proinflammatory cytokines and chemokines following PHA stimulation compared to controls

We used flow cytometry to phenotype PBMCs before and after PHA stimulation. A panel of fluorophore-conjugated antibodies was used to distinguish monocytes (CD45 + CD3-CD14 +), B cells (CD45 + CD14-CD3-CD19 +), CD4 + T cells (CD45 + CD3 + CD14-CD4 + CD8-) and CD8 + T cells (CD45 + CD3 + CD14-CD4-CD8 +). CD8 + T cells were separated into two types: those with the co-stimulatory receptor CD28 (CD45 + CD3 + CD14-CD4-CD8 + CD28 +) and those that have lost CD28 expression (CD45 + CD3 + CD14-CD4-CD8 + CD28-).

Flow cytometry analysis of unstimulated PBMCs showed no difference between caregivers and control mothers in the frequencies of CD4 + T cells, CD8 + CD28 + and CD8 + CD28- T cells, CD19 + B cells or CD14 + monocytes (Supplemental Fig. S1 A). After five days of PHA stimulation and culturing, the cell population was mainly composed of T cells with a minor proportion of B cells (Supplemental Fig. S1B).

Table 2
Effect sizes (Cohen's d) of gene expression differences between caregivers and controls: unadjusted and BMI-adjusted.

Gene	Cohen's d (unadjusted)	Cohen's d (BMI adjusted)
CD28	**0.97****	**0.76***
AKT	**0.74***	**0.79***
CEBPA	**0.68***	0.55
CEBPB	**0.73***	0.69
COX-2	**0.73***	0.51
CREB1	**0.92****	**0.85***
CXCL1	**0.90****	**0.73***
CXCL2	**0.97****	**0.76***
ETS1	**0.75***	**0.69***
Erk1	**0.71***	**0.66***
FOXP3	**0.72***	0.64
IFNγ	0.52	0.45
IL-10	0.37	0.41
IL-17A	0.62	0.40
IL-17C	**0.85***	**0.74***
IL-1β	**0.86****	**0.67***
IL-2	0.28	0.45
IL-4	0.25	0.25
IL-6	**0.96****	**0.78***
IL-6R	**0.91****	**0.84***
IL-8	**0.99****	**0.75***
NFκB	**1.05****	**0.92***
Osteopontin	**0.63****	0.45
PIK3CA	**0.99****	**0.91****
PIK3CD	**0.75***	**0.72***
RELA	**0.91****	0.75
TGFβ	0.67	0.61

#: Effect sizes without asterisks are not statistically significantly different between groups.
* $p < 0.05$.
** $p < 0.01$.

However, some differences in cell population emerged. Caregivers showed a lower percentage of B cells compared to controls and a higher percentage of CD8+CD28+ T cells (Supplemental Fig. S1B). The increase in CD8+CD28+ T cells is also revealed in higher mRNA expression levels of CD28 in the total population after stimulation (Supplemental Fig. S1C).

We selected a panel of 54 key genes involved in inflammation and immunity functions and measured the RNA expression levels in the unstimulated and stimulated PBMCs (Supplemental Table S1). While no group difference was seen in any of the 54 genes in the unstimulated PBMCs, caregivers differed from controls in a set of genes following PHA stimulation. We present these differences below in the context of gene function groups. The expression levels of 27 genes are discussed, with some genes differ between caregivers and controls, while others do not. First, consistent with earlier reports in monocytes (Miller et al., 2008, 2014), the expression levels of the major proinflammatory transcription factor NF-κB and its related protein RelA were increased in caregivers in the stimulated PBMCs (Fig. 1A). Target genes of NF-κB, proinflammatory cytokines IL-1β, IL-6 and its receptor IL-6R (Fig. 1B), inflammatory chemokines IL-8, CXCL1 and CXCL2, were also elevated in caregivers relative to controls (Fig. 1C). Taken together, these gene expression patterns suggest an exaggerated proinflammatory reactivity response in PBMCs from caregivers.

3.3. Caregivers have increased levels of Th17 and Treg cytokines after in vitro stimulation

Earlier work indicated that there is a shift from Th1 type to Th2 type CD4 + T cells in chronically stressed caregivers (Damjanovic et al., 2007; Glaser et al., 2001). We examined RNA expression levels of key elements for four major CD4 + T cell subsets: Th1, Th2, Treg and Th17 T cells. There was no statistically significant group difference in the expression levels of IL-2 and IFNγ, important cytokines secreted by the Th1 type CD4+ and CD8 + T cells, or IL-4, an important cytokine secreted by the Th2 type CD4 + T cells (Fig. 2A). The levels of IL-10, an anti-inflammatory cytokine (Kubo and Motomura, 2012) were also similar in caregivers and controls (Fig. 2A). Interestingly, IL-17 A and IL-17C, cytokines secreted by the Th17 type T cells, were increased in caregivers, with IL-17C achieving statistical significance (Fig. 2B). Osteopontin, a proinflammatory immune regulator that can upregulate IL-17 production (Zheng et al., 2012), was also increased in caregivers relative to controls (Fig. 2C) as was FOXP3, the master transcriptional factor for the regulatory T cells, and TGFβ, which is a cytokine involved in both the Th17 cell differentiation and regulatory T cell function (Fig. 2D). Taken together, caregivers exhibited an immune profile suggesting exaggerated response to stimulation resulting in increased Th17 and Treg CD4 + T cell differentiation, while Th1 and Th2 cytokine expression was similar to control participants.

3.4. PBMCs from caregivers have increased expression levels of COX-2 and genes in COX-2 signaling networks after in vitro stimulation

Cyclooxygenease-2 (COX-2) is a key, rate-limiting enzyme in the synthesis of prostaglandin, a major inflammatory mediator involved in many diseases and is upregulated during inflammation and downregulated by glucocorticoids (Crofford, 1997; Smith et al., 1996). While monocytes/macrophages are the main leukocyte cell type expressing COX-2, T cells are also reported to express it (Pablos et al., 1999). In our study, we observed no difference in COX-2 mRNA levels in the unstimulated PBMCs between caregiver and controls, but PBMCs stimulated by PHA from caregivers expressed higher levels of COX-2 mRNA (Fig. 3A). The expression levels of a list of genes involved in the intracellular signaling of COX-2 mRNA upregulation (Tsatsanis et al., 2006) were also increased in caregivers. These included: PI3 kinase C subunits A, D and Akt, mitogen-activated protein kinase (MAPK) Erk1 (Fig. 3B), and several transcriptional factors found at the COX-2 promoter CCAAT/enhancer-binding protein alpha and beta (CEBPA and CEBPB) and two transcriptional factors CREB1 and Est1 (Fig. 3C) (Tsatsanis et al., 2006).

3.5. Effects of BMI adjustment on gene expression

Given that caregivers have higher BMI than the control mothers on average, we also conducted group comparisons while covarying BMI. The differences remained for most genes, but not all. As summarized in Table 2, for most genes, the effect size was attenuated after BMI adjustment. Statistical difference remained for many genes, but for some genes, the attenuated effect size eliminated statistical significance. This analysis suggested that increased BMI in caregivers may serve as a partial link between caregiving stress and increased expression levels in these genes.

3.6. Expression levels of proinflammatory genes following in vitro stimulation are associated with shorter telomere length 15 months later

One of the downstream consequences of chronic inflammation is telomere dysfunction and cellular senescence. Although age-adjusted TL is shorter in caregivers *vs*. controls at both baseline and 15 month (Cohen's $d = 0.345$ $p = 0.306$ for baseline and Cohen's $d = 0.504$, $p = 0.214$ for 15 month), the difference does not reach statistical significance, probably due to small sample size. We examined whether the expression levels of the proinflammatory genes following PHA stimulation described above were associated with telomere length across the whole sample of 39 participants. Cross-sectionally, TL length is correlated with expression levels of three genes, Erk1, CEBPB and PIK3CA. Examination of the 15-month TL data showed a more prominent pattern of association of several, but not all, of the key inflammatory genes inversely correlated with telomere length in PBMCs collected 15 months later (Table 3). Because it was previously reported that

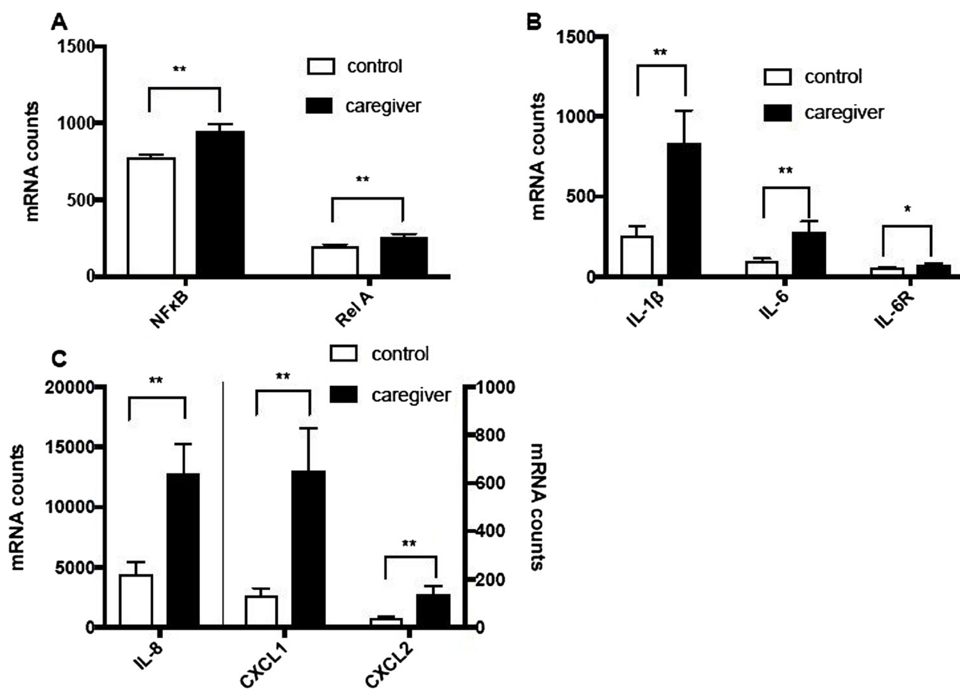

Fig. 1. PBMCs from caregivers have increased RNA levels of proinflammatory cytokines and chemokines. Average and standard deviation of the counts of RNA molecules in the caregivers and controls are shown. A. Increased RNA expression of the master proinflammatory transcriptional regulator NF-κB and its partner RelA in caregivers. B. Increased RNA expression of proinflammatory cytokines IL1-β, IL-6 and IL-6 receptor (IL-6R). C. Increased RNA expression of proinflammatory chemokines IL-8, CXCL1 and CXCL2. The Y axis for CXCL1 and CXCL2 is shown on the right. *$p < 0.05$, **$p < 0.01$.

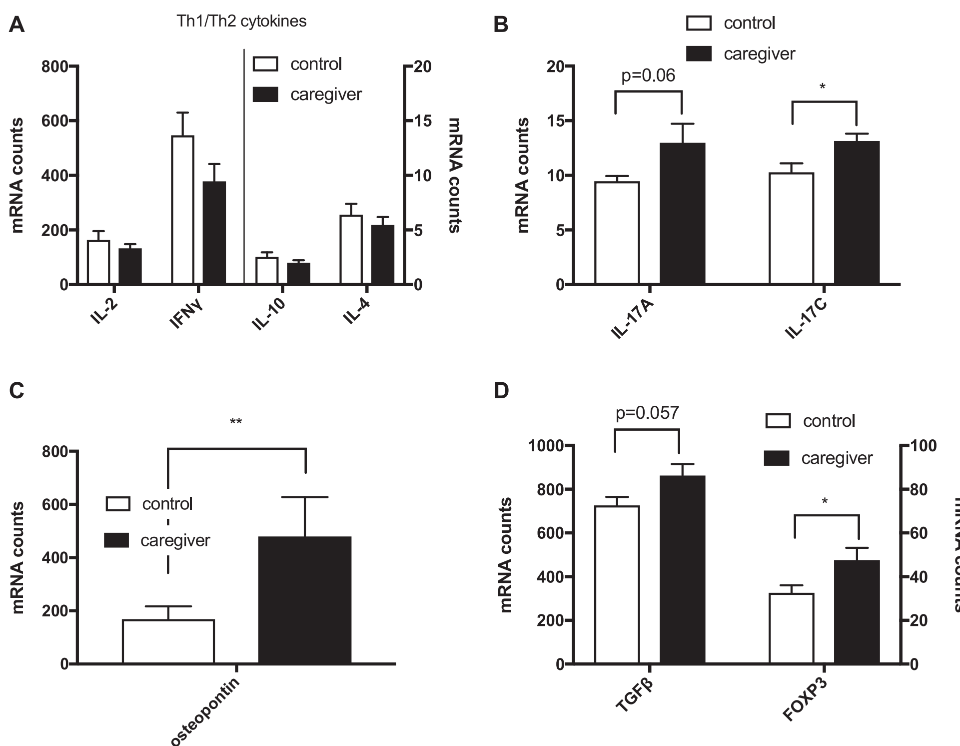

Fig. 2. Caregivers have increased RNA levels of Th17 and Treg cytokines. Average and standard deviation of the counts of RNA molecules in the caregivers and controls from the NanoString experiment are shown. A. No difference between caregiver and control in Th1 (IL-2, IFNγ) and Th2 (IL-4, IL-10) type cytokines. The Y axis for IL-10 and IL-4 is shown on the right. B. Increased levels of IL-17A and IL-17C in caregivers. C. Increased levels of osteopontin in caregivers. D. Increased levels of TGFβ and FOXP3 in caregivers. The Y axis for FOXP3 is shown on the right. *$p < 0.05$, **$p < 0.01$.

telomere length change negatively correlates with baseline telomere length (Aviv et al., 2009; Farzaneh-Far et al., 2010; Nordfjall et al., 2009), we examined the associations between expression levels of proinflammatory genes and 15-month telomere length after statistically adjusting for baseline telomere length. The results were unchanged (Supplemental Table S2).

4. Discussion

Chronic psychological stress is linked to systemic inflammation, an important contributor to major diseases of aging (Cohen et al., 2007). One potential mechanism associated with inflammation-related disease is accelerated telomere shortening. Although prior work has established key associations between stress-induced chronic inflammation and telomere shortening (Amsellem et al., 2011; Lopizzo et al., 2017; Masi et al., 2012; O'Donovan et al., 2011; Wolkowitz et al., 2010), no human study to date has reported a link between increased inflammation and shorter telomere length *in vivo* measured prospectively. Consistent with, and extending findings in monocytes of chronically stressed samples, we found that PBMCs of caregivers, as compared to controls, showed increased levels of gene expression in inflammation pathways including the master inflammation regulator NF-κB and its target

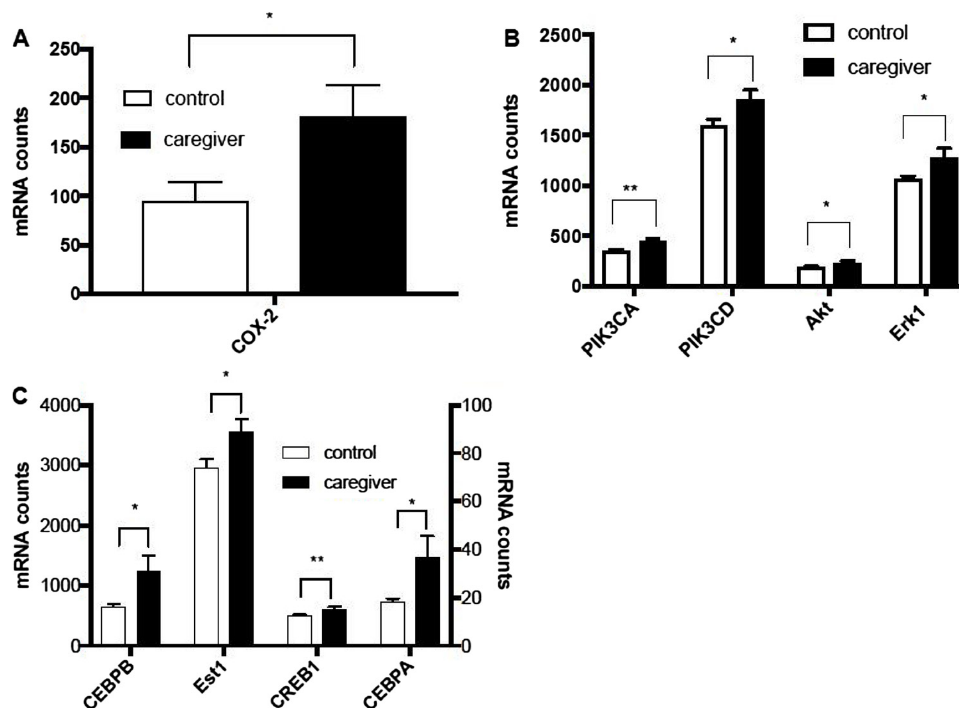

Fig. 3. Caregivers have increased RNA levels COX-2 and its signaling pathway genes. The Y axis for CEBPA is shown on the right. *p < 0.05, **p < 0.01.

Table 3
Pearson Correlations between key inflammatory genes related to chronic stress and PBMC Telomere length 15 months later.

Gene	Age-adjusted Baseline TL		Age-adjusted 15-month TL	
	Pearson correlation (r)	p value	Pearson correlation (r)	p value
NF-κB	-0.375	0.026	**-0.455 − 0.455**	**0.007****
IL-1β	− 0.240	0.165	**− 0.435**	**0.012***
IL-6	− 0.109	0.533	**− 0.351**	**0.042***
IL-6R	− 0.235	0.174	**− 0.404**	**0.018***
IL-8	− 0.305	0.075	**− 0.471**	**0.005****
IL-17C	− 0.237	0.171	**− 0.501**	**0.003****
Erk1	**− 0.368**	**0.03***	**− 0.442**	**0.009****
CREB1	− 0.314	0.066	**− 0.372**	**0.031***
CEBPA	− 0.242	0.161	**− 0.358**	**0.038***
CEBPB	**− 0.397**	**0.018***	**− 0.401**	**0.019***
PIK3CA	**− 0.338**	**0.047***	**− 0.430**	**0.011***
CD28	− 0.169	0.331	**− 0.363**	**0.035***
PIK3CD	− 0.228	0.187	− 0.325	0.061
ETS1	− 0.162	0.353	− 0.313	0.072
RELA	− 0.236	0.173	− 0.298	0.087
AKT	− 0.287	0.094	− 0.337	0.051
COX2	− 0.098	0.576	− 0.305	0.08
CXCL1	− 0.138	0.431	− 0.337	0.052
CXCL2	− 0.156	0.371	− 0.315	0.07
IL-17A	0.020	0.91	− 0.16	0.366
Osteopontin	0.074	0.672	− 0.296	0.090
IFNγ	− 0.222	0.200	− 0.156	0.377
IL10	− 0.120	0.493	− 0.094	0.597
IL17A	0.0198	0.91	− 0.16	0.366
IL2	− 0.056	0.749	0.259	0.139
IL4	− 0.115	0.51	− 0.0716	0.688
TGFβ	− 0.227	0.19	− 0.332	0.055

* $p < 0.05$.
** $p < 0.01$.

proinflammatory cytokines and chemokines following stimulation by PHA. Further, the caregiver group expressed higher levels of genes that are key to the Th17 subset, a T cell type linked to depression (reviewed in (Slyepchenko et al., 2016)). RNA expression of several of these inflammatory genes was correlated with shorter telomere length in PBMCs collected from the participants 15 months later, independent of baseline telomere length. These novel findings demonstrate prospectively that the exaggerated inflammatory response of immune cells predicts shorter telomeres long-term *in vivo*.

Previous research has demonstrated that the chronic stress of caregiving is linked to diminished immune function marked by reduced antibody titers to vaccination (Gallagher et al., 2009; Glaser et al., 2000; Li et al., 2007), less proliferation of PBMCs when stimulated with mitogen *in vitro* (Castle et al., 1995; Damjanovic et al., 2007) and elevated circulating proinflammatory cytokines IL-6 and TNF-α (Castle et al., 1995; Damjanovic et al., 2007). Extending these earlier findings, we found increased mRNA expression of key candidate genes for inflammatory pathways in our sample of high stress caregivers of children with autism spectrum disorders compared to low stress control mothers of neurotypical children. These elevated genes include the master inflammation transcriptional factor NF-κB and its associated protein RelA, cytokines IL-1β, and IL-6 the IL-6 receptor, IL-6R, as well as chemokines IL-8, CXCL1 and CXCL2.

Several studies have examined how psychological stress impacts T cell polarization and found a shift from Th1to Th2 cytokine production. In caregivers of dementia patients, the frequency of IL-10-expressing cells was higher in lymphocytes obtained from caregivers than control subjects, while the frequency of IL-2+ and IFNγ+ (Th1 type) cells did not differ between caregivers and controls (Glaser et al., 2001). *In vitro* stimulated PBMCs from dementia caregivers expressed more TNF-α and IL-10, but levels of IL-2, IL-4, IL-6, IL-8, GM-CSF, and IFNγ were not different (Damjanovic et al., 2007). In another study, stimulated PBMCs from adults who presumably had higher prenatal stress exposure were shown to have higher levels of IL-4, IL-6, IL-10, but lower levels of IFNγ (Entringer et al., 2008). In the current study, while there was no difference between caregivers and controls in mRNA expression levels of Th1 cytokines (IL-2 and IFNγ) and Th2 cytokines (IL-4), caregivers expressed higher mRNA levels of IL-17 A and IL-17C, cytokines specific to the Th17 T cell types, and FOXP3, a master transcription factor for regulatory T cells. Additionally, TGFβ, a cytokine involved in differentiation of both Th17 and Treg cells, was increased in caregivers compared to controls. While the normal functions of Th17 T cells are to

fight extracellular bacteria and fungi infection (Onishi and Gaffen, 2010), they also play an important role in the pathophysiology of an array of diseases including major depressive disorder (MDD) (Slyepchenko et al., 2016). As chronic stress is often the precursor of MDD, it will be interesting to determine whether IL-17 plays a role in stress-induced depression. Some of these findings were diminished when adjusting for BMI, suggesting that stress related adiposity may be a partial mechanism for the Th17 pathway, but most findings remained significant.

The role of psychological stress in regulatory T cells has been reported by several groups. In rodent models, chronic stress is associated with increased expression FOXP3 RNA as well as TGFβ RNA (Hong et al., 2013) and increased proportions of Treg cells (Rangassamy et al., 2016). In humans, prior findings are inconsistent. While one paper reported decreased Treg cells in young healthy males undergoing a mental stressor (Freier et al., 2010), increased Treg cells were observed in older men and women and were associated with inflammatory and neuroendocrine responses to acute psychological stress and poorer health status (Ronaldson et al., 2016). Our findings of increased TGFβ and FOXP3 RNA expressions are consistent with the rodent models and the Ronaldson et al report. The increased levels of Treg cells, concomitant with the increased proportions of Th17 may indicate a defense mechanism against increased inflammation, as suggested by Ronaldson et al (Ronaldson et al., 2016).

Loss of the CD28 costimulatory molecule, shortened telomeres and elevated production of pro-inflammatory cytokines are associated with T cell senescence (Chou and Effros, 2013). Given the prior reported association of short telomere and psychological stress, we expected that caregivers may have a higher percentage of CD8 + CD28- T cells. However, there was no difference in the percentage of CD8 + CD28 + or CD8 + CD28- T cells between caregivers and controls in the unstimulated PBMCs. This could be due to the small sample size, or the relative young age of our study population. Surprisingly, we observed an increase in the proportion of CD8 + CD28 + T cells in caregivers compared to control mothers after 5 days of *in vitro* stimulation and culturing. This is also revealed in higher RNA expression levels of CD28 in the total population after stimulation. Since the percentages of cell types were only measured at one time point (5 days after PHA stimulation), it is not clear how the CD8 + CD28 + T cell proportion changes during long term *in vitro* culturing.

Another novel finding in our study is the increased COX-2 signaling pathway in caregivers following *in vitro* stimulation. As the rate-limiting enzyme in the production of prostaglandin, a central inflammatory mediator involved in many diseases, expression of COX-2 is induced by proinflammatory stimuli such as IL-1β and IL-6, and regulated by several transcription factors including the cyclic-AMP response element binding protein (CREB1) and NF-κB (Tsatsanis et al., 2006). In the current study, we found increased mRNA expression following *in vitro* stimulation in several key molecules in the COX-2 pathway including IL-1β, IL-6, Erk1, CREB1, NF-κB/RelA, and Est 1. Prostaglandin E2 (PGE2) regulates multiple aspects of immune response (Kalinski, 2012). Notably, PGE2 promotes Th17 mediated immunity (Kalinski, 2012), which is consistent with our findings of increased expression of Th17 mediators IL-17 A and IL-17C.

Psychological stress is associated with lowered pain sensation thresholds and enhanced pain perception (Lightman, 2008). Prostaglandins sensitize peripheral sensory nerve endings at the site of inflammation to transmit pain signals. Induction of COX-2 worsens pain perception to hyperalgesia levels (Johnson and Greenwood-Van Meerveld, 2014). Although our results were with PBMCs, thus do not directly address the expression level of COX-2 expression at the peripheral sensory nerve or spinal cord, they do suggest a possible mechanism of enhanced pain perception experienced in chronic stress.

Telomere shortening induced replicative senescence is one of multiple pathways that have been proposed as contributors to chronic inflammation. Senescent cells secrete an array of inflammatory cytokines, chemokines, growth factors, and proteases known as the senescence associated secretory phenotype (SASP) (Zhu et al., 2014). Interestingly, recent work from a mouse model where systematic chronic inflammation was induced by knocking out one repressive subunit of the NF-κB pathway (Jurk et al., 2014) showed accumulation of telomere dysfunctional senescent cells, suggesting that chronic inflammation can lead to telomere dysfunction. This bi-directional relationship between chronic inflammation and telomere dysfunction has not been fully explored in humans despite reports of cross-sectional correlations of inflammation marker and shorter telomere length by various groups. Our finding that mRNA expression levels of multiple inflammatory genes following *in vitro* stimulation predict shorter telomere length 15 months later suggests that elevated inflammation in response to typical immune challenges and possibly stressors (sterile immune responses) may contribute to future telomere shortening. Increased immune cell proliferation and/or reactive oxygen species (ROS) are potential mechanisms of how chronic inflammation may promote telomere shortening and need to be further investigated.

Our study has several limitations. This study is a pilot study with a small sample size of 20 caregivers and 19 controls. We undertook a statistical approach aimed at exploratory discoveries, namely, we compared groups for each individual gene for a list of 54 preselected immune function genes without correcting for potential false discovery rate. However, our findings that multiple genes that belong to the same pathway, especially for proinflammation genes and the COX-2 pathway, are simultaneously elevated strengthen the provisional conclusion that these changes are biologically meaningful despite the small sample size and exploratory-nature of the study. Further, the lack of gene expression difference in the unstimulated cells between caregivers and controls argues favorably that the differences seen in stimulated cells are not due to type I error. However, the possibility of type I errors cannot be completely ruled out. The differential expressions (or lack thereof) of specific proinflammatory genes and pathways between high and low stressed adults needs to be replicated in larger studies to confirm the conclusions of the current study. We performed the multiple comparisons using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with false discovery rate Q = 5% as recommended by Graphpad Prism. This analysis showed that a list of 13 gene remained significant after correction for false discovery rate. We present the multi-comparison results as Supplemental Table S3. Due to the small sample size, we analyzed and presented the data based on caregiver status (mothers of autistic children *vs*. control mothers), whether the chronicity of the caregiving stress is associated the expression levels of the inflammation genes reported here needs to be examined in a larger study. As a preliminary study, we chose a selected list of key genes in inflammation and immunity for gene expression analysis. Thus, the study lacks the comprehensiveness of whole transcriptome analysis. Our experimental setting is an *in vitro* culture system where PBMCs were stimulated with the mitogen PHA to mimic non-specific stimuli. The purpose was to examine the full stimulatory potential in these cells regardless of the type of stimulus. This allowed us to discover gene expression differences that were not detected with unstimulated PBMC. But to be relevant to humans, the results need to be confirmed by research in *in vivo* settings such as infections. The caregivers have higher BMI, and this appears to partially explain some variance in the pattern of findings. Whether and how BMI mediates the difference in gene expression levels between caregivers and controls would need to be reexamined in larger samples with a larger range of BMI. Additionally, the study did not control for menstrual cycle stage, which can potentially influence gene expression patterns (Groothuis et al., 2007). Finally, we only measured mRNA levels. Analysis at the protein levels and functional assays is needed to further investigate the significance of the mRNA levels changes we observed. Nevertheless, the novel findings presented here serve as the first step towards a better understanding of the complex molecular changes associated with chronic stress.

5. Conclusions

Gene expression analysis of a panel of inflammation and immune function genes in PBMCs from mothers of autistic children (caregivers) and mothers of neurotypical children (controls) in an *in vitro* culture system showed that caregivers have increased mRNA levels in a list of inflammation genes that include the master inflammatory regulator NF-κB and its proinflammatory targets, Th17 T cell differentiation and COX-2 pathway. Higher expression levels of proinflammatory genes were associated with shorter telomere length in PBMCs from blood drawn 15 months later, even after adjusting for baseline levels. These results provide preliminary evidence that chronic stress of caregiving is associated with an exaggerated inflammatory response in PBMCs, which in turn is associated with shorter telomere length measured from PBMCs collected prospectively.

Declaration of interest

Jue Lin is a co-founder of Telomere Diagnostics Inc. and serves on its scientific advisory board. The company plays no role in the current study. The other authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.06.020.

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