

# Stress-induced modulation of NF- $\kappa$ B activation, inflammation-associated gene expression, and cytokine levels in blood of healthy men

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## A B S T R A C T

Acute psychosocial stress stimulates transient increases in circulating pro-inflammatory plasma cytokines, but little is known about stress effects on anti-inflammatory cytokines or underlying mechanisms. We investigated the stress kinetics and interrelations of pro- and anti-inflammatory measures on the transcriptional and protein level.

Forty-five healthy men were randomly assigned to either a stress or control group. While the stress group underwent an acute psychosocial stress task, the second group participated in a non-stress control condition. We repeatedly measured before and up to 120 min after stress DNA binding activity of the pro-inflammatory transcription factor NF- $\kappa$ B (NF- $\kappa$ B-BA) in peripheral blood mononuclear cells, whole-blood mRNA levels of NF- $\kappa$ B, its inhibitor I $\kappa$ B $\alpha$ , and of the pro-inflammatory cytokines interleukin (IL)-1 $\beta$  and IL-6, and the anti-inflammatory cytokine IL-10. We also repeatedly measured plasma levels of IL-1 $\beta$ , IL-6, and IL-10.

Compared to non-stress, acute stress induced significant and rapid increases in NF- $\kappa$ B-BA and delayed increases in plasma IL-6 and mRNA of IL-1 $\beta$ , IL-6, and I $\kappa$ B $\alpha$  ( $p$ 's < .045). In the stress group, significant increases over time were also observed for NF- $\kappa$ B mRNA and plasma IL-1 $\beta$  and IL-10 ( $p$ 's < .055). NF- $\kappa$ B-BA correlated significantly with mRNA of IL-1 $\beta$  ( $r = .52$ ,  $p = .002$ ), NF- $\kappa$ B ( $r = .48$ ,  $p = .004$ ), and I $\kappa$ B $\alpha$  ( $r = .42$ ,  $p = .013$ ), and marginally with IL-6 mRNA ( $r = .31$ ,  $p = .11$ ). Plasma cytokines did not relate to NF- $\kappa$ B-BA or mRNA levels of the respective cytokines.

Our data suggest that stress induces increases in NF- $\kappa$ B-BA that relate to subsequent mRNA expression of pro-inflammatory, but not anti-inflammatory cytokines, and of regulatory-cytoplasmic-proteins. The stress-induced increases in plasma cytokines do not seem to derive from de novo synthesis in circulating blood cells.

### Keywords:

Stress  
TSST  
mRNA  
Cytokines  
Interleukin-1 $\beta$   
Interleukin-6  
Interleukin-10  
NF- $\kappa$ B  
I $\kappa$ B $\alpha$

## 1. Introduction

Acute psychosocial stress is a potent stimulus of transient increases in peripheral inflammatory markers with presumed consequences for long-term inflammatory regulation and health (Rohleder, 2014). The most consistent transient increases were

found for the two pro-inflammatory cytokines interleukin (IL)-6 and, to a lesser extent, IL-1 $\beta$ , both measured in plasma 30–120 min following acute psychosocial stress (Rohleder, 2014; Steptoe et al., 2007). Notably, inflammatory cytokines are glycosylated polypeptides secreted by immune but also a range of other human cells. Compared to pro-inflammatory cytokines, considerably less is known regarding stress-related changes in anti-inflammatory cytokines, such as IL-10. To date, human studies investigating IL-10 responses to acute psychosocial stress are few in number and show contradictory results. While one study reported increases in IL-10 plasma levels 30 min after acute

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psychosocial stress in young women (Altemus et al., 2001), two other studies found plasma levels of IL-10 to not significantly change for a period of up to 120 min post-stress in young women (Fredericks et al., 2010) and in a gender-mixed group of middle-aged participants (de Brouwer et al., 2014).

The mechanisms underlying the well-documented stimulating effects of acute psychosocial stress on pro-inflammatory plasma cytokines are beginning to be understood. Studies in ex vivo isolated human peripheral mononuclear immune cells (PBMCs) consistently revealed an intracellular level rapid and strong increases in deoxyribonucleic acid (DNA) binding activity of the inflammatory transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) with peak levels occurring 10 min after acute psychosocial stress (Bierhaus et al., 2003; Pace et al., 2006; Wolf et al., 2009) although not unequivocally so (Pace et al., 2006). Notably, activated NF- $\kappa$ B promotes the expression of numerous genes, including genes coding for the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 (Pahl, 1999), by binding to specific DNA sequences. It follows from these studies that stress-induced NF- $\kappa$ B-regulated inflammatory gene expression may provide a mechanism by which stress affects plasma levels of IL-1 $\beta$  and IL-6. In support of this notion, Bierhaus et al. (2003) reported dose-dependent increases in both NF- $\kappa$ B binding activity to DNA and NF- $\kappa$ B-regulated gene expression of IL-6 (i.e. messenger ribonucleic acid, mRNA) in cells of the human acute monocytic leukemia (THP1) cell line after stimulation with physiological concentrations of norepinephrine. Further support comes from the hitherto only human study which quantitatively assessed the effects of acute psychosocial stress on mRNA levels of ex vivo isolated PBMCs (Brydon et al., 2005). In that study, stress-induced increases in mRNA levels of IL-1 $\beta$ , but not IL-6, were found in PBMCs of healthy men, with highest IL-1 $\beta$  mRNA gene expression observed 120 min after stress. In agreement with this, increased expression of genes regulated by the inflammatory transcription pathways of NF- $\kappa$ B and Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) were observed 30 min after acute psychosocial stress as compared to baseline in ex vivo isolated PBMCs of ten healthy men using genome-wide microarrays (Nater et al., 2009). However, to the best of our knowledge, no human study has previously examined stress reactivity of both NF- $\kappa$ B binding activity to DNA and inflammatory cytokine gene expression simultaneously. Accordingly, no studies have been testing associations between stress-induced changes in NF- $\kappa$ B DNA binding activity, cytokine mRNA, and plasma levels of inflammatory cytokines in humans.

We therefore investigated in healthy men the effects of acute psychosocial stress vs. a non-stress control condition on repeatedly measured NF- $\kappa$ B DNA binding activity. In addition, we repeatedly assessed mRNA and plasma levels of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6, and of the anti-inflammatory cytokine IL-10 up to 120 min after stress/non-stress cessation. We primarily aimed to elucidate the stress kinetics of each inflammatory measure alone and in relation to other measures. To further explore underlying mechanisms of stress-induced inflammatory activity, we additionally analyzed mRNA levels of NF- $\kappa$ B and inhibitory kappa-b ( $\text{I}\kappa\text{B}$ ) $\alpha$  as the inhibitor of NF- $\kappa$ B activation (Baldwin, 1996). The sampling interval for every inflammatory measure was chosen based on previous stress studies and in part further clarified by kinetic testing. Our main hypotheses were that (1) acute psychosocial stress induces rapid increases in NF- $\kappa$ B DNA binding activity, as well as delayed increases in both mRNA and plasma levels of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6, and that (2) direct associations are found of highest levels of NF- $\kappa$ B DNA binding activity with mRNA and plasma proteins of IL-1 $\beta$  and IL-6, as well as of mRNA of pro-inflammatory cytokines with plasma levels of the respective cytokine.

## 2. Materials and methods

### 2.1. Study participants

This study has been conducted within a project assessing the stress reactivity of immune measures (Kuebler et al., 2013). For this part of the project, we recruited a total of 45 healthy men between 20 and 50 years of age. We assigned 25 participants to the stress and 20 participants to the control group. Group assignment was performed randomly and age-matched ( $\pm 2$  years) until the control group was complete. Participants were in good physical and mental health, as confirmed by a telephone interview. Explicit exclusion criteria were: regular exercise more than 12 h a week, smoking, alcohol and illicit drug abuse, any heart disease, varicosis or thrombotic diseases, elevated blood sugar and diabetes, elevated cholesterol, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, and current infectious diseases. If the personal or medication history was not conclusive, the participants' primary care physician was contacted for verification.

The Ethics Committee of the State of Zurich, Switzerland, formally approved the research protocol. All participants provided written informed consent before participating in the study. Recruitment was carried out with the aid of the Swiss Red Cross of the Canton of Zurich and through advertisements.

### 2.2. Study protocol

Participants of the stress and control group reported to the laboratory by 10 a.m. and had to abstain from extensive physical exercise, alcohol, and caffeinated beverages during the previous 24 h. Participants were given calorically standardized food with comparable amounts of macro-nutritional composition (i.e. fat, protein, and carbohydrate) and an indwelling venous catheter was inserted. After a resting period of 165 min, participants of the stress group were exposed to the TSST (Kirschbaum et al., 1993), which comprises a short introduction followed by a 5-min preparation period, a 5-min mock job interview, and a 5-min mental arithmetic task (serial subtraction) in front of an unknown panel of two persons. Participants of the control group were not exposed to the TSST but were required to stand in an empty room for 10 min. Before and after TSST/non-stress, participants remained seated in a quiet room.

Blood samples for NF- $\kappa$ B binding activity measurements were obtained immediately before (serving as baseline levels) and 1, 10, 20, 60, 90, and 120 min after TSST/non-stress cessation. We decided for these time points to assess the immediate peak as previously published (Bierhaus et al., 2003; Wolf et al., 2009) but also potential increases beyond 60 min after stress cessation since this has not previously been investigated. In a testing sample of seven participants, blood samples for mRNA measurements were taken immediately before (baseline) and 10, 20, 60, 90, and 120 min after TSST cessation to test for the stress kinetics of IL-1 $\beta$  gene expression reported by Brydon et al. (2005) in more detail. Based on the results of this kinetics testing ( $n = 7$ ; see Fig. 3A), we determined mRNA gene expression of IL-1 $\beta$ , IL-6, IL-10, NF- $\kappa$ B, and  $\text{I}\kappa\text{B}$  $\alpha$  in all participants immediately before (baseline), and 60 and 120 min after TSST/non-stress cessation. To determine protein plasma levels of the cytokines IL-1 $\beta$ , IL-6, and IL-10, blood samples were taken immediately before (baseline) and 10, 60, 90, and 120 min after TSST/non-stress cessation. For determination of salivary free cortisol levels, samples of saliva were collected immediately before (baseline) and 1, 10, 20, 30, 45, and 60 min after TSST/non-stress cessation. At the end of the experiment, participants

were debriefed and participation was remunerated with 175 Swiss Francs.

### 2.3. Biochemical analyses

Biochemical analyses were performed in the Biochemical Laboratory of the Institute of Psychology of the University of Zurich. In terms of immunoassays all samples of a person were analyzed on the same microtiter plate.

#### 2.3.1. NF- $\kappa$ B binding activity to DNA

To determine NF- $\kappa$ B binding activity to DNA, we used an adapted version of the Nuclear Extract Kit (Active Motif, La Hulpe, Belgium) in combination with the TransAM NF $\kappa$ B p65 Transcription Factor Assay Kit (Active Motif, La Hulpe, Belgium). The adapted Nuclear Extract Kit version was used to prepare whole cell extracts since this allows for quantification of activated NF- $\kappa$ B in the nucleus and in the cytoplasm, and thus for complete quantification of a cell's activated NF- $\kappa$ B. Notably, the TransAM assay is based on double-stranded oligonucleotides with NF- $\kappa$ B consensus binding sites in combination with enzyme-labeled NF- $\kappa$ B specific antibodies. Active but not inactive NF- $\kappa$ B obtained in whole-cell extracts binds to these oligonucleotides and can be subsequently detected.

In detail, 9 ml of blood were collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany), immediately layered on top of 10 ml Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden), and centrifuged for 20 min at 300g and 20 °C. After centrifugation, the interface containing PBMCs was removed, washed twice in ice-cold phosphate buffer saline (PBS; Sigma-Aldrich, Buchs, Switzerland), resuspended in 1000  $\mu$ l ice-cold PBS/Phosphatase Inhibitors (Active Motif, La Hulpe, Belgium), and cells were counted using a cell counter (KX-21N, Sysmex Digitana AG, Horgen, Switzerland). Next,  $3.0 \times 10^6$  ml<sup>-1</sup> PBMCs were transferred into a 1.5 ml pre-chilled tube and centrifuged in a microcentrifuge (Heraeus Biofuge fresco, Thermo Fisher Scientific, Reinach, Switzerland) for 4 min at 8100 rpm and 4 °C. After centrifugation, the supernatant was discarded and the cell pellet was stored at -80 °C for 30 min. Next, the cell pellet was resuspended in 300  $\mu$ l Complete Lysis Buffer (Active Motif, La Hulpe, Belgium) and vortexed for 10 s at highest setting. After 30 min at -80 °C, the resulting suspension was incubated for 60 min on ice on a rocking platform set at 150 rpm and vortexed every 5 min for 15 s at highest setting. Subsequently, the suspension was centrifuged for 20 min at 14,000 rpm and 4 °C. The supernatant containing the resulting cell extract was transferred into a pre-chilled 1.5 ml tube and stored at -80 °C until analysis with the TransAM NF $\kappa$ B p65 Transcription Factor Assay Kit according to the manufacturer's protocol.

NF- $\kappa$ B binding activity was determined in 20 participants of each subject group (i.e. stress and control group) following implementation of the assay procedure. One participant of the stress group and one participant of the control group had to be excluded because of technical problems with assaying, rendering a final sample of 19 participants of the stress group and 19 participants of the control group for NF- $\kappa$ B DNA binding activity measurements.

#### 2.3.2. Gene expression

For quantification of mRNA levels of IL-1 $\beta$ , IL-6, IL-10, NF- $\kappa$ B, and I $\kappa$ B $\alpha$ , venous blood was drawn in PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) and stored at -80 °C until analysis. Total RNA purification was performed using the PAXgene Blood RNA Kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland), followed by reverse transcription with High Capacity RNA-to-cDNA Kit (Applied Biosystems, Zug, Switzerland). The

quantitative real-time (RT)-PCR was conducted using TaqMan Fast Advanced Master Mix (Applied Biosystems, Zug, Switzerland) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Zug, Switzerland). mRNA was measured using the following TaqMan Gene Expression Assays (Applied Biosystems, Zug, Switzerland): Hs01555410\_m1 for IL-1 $\beta$ , Hs00985639\_m1 for IL-6, Hs00961622\_m1 for IL-10, Hs00765730\_m1 for NF- $\kappa$ B, Hs00153283\_m1 for I $\kappa$ B $\alpha$ , and Hs99999905\_m1 for GAPDH. GAPDH was amplified as reference gene. Relative gene expression was calculated by using the  $2^{-\Delta\Delta C_T}$  method (Schmittgen and Livak, 2008). All kits and assays were performed according to the manufacturers' protocol.

GAPDH mRNA levels of four participants of the stress group could not be determined due to technical problems. Moreover, in three participants of the stress group and two participants of the control group gene-specific detection problems occurred, rendering the following sample sizes for mRNA analyses (stress vs. control group): IL-1 $\beta$ , 21 vs. 20; IL-6, 18 vs. 18; IL-10, 18 vs. 20; NF- $\kappa$ B, 21 vs. 20; and I $\kappa$ B $\alpha$ , 21 vs. 20.

#### 2.3.3. Cytokines

For quantification of the cytokines IL-1 $\beta$ , IL-6, and IL-10, venous blood was drawn in EDTA-coated monovettes (Sarstedt, Numbrecht, Germany), and immediately centrifuged for 10 min at 2000g and 4 °C. Obtained plasma was stored at -80 °C until analysis. Plasma cytokine levels were determined with a high sensitivity sandwich immunoassay (Meso Scale Discovery (MSD), Rockville, USA) with intra- and inter-assay coefficients of variance (CVs) of 4.1% and 7.7% for IL-1 $\beta$ , 4.5% and 7.3% for IL-6, and 3.7% and 10.1% for IL-10. Detection limits were as follows: IL-1 $\beta$ : 0.041 pg/ml, IL-6: 0.117 pg/ml, IL-10: 0.046 pg/ml. Values below sensitivity were substituted with half the detection limit where appropriate.

Due to technical problems with assaying, data of IL-1 $\beta$  and IL-6 were missing in some participants rendering the following sample sizes for cytokine analyses (stress vs. control group): IL-1 $\beta$ , 21 vs. 16; IL-6, 23 vs. 20; and IL-10, 25 vs. 20. Notably, IL-1 $\beta$  levels were very low and without substitution of levels below the detection limit we could only obtain complete and measurable IL-1 $\beta$  levels in 18 participants from the stress group and in 13 participants from the control group. Therefore, IL-1 $\beta$  plasma results are to be interpreted with caution.

#### 2.3.4. Cortisol

For cortisol assessment, saliva samples were collected in salivettes (Sarstedt, Sevelen, Switzerland) and stored at -20 °C until analysis in the Biochemical Laboratory, Institute of Psychology, University of Zurich. Thawed saliva samples were centrifuged at 2000g, yielding low-viscosity saliva. Salivary free cortisol concentrations were determined using a commercial chemiluminescence immunoassay with high sensitivity of 0.16 ng/ml (LIA) (IBL, Hamburg, Germany). Inter- and intra CVs were below 11.5% and 7.7%, respectively.

### 2.4. Statistical analyses

Data were analyzed using SPSS Inc. version 20.0 for Mac OS X (Chicago, IL, USA) and presented as mean  $\pm$  SEM. All tests were two-tailed with the significance level set at  $p \leq .05$  and the level of borderline significance set at  $p \leq .10$ .

G\*power 3.1 analysis suggested that a total sample size of  $N=40$  is needed to detect an interaction between group and repeated inflammatory parameters with an expected medium effect size varying between  $f=0.22$  (3 repetitions, mRNA expression, observed minimum inter-correlation among repeated measures of  $r=.40$ ) and  $f=0.20$  (5 repetitions, plasma cytokine

levels, observed minimum  $r = .34$ ) in general linear models with repeated measures with  $\alpha = .05$  and a power of .80.

We calculated body mass index (BMI) as the weight in kilograms divided by height in meters squared. Since platelets are not negligible as a source of NF- $\kappa$ B (Schattner, 2013), NF- $\kappa$ B binding activity levels were corrected for the ratio of platelet to leukocyte numbers (WBC/PLT ratio) counted in our cell pellets by KX-21N after Ficoll purification, while taking into account the baseline WBC/PLT ratio. To rule out confounding effects of inter-assay baseline differences, corrected NF- $\kappa$ B binding activity levels were calculated as percentage changes from baseline following previous methods (Pace et al., 2006). We also controlled for the positive control (2.5  $\mu$ g Jurkat [TPA + Cl] nuclear extract/well) as a covariate in all analyses with NF- $\kappa$ B binding activity as the dependent variable.

For NF- $\kappa$ B binding activity, mRNA levels, and plasma cytokine levels, areas under the curve with respect to increase (AUCs) using the trapezoid formula were calculated from baseline to highest responses following stress (i.e. from baseline to 10 min post-stress/non-stress for NF- $\kappa$ B binding activity [AUC<sub>NF- $\kappa$ B-BA</sub>], from baseline to 120 min post-stress/non-stress for all mRNA parameters [AUC<sub>mRNA IL-1 $\beta$ , etc.</sub>], and from baseline to 90 min post-stress/non-stress for plasma cytokine levels [AUC<sub>plasma IL-1 $\beta$ , etc.</sub>]) (Pruessner et al., 2003).

Prior to statistical analyses, data were tested for normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests in both groups. Skewed salivary cortisol levels, plasma IL-1 $\beta$  and IL-10 levels, and AUC<sub>plasma IL-10</sub> levels were logarithmically transformed and normal distribution was verified. While modeling and testing was performed with log-transformed data we provide original data for group characteristics and in figures for reasons of clarity.

Univariate analyses of variance (ANOVAs) were calculated to test for group differences in terms of demographic measures, and baseline levels of salivary cortisol and plasma cytokines.

To test whether the stressor evoked a significant neuroendocrine stress response, we calculated repeated measures ANOVA with group (stress group vs. control group) as the independent variable and the seven time points at which cortisol was measured as repeated dependent variable.

To test whether the stressor evoked a significant inflammatory stress response, we calculated repeated measures ANOVAs with group as the independent variable; the seven, three, and five time points at which NF- $\kappa$ B binding activity, mRNA levels, and plasma cytokine levels were measured served as repeated dependent variables. To rule out potential confounding influences on inflammatory markers (Wirtz et al., 2008; Wolf et al., 2009) we controlled for age and BMI as covariates. Further testing of effects in inflammatory measures comprised re-analyses of all repeated measures ANOVAs separately for each subject group.

To test for associations between NF- $\kappa$ B binding activity, mRNA, and plasma cytokine levels, we calculated Pearson's product-moment correlations using the respective AUC peak reactivity scores.

### 3. Results

#### 3.1. Participants' characteristics

The study groups did not significantly differ in age (stress group: 36.3  $\pm$  1.9, control group: 35.1  $\pm$  1.8;  $p = .66$ ) and BMI (stress group: 24.3  $\pm$  0.8, control group: 24.9  $\pm$  0.5;  $p = .61$ ), nor did they differ in baseline levels of cortisol ( $p = .45$ , see Fig. 1), or cytokines ( $p$ 's > .43, see Fig. 4A-C).

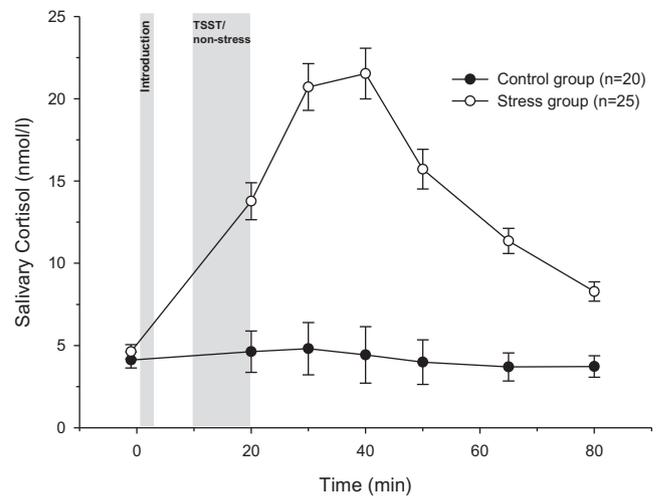


Fig. 1. Salivary cortisol over time in the stress and the control group. Values are given as means  $\pm$  SEM.

#### 3.2. Validation check of the stress protocol

As compared to the non-stress control group, the stress group showed significant increases in salivary cortisol (group-by-time interaction:  $F(3.1, 135.2) = 49.15$ ,  $p < .001$ ,  $f = 1.07$ ; see Fig. 1).

#### 3.3. NF- $\kappa$ B binding activity to DNA in the stress and the control group

The study groups significantly differed in NF- $\kappa$ B binding activity over time ( $p = .007$ , see Fig. 2 and Table 1), whereby NF- $\kappa$ B binding activity levels were highest at 1 and 10 min after stress. This result did not significantly change without controlling for age and BMI ( $p = .003$ ). Separate re-analyses in each subject group revealed that NF- $\kappa$ B binding activity significantly increased from baseline to 10 min after stress/non-stress in the stress group (main effect time:  $F(2.0, 32.0) = 3.87$ ,  $p = .031$ ,  $f = 0.49$ ) but not in the control group (main effect time:  $p = .24$ ).

#### 3.4. Gene expression

##### 3.4.1. Identification of gene expression stress kinetics

As illustrated in Fig. 3A, IL-1 $\beta$  mRNA levels did not increase within the first 60 min after stress cessation and were highest

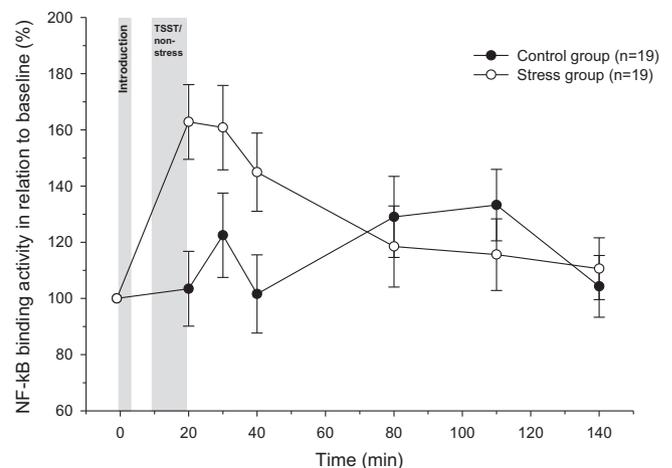


Fig. 2. NF- $\kappa$ B binding activity over time in the stress and the control group. Age and BMI were controlled. Values are given as means  $\pm$  SEM.

**Table 1**

Group-by-time interactions of repeated measures AN(C)OVAs with group as independent variable and inflammatory markers as repeated dependent variables.

Group-by-time interaction	AN(C)OVA with (and without) controlling for age and BMI		
	<i>F</i>	<i>p</i>	<i>f</i>
NF-κB-BA mRNA	IL-1β	3.03 (3.41)	.007 (.003)
	IL-6	3.49 (3.86)	.044 (.036)
	IL-10	3.49 (2.85)	.036 (.066)
	NF-κB		.52 (.52)
	IκBα	4.02 (4.43)	.22 (.17)
Plasma	IκBα		.022 (.015)
	IL-1β		.93 (.92)
	IL-6	3.20 (3.19)	.038 (.040)
	IL-10		.44 (.44)

Note: AN(C)OVAs = analyses of (co)variance; BMI = body mass index; NF-κB-BA = NF-κB binding activity to DNA; IL = interleukin. Effect size conventions: *f*: .10 = small, .25 = medium, .40 = large.

120 min after stress. Based on these pilot data, we decided for analyzing gene expression immediately before stress induction, as well as 60 min, and 120 min after stress cessation in the whole group of study participants.

#### 3.4.2. Gene expression in the stress and the control group

Compared to the control condition, stress induced significant increases in mRNA levels of IL-1β ( $p = .044$ ), IL-6 ( $p = .036$ ), and IκBα ( $p = .022$ ) with the highest mRNA gene expression observed 120 min after stress (see Fig. 3B, C, and F and Table 1). In terms of IL-10 and NF-κB mRNA levels, there were no significant group differences over time ( $p$ 's > .21, see Fig. 3D and E and Table 1). The interaction effect of IL-6 became of borderline significance without controlling for age and BMI ( $p = .066$ ), but none of the other mRNA results significantly changed (see Table 1).

Separate re-analyses in each subject group revealed increases in mRNA levels of IL-1β, NF-κB, and IκBα over time in the stress group (main effect time IL-1β:  $F(1.4, 27.8) = 6.0$ ,  $p = .013$ ,  $f = 0.55$ ; main effect time NF-κB:  $F(1.3, 26.0) = 3.75$ ,  $p = .054$ ,  $f = 0.43$ ; main effect time IκBα:  $F(1.9, 38.7) = 5.31$ ,  $p = .010$ ,  $f = 0.51$ ), but not in the control group ( $p$ 's > .41). No significant time effects were observed in IL-6 and IL-10 mRNA levels, either in the stress (main effect time IL-6:  $p = .18$ ; main effect time IL-10:  $p = .22$ ) or control group (main effect time IL-6:  $p = .13$ ; main effect time IL-10:  $p = .92$ ).

#### 3.5. Cytokine plasma levels

Study groups significantly differed in plasma IL-6 levels over time ( $p = .038$ ; see Fig. 4B and Table 1) with highest levels seen at 90 min after stress. This result did not significantly change without controlling for age and BMI ( $p = .040$ ). In terms of plasma IL-1β and IL-10, no group-by-time interaction effects were observed, with and without controlling for age and BMI ( $p$ 's > .43, see Fig. 4A and C and Table 1).

Separate re-analyses in each subject group revealed increases in plasma IL-6 over time in both the stress (main effect time:  $F(2.0, 44.7) = 11.38$ ,  $p < .001$ ,  $f = 0.72$ ) and the control group (main effect time:  $F(2.2, 42.4) = 6.93$ ,  $p = .002$ ,  $f = 0.60$ ). Additionally, significant increases in plasma IL-1β and IL-10 levels over time were observed in the stress group (main effect time IL-1β:  $F(3.7, 74.1) = 4.23$ ,  $p = .005$ ,  $f = 0.46$ ; main effect time IL-10:  $F(3.5, 83.0) = 5.86$ ,  $p = .001$ ,  $f = 0.49$ ) but did not reach statistical significance in the control group (main effect time IL-1β:  $p = .13$ ; main effect time IL-10:  $p = .12$ ).

#### 3.6. Associations between NF-κB binding activity to DNA, mRNA, and cytokine levels

Higher peak reactivity of NF-κB binding activity correlated significantly with higher mRNA peak reactivity levels of IL-1β

( $r [AUC_{NF-κB-BA}/AUC_{mRNA\ IL-1β}] = .52$ ,  $p = .002$ ), NF-κB ( $r [AUC_{NF-κB-BA}/AUC_{mRNA\ NF-κB}] = .48$ ,  $p = .004$ ), and IκBα ( $r [AUC_{NF-κB-BA}/AUC_{mRNA\ IκBα}] = .42$ ,  $p = .013$ ). While IL-10 mRNA peak reactivity did not relate to peak reactivity of NF-κB binding activity ( $r [AUC_{NF-κB-BA}/AUC_{mRNA\ IL-10}] = -.16$ ,  $p = .40$ ), higher IL-6 mRNA related to higher NF-κB binding activity approaching borderline significance ( $r [AUC_{NF-κB-BA}/AUC_{mRNA\ IL-6}] = .31$ ,  $p = .11$ ).

Plasma cytokine peak reactivity levels of IL-1β, IL-6, and IL-10 were neither associated with mRNA peak reactivity levels of the respective cytokine ( $r [AUC_{plasma\ IL-1β}/AUC_{mRNA\ IL-1β}] = -.06$ ,  $p = .72$ ;  $r [AUC_{plasma\ IL-6}/AUC_{mRNA\ IL-6}] = .04$ ,  $p = .83$ ;  $r [AUC_{plasma\ IL-10}/AUC_{mRNA\ IL-10}] = .34$ ,  $p = .085$ ) nor with peak reactivity of NF-κB binding activity ( $r [AUC_{plasma\ IL-1β}/AUC_{NF-κB-BA}] = -.23$ ,  $p = .20$ ;  $r [AUC_{plasma\ IL-6}/AUC_{NF-κB-BA}] = -.00$ ,  $p = .99$ ;  $r [AUC_{plasma\ IL-10}/AUC_{NF-κB-BA}] = .13$ ,  $p = .53$ ).

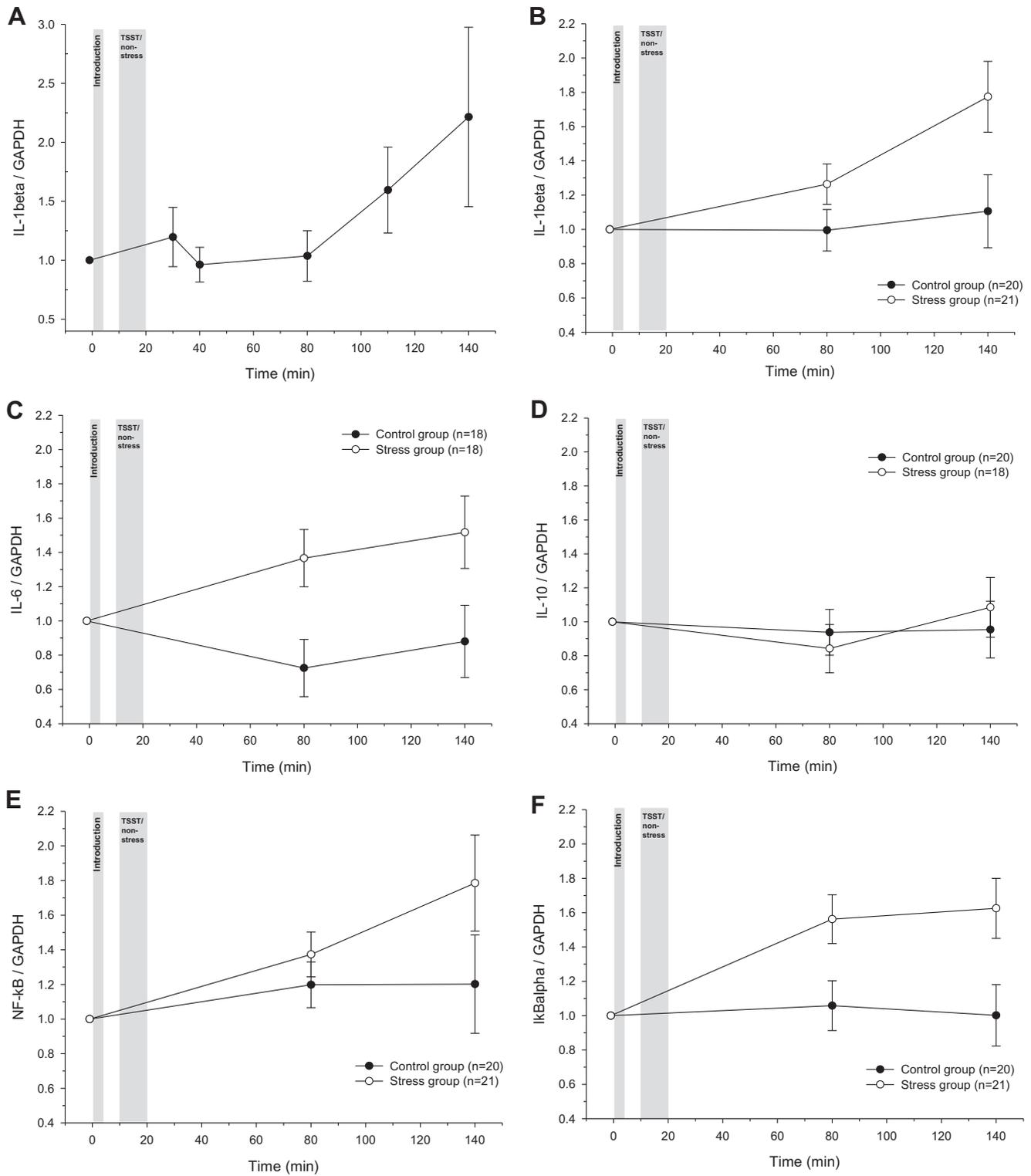
## 4. Discussion

This is the first human study to investigate the stress kinetics and interrelations of NF-κB binding activity (BA) in PBMCs, in addition to plasma and whole-blood mRNA levels of pro- and anti-inflammatory measures.

### 4.1. Stress kinetics

Regarding stress kinetics, we found in healthy men a rapid increase in NF-κB-BA immediately after exposure to acute psychosocial stress as compared to the control condition with recovery to baseline levels 60 min after stress cessation. In addition, as compared to the non-stress condition, stress exposure induced delayed increases in mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 with highest levels observed at 120 min after stress cessation, as well as in plasma IL-6 levels peaking at 90 min after stress cessation. In the stress group we found increases in IL-1β plasma levels peaking 90 min after stress cessation and in NF-κB mRNA with highest levels observed at 120 min post stress. In terms of anti-inflammatory measures under study, we found in the stress group delayed increases in plasma levels but not in mRNA levels of IL-10. As compared to non-stress, stress exposure induced increases in mRNA expression of the inhibitor protein IκBα with highest levels 120 min post stress. The significant effects of stress on inflammatory measures were of medium to large effect size and independent of age and BMI. These findings suggest that psychosocial stress not only induces increases in circulating pro-inflammatory but also in anti-inflammatory measures both on the transcriptional and protein levels.

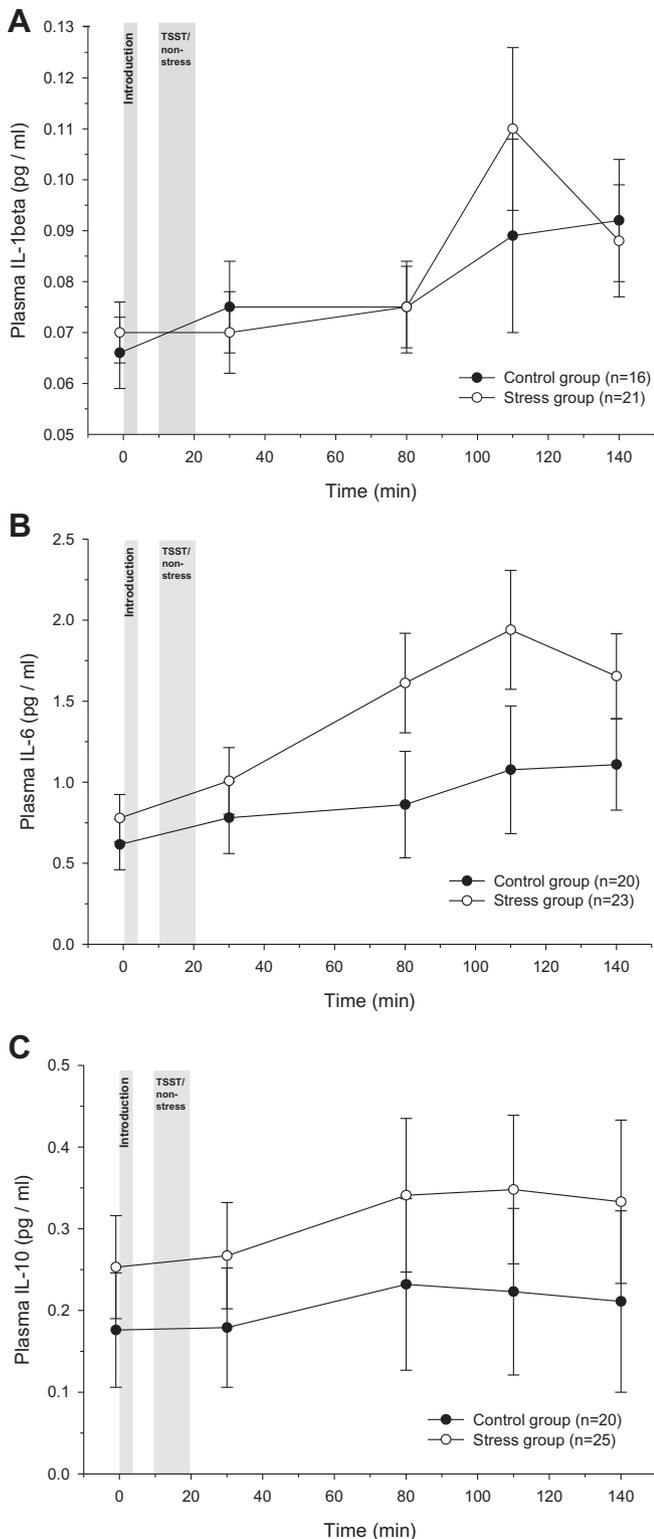
Our stress kinetics findings of plasma IL-1β and IL-6, mRNA IL-1β, and NF-κB-BA in whole-cell extracts of PBMCs corroborate previous observations (Bierhaus et al., 2003; Brydon et al., 2005; Pace et al., 2006; Steptoe et al., 2007; Wolf et al., 2009). Moreover,



**Fig. 3.** A to F. Course of mRNA levels over time. IL-1 $\beta$  mRNA levels before and after psychosocial stress (Trier Social Stress Test, TSST) in the gene expression testing sample ( $N = 7$ , A). mRNA levels of IL-1 $\beta$  (B), IL-6 (C), IL-10 (D), NF- $\kappa$ B (E), and I $\kappa$ B $\alpha$  (F) over time in the stress and the control group. Age and BMI were controlled. Values are given as means  $\pm$  SEM.

because NF- $\kappa$ B-BA stress reactivity has been hitherto determined in nuclear but not nuclear and cytoplasmic or whole-cell extract of PBMCs (Bierhaus et al., 2003; Pace et al., 2006; Wolf et al., 2009), our NF- $\kappa$ B-BA stress reactivity findings extend previous observations to whole-cell extracts. In contrast to Brydon et al. (2005), we observed stress-induced increases in IL-6 mRNA in

addition to increases in mRNA levels of I $\kappa$ B $\alpha$  and NF- $\kappa$ B. Methodological differences might account for this discrepancy. Interestingly, mRNA levels of IL-1 $\beta$ , IL-6, NF- $\kappa$ B, and I $\kappa$ B $\alpha$  revealed similar kinetics with highest levels 120 min after stress cessation, suggesting that peak levels may occur later than 120 min after stress cessation. Therefore, future research would benefit from



**Fig. 4.** A to C. Plasma levels of IL-1 $\beta$  (A), IL-6 (B), and IL-10 (C) over time in the stress and the control group. Age and BMI were controlled. Values are given as means  $\pm$  SEM.

longer post-stress monitoring periods. In previous studies changes in IL-10 to acute psychosocial stress were not consistently seen (Altemus et al., 2001; de Brouwer et al., 2014; Fredericks et al., 2010), although none of these studies investigated men only.

#### 4.2. Interrelations between NF- $\kappa$ B binding activity, mRNA, and cytokine levels

In terms of interrelations of transcriptional inflammatory measures, greater stress-induced NF- $\kappa$ B-BA (i.e. active NF- $\kappa$ B) was associated with higher delayed stress-induced increases in mRNA levels of the NF- $\kappa$ B-regulated cytokines IL-1 $\beta$  and IL-6. In addition, higher stress-induced changes in active NF- $\kappa$ B were also associated with higher mRNA levels of NF- $\kappa$ B and I $\kappa$ B $\alpha$ .

In contrast to our hypothesis regarding interrelations between transcriptional and plasma inflammatory measures, we could not find stress-induced increases in cytokine plasma levels to be associated with stress-induced increases in NF- $\kappa$ B-BA or with mRNA levels of the respective cytokine. This lack of an association and the fact that plasma cytokines peaked earlier than cytoplasmic measures suggests that the stress-induced increases in plasma cytokines are unlikely to derive from de novo synthesis by circulating immune cells. Previous research indeed suggests a complex regulatory mechanism from cytokine mRNA transcription to cytokine production and subsequent secretion in ex vivo isolated human monocyte derived macrophages upon in vitro stimulation with lipopolysaccharide (LPS) (Zhong et al., 1993). Notably, in humans the temporal relation between mRNA level increases and its resulting protein production and release is to the best of our knowledge unknown. So far, we can only speculate about the sources of stress-induced increases in plasma cytokines. It remains to be tested whether increases in plasma cytokines result from intracellularly stored cytokines in circulating immune cells or from other stress-sensitive cytokine producing cell types, such as adipocytes (Mohamed-Ali et al., 2001), endothelial cells (Gornikiewicz et al., 2000) or adrenal zona glomerulosa cells (Judd and MacLeod, 1995). Future research is needed to identify the sources of the stress-induced plasma cytokines, and to investigate whether, and if so, when increases in plasma cytokines result from stress-induced increases in NF- $\kappa$ B-BA and subsequent cytokine mRNA levels of circulating blood cells.

#### 4.3. Integrative model

Based on our results and those from the literature, we propose an integrative model with the following cascade of stress-induced events: Acute psychosocial stress immediately induces activation of NF- $\kappa$ B supposedly by degradation of the cytoplasmic NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  in blood cells. After translocation from cytoplasm to the nucleus, activated NF- $\kappa$ B up-regulates mRNA expression of pro-inflammatory, but not anti-inflammatory cytokines, with the aim of later cytokine production. In addition, activated NF- $\kappa$ B also induces the expression of I $\kappa$ B $\alpha$  and NF- $\kappa$ B subunits, thereby limiting its own activation and restoring its unstimulated but stress-responsive cytoplasmic basal state. Notably, this proposed autoregulatory feedback mechanism of NF- $\kappa$ B has been observed in cell lines exposed to different NF- $\kappa$ B stimulating agents (Brown et al., 1993; Lombardi et al., 1995; Sun et al., 1993).

The proposed cascade of events may represent a well-orchestrated effort of the organism to deal with a stressful situation. However, it may also contribute to negative health outcomes. More specifically, according to the allostatic load theory, effects of sustained or repeated stress may accumulate over time and result in dysfunctions in stress response systems (Goldstein and McEwen, 2002). These dysfunctions, in turn, have been linked consistently to negative health outcomes, specifically those associated with inflammatory processes, including cardiovascular diseases (e.g., atherosclerosis) and depression (Chrousos and Gold, 1998; Goldstein and McEwen, 2002). As such, the current findings contribute to our understanding of the downstream effects of stress

on immune processes and may provide a reference for characterizing health-relevant dysfunctions in immune stress responses.

#### 4.4. Strengths and limitations of the study

The present study has several strengths: It is the first study to assess stress reactivity of several inflammatory measures on different transcriptional levels and in plasma within the same person. Also, our study design included a resting control group of an appropriate sample size. In addition, we used whole-cell extracts that allowed us to determine stress-induced changes in total active NF- $\kappa$ B of a cell in both the nuclear and cytoplasmic cell fraction. Moreover, total RNA was extracted from whole blood collected in PAXgene tubes, which stabilize mRNA during blood collection and storage and thus allow for reliable gene expression results (Rainen et al., 2002). Furthermore, potential confounding factors were controlled in our statistical analyses by a priori entering age and BMI as covariates and by restricting recruitment to men only. We also used a highly standardized and potent stress test that reliably induces physiological stress responses (Dickerson and Kemeny, 2004). Our study also has limitations. First, the generalization of our study's findings might be limited to healthy men aged 20–50 years, and the cytokines IL-1 $\beta$ , IL-6, and IL-10. It remains to be elucidated whether our findings also apply to women, men younger or older than 20 to 50 years, patients suffering from a disease, other populations, or inflammatory cytokines other than IL-1 $\beta$ , IL-6, and IL-10. Second, our sample size was relatively small and therefore our data should be interpreted with caution and need to be confirmed in future studies. Third, we investigated humans and thus our inter-relational assessment of the different ex vivo derived inflammatory measures was of correlational nature; in other words, our study design did not allow us to investigate causal associations of stress-induced changes in NF- $\kappa$ B binding activity, cytokine mRNA, and plasma levels of cytokines by means of parameter-specific experimental manipulations as possible in animal or in vitro studies. Fourth, although in our NF- $\kappa$ B-BA analyses we standardized the number of PBMCs, we cannot completely rule out potential influences of shifts in lymphocyte redistribution or subpopulations as observed in reaction to strenuous exercise (Richlin et al., 2004). However, two previous studies could not detect confounding effects of psychosocial stress-induced shifts in lymphocyte redistribution or subpopulations on NF- $\kappa$ B-BA (Pace et al., 2006; Wolf et al., 2009). Moreover, we observed comparably strong stress-induced increases in NF- $\kappa$ B-BA 1 and 10 min after TSST suggesting only a small influence of the redistribution phenomenon on NF- $\kappa$ B-BA, considering that stress-induced redistribution in leukocyte subsets differs considerably between 1 and 10 min after TSST (Buske-Kirschbaum et al., 2007). Fifth, because plasma cytokine levels measured by multiplex technology can be influenced by the used multiplex platform or multiplex assay (Toedter et al., 2008), our absolute plasma cytokine levels should be interpreted with caution.

#### 4.5. Conclusion

Our findings suggest that psychosocial stress induces increases in the binding activity of the pro-inflammatory transcription factor NF- $\kappa$ B that relate to subsequent delayed mRNA expression of pro-inflammatory but not anti-inflammatory cytokines and regulatory cytoplasmic proteins starting within 2 h from stress cessation. The observed delayed stress-induced increases in plasma levels of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and of the anti-inflammatory cytokine IL-10 are unlikely to derive from de novo synthesis in circulating blood cells. Future studies are needed to investigate the mechanisms underlying the kinetics and interrelations of

stress-induced changes in NF- $\kappa$ B binding activity, cytokine mRNA, and plasma levels of cytokines.

#### Conflict of interest statement

All authors declare that there are no conflicts of interest.

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#### References

- Altemus, M., Rao, B., Dhabhar, F.S., Ding, W., Granstein, R.D., 2001. Stress-induced changes in skin barrier function in healthy women. *J. Invest. Dermatol.* 117, 309–317.
- Baldwin Jr., A.S., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14, 649–683.
- Bierhaus, A., Wolf, J., Andrassy, M., Rohleder, N., Humpert, P.M., Petrov, D., Ferstl, R., von Eynatten, M., Wendt, T., Rudofsky, G., Joswig, M., Morcos, M., Schwaninger, M., McEwen, B., Kirschbaum, C., Nawroth, P.P., 2003. A mechanism converting psychosocial stress into mononuclear cell activation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1920–1925.
- Brown, K., Park, S., Kanno, T., Franzoso, G., Siebenlist, U., 1993. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc. Natl. Acad. Sci. U.S.A.* 90, 2532–2536.
- Brydon, L., Edwards, S., Jia, H., Mohamed-Ali, V., Zachary, I., Martin, J.F., Steptoe, A., 2005. Psychological stress activates interleukin-1beta gene expression in human mononuclear cells. *Brain Behav. Immun.* 19, 540–546.
- Buske-Kirschbaum, A., Kern, S., Ebrecht, M., Hellhammer, D.H., 2007. Altered distribution of leukocyte subsets and cytokine production in response to acute psychosocial stress in patients with psoriasis vulgaris. *Brain Behav. Immun.* 21, 92–99.
- Chrousos, G.P., Gold, P.W., 1998. A healthy body in a healthy mind – and vice versa – the damaging power of “uncontrollable” stress. *J. Clin. Endocrinol. Metabol.* 83, 1842–1845.
- de Brouwer, S.J., van Middendorp, H., Stormink, C., Kraaijaak, F.W., Joosten, I., Radstake, T.R., de Jong, E.M., Schalkwijk, J., Donders, A.R., Eijsbouts, A., van de Kerkhof, P.C., van Riel, P.L., Evers, A.W., 2014. Immune responses to stress in rheumatoid arthritis and psoriasis. *Rheumatology*.
- Dickerson, S.S., Kemeny, M.E., 2004. Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychol. Bull.* 130, 355–391.
- Fredericks, C.A., Drabant, E.M., Edge, M.D., Tillie, J.M., Hallmayer, J., Ramel, W., Kuo, J.R., Mackey, S., Gross, J.J., Dhabhar, F.S., 2010. Healthy young women with serotonin transporter 5S polymorphism show a pro-inflammatory bias under resting and stress conditions. *Brain Behav. Immun.* 24, 350–357.
- Goldstein, D.S., McEwen, B., 2002. Allostatics, homeostasis, and the nature of stress. *Stress* 5, 55–58.
- Gornikiewicz, A., Sautner, T., Brostjan, C., Schmierer, B., Fugger, R., Roth, E., Muhlbacher, F., Bergmann, M., 2000. Catecholamines up-regulate lipopolysaccharide-induced IL-6 production in human microvascular endothelial cells. *FASEB J.* 14, 1093–1100.
- Judd, A.M., MacLeod, R.M., 1995. Differential release of tumor necrosis factor and IL-6 from adrenal zona glomerulosa cells in vitro. *Am. J. Physiol.* 268, E114–120.
- Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The ‘Trier Social Stress Test’ – a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology* 28, 76–81.
- Kuebler, U., Wirtz, P.H., Sakai, M., Stemmer, A., Ehlert, U., 2013. Acute stress reduces wound-induced activation of microbicidal potential of ex vivo isolated human monocyte-derived macrophages. *PLoS One* 8, e55875.
- Lombardi, L., Ciana, P., Cappellini, C., Trecca, D., Guerrini, L., Migliozza, A., Maiolo, A.T., Neri, A., 1995. Structural and functional characterization of the promoter regions of the NFkB2 gene. *Nucleic Acids Res.* 23, 2328–2336.
- Mohamed-Ali, V., Flower, L., Sethi, J., Hotamisligil, G., Gray, R., Humphries, S.E., York, D.A., Pinkney, J., 2001. Beta-adrenergic regulation of IL-6 release from adipose tissue: in vivo and in vitro studies. *J. Clin. Endocrinol. Metabol.* 86, 5864–5869.
- Nater, U.M., Whistler, T., Lonergan, W., Mletzko, T., Vernon, S.D., Heim, C., 2009. Impact of acute psychosocial stress on peripheral blood gene expression pathways in healthy men. *Biol. Psychol.* 82, 125–132.
- Pace, T.W., Mletzko, T.C., Alagbe, O., Musselman, D.L., Nemeroff, C.B., Miller, A.H., Heim, C.M., 2006. Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. *Am. J. Psychiatry* 163, 1630–1633.
- Pahl, H.L., 1999. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18, 6853–6866.
- Pruessner, J.C., Kirschbaum, C., Meinlschmid, G., Hellhammer, D.H., 2003. Two formulas for computation of the area under the curve represent measures of

- total hormone concentration versus time-dependent change. *Psychoneuroendocrinology* 28, 916–931.
- Rainen, L., Oelmueller, U., Jurgensen, S., Wyrich, R., Ballas, C., Schram, J., Herdman, C., Bankaitis-Davis, D., Nicholls, N., Trollinger, D., Tryon, V., 2002. Stabilization of mRNA expression in whole blood samples. *Clin. Chem.* 48, 1883–1890.
- Richlin, V.A., Arevalo, J.M., Zack, J.A., Cole, S.W., 2004. Stress-induced enhancement of NF-kappaB DNA-binding in the peripheral blood leukocyte pool: effects of lymphocyte redistribution. *Brain Behav. Immun.* 18, 231–237.
- Rohleder, N., 2014. Stimulation of systemic low-grade inflammation by psychosocial stress. *Psychosom. Med.* 76, 181–189.
- Schattner, M., 2013. Role of NF-kappaB pathway on platelet activation. *Circ. Res.* 113, e92.
- Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3, 1101–1108.
- Stephens, A., Hamer, M., Chida, Y., 2007. The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. *Brain Behav. Immun.* 21, 901–912.
- Sun, S.C., Ganchi, P.A., Ballard, D.W., Greene, W.C., 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science* 259, 1912–1915.
- Toedter, G., Hayden, K., Wagner, C., Brodmerkel, C., 2008. Simultaneous detection of eight analytes in human serum by two commercially available platforms for multiplex cytokine analysis. *Clinical Vaccine Immunol.* 15, 42–48.
- Wirtz, P.H., Ehlert, U., Emini, L., Suter, T., 2008. Higher body mass index (BMI) is associated with reduced glucocorticoid inhibition of inflammatory cytokine production following acute psychosocial stress in men. *Psychoneuroendocrinology* 33, 1102–1110.
- Wolf, J.M., Rohleder, N., Bierhaus, A., Nawroth, P.P., Kirschbaum, C., 2009. Determinants of the NF-kappaB response to acute psychosocial stress in humans. *Brain Behav. Immun.* 23, 742–749.
- Zhong, W.W., Burke, P.A., Hand, A.T., Walsh, M.J., Hughes, L.A., Forse, R.A., 1993. Regulation of cytokine mRNA expression in lipopolysaccharide-stimulated human macrophages. *Arch. Surg.* 128, 158–163 (discussion 163–154).