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Investigating the effects of childhood maltreatment on pro-inflammatory signaling: The influence of cortisol and DHEA on cytokine secretion *ex vivo*

Martha Leonie Geiger[a,*,1], Christina Boeck[a,*,1], Alexandra Maria Koenig[a], Katharina Schury[a], Christiane Waller[b,d], Stephan Kolassa[c], Alexander Karabatsiakis[a], Iris- Tatjana Kolassa[a]

[a] Clinical & Biological Psychology, Institute of Psychology and Education, Ulm University, Albert-Einstein-Allee 47, 89081 Ulm, Germany
[b] Department of Psychosomatic Medicine and Psychotherapy, University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany
[c] SAP Switzerland AG, Tägerwilen, Switzerland
[d] Department of Psychosomatic Medicine and Psychotherapy, Paracelsus Medical University, Nuremberg General Hospital, Germany

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ABSTRACT

Childhood maltreatment (CM) is associated with chronic low-grade inflammation and an increased risk for the development of adverse mental and physical health outcomes in CM-affected adults. Differences in cortisol signaling were described to contribute to this pro-inflammatory phenotype. We investigated in a study cohort of 13 postpartum women with and 12 postpartum women without CM whether treatment of peripheral blood mononuclear cells (PBMC) with cortisol, the anti-glucocorticoid hormone dehydroepiandrosterone (DHEA), or co-treatment with both differentially affected pro-inflammatory cytokine release *ex vivo*. The childhood trauma questionnaire was used to retrospectively assess CM and the severity of CM experiences (maltreatment load). PBMC of maltreated women (CM+) showed in all conditions an increase in pro-inflammatory cytokine secretion compared to PBMC of the control group (CM-), which was correlated with the maltreatment load. *Ex vivo* stimulation analyses provided preliminary evidence for a differential responsivity of PBMC in CM+ and CM- women to cortisol regarding TNF-α secretion, but no difference in the responsivity to DHEA treatment. The results of the co-treatment with cortisol and DHEA support the hypothesis that cortisol and DHEA interact in the modulation of inflammatory processes.

1. Introduction

The experience of childhood maltreatment (CM) – either in the form of physical, emotional and/or sexual abuse, or in the form of physical and/or emotional neglect – can have lifelong consequences for both, mental and physical health (Nemeroff, 2016). The manifestation of adverse health outcomes seems to depend in a cumulative manner on the severity and frequency of CM experiences, i.e., maltreatment load (Kolassa & Schury, 2012). CM is not only associated with an increased risk for psychiatric disorders such as posttraumatic stress disorder (PTSD), major depression and anxiety disorders, but also with an increased risk for the premature onset of physical diseases including cardiovascular diseases, diabetes and even cancer (Nemeroff, 2016). A large body of literature suggests that inflammatory processes, which are majorly regulated by the hypothalamic-pituitary-adrenal (HPA) axis, play a central role in the pathophysiology of these disorders.

1.1. Role of the HPA axis in inflammatory processes

Upon HPA-axis activation, the steroid hormone cortisol is secreted from the adrenal glands into the blood stream and mediates its immune-regulatory effects via binding to glucocorticoid receptors (GR) on target cells and tissues (Guilliams & Edwards, 2010). Besides cortisol, the steroid hormone dehydroepiandrosterone (DHEA) is also released from the adrenal glands following HPA axis activation (Endoh, Kristiansen, Casson, Buster, & Hornsby, 1996). DHEA exerts not only anti-oxidant (Bastianetto, Ramassamy, Poirier, & Quirion, 1999), neuroprotective (Bastianetto et al., 1999; Cardounel, Regelson, & Kalimi, 1999), and immune-enhancing actions (Daynes, Dudley, & Araneo, 1990), but also anti-glucocorticoid effects. However, the exact molecular mechanisms underlying the anti-glucocorticoid effect of DHEA remain poorly understood. There is evidence suggesting that DHEA may inhibit the nuclear translocation of the GR (Cardounel, Regelson, & Kalimi, 1999) and up-regulate the gene expression of the inactive GRβ isoform, which acts as dominant-negative

* Corresponding authors. Clinical & Biological Psychology, Institute of Psychology and Education, Ulm University, Albert-Einstein-Allee 47, 89081 Ulm, Germany.
E-mail addresses: christina.boeck@uni-ulm.de (C. Boeck), iris.kolassa@uni-ulm.de (I.-.T. Kolassa).
[1] These authors contributed equally to this work.

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suppressor of the functionally active GRα isoform (Pinto et al., 2015). Furthermore, DHEA was described to inhibit gene expression (Apostolova, Schweizer, Balazs, Kostadinova, & Odermatt, 2005) and activity of *11β-hydroxysteroid dehydrogenase 1* (Hennebert, Chalbot, Alran, & Morfin, 2007), an enzyme that converts the hormonally inactive cortisone to its bioactive form cortisol. DHEA metabolites may further engage in anti-glucocorticoid actions, as estrogens were described to inhibit GR gene expression (Turner, 1997), while androgens compete for binding sites of GR target genes (Rundlett & Miesfeld, 1995). Furthermore, their function is central for the DHEA-induced stimulation of GRβ expression in immune cells (Corsini et al., 2016). In sum, these findings imply that DHEA has the potential to influence GR signaling, supporting the hypothesis that both steroid hormones might interactively modulate inflammatory processes.

1.2. Childhood maltreatment and HPA axis dysregulation

Experiencing CM can affect the development of the endocrine stress response system and induce long-lasting alterations in HPA axis signaling and regulation (for a review see Panzer, 2008). While studies on basal cortisol and DHEA levels have produced inconsistent findings reporting both increases (DHEA: Kellner et al., 2010; Yehuda, 2006), decreases (cortisol: Bunea, Szentágotai-Tătar, & Miu, 2017; Heim, Newport, Bonsall, Miller, & Nemeroff, 2003) and no alterations (cortisol: Carpenter et al., 2007; Heim et al., 2000; DHEA: Pico-Alfonso, Garcia-Linares, Celda-Navarro, Herbert, & Martinez, 2004; Van Voorhees, Dennis, Calhoun, & Beckham, 2014) in blood and saliva hormone levels in CM, mounting evidence supports the hypothesis that it is more significant how target tissues respond to these hormones rather than alterations in the circulating hormone levels *per se* (Cohen et al., 2012; Schwaiger et al., 2016). On a cellular level, traumatic stress exposure during childhood has been linked to epigenetic DNA modifications that affect the expression and sensitivity of the GR (hypermethylation within the promoter region of the GR gene *NR3C1* (Tyrka, Price, Marsit, Walters, & Carpenter, 2012; Van Der Knaap et al., 2014), hypomethylation of the gene *FKBP5* encoding for the GR co-chaperone FK506 binding protein 51 (Klengel et al., 2013; Tyrka et al., 2015)) as well as to a shift in GR isoforms, favoring GRβ over GRα expression in peripheral blood mononuclear cells (PBMC) (Derijk et al., 2001; Gola et al., 2014). Together these biological alterations may promote a state of GR resistance, reducing the sensitivity of target tissues to the generally immune-suppressive actions induced by cortisol. This might result in a phenotype of chronic low-grade inflammation and offer an explanation for the increased risk of inflammation-associated disorders in individuals with a history of CM (Cohen et al., 2012; Guilliams & Edwards, 2010). In line with this hypothesis, we showed previously that CM was dose-dependently associated with increased inflammation in postpartum women (Boeck et al., 2016). Ehrlich, Ross, Chen, and Miller (2016) further showed that adverse early life experiences were over a period of 2.5 years persistently associated with a higher odds ratio for such a so-called pro-inflammatory phenotype. The results showed a robust increase in the secretion of the pro-inflammatory cytokine interleukin (IL)−6 following the stimulation of PBMC with the bacterial mitogen lipopolysaccharide (LPS). Additionally, they found a concomitant decrease in the sensitivity of PBMC to the suppressive effects of cortisol treatment on LPS-stimulated cytokine secretion *in vitro*. In addition to the results from LPS stimulation, unstimulated PBMC from individuals with PTSD already secreted spontaneously higher levels of IL-1β, IL-6, and tumor necrosis factor α (TNF-α) compared to non-traumatized healthy controls (Gola et al., 2013). Taking the increase in spontaneous cytokine secretion into account, the net effect of LPS stimulation on pro-inflammatory cytokine release did thereby no longer differ between the PTSD and the control group. Based on these findings, we hypothesized that the experience of traumatic stress might be associated with a pre-activation of immune cells in vivo that is potentially caused by alterations in cellular signaling cascades that regulate inflammatory processes.

1.3. Aims of the study

This study aimed at investigating whether the suggested pro-inflammatory phenotype of PBMC observed in postpartum women with a history of CM (Boeck et al., 2016) is associated with differences in the responsivity of PBMC to the regulatory steroid hormones cortisol and DHEA. Moreover, we hypothesized that the secretion of the selected pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) by PBMC would be higher depending on the maltreatment load. Furthermore, we expected that a history of CM would be associated with differences in the responsivity of PBMC, as assessed by the release of pro-inflammatory cytokines, to the treatment with cortisol, DHEA and a combination of both cortisol and DHEA (1:1).

2. Methods and Materials

2.1. Participants

The data presented here were part of a longitudinal project investigating the psychological and biological mechanisms in the transgenerational transmission of CM experiences in mother-infant-dyads (see also Boeck et al., 2016; Koenig et al., 2016). The study was conducted in line with the Declaration of Helsinki (World Medical Association, 2013) and was approved by the local ethics committee. Exclusion criteria were an age below 18 years, insufficient knowledge of the German language, reported drug consumption, lifetime psychotic disorders, and severe complications during delivery as well as severe health problems of mother and/or child. Two-hundred and forty women provided written informed consent within a maximum of six days after parturition (t0, see Fig. 1 for overview of study recruitment).

The initial assessment of personalized data at t0 by trained interviewers included basic socio-demographic information and a screening for CM using the German short version of the *Childhood Trauma Questionnaire* (CTQ; Bader, Hänny, Schäfer, Neuckel, & Kuhl, 2009). The CTQ is a self-report questionnaire, which assesses the experiences of physical, sexual and emotional abuse, as well as physical and emotional neglect before the age of 18 years. The sum score of all CTQ subscales was applied as a measure for maltreatment load. Based on the validated cut-off criteria of the CTQ (Bernstein & Fink, 1998), reported CM experiences were classified as “no”, “low”, “moderate” and “severe” in each of the five subscales. Three months postpartum (t1), further sociodemographic and medical data were assessed and peripheral blood samples could be drawn from 58 study participants (failure of blood sampling for PBMC isolation in seven cases and failure of blood sampling for serum separation in two cases due to technical reasons). As obesity is known to be associated with higher cytokine secretion (Das, 2001), a body mass index (BMI) of $> 30\,kg/m^2$ was set as an exclusion criterion for the investigation of CM-associated changes in *ex vivo* cytokine release, which lead to the exclusion of five study participants.

In total, fourteen of the 53 women, who provided peripheral blood samples at t1 and had a BMI $< 30\,kg/m^2$, reported moderate or severe CM experiences in at least one of the CTQ subscales and were therefore classified as positive for a history of CM (CM+). Out of the remaining 39 women (CM-), 14 women with a comparable age and body mass-index (BMI) were matched to these 14 women from the CM+ group. None of the study participants reported any immunologically relevant acute physical diseases.

2.2. Blood collection and sample processing

To minimize circadian rhythm-based fluctuation on endocrine hormones, 30 ml peripheral blood were collected between 12:30 p.m. and

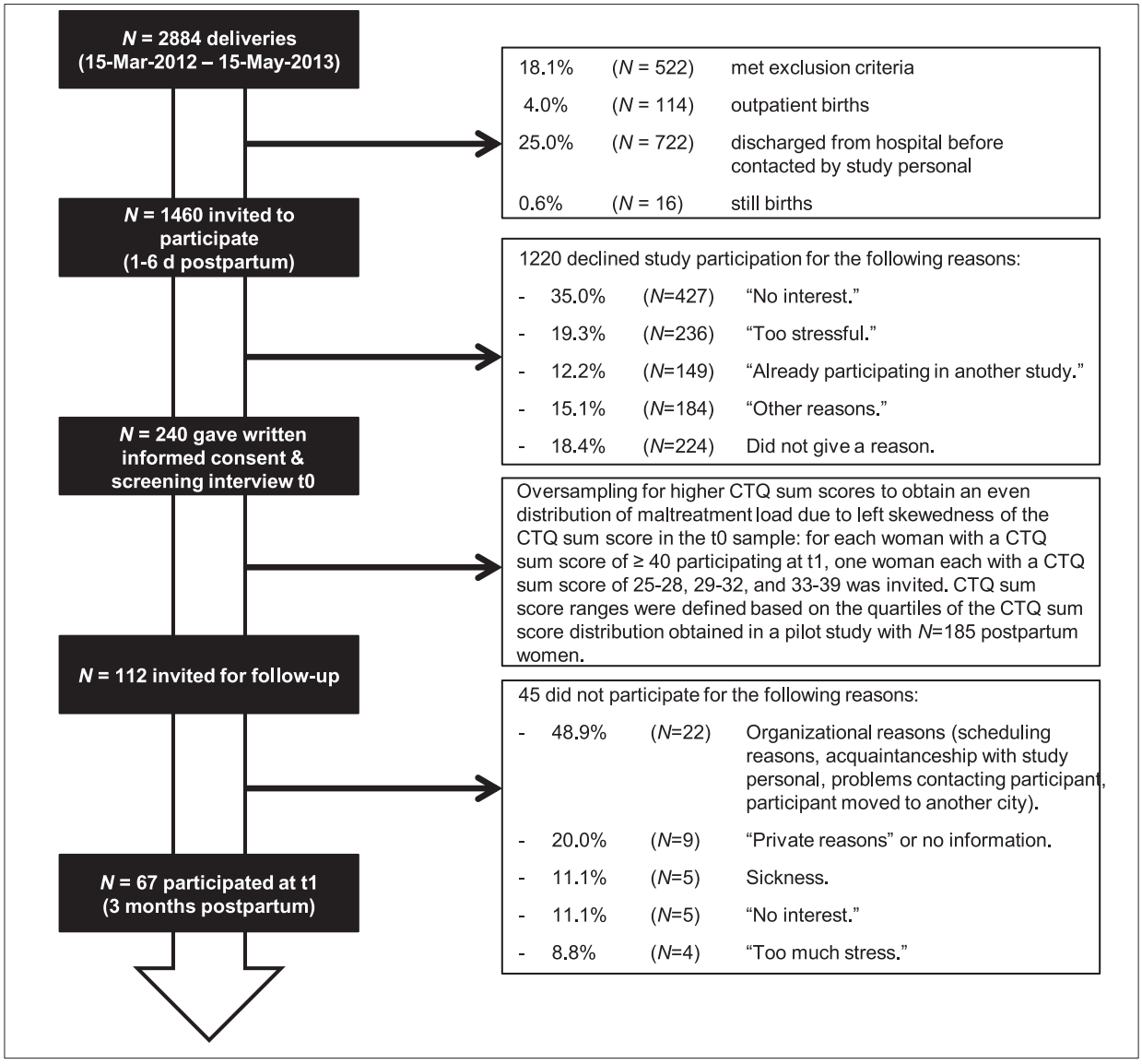

Fig. 1. Overview over study flow, recruitment procedure and withdrawal rates.

2:00 p.m. The two groups did not differ with respect to time of blood collection (Table 1). Immediately after blood drawings, PBMC were isolated using Ficoll-Hypaque gradient centrifugation (GE Healthcare, Chalfon St Giles, UK) according to the manufacturer's protocol. The isolated PBMC were frozen at -80°C in standard cryo-protective freezing medium (dimethyl sulphoxide: Sigma-Aldrich, St. Louis, MO, USA; fetal calf serum: Sigma-Aldrich; dilution: 1:10) until further analysis. For serum sampling, another 7.5 ml peripheral blood were drawn into pre-chilled (4°C) Z-Gel monovettes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged at 3000 *g* for 10 minutes at 4°C and aliquots of 250 µl were stored at -80°C.

2.3. Analysis of endocrine levels in serum

Serum aliquots were shipped on dry ice for the quantification of cortisol and DHEA levels via luminescence immunoassays (IBL international, Hamburg, Germany) to the laboratory of C. Kirschbaum (University of Dresden, Germany). As approximately 90% of serum cortisol is bound to corticosteroid-binding globulin (CBG), we additionally assessed serum levels of CBG by sandwich enzyme immunoassay (IBL International) according to the manufacturer's protocol. Mean inter-assay precision was described with an intra-assay coefficient of variation (CV) of 2.7% - 4% and the mean inter-assay precision as 6.3% - 7.2% CV by the manufacturer for cortisol, an intra-assay CV of 3.9% - 7.6% and the mean inter-assay precision as 5.1% – 10.4% CV for DHEA and an intra-assay CV of 1.2% - 2.2% and the mean inter-assay precision as 6.8% – 7.3% CV for CBG.

2.4. Cell culture and treatment

Frozen PBMC were first thawed by resuspending the frozen cells with pre-warmed phosphate-buffered saline (PBS; Life Technologies, Carlsbad, California, U.S.A.) supplemented with 2% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), centrifuged at 270 *g* for 10 min, resuspended with fresh PBS and the amount of living cells was counted using trypan blue staining. In one case, the percentage amount of dead cells in the thawed sample exceeded 20% (in all other cases > 95% living cells), which led to the exclusion of this sample. PBMC were washed with PBS with 300 g for 10 min and diluted to a final concentration of 1×10^6 living cells per ml culture medium RPMI 1640 without phenol red (Life technologies, Carlsbad, CA, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin (PAA Laboratories, Pasching, Austria). PBMC were incubated overnight at 37°C and 5% CO_2 in 12-well flat-bottom culture plates (Corning - Life

Table 1
Socio-demographic and clinical data of $N = 25$ study participants.

	Total	Groups		W/χ^2	r [d]	p
	($N = 25$)	CM+ ($N = 13$)	CM- ($N = 12$)			
Demographics						
Age (years)	32.0 ± 6.1	31.1 ± 6.1	33.1 ± 6.2	93.5	−0.16	.41
BMI (kg/m^2)	23.5 ± 2.4	23.8 ± 2.6	23.2 ± 2.4	71	−0.07	.73
Smoking status (yes, *N* (%)) [a]	5 (21.7)	4 (36.4)	1 (8.3)	1.26		.26
Caucasian ethnicity (yes, *N* (%))	24 (95)	13 (100)	11 (92)	0.81		.37
University degree (yes, *N* (%))	10 (40)	4 (36.4)	6 (50)	0.33		.57
Vaginal delivery (yes, *N* (%))	23 (100)	13 (100)	10 (83.3)	0.64		.43
Breastfeeding (yes, N(%))	21 (84)	9 (69.2)	12 (100)	2.40		.12
Maltreatment load						
CTQ sum score	42.6 ± 14.6	53.3 ± 12.6	31.1 ± 3.5	2	−0.82	<0.01
Emotional abuse sum score	9.7 ± 5.6	12.9 ± 6.1	6.3 ± 1.4	22.5	−0.61	<0.01
Physical abuse sum score	7.1 ± 4.0	8.8 ± 5.0	5.3 ± 0.5	48	−0.36	.07
Sexual abuse sum score	7.0 ± 4.5	8.8 ± 5.8	5.0 ± 0	42	−0.52	<0.01
Emotional neglect sum score	12.0 ± 4.8	14.8 ± 4.8	9.0 ± 2.5	25	−0.57	<0.01
Physical neglect sum score	6.9 ± 3.0	8.2 ± 3.7	5.6 ± 1.0	45	−0.38	.06
Chronic illnesses						
Thyroid disease (yes, *N* (%))	4 (12)	2 (15.4)	2 (16.7)			
Hypertension (yes, *N* (%))	1 (4)	1 (7.7)	0			
Chronic bronchitis (yes, *N* (%))	1 (4)	1 (7.7)	0			
Colitis ulcerosa (yes, *N* (%))	1 (4)	0	1(8.3)			
Allergy (yes, *N* (%))	1 (4)	1 (7.7)	0			
Medication intake						
L-Thyroxin (yes, *N* (%))	4 (16)	2 (15.4)	2 (16.7)			
Antidepressants (yes, *N* (%))	5 (20)	3 (23.1)	2 (16.7)			
Steroid hormone levels						
Cortisol serum level (ng/ml)	247.1 ± 71.6	228.3 ± 42.7	267.4 ± 91.2	98	−0.21	.29
CBG serum level (µg/ml) [b]	34.3 ± 6.5	33.4 ± 8.4	35.2 ± 3.6	107	−0.31	.12
DHEA serum level (ng/ml)	6.5 ± 4.2	7.0 ± 4.7	5.9 ± 3.7	69	−0.09	.65
Time of blood collection [c]	12.9 ± 0.4	13.0 ± 0.4	12.9 ± 0.4	77	−0.01	.98
PBMC subset composition (% within the living cell fraction)%						
T cells	57.8 ± 9.8	60.1 ± 9.2	55.3 ± 10.2	53	−0.27	.18
Cytotoxic T cells (CD8$^+$)	15.7 ± 6.2	16.1 ± 4.9	15.3 ± 7.6	66	−0.12	.54
Non-cytotoxic T cells (CD8$^-$)	42.1 ± 7.6	44.0 ± 8.0	39.9 ± 6.7	51.2	−0.28	.16
Monocytes	10.6 ± 3.9	10.4 ± 4.7	10.9 ± 3.0	93.5	−0.16	.41
B and NK cells	31.6 ± 8.1	29.6 ± 7.1	33.8 ± 8.8	99.5	−0.23	.25

All values are given as mean ± standard deviation, if not stated otherwise.
CM = Childhood maltreatment, BMI = *Body mass index*, CTQ = *Childhood Trauma Questionnaire*, CBG = Cortisol-binding globulin, DHEA = Dehydroepiandrosterone, NK cells = Natural killer cells, PBMC = Peripheral blood mononuclear cells.
[a] : $N = 23$, $N\ (CM+) = 11$, $N\ (CM-) = 12$
[b] : After exclusion of one outlier (> 3.5 SD from mean) in CM+ group: 31.3 ± 4.2 µg/ml [$W = 107$, $r = -0.40$, $p = .045$ vs. CM-]
[c] : hours from midnight
[d] : effect size

Sciences, Durham, NC, USA). The medium was replaced after this recovery phase by fresh culture medium supplemented with either cortisol or DHEA separately - each with a final concentration of 10 µM per well - or a combination of both with a concentration of 10 µM each. All steroid hormones were purchased from Sigma-Aldrich and were dissolved in DMSO (Sigma-Aldrich, Taufenkirchen, Germany). After an incubation period of 24 h, cell supernatants were collected by centrifugation at 300 *g* for 10 min and aliquots of 600 µl were immediately stored at −80°C until further analysis. Exploratory analyses on separate samples ($N = 4$) revealed that cell viability was not significantly affected by steroid hormone treatment (percentages of living cells following the incubation with cortisol, DHEA, co-treatment with cortisol and DHEA as well as without hormone treatment were all > 93%).

2.5. Flow cytometry analysis of PBMC composition

To control for possible differences in the subcellular composition of PBMC between individuals with and without CM, the fractions of monocytes (CD14$^+$), T cells (CD3$^+$), and B and NK cells (CD3$^-$CD14$^-$) were quantitatively analyzed by flow cytometry. The fraction of T cells

Table 2
Pro-inflammatory cytokine levels in cell culture medium of unstimulated PBMC and following treatment with cortisol, DHEA, and co-stimulation with cortisol and DHEA.

	Treatment	Total (N = 25)	Groups		W	r [a]	p
			CM+ (N = 13)	CM- (N = 12)			
IL-1β (pg/ml)	Unstimulated	194.8 (353.5)	411.9 (489.1)	125.6 (134.9)	38	−0.43	.03
	Cortisol	204.4 (343.3)	426.9 (519.2)	101.6 (155.0)	38	−0.43	.03
	DHEA	288.8 (487.9)	626.6 (662.3)	198.3 (137.5)	38	−0.43	.03
	Cortisol + DHEA	161.0 (339.9)	350 (368.5)	90.4 (111.0)	32	−0.51	.01
IL-6 (pg/ml)	Unstimulated	1003.5 (1598.9)	1933.3 (2486.2)	775.6 (607.8)	35	−0.47	.02
	Cortisol	1585.0 (2129.9)	2317.6 (1284.1)	768 (989.1)	35	−0.47	.02
	DHEA	1590.4 (2197.2)	2486.1 (2579.0)	1057.1 (789.5)	28	−0.56	.01
	Cortisol + DHEA	1174.7 (1649.1)	2348.9 (2033.3)	719.9 (696.5)	27	−0.57	<0.01
TNF-α (pg/ml)	Unstimulated	237.1 (417.8)	441.3 (413.0)	191 (154.1)	34	−0.48	.02
	Cortisol	189.4 (321.1)	384.4 (263.0)	114.8 (134.6)	30	−0.53	.01
	DHEA	128.7 (193.9)	280.0 (255.5)	96.1 (52.5)	30	−0.53	.01
	Cortisol + DHEA	100.8 (232.1)	278.9 (251.7)	74.2 (54.7)	28	−0.56	.01

All measures are reported as median (interquartile range).
CM = Childhood maltreatment, DHEA = Dehydroepiandrosterone, IL = Interleukin, TNF-α = Tumor necrosis factor α.
[a] : effect size.

was further separated into cytotoxic T cells ($CD3^+CD8^+$) and non-cytotoxic T cells ($CD3^+CD8^-$, mainly representing T-helper cells). All antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany) and cells were stained according to the manufacturer's protocol. Flow cytometry analysis was performed on a BD FACSAria III and data were processed with BD FACSDIVA software (BD Biosciences, Heidelberg, Germany).

2.6. Determination of selected cytokine levels with bead-based multiplex assays

For the analysis of cytokine levels, cell supernatants were thawed batch-wise and prepared according to the manufacturer's protocol for luminex bead-based multiplex assay analysis (Bio-Rad Laboratories, Hercules, CA, USA). The levels of IL1-β, IL-6, and TNF-α were quantified in duplicates using a microplate reader (Bio-Plex 200 System, Bio-Rad Laboratories). The software Bio-Plex Manager™ was used to calculate the absolute cytokine levels based on the fluorescence intensity of the respective cytokine standards. Assay detection range for the analyzed cytokines were 0.8–13,000 pg/ml for IL-1β, 0.5-7640 pg/ml for IL-6 and 0.9–13,879 pg/ml for TNF-α. Mean inter-assay precision was described with a coefficient of variation (CV) of 5% and the mean intra-assay precision as 3% CV for the analyzed cytokines (Gupta et al., 2000).

Two women of the CM- group showed an average 3.5 times higher cytokine secretion in both the unstimulated and stimulated conditions than the remaining study cohort. Analysis of sociodemographic data revealed that these women had experienced a case of death of a close family member within the past three months. As current grief was already described to be associated with enhanced plasma levels of pro-inflammatory cytokines (Schultze-Florey et al., 2012), these women were excluded from all further analyses. In the remaining study sample, no cases of death of a close family member were reported.

2.7. Statistical analysis

Statistical analyses were performed with *R 3.2.0* (R Core Team, 2015) and the alpha level for statistical significance was set to $p \leq 0.05$. To account for the small sample size (N = 25), all data were log-transformed (base 10) and non-parametric tests were used for group comparisons and correlation analyses. To analyze the association of CM with spontaneous and stimulated secretion of pro-inflammatory cytokines, and for the analysis of descriptive data, PBMC subset composition and cortisol, CBG, and DHEA serum levels, *Wilcoxon Rank Sum tests* were applied. To test the hypothesis that *ex vivo* secretion of pro-inflammatory cytokines was positively associated with maltreatment load, one-tailed *Kendall's τ* correlations were calculated. The effects of steroid hormone treatments were analyzed using linear mixed effects models with the R package "multcomp" (Hothorn, Bretz, & Westfall, 2008), modeling an interaction effect between group and treatment to test for CM-associated differences in the responsivity of PBMC to treatment with cortisol, DHEA and co-treatment with both cortisol and DHEA.

3. Results

3.1. Study cohort

Descriptive statistics for socio-demographic and clinical variables are summarized in Table 1. The age- and BMI-matched CM+ and CM- group did not differ with respect to current smoking status and socio-economic status (as estimated by the number of study participants holding a university degree). Furthermore, there were no group differences in the serum levels of cortisol and DHEA, as well as the subset composition of PBMC (see Table 1). The CM+ group presented, however, reduced serum levels of CBG, which was significant when one outlier (> 3.5 SD from the group mean) was excluded from the analysis.

3.2. Increased pro-inflammatory cytokine release with CM

Extending previous results from the same study cohort on spontaneous cytokine secretion (Boeck et al., 2016), the CM+ group showed not only significantly higher levels of pro-inflammatory cytokine (IL-6, IL-1β, TNF-α) secretion compared to the CM- group in the unstimulated condition, but also after stimulation with cortisol, DHEA, as well as with both cortisol and DHEA (see Table 2 and Fig. 2). Moreover, the CTQ sum score over the whole study cohort correlated positively with both the spontaneous and the stimulated secretion of IL-1β, IL-6, and TNF-α in each of the incubation conditions (Fig. 3).

3.3. Responsivity of PBMC to cortisol and DHEA treatment

Changes in cytokine release compared to unstimulated secretion

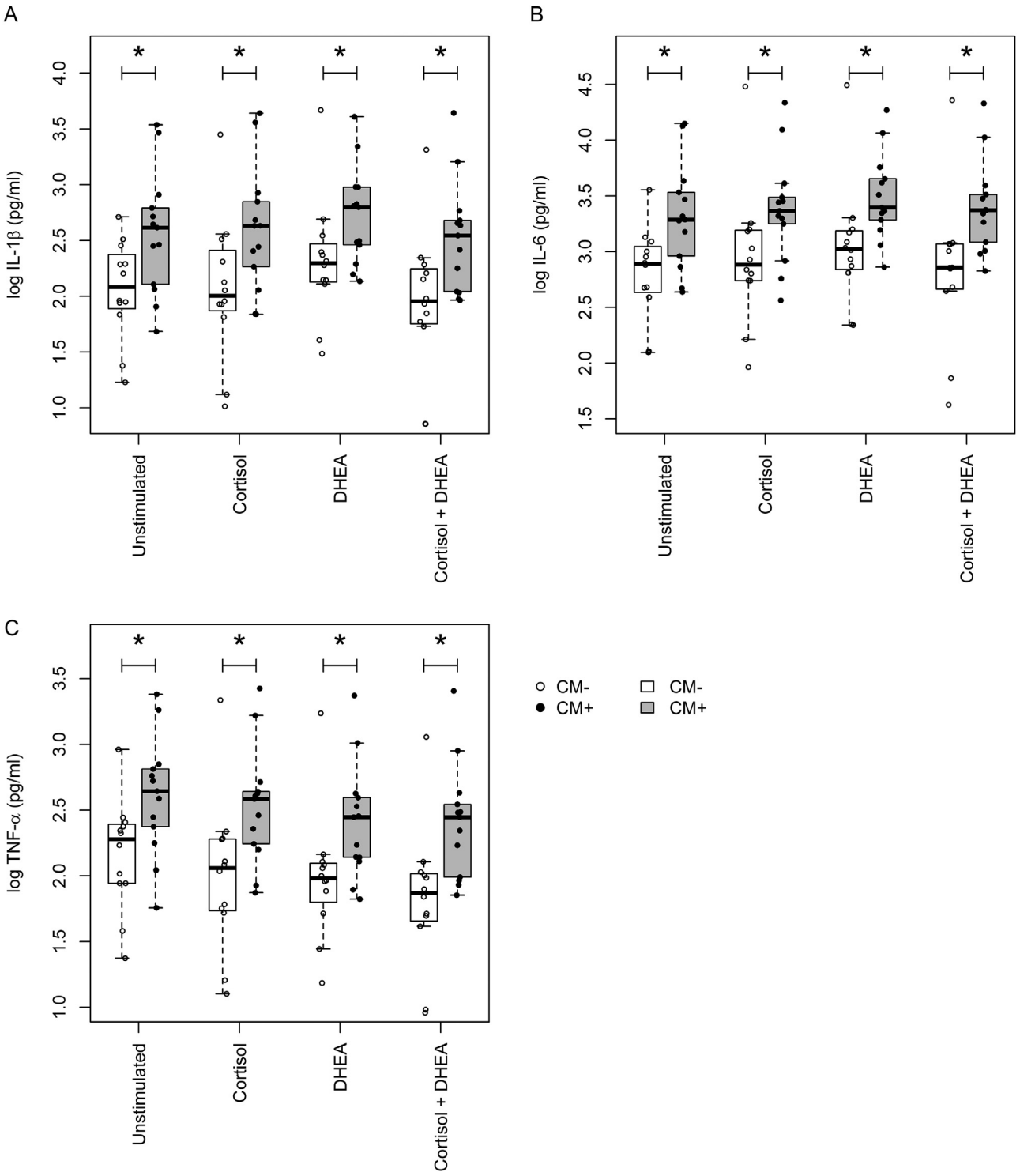

Fig. 2. Group comparisons of the secretion of A) IL-1β, B) IL-6, and C) TNF-α by unstimulated PBMC and after stimulation with 10 μM cortisol, 10 μM DHEA and costimulation with 10 μM cortisol and 10 μM DHEA. CM+ = women with a history of childhood maltreatment (N = 13), CM- = women without a history of childhood maltreatment (N = 12), DHEA = Dehydroepiandrosterone, IL-1β = Interleukin-1β, IL-6 = Interleukin-6, TNF-α = Tumor necrosis factor alpha, log = log-transformed values are displayed (base 10). * $p < .05$.

were analyzed with linear mixed effects models. Modeling an interaction effect between Group and Treatment revealed only a marginally significant effect for TNF-α (TNF-α: $p = .10$), while no statistically significant effects were found for IL-1β and IL-6 (IL-1β: $p = .17$; IL-6: $p = .25$). The main effects of both Group and Treatment were, however, significant for each of the three pro-inflammatory cytokines (all $p < 0.0001$). Exploratory analyses of treatment effects in the CM+ and CM- groups by linear mixed effects models revealed that treatment with cortisol significantly suppressed the TNF-α secretion, whereas it did not influence cytokine release in the CM+ group (Fig. 1 and summarized in Table 3). Across both groups, treatment with DHEA significantly enhanced the secretion of IL-1β and IL-6, while TNF-α secretion was substantially decreased. The stimulatory effect of DHEA on the secretion of IL-1β and IL-6 disappeared, when PBMC of the CM+ and the

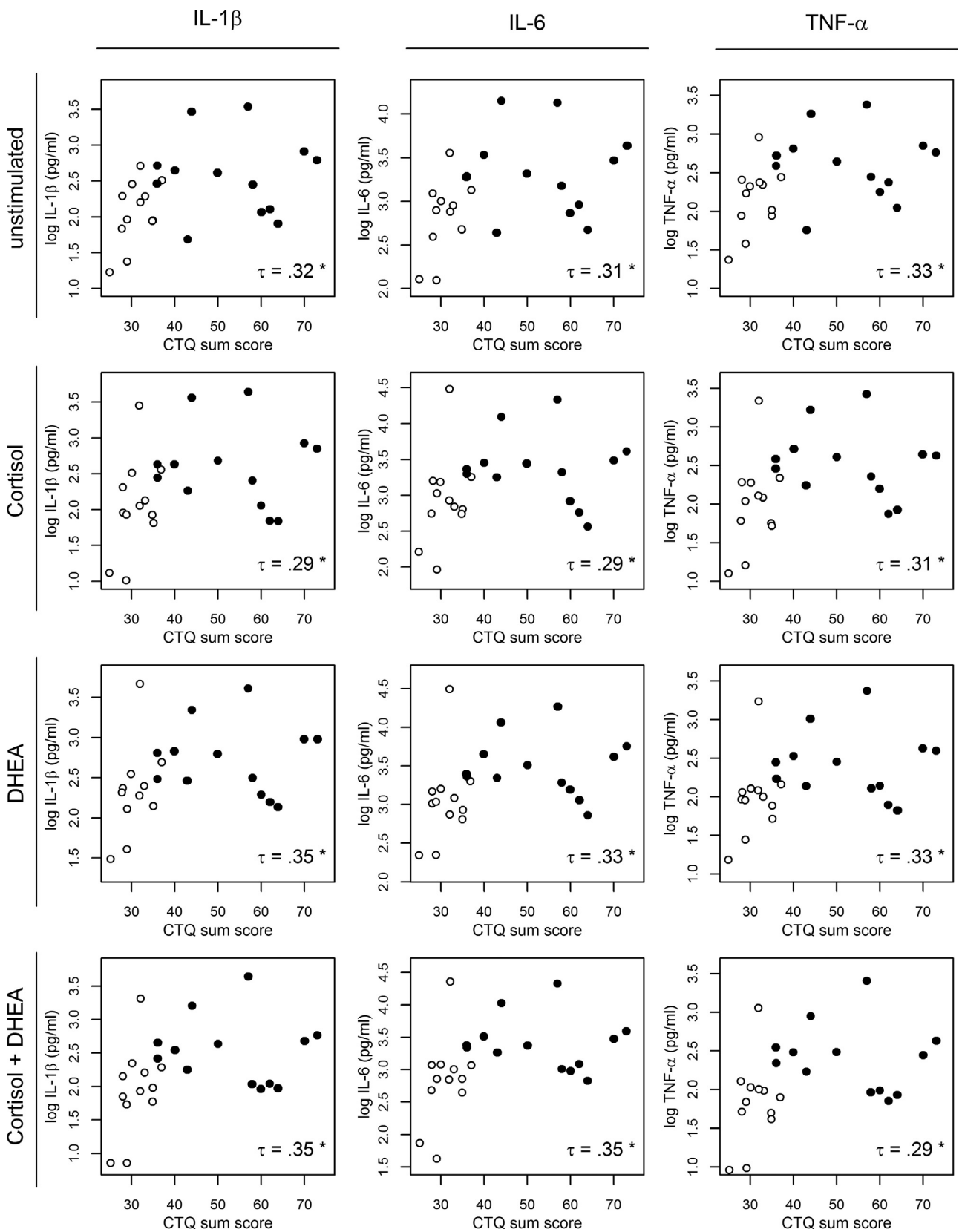

Fig. 3. Scatterplots for the association between maltreatment load as represented by the CTQ sum score and cytokine secretion by unstimulated PBMC and after stimulation with 10 μM cortisol, 10 μM DHEA and co-stimulation with 10 μM cortisol and 10 μM DHEA. Filled circles: CM+ = Women with a history of childhood maltreatment (N = 13), open circles: CM- = Women without a history of childhood maltreatment (N = 12). DHEA = Dehydroepiandrosterone, IL-1β = Interleukin-1beta, IL-6 = Interleukin-6, TNF-α = Tumor necrosis factor alpha log = log-transformed values are displayed (base 10). Kendalls τ correlation coefficients are displayed, * $p < .05$.

Table 3
Linear mixed effects models for the investigation of hormone stimulation on cytokine secretion in the CM+ and CM- group.

		CM+				CM-			
	Treatment	*F (df)*	*b* [a]	*z*	*p*	*F (df)*	*b* [a]	*z*	*p*
IL-1β	in total	91.0 (4,36)			< 0.0001	51.0 (4,33)			< 0.0001
	Cortisol		−0.07	−0.63	0.86		−0.01	−0.02	1
	DHEA		−0.36	−3.12	< 0.01		−0.58	−4.05	< 0.01
	Cortisol + DHEA		0.1	0.83	0.74		0.28	1.98	.12
IL-6	in total	204.5 (4,36)			< 0.0001	87.2 (4,33)			< 0.0001
	Cortisol		−0.14	−1.33	0.4		−0.33	−2.2	.07
	DHEA		−0.4	−3.82	< 0.01		−0.57	−3.77	< 0.01
	Cortisol +DHEA	−0.19	−1.82	0.17		−0.06	−0.39	.96	
TNF-α	in total	115.7 (4,36)			< 0.0001	60.5 (4,33)			< 0.0001
	Cortisol		0.18	1.59	0.26		0.37	3.46	< 0.01
	DHEA		0.45	3.93	< 0.01		0.41	3.87	< 0.01
	Cortisol + DHEA		0.47	4.12	< 0.01		0.78	7.31	< 0.01

CM = Childhood maltreatment, DHEA = Dehydroepiandrosterone, IL = Interleukin, TNF-α = Tumor necrosis factor α.
[a] : A negative estimate (*b)* indicates an increase in cytokine secretion compared to spontaneous cytokine secretion, and a positive estimate (*b)* indicates a decrease in cytokine secretion compared to spontaneous cytokine secretion.

CM- group were co-stimulated with cortisol. In the case of TNF-α, co-stimulation of PBMC with cortisol and DHEA led, however, to an even higher reduction in TNF-α secretion across both groups.

4. Discussion

The present results confirm previous reports on increased pro-inflammatory signaling by PBMC in individuals with CM. Both with and without stimulation, the secretion of IL-1β, IL-6, and TNF-α was significantly higher among individuals with CM experiences than among those without CM. Furthermore, the CTQ sum score correlated positively with the level of pro-inflammatory cytokine secretion. These findings might indicate a pre-activation of PBMC *in vivo*. The CM + and CM- group did not show significant differences in serum cortisol and DHEA levels. However, the decrease in serum CBG levels indicated an increased fraction of free, bioactive cortisol in the CM + compared to the CM- group. *Ex vivo* stimulation analyses provided preliminary evidence for a differential responsivity of PBMC in CM + and CM- women to cortisol regarding TNF-α secretion, but no difference in the responsivity to DHEA treatment. The results of the co-treatment with cortisol and DHEA support the hypothesis that cortisol and DHEA interact in the modulation of inflammatory processes.

4.1. CM is associated with higher pro-inflammatory signaling of PBMC

This study shows an association between a history of CM and pro-inflammatory signaling of PBMC. Women with a history of CM showed an increased spontaneous secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α into the cell culture medium compared to non-exposed women. Confirming a dose-response relationship, a higher maltreatment load was associated with a significantly higher spontaneous cytokine release and a significantly higher release of cytokines after stimulation with cortisol, DHEA as well as cortisol in combination with DHEA.

The results of this study are in line with Gola et al. (2013), who investigated cytokine secretion in traumatized individuals with PTSD and who observed the same pattern of increased spontaneous IL-1β, IL-6 and TNF-α secretion by PBMC of PTSD patients. Previous studies showed, however, that PBMC from PTSD patients did not differ in the secretion of IL-1β (Gola et al., 2013) and TNF-α (Gola et al., 2013; Rohleder, Joksimovic, Wolf, & Kirschbaum, 2004) from control subjects if PBMC were stimulated with LPS. In contrast to these results, Lopes et al. (2012) reported a reduced secretion of TNF-α following immune activation with phytohemagglutinin (PHA) in female patients with recurrent Major Depressive Disorder (MDD), current symptoms of PTSD and a history of CM, whereas PHA-stimulated secretion of IL-2, IL-4, IL-6, IL-10 and IFN-γ did not differ from MDD patients without a history of CM and without current PTSD symptoms. In our study, we did not analyze CM-dependent alterations in cytokine secretion following the activation of PBMC as induced by the immunogens LPS or PHA. Future studies are warranted to investigate whether the present findings can be translated to a state of antigen-induced immune activation or whether immune cells of CM+ women specifically differ with respect to the unstimulated release of pro-inflammatory cytokines, suggesting a basal pro-inflammatory milieu in the absence of immune activation. Due to the cross-sectional study design, it also remains to be elucidated whether the increase in cytokine release represents a general state of chronic low-grade inflammation that persists over time. Supporting this hypothesis, Ehrlich et al. (2016) recently showed that adolescents with a history of adverse early life events were consistently at a higher risk to show a pro-inflammatory phenotype over a period of 2.5 years. As blood samples were collected three months postpartum, it is, however, also possible that the observed increase in pro-inflammatory signaling with CM might be driven by a delay in wound healing processes, which was already described to be associated with chronic and perceived stress states (Gouin & Kiecolt-Glaser, 2011).

4.2. Serum CBG, but not cortisol and DHEA levels differ with CM

Analyses of serum cortisol and DHEA levels revealed no differences between the CM+ and CM- group. Cortisol in serum is, however, mainly bound to CBG (about 80-90%) and only the free, unbound fraction of cortisol can translocate into target cells and bind to GR. The analyses of the biological sequelae of early life stress in animals already showed a reduction in CBG levels in rodents following maternal separation (Viau, Sharma, & Meaney, 1996). Our data indicate that serum CBG levels were reduced in individuals with CM, which might translate to higher levels of unbound cortisol. We speculate that this might represent a counter-regulatory mechanism to dampen the pro-inflammatory milieu that is associated with CM.

4.3. CM is associated with changes in the responsivity of PBMC to cortisol but not DHEA

Ex vivo stimulation analyses revealed that PBMC of CM+ women were less sensitive to cortisol exposure than the PBMC of CM- women:

while cortisol suppressed the secretion of TNF-α in the CM- group, the treatment did not influence the release of pro-inflammatory cytokines in the CM+ group. In contrast, treatment with cortisol did not significantly inhibit IL-6 and IL-1β secretion in the CM- group. This finding might indicate a shift from a Th1 (e.g. TNF-α) to a Th2 (e.g. IL-6) immune response. This shift has previously been associated with cortisol (Elenkov & Chrousos, 1999). Cortisol mediates its effects via binding to two types of receptors, the GR and the mineralocorticoid receptor (MR), which are both expressed in peripheral immune cells. Alterations in GR responsivity are well-described in association with CM and might underlie the observed differential reactivity of PBMC to cortisol treatment. While epigenetic and functional changes affecting GR signaling were extensively studied in the context of CM, data on the MR is sparse and produced controversial results so far. It is, however, possible that alterations in MR signaling might also contribute to the observed reduction in cortisol responsivity in the CM+ group.

CM+ and CM- women did not differ with respect to DHEA-stimulated cytokine secretion. *Ex vivo* treatment with DHEA induced in PBMC of both CM+ and CM- women an increase in IL-1β and IL-6 secretion, while the release of TNF-α was suppressed. In contrast to this observation, DHEA was previously reported to support a shift from a Th2 to a Th1 immune response (Reza, 2009; Straub et al., 2002). The influence of DHEA appears, however, to be dependent on the dosage applied. Straub et al. (1998) showed that the effects of DHEA on cytokine secretion followed a U-shaped curve, with nM concentrations inhibiting IL-6 release, while concentrations in the μM range increased IL-6 secretion of LPS-stimulated PBMC. Over all dosages applied, there was no influence of DHEA on the release of TNF-α by LPS-stimulated PBMC (Straub et al., 1998). In accordance with our results, stimulation of human spleen cells with increasing concentrations of DHEA (10^{-10} to 10^{-4} M) inhibited the release of various cytokines (e.g TNF-α), while IL-6 was resistant to the inhibitory effect of DHEA (Young, Skibinski, Skibinska, Mason, & James, 2001). As it has been shown that the influence of DHEA on cytokine secretion is independent of the GR (Straub et al., 1998) and CM+ and CM- women did not differ in their responsivity to DHEA, we hypothesize that a history of CM is associated with cellular alterations specifically affecting GR-mediated signaling. In line with our results, Lopes et al. (2012) reported that PHA-stimulated PBMC of depressed patients with a history of CM were less sensitive to the inhibitory effects of the selective GR agonist dexamethasone on T cell proliferation, while high concentrations (10^{-6} to 10^{-4} M) of the sulfate ester of DHEA (DHEA-S) did not induce substantially different effects compared to PBMC of MDD patients without CM. How DHEA exerts its direct immune-modulatory effects on a biomolecular level is not completely understood. It has been suggested that DHEA activates multiple receptors (Webb, Geoghegan, Prough, & Michael Miller, 2006) including membrane-associated receptors (Liu & Dillon, 2002) and intracellular DHEA binding sites, which have been reported in human T lymphocytes (Okabe et al., 1995). As DHEA is the precursor of several bioactive metabolites (e.g., androgens, estrogens), it is also possible that the intracellular metabolism of DHEA contributes to its immune-modulatory effects.

This study is the first to additionally investigate the influence of a direct co-stimulation of PBMC with both cortisol and DHEA. Cytokine release after co-treatment did not differ between CM+ and CM- women and showed across both groups a cytokine-specific pattern: while the stimulatory effect of DHEA on IL-1β and IL-6 secretion disappeared, the inhibitory effect of DHEA on the release of TNF-α was even potentiated under co-treatment with cortisol. Together these results support the perspective that these two hormones interact in the modulation of inflammatory processes. Moreover, the finding of an additive inhibitory effect of cortisol and DHEA on TNF-α secretion provides further evidence for a specific effect of DHEA on TNF-α, which differs from its influence on IL-1β and IL-6 secretion.

4.4. Limitations and future perspectives

The investigated sample size was relatively small; however, (1) the consistent findings for all three cytokines and (2) the positive correlations with maltreatment load as well as (3) the consistency of the results with findings from previous studies argue for the strengths of the results. It is noteworthy that we found such significant relations between cytokine secretion and CM in a study cohort consisting of healthy women mainly reporting a high socio-economic status and low to moderate maltreatment load. Based on the observed cumulative effect of maltreatment load, we would expect even stronger effects in a sample with severe CM experiences. The small sample size precluded the inclusion of potentially confounding covariates into the statistical analyses. To minimize the effects of age and BMI, two factors that are known to influence inflammatory processes, study participants with a BMI over 30 kg/m^2 were excluded and the CM+ and CM- group were matched for age and BMI. Future replication studies with larger study cohorts should consider these factors as potential confounders.

As blood sampling took place three months postpartum, future studies including male and non-postpartum female study participants are needed to confirm the generalizability of the presented results. Pregnancy and especially the early postpartum period were reported to be associated with higher levels of inflammation (Christian & Porter, 2014). While cortisol levels are high during the last trimester of pregnancy, the drop after delivery is thought to further promote this pro-inflammatory milieu. Jung et al. (2011) showed, however, that plasma cortisol already returned to normal levels two to three months postpartum. Future studies including male study participants should, however, also investigate potential gender-differences in pro-inflammatory signaling and stress-response regulation in the context of CM. In this study, we assessed serum cortisol and DHEA levels only at a single time point in the early afternoon. The assessment was, however, standardized with respect to sampling time and mean time of blood drawings did not differ between the two groups. We stimulated the PBMC for a standardized period of 24 hours with supra-physiological doses of cortisol and DHEA. Depending on the concentration and duration of exposure, cortisol can exert diverse immune-modulatory effects, ranging from immune-enhancing to immunosuppressive actions (Dhabhar, 2002). We found preliminary evidence for the interactive effects of cortisol and DHEA, however, the application of various concentrations of cortisol, DHEA and a combination of both is needed to provide a conclusive evaluation of the presumably dose-dependent interactive hormonal effects on the modulation of inflammatory processes. Additionally, varying ratios of cortisol to DHEA might improve the knowledge of the biochemical interaction of the two bioactive molecules. To gain a better understanding of the biological mechanisms underlying the pro-inflammatory phenotype associated with CM, future studies should additionally account for differences in GR density and function (e.g., by testing CM-related alterations in GR suppression under dexamethasone treatment). Furthermore, as inflammatory processes are regulated by the interplay between pro- and anti-inflammatory cytokines, future studies are warranted that also investigate differences in anti-inflammatory cytokine secretion with CM.

5. Conclusion

In summary, these results suggest that the experience of maltreatment during childhood is associated with a pro-inflammatory phenotype. This phenotype was represented by higher spontaneous secretion of pro-inflammatory cytokines and a reduced responsivity of PBMC to cortisol with regard to TNF-α secretion. More severe CM experiences were associated with higher levels of inflammation, indicating a dose-response relationship. Co-treatment of PBMC with cortisol and DHEA alleviated the stimulatory effect of DHEA on Th1 cytokine release (IL-1β, IL-6), while exacerbating the effect on Th2 cytokine suppression (TNF-α). CM seems to specifically affect cortisol signaling at the cellular

level, as there were no group differences in the cytokine response of PBMC following treatment with DHEA. Together, these findings provide further evidence that CM is associated with long-lasting consequences on the immune system, which might be the cause for the high prevalence of adverse physical health outcomes among affected individuals.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Conflict of interest

All authors report no conflicts of interest in relation to this work.

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Authors' contributions

This study was part of a pilot for the project "My childhood – your childhood", funded by the BMBF between 2013 and 2016. The project was conceptualized by ITK and AK. The design of the present study was conceptualized by AK. Study participants were recruited by KS. She further performed diagnostic interviews and collected the psychological data. MG and CB co-designed and realized the experiments. Cell culture work and cytokine assays were performed by MG under the supervision of CB. Statistical analyses were performed by MG with support from AMK and SK. MG and CB analyzed and interpreted the data and wrote the first draft of the manuscript. All authors read, revised and approved the final version of the manuscript.

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