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Natural killer cell cytotoxicity and lymphocyte perforin expression in veterans with posttraumatic stress disorder

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ABSTRACT

Objective: To examine the effect of posttraumatic stress disorder (PTSD) on the measures of immune function and the hypothalamic–pituitary–adrenal axis components, and to determine whether additional life stressors affect measured variables.
Methods: We simultaneously examined the natural killer cell cytotoxicity (NKCC), perforin and glucocorticoid receptor (GCR) expression in natural killer (NK) and cytotoxic T (CD8) cells, as well as serum cortisol concentration in a group of Croatian war veterans with chronic, combat-related PTSD ($n=29$) and a group of healthy, age-matched men ($n=13$). PTSD patients were divided into two subgroups: compensation-seeking ($n=15$) and retired or compensation non-seeking ($n=14$) subjects. The former includes those involved in the process of getting disability-based army retirement as an additional life stressor.
Results: NKCC was decreased in both PTSD groups when compared to controls. Impairment of NKCC could not be attributed to the perforin expression as perforin was not decreased in comparison to controls. Moreover, the increased level of perforin was recorded in NK cells of retired PTSD subjects. Both PTSD groups shared an increased relative quantity of GCR in lymphocytes, whereas no difference between the groups in the baseline levels of serum cortisol was observed.
Conclusions: Diminished NKCC was not accompanied by perforin insufficiency in PTSD subjects, and other causes should be examined. An additional life stressor does not contribute considerably to either immune or endocrine system related changes.

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1. Introduction

Natural killer cell cytotoxicity (NKCC) has been shown to be extremely vulnerable to various stressors and psychological changes. A meta-analysis of more than 300 studies on the impact of psychological stress on the human immune system showed that impaired NKCC is associated with the state of chronic stress (Segerstrom and Miller, 2004). Similar findings were observed in major depression (Jung and Irwin, 1999), anxiety (Borella et al., 1999), schizophrenia (Abdeljaber et al., 1994) and chronic fatigue syndrome (Maher et al., 2005). Studies of NKCC in posttraumatic stress disorder (PTSD) are relatively sparse and inconsistent results were reported (Ironson et al., 1997; Kawamura et al., 2001; Laudenslager et al., 1998; Mosnaim et al., 1993; Vidović et al., 2007). A study with Vietnam veterans reported no differences in baseline NKCC and decreased NKCC in PTSD sufferers after *in vitro* methionine–enkephaline challenge (Mosnaim et al., 1993), while another study observed an increased NK activity in Vietnam veterans (Laudenslager et al., 1998). PTSD symptom-associated decreases of NKCC were observed in gender-mixed Hurricane Andrew victims shortly after traumatic experiences (Ironson et al., 1997). NKCC was also lower in Japanese male workers identified with a past history of PTSD when compared to men who experienced similar trauma but didn't develop PTSD (Kawamura et al., 2001). When these and other trauma-related studies, not including diagnosis of PTSD (Delahanty

Abbreviations: ANCOVA, analysis of covariance; BDI, Beck Depression Inventory; CD, cluster of differentiation; CTL, cytotoxic T lymphocytes; DEX, dexamethasone; DPBS, Dullbeco's phosphate buffer saline; DSM, Diagnostic and Statistical Manual of Mental Disorders; DTH, delayed-type hypersensitivity; E:T, effector to target cell ratio; FCS, fetal calf serum; FDR, false discovery rate; FITC, fluorescein isothiocyanate; GCR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; IES-R, Impact of Event Scale—Revised; IL, interleukin; MANCOVA, multivariate analysis of covariance; m.f.i., mean fluorescence intensity; M-PTSD, Mississippi Scale for Combat-Related PTSD; NK, natural killer; NKCC, natural killer cell cytotoxicity; PBMC, peripheral blood mononuclear cells; PE, phycoerytrin; PerCP, peridinin chlorophyll protein; PTSD, posttraumatic stress disorder; STAI, Spilberger State-Trait Anxiety Inventory; WB, whole blood.

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et al., 1997; Solomon et al., 1997), are considered, there are indications that an acute stress response may be associated with increased NKCC followed by a decline if the stressor persists over time (Ironson et al., 2007). However, the overall picture in PTSD is still inconsistent, and the discrepancies between these findings may be due to differences in the type of stressor, the duration of trauma, age at which trauma occurred, the time between trauma and assessment, the severity of trauma and additional life stressors.

Cytolytic activity of NK cells and cytotoxic T lymphocytes (CTL), which participate in the killing of virus-infected-, tumor-, and allogenic cells through close contact (Voskoboinik and Trapani, 2006), is dependent upon functional perforin, a pore forming protein found in the exocytic granules of those cells. It is reasonable to assume that perforin insufficiency might be an underlying mechanism of impaired NKCC observed in PTSD (Škarpa et al., 2001). However, simultaneous examination of NKCC and expression of intracellular perforin levels in cytotoxic cells of PTSD sufferers have not been reported previously.

Acute or chronic exposure to physical and/or psychological stressors affects the immune system and *vice versa* as activation of immune cells can cause physiological, behavioral and affective changes (Ader et al., 1995). PTSD is associated with psychological, neuroendocrine (Wessa and Rohleder, 2007) and immune (Altemus et al., 2006; Stam, 2007; Gill et al., 2009) changes. It has been hypothesized that long-term effects of prolonged response to stress or malfunction in the stress system can result in pathologic outcomes over time (McEwen and Seeman, 1999); yet the mechanisms remain elusive. Hypothalamic–pituitary–adrenal (HPA) axis disturbances and consequent changes in the immune system may be the key mediators in this process. Various studies have demonstrated a decreased cortisol output and an elevated glucocorticoid receptor (GCR) expression in PTSD patients (reviewed by de Kloet et al., 2006). Furthermore, many studies showed that PTSD patients exhibited an enhanced feedback sensitivity of HPA-axis as indicated by enhanced cortisol suppression after low-dose dexamethasone (DEX) administration, suggesting enhanced GCR function (de Kloet et al., 2006; Yehuda et al., 1995). Increased GCR sensitivity is also supported by the finding that less DEX is needed to suppress pro-inflammatory cytokine production in the immune cells of PTSD patients (Rohleder et al., 2004). However, many other studies did not support those findings (de Kloet et al., 2007; Gotovac et al., 2003; Lindley et al., 2004; Vidović et al., 2007), suggesting that HPA-axis profile may depend on various factors such as trauma type and/or duration of PTSD and additional stressors. Moreover, it has been shown that trauma exposure alone is sufficient to induce changes in GCR expression (de Kloet et al., 2007) and enhanced cortisol suppression following DEX (de Kloet et al., 2008). Besides, while numerous studies to date have assessed the components of HPA-axis or immune system changes in PTSD separately (reviewed in Ironson et al., 2007), a few have addressed those systems simultaneously (de Kloet et al., 2007; Gill et al., 2008; Lemieux et al. 2008; Rohleder et al., 2004; Vidović et al., 2007), with the aim of elucidating the influence of PTSD on glucocorticoid immune regulation.

Recurrent activation of stress response in PTSD, e.g. through recollections and hyperarousal (American Psychiatric Association, 2000), might have long-term consequences on the immune function far beyond the time when an actual event had happened (Kawamura et al., 2001). It has been shown that antecedent chronic life stress modulates immune reactivity to additional acute stressors (Benschop et al., 1994; Brosschot et al., 1994; Pike et al., 1997; Glover et al., 2005). Additional life stress (Benyamini and Solomon, 2005), such as unemployment and low socioeconomic status (McCarren et al., 1995), disruption of relationships (Prigerson et al., 2001), post-war adjustment difficulties and increased somatic symptoms and/or illnesses (Boscarino, 2004; Schnurr and Jankowski, 1999) were significantly associated with combat-related PTSD. Research among Croatian war veterans with PTSD showed similar results, such as enhanced social dysfunctions (Gregurek et al., 2001; Vukšić-Mihaljević et al., 1998), frequent psychosomatic complaints (Proroković et al., 2005), psychotic comorbidity (Kozarić-Kovačić and Borovečki, 2005) and increased risk of cardiovascular diseases (Karlović et al., 2004). Furthermore, Croatian war veterans with PTSD describe the administrative process of compensation-seeking as extremely stressful, long-lasting, disparaging and a very exhausting process (Vukušić et al., 2003). They experience a lack of understanding, isolation, injustice and often complain that the process "has made things worse" for them. This position is fairly common among combat-related PTSD population, and we presumed that it could be considered as an additional life stressor.

We hypothesized that chronic PTSD patients would have altered cytotoxic function, especially in the presence of an additional life stressor. To evaluate the cytotoxic capacity of immune cells, we determined NKCC and intracellular perforin expression as an indirect measure of NK ($CD3^-CD16^+$, $CD3^-CD56^+$) and CTL ($CD3^+CD8^+$) cytotoxic potential. At the same time, baseline serum cortisol levels and GCR expression in lymphocytes were also assessed. To examine the possible influence of additional life stress experience on biological variables, PTSD patients were subdivided into two groups according to their status of being retired or being involved in the process of seeking military pension.

2. Subjects and methods

2.1. Subjects

We examined a group of Croatian war veterans with chronic, combat-related PTSD ($n=29$) and a group of healthy controls ($n=13$). The PTSD patients were all men, recruited from an outpatient program at the Department of Psychological Medicine at the University Hospital Center Zagreb, and were divided into two subgroups: a) retired, compensation non-seeking group ($n=14$) and b) compensation-seeking group ($n=15$) which included patients who were in the bureaucratic process of getting mental disability-based army retirement. We considered that process as an additional life stressor. Exclusion criteria were other major life stress experiences (i.e. income loss, divorce, and death of a beloved person) during the year prior to examination, or drug or alcohol abuse. Three subjects from the compensation-seeking group and two retired subjects had been divorced for years before the study and another five subjects seeking pension were officially unemployed but had a steady income through welfare or part-time jobs.

Prior to blood drawing, all study participants were examined and interviewed by an experienced clinician (a psychiatrist), and relevant data for the purpose of this study were recorded on the basis of their histories. The subjects were then asked to complete questionnaires related to demographic data. In addition, the patients completed the scales related to PTSD, depression and anxiety symptoms.

During the psychiatric interview, patients reported the absence of any previous psychiatric disorders, and were not diagnosed with major depression or other psychiatric comorbidity. Multimodal assessment approach was used in the diagnosis of PTSD (Keane et al., 1996.) All patients met the ICD-10 (World Health Organisation, 1992) PTSD criteria, the official classification in Croatian psychiatric practice, as well as DSM-IV (American Psychiatric Association, 2000) criteria. Characteristics of traumatic events are summarized in Table 2. During the previous year, the diagnosis of PTSD had been confirmed using Clinician-Administered PTSD Scale (CAPS) (Blake et al., 1995). For the purpose of this study, the severity of symptoms during the week preceding the interview was assessed by the 39-item Mississippi Scale for Combat-Related PTSD (M-PTSD) (Keane et al., 1988) and Impact of Event Scale—Revised (IES-R) (Weiss and Marmar, 1997). According to M-PTSD, every patient scored above the cut-off value (107) for diagnosis of PTSD (Keane et al., 1988), and only three patients had scores less than 121, the cut-off value that was proposed later (Lyons et al., 1994). Depression and anxiety symptoms were checked with Beck Depression

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Inventory (BDI) (Beck et al., 1996) and Spielberger State-Trait Anxiety Inventory (STAI-T, STAI-S) (Spielberger et al., 1970).

The healthy control subjects were recruited among hospital personnel and staff from the Institute of Immunology and matched by sex and age to PTSD patients. Five out of thirteen controls reported combat experience but did not experience a traumatic event that could induce PTSD as defined by DSM-IV criterion A. Healthy controls had negative histories of any psychiatric disorders and no symptoms or signs of current psychiatric disease.

At the time of sampling, all study subjects were free from any psychotropic medication for at least one month and did not suffer from any infectious, allergic or endocrine disorder. They had no symptoms or signs of acute or chronic physical illness.

The Ethics Committee of University Hospital Center Zagreb approved the study protocol. A written informed consent was provided by all subjects after the study design had been fully explained, and prior to undergoing psychiatric evaluation and blood collection.

2.2. Methods

2.2.1. Samples

Peripheral blood samples were obtained from patients with PTSD and healthy controls by venipuncture between 7:00 and 8:00 a.m. Ten milliliters of blood were collected in Vacutainer tubes with heparin and 5 mL in tubes without anticoagulant for serum separation (Becton Dickinson (BD), Vacutainer Systems, Plymouth, UK). Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll–Paque™ Plus density gradient (Amersham Biosciences, Uppsala, Sweden), washed twice and resuspended in RPMI 1640 (Institute of Immunology, Zagreb, Croatia) supplemented with 10% fetal calf serum (FCS, Dipro, Wiener Neudorf, Austria) and 50 mg/L of gentamicin (Sigma, St. Louis, MO). The heparinized whole blood (WB) was used for flow cytometric analyses and PBMCs were used for *in vitro* test of NK cell cytotoxic activity. The sera were separated from clotted blood and stored at −80 °C until cortisol determination.

2.2.2. NK cell cytotoxicity assay

NK cell cytotoxic activity was assessed by the chromium-51 (^{51}Cr) release assay. The NK-sensitive K562 erythroleukemia cell line was used as target cells. They were labeled with $Na_2^{51}CrO_4$ (Amersham, Little Chalfont, UK; 3.7 MBq/3×10^6 cells) for 60 min at 37 °C, washed three times in RPMI 1640 and adjusted to 2×10^5/mL with RPMI 1640 containing 10% FCS. Effector cells (PBMC) were adjusted to 5×10^6/mL and serial 2-fold dilutions were prepared in complete medium. Three effectors to target (E:T) cell ratios (100:1, 50:1, and 25:1) were prepared in triplicate by mixing 200 μL of PBMC dilutions and 50 μL of target cells. After a 3-h incubation at 37 °C in a 5% CO_2 atmosphere, supernatants were harvested and radioactivity was assessed by a gamma-counter (1272 CliniGama, LKB Wallac, Turku, Finland). Spontaneous release and maximal release were determined in supernatants after incubating target cells without effector cells or by lysing target cells with Triton X-100 (Sigma, St. Louis, MO), respectively. The percentage of cytotoxicity was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)]×100. Results for all three ratios exhibited a high correlation, and comparisons between groups based on the separate measures of cytotoxicity were in full agreement. To minimize the number of comparisons (and consequently the type I error rate), a unique measure of NK cell activity was calculated by averaging the lysis across the three E:T ratios. A similar approach has already been used (Bartlett et al., 2001).

2.2.3. Immunophenotyping

Lymphocyte counts were determined by flow cytometry using FlowCount™ Fluorospheres (Coulter Corporation, Miami, FL) according to the manufacturer's protocol. The relative numbers of NK and cytotoxic T cells, as well as perforin expression in these cell populations were determined by three-color intracellular staining protocol for flow cytometry. The staining of cell-specific surface molecules in the WB samples was performed first with anti-human antibodies (5 μL each *per* 50 μL of WB): CD3-phycoerythrin (CD3-PE) or CD3-peridinin chlorophyll protein (CD3-PerCP), CD8-PerCP, CD16-PE, and CD56-PE. The mouse IgG_1 conjugated with FITC, PE or PerCP (all from BD Biosciences, Erembodegem-Aalst, Belgium) were used as isotype-specific controls. After 15 min of incubation on ice, the samples were washed in Dullbeco's phosphate buffer saline (DPBS) supplemented with 4% FCS (Dipro). The erythrocytes were removed with FACSLysing solution (BD Biosciences, San Jose, CA) and cells were washed. Fixation was done with 1% formaldehyde in DPBS for 15 min at 4 °C, and cells were then washed twice. The cells were permeabilized with cold 0.1% saponin in DPBS with 1% FCS and 0.1% NaN_3 (permeabilization buffer) and subsequently stained with FITC-conjugated mouse IgG_{2b} anti-perforin or appropriate isotype control (BD PharMingen, San Diego, CA) antibodies for 30 min at 4 °C. Finally, the cells were washed in permeabilization buffer, followed by a wash in DPBS with 4% FCS, and resuspended in 500 μL DPBS with 4% FCS.

The staining of intracellular GCR was performed following the same protocol but without the surface immunophenotyping step. The monoclonal antibody used was FITC-conjugated anti-GCR (mouse IgG_1, clone No. 5E4-B1) described by Berki et al. (1998). The antibody was diluted in permeabilization buffer to a ratio of 1:500, and 5 μL were added to the cell pellet after centrifugation in the permeabilization buffer. The optimal concentration (2.66 μg/mL) of anti-GCR monoclonal antibody was predetermined.

All samples were run on a FACSCalibur flow cytometer and analyzed with the CellQuest software (both BD Immunocytometry Systems, San Jose, CA). The following gates were used to distinguish the populations of interest: lymphocytes were identified and gated on forward *vs.* side scatter (FSC/SSC) plots, while cytotoxic *T*-cells and NK cells were gated on PE *vs.* PerCP plots defined as $CD3^+CD8^+$, and $CD3^-CD56^+$ or $CD3^-CD16^+$ cells, respectively. The percentage was calculated relative to the number of events in the lymphocyte gate. The FITC-fluorescence intensity of GCR and perforin in cell subpopulations or isotype controls were determined in histogram plots. The relative quantities of intracellular markers were expressed by mean fluorescence intensity (m.f.i.) calculated as the difference between mean channel-values of specific and isotype control staining.

2.2.4. Serum cortisol level

Serum concentrations of total cortisol were assessed in duplicate by an enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments, Germany). Procedures were conducted according to the manufacturer's instructions.

2.3. Statistical analysis

All data were checked for the presence of outliers and extreme values. Distribution analyses using Shapiro–Wilks' tests revealed deviation from normality in some variables. Variables that did not follow Gaussian distribution were transformed to meet the assumptions of multivariate parametric procedures (homogeneity of variances were checked using Levene's test and equality of covariance matrices for MANCOVA were assessed with Box's M test). Transformations were performed as follows: Box Cox for perforin expression in $CD3^-CD16^+$ and $CD3^-CD56^+$ cells, as well as NKCC, and cube transformation for lymphocyte GCR expression. Because the percentage of $CD3^-CD56^+$ cells could not be normalized, and since data on the absolute numbers of lymphocytes, due to technical reasons, were only available for a number of study subjects (compensation-seeking group, $n=9$; retired group, $n=4$; healthy controls, $n=13$) and did not satisfy the criteria for missing data imputation, groups were compared with Kruskal Wallis test followed by a median *Z* test. Back-transformed means and 95% confidence intervals of the means were

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reported to preserve characteristics of the initial distribution. When the non-parametric procedure was used, data were presented by median and interquartile range (IQR). Characteristics of the groups and trauma characteristics among patients were compared using χ^2-test for categorical variables and ANOVA, Mann–Whitney or independent sample *t*-test for continuous variables. Subsequent analyses were conducted to identify potential confounders. Independent sample *t*-tests were performed to examine differences in biological variables between smokers and non-smokers, and correlation analyses were used to determine associations of age with biological variables. Based on these analyses as well as on previous research, age was used as a covariate in the MANCOVA to analyze between-group differences in biological variables. All *P* values from subsequent ANCOVAs were corrected by the Bonferroni procedure, and *post hoc* Tukey honest significant difference tests were used to determine group differences. Partial correlations controlling for age were performed to determine the relationship of psychological test scores with biological variables and endocrine variables with immune variables. Statistical analyses were performed with Statistica v6 (StatSoft, Inc, Tulsa, OK).

3. Results

3.1. Sample characteristics

The groups were similar in size, age, marital status and education (Table 1). All subjects in the group of healthy controls reported average monthly income (Table 1) while retired patients and compensation-seeking group did not differ in self-reported income ($\chi^2=0.967$, $P=0.617$). Correlations performed with the immune and endocrine variables revealed an association of age with the expression of perforin in NK cells ($CD3^-CD16^+$: $r=0.624$, $P<.001$; and $CD3^-CD56^+$: $r=0.621$, $P<.001$). Although no other biological variable correlated significantly with age, we used it as a covariate in subsequent analyses because of the well-known influence of aging on the immune function (Gruver et al., 2007) as well as on the HPA-axis activity (Chahal and Drake, 2007).

3.2. PTSD symptoms

The groups diagnosed with PTSD were similar in the duration of the disorder as indicated by the lack of difference in the time elapsed from the diagnosis of PTSD and the self-reported duration of symptoms (Table 1). Duration of exposure to the traumatic environment was also similar between the two groups since they did not differ in the time spent in the army (Table 1). The time from the diagnosis, the duration of symptoms and the time spent in the army did not correlate with biological variables.

Retired patients and compensation-seeking patients had experienced similar traumatic events (Table 2). All patients had pronounced PTSD symptoms as indicated by M-PTSD and IES-R scores, yet retired patients did not differ from compensation-seeking patients (Table 1). The severity of the symptoms did not correlate with either immune or endocrine variables in PTSD patients.

3.3. Depression and anxiety

Patients exhibited a high level of depression symptoms as indicated by BDI scores, but no difference was found between retired patients and compensation-seeking patients (33.3 ± 12.5 *vs.* 35.2 ± 9.9, $t=-0.458$, $P=.404$). Retired patients tended to have slightly higher, but not statistically significant, state anxiety as well as higher trait anxiety scores (62.4 ± 10.0 *vs.* 56.1 ± 8.9, $t=1.794$, $P=.084$ and 57.7 ± 7.4 *vs.* 53.6 ± 6.3, $t=1.620$, $P=.110$ respectively). Since no correlation was found for biological variables, we did not consider that anxiety would have confounded the comparison of immune and endocrine variables in PTSD patients.

3.4. Immune and endocrine measures

Group comparisons are summarized in Table 3. Both PTSD groups had higher lymphocyte counts compared to healthy controls, but the results must be interpreted with caution since the data from only a limited number of patients were available. The groups did not differ in the percentage of circulating NK ($CD3^-CD56^+$, $CD3^-CD16^+$) and

Table 1
Characteristics of study subjects.

Variable	PTSD patients		Healthy controls ($n=13$)	Statistics[d]
	Retired ($n=14$)	Seeking pension ($n=15$)		
Age [a]	39.5 (5.8)	38.13 (6.2)	38.00 (6.2)	$F=0.263$, $P=0.770$
Smoking [b]	5 (36)	9 (60)	6 (46)	$\chi^2=1.728$, $P=0.421$
Marital status [b]				
Married	11 (79)	9 (60)	11 (85)	$\chi^2=7.901$, $P=0.095$
Single	1 (7)	3 (20)	2 (15)	
Divorced	2 (14)	3 (20)	0 (0)	
Education [b]				
Elementary school	4 (29)	2(13)	0 (0)	$\chi^2=3.930$, $P=0.416$
High school	9 (64)	11 (73)	8 (62)	
University	1 (7)	2 (13)	5 (38)	
Monthly income [b]				
Above average	0 (0)	1 (6)	0 (0)	$\chi^2=9.828$, $P=0.043$
Average	8 (57)	8 (53)	13 (100)	
Below average	6 (43)	6 (40)	0 (0)	
Years spent in army [c]	5.7 (4.5–6.6)	5.8 (5.0–9.0)		$U=78.0$, $P=0.239$
Years since diagnosis [c]	6.0 (5.0–6.0)	4.0 (1.0–6.0)		$U=23.0$, $P=0.242$
Duration of symptoms (years)[c]	8.0 (7.0–9.0)	8.0 (5.0–8.0)		$U=36.5$, $P=0.142$
Mississippi Scale for Combat-related PTSD [a]	147.9 (18.8)	142.3 (16.6)		$t=0.850$, $P=0.403$
Impact of event scale—revised [a]				
Intrusion	28.1 (3.1)	27.2 (3.9)		$t=0.642$, $P=0.526$
Avoidance	17.6 (6.8)	20.6 (4.6)		$t=1.382$., $P=0.178$
Hyperarousal	21.9 (2.2)	21.7 (2.9)		$t=0.146$, $P=0.885$
Total	67.3 (7.4)	69.3 (7.9)		$t=0.692$, $P=0.495$

[a] Means (SD).
[b] Numbers (%).
[c] Medians (interquartile range).
[d] ANOVA (*F* statistics), Pearson's χ^2-test (χ^2 statistics), Mann–Whitney's test (*U* statistics), independent sample *t*-test.

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Table 2
Traumas experienced by PTSD patients. Numbers and (percentages) of patients and Pearson's chi-square (χ^2) statistics are shown.

Trauma	Retired (n = 14)	Compensation-seeking (n = 15)	χ^2	P
Being wounded or injured	5 (36)	2 (13)	1.981	.159
Witnessing wounding	14 (100)	15 (100)		
Witnessing death	14 (100)	15 (100)		
Witnessing grotesque death (massacre)	13 (93)	14 (93)	0.003	.960
Witnessing torture	3 (21)	4 (27)	0.109	.742
Being tortured or being taken captive	0 (0)	3 (20)	3.123	.078
Serving on graves	11 (79)	8 (53)	2.042	.153
Forced to leave home/family	2 (14)	2 (13)	0.006	.941
Threatened with death	8 (57)	7 (47)	0.318	.573
Ambush, sniper, mine field experience	13 (93)	15 (100)	1.110	.292

cytotoxic T ($CD3^+CD8^+$) cells. Perforin expression in $CD3^-CD56^+$ and $CD3^-CD16^+$ cells was highest in retired patients. Expression of perforin in $CD3^+CD8^+$ cells seems to have been lower compared to $CD3^-CD16^+$ and $CD3^-CD56^+$ cells in all three groups but followed a similar pattern between the groups (i.e. higher levels in retired patients compared to compensation-seeking patients or healthy controls, although the difference was not significant). In contrast to perforin expression, both PTSD groups had lower mean NKCC compared to healthy volunteers. Moreover, with regard to age, NKCC positively correlated with perforin expression in $CD3^-CD16^+$ cells of healthy volunteers ($r = 0.658$, $P = .020$) but not in those from retired patients ($r = 0.174$, $P = .552$) or compensation-seeking patients ($r = 0.065$, $P = .832$).

A higher expression of GCR in lymphocytes was found in both groups of PTSD patients compared to healthy volunteers. However, no difference was observed in serum cortisol concentrations between the groups.

Age controlled partial correlations of immune variables with endocrine variables did not reveal any significant associations.

4. Discussion

Our results indicate that chronic, long-lasting, combat-related PTSD is associated with impaired NKCC and an increased level of GCR in lymphocytes. Additional stress had no major influence on the immune and/or endocrine variables studied in PTSD sufferers. Since NK cells have an important role fighting viruses and tumor cells, and since GCR mediates modulation of the immune system by glucocorticoids, these findings provide further insights into the potential pathways connecting PTSD and adverse health outcomes (Boscarino, 2004; Schnurr and Green, 2004).

Research of the immune system in PTSD in general has revealed activation of the immune system. An enhanced delayed-type hypersensitivity (DTH) reaction (Altemus et al., 2006; Boscarino, 2004; Watson-Burges et al., 1993), increased levels of pro-inflammatory cytokines (Baker et al., 2001; Hoge et al., 2009; Maes et al., 1999; Pervanidou et al., 2007; Spivak et al., 1997; Tucker et al., 2004; von Kanel et al., 2007), elevated total lymphocyte count (Boscarino and Chang, 1999), increased percentage of peripheral blood CD4, CD16 and CD56 (Škarpa et al., 2001) or CD3, CD4 and CD8 subsets (Wilson et al., 1999) and increased percentage of perforin-positive cells (Škarpa et al., 2001) were reported. We noticed that PTSD patients tended to have increased lymphocyte counts. However, as this observation was based on a limited amount of data due to technical reasons, this finding was considered unreliable and therefore valid conclusion could not be made. Nevertheless, our previous research (Vidović et al., 2007) revealed increased lymphocyte counts in Croatian combat veterans without changes in the percentage of lymphocyte subpopulations. These data suggest that a generally increased lymphocyte trafficking is most likely due to hyperactivity of the sympathetic nervous system in PTSD sufferers (Elenkov et al., 2000). However, others have reported no difference in peripheral blood lymphocyte enumerative variables at baseline conditions in PTSD-symptomatic mothers of pediatric cancer survivors (Glover et al., 2005) and in combat (Laudenslager et al., 1998) or childhood sexual abuse related chronic PTSD (Wilson et al., 1999). Our finding, on the other hand, is consistent with the report of Boscarino and Chang (1999) that showed elevated leukocyte counts in a sample of over 2000 veterans. Whether this elevation reflects excessive inflammation in PTSD (Gill et al., 2009) is yet to be determined.

On the contrary, the result of decreased NKCC in both PTSD groups suggests an impaired immune function. Due to the essential role of perforin in the NK cytotoxic function and greater vulnerability of K562 cell line to granule-mediated killing than to the Fas-pathway (Munker et al., 1997), decreased NKCC in PTSD would be expected to result

Table 3
Between-group comparison of immune and endocrine variables.

	PTSD patients			Statistics[b]	
Variables[a]	Retired ($n = 14$)	Compensation-seeking ($n = 15$)	Healthy controls ($n = 13$)	F/H	P
Lymphocytes/mL [c]	3769.2 (2176.9–5015.8)	3337.6 (1987.1–7655.7)	1404.3 (1049.7–1777.5)*	14.249	0.001
$CD3^-CD56^+$ (%)	13.0 (7.0–18.0)	9.0 (7.0–11.0)	14.0 (11.0–17.0)	6.094	0.051
$CD3^-CD16^+$ (%)	3.9 (2.8)	3.1 (2.5)	1.5 (2.2)	3.125	0.055
$CD3^+CD8^+$ (%)	27.9 (10.4)	30.1 (12.9)	27.5 (6.4)	0.244	0.784
Perforin expression in					
$CD3^-CD56^+$ (m.f.i.)	127.5 (119.0–137.0)[d]	122.0 (88.0–133.0)	100.0 (95.0–109.0)	6.146	0.005
$CD3^-CD16^+$ (m.f.i.)	120.0 (113.0–139.0)[d]	111.0 (82.0–133.0)	99.0 (91.0–113.0)	5.899	0.006
$CD3^+CD8^+$ (m.f.i.)	80.6 (17.6)	69.7 (17.9)	69.15 (11.8)	1.990	0.151
NKCC (mean %)	37.0 (15.7–49.7)	34.3 (17.0–51.3)	58.1 (56.7–60.0)[e]	6.479	0.004
Ly GCR (m.f.i.)	92.5 (85.0–100.0)	99.0 (87.0–101.0)	64.0 (56.0–79.0)[f]	18.161	<0.001
Cortisol (μg/100 mL)	13.2 (4.1)	13.2 (6.0)	11.4 (4.5)	0.698	0.440

Ly GCR = lymphocyte glucocorticoid receptor expression; m.f.i. = mean fluorescence intensity; NKCC = natural killer cell cytotoxicity.

[a] Variables following Gaussian distribution are presented as mean and standard deviation in parentheses. For variables normalized by transformations back-transformed means and 95% confidence intervals of the mean are presented in parentheses. Variables that could not be normalized (Lymphocytes and $CD3^-CD56^+$ (%)) are presented by median (lower quartile – upper quartile) and analyzed using Kruskal Wallis' test.

[b] Results from Kruskal Wallis' tests (H) or ANCOVAs (F) controlling for age. Prior to ANCOVAs, all variables except for lymphocytes/ml and $CD3^-CD56^+$ (%) were included in MANCOVA (Wilks lambda = 0.292, $F(12,66) = 4.676$, $P < 0.001$). *Post hoc* analyses were performed with Tukey honestly significant difference or median Z test and probabilities indicated by asterisk(s).

[c] Data were available for the limited number of participants (retired, $n = 4$; seeking pension, $n = 9$; healthy controls, $n = 13$).

[d] $P < 0.05$, comparison with either of the other two groups.

[e] $P < 0.01$, comparison with either of the other two groups.

[f] $P < 0.001$, comparison with either of the other two groups.

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from a reduced intracellular perforin content in $CD16^+$ or $CD56^+$ cells. In the present study, we showed that decreased NKCC stemmed from neither reduced percentage of NK cells nor decreased perforin content. In spite of impaired NK function in both PTSD groups, compensation-seeking patients had unchanged level of perforin compared to controls, while retired patients had even higher perforin expression compared to the other two groups. Therefore, other reasons, besides the quantity of perforin, for impaired NK lysis should be considered. Those reasons include functional defects of perforin, granzyme or NK-target cell interaction. NK cells might form improper secretory synapses, downregulate adhesion molecules or produce insufficient amount of granzymes, while perforin defects result in a reduced ability to bind to target cell membranes or to polymerize at the target cell surface upon its release into the synaptic cleft (Voskoboinik and Trapani, 2006). Modulation of NK (as well as CTL) activity, NK receptor expression, capacity to form conjugates and mRNA transcription of perforin and granzyme genes by different stressors were described in mice (Li et al., 2005) or in human NK cell lines cultured in the presence of cortisol (Zhou et al., 1997). We previously reported that higher expression of GCR in NK cells compared to any other lymphocyte subpopulation might be the reason for pronounced sensitivity of NKCC to stress and cortisol (Gotovac et al., 2003). In the present study, we did not find any correlations of NKCC with other immune-related variables, either with the self-reported measures of PTSD, anxiety and depression symptoms, or with the variables related to the components of HPA-axis (cortisol and GCR). Alternatively, sympathetic activation with an increased catecholamine level, a common finding in PTSD (Southwick et al., 1999), might have been partly responsible for impaired NKCC. Although earlier studies reported an increase in NK cell activity in response to acute stress (Schedlowski et al., 1993) or to catecholamine administration (Schedlowski et al., 1996), others reported epinephrine-mediated decrease in NK cell activity during exercise (Kappel et al., 1991). Additionally, in vitro treatment of NK cells with catecholamines suppressed the NK cell activity and affected perforin expression (Dokur et al., 2004).

Elevated perforin content found in retired patients with PTSD together with increased percentage of perforin-positive cells in combat-related PTSD veterans reported previously (Škarpa et al., 2001) seem to be remarkably intriguing. Although the mechanisms responsible for the increased perforin levels remains unknown, this result might be interpreted in line with other reports on overactivity of some components of the immune system in PTSD mentioned earlier (Boscarino, 2004; Boscarino and Chang, 1999; Maes et al., 1999; Spivak et al., 1997; Škarpa et al., 2001; Watson-Burges et al., 1993; Wilson et al., 1999). The evidence suggests that combat-related PTSD is associated with increased prevalence of diverse autoimmune diseases, e.g. rheumatoid arthritis and psoriasis (Boscarino, 2004). It was shown that perforin expression is upregulated in the cells from psoriatic lesions (Kaštelan et al., 2004) or in patients with rheumatoid arthritis (Gulan et al., 2003) so that the potential self-damage from perforin overexpression should be kept in mind (Voskoboinik and Trapani, 2006). Although retired patients and compensation-seeking patients did not differ in PTSD, depression and anxiety symptoms and marginally higher state anxiety in retired patients indicate that elevated perforin expression might have been stress-related. Additionally, compensation-seekers more frequently over-report their PTSD symptoms (Freeman et al., 2008; Frueh et al., 2000; Kozarić-Kovačić et al., 2004). This leads to the possibility that retired patients actually had more pronounced PTSD symptoms with additional influence on perforin expression.

However, compensation-seeking as an additional stressor had no major impact on the variables tested in this study. On the contrary, Glover et al. (2005) reported a significantly lower increase in the percentage of NK cells in PTSD-symptomatic mothers of pediatric cancer survivors compared to mothers of healthy children after laboratory, mental arithmetic stressor challenge. Differences in immune responses to additional challenges were also demonstrated in studies of chronically stressed persons (Pike et al., 1997), men who reported very high daily hassles (Benschop et al., 1994) and male teachers with accumulated life stress experience (Brosschot et al., 1994). It is possible that enduring overactivity of the stress system changes its function and/or diminishes sensitivity to additional stressors (McEwen and Seeman, 1999). In other words, biological changes in our PTSD patients may have influenced the stress system in a way that it was not able to respond to new stressors adequately. Alternatively, it could be guessed that additional stress (e.g. process of retirement) had cumulative and/or delayed effects that would become evident later in the "recovery" state.

Regardless of whether the examined veterans were retired or in the process of compensation-seeking, the relative quantity of GCR in their lymphocytes was increased while cortisol levels in serum were unchanged. Together with the results of impaired NKCC, these data indicate that immune cells of PTSD patients may be more sensitive to suppressive effects of cortisol. Our results are in keeping with the common findings of increased GCR expression in PTSD patients and with the prevalent hypothesis that PTSD is associated with an enhanced negative feedback inhibition at one or more sites along the HPA-axis (reviewed in de Kloet et al., 2006). This hypothesis is mainly supported by findings in PTSD patients decades after traumatic experience (Newport et al., 2004; Stein et al., 1997; Yehuda et al., 1995, 2002). We reported that Croatian combat veterans had increased cortisol levels and lower lymphocyte GCR expression at approximately six years after the traumatic experience (Gotovac et al., 2003). Besides, we showed that there was no difference twelve years after the initial trauma in lymphocyte GCR expression compared to controls and that GCR expression positively correlated with time (years) elapsed from the trauma (Vidović et al., 2007). These results could imply that, due to allostatic load, PTSD patients would eventually develop a profile of HPA-axis activity like the one observed in studies carried out decades following the trauma (Segerstrom and Miller, 2004). As we were unable to identify the exact time that elapsed since the initial trauma, we considered the self-reported duration of symptoms (median eight years) to be indicative of the duration of PTSD. During that time, i.e. earlier than our previous study indicated, PTSD patients developed a profile of increased levels of GCR (suggesting an increased sensitivity to cortisol) and decreased NKCC (suggesting immune suppression). Differences in the severity of trauma and/or PTSD symptoms might have been the reason for an earlier development of this condition. Our future longitudinal study will address this question.

One of the major limitations of this study was a relatively small sample size. Larger sample sizes are needed to obtain adequate statistical power to confirm the changes observed. Although our aim was to examine the impact of life stressors, more controlled laboratory stressors with acute, individualized stress stimuli would have given a better insight into the stress-related immune reactivity of PTSD patients. Furthermore, we failed to discern the particular contribution of additional stressors by means of the psychological instruments applied. We relied on observations and patient's reports that the process of seeking compensation was a very stressful and exhausting experience. Nevertheless, although patients were divided into two groups according to compensation-seeking, most of the observed changes applied to the PTSD group as a whole, confirming the effect of the disease itself on measured variables. Another limitation of our findings relates to our assessment of current PTSD symptoms, which relies on self-report scales rather than on clinician-administered diagnostic interviews. This particularly applies to compensation-seeking veterans since it has been shown that they tend to over-report the symptoms and that their compensation-seeking status influences the diagnosis, treatment and outcome of their disease (Gold and Frueh, 1999). As in many studies in the field, variables that were found to be important modulators, such as disease

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duration and the magnitude of a stressor, host factors (genetic predisposition, nutritional state, and experience) and the ability of an individual to cope with stressful situation (Ader et al., 1995; Olff et al., 2005; Segerstrom and Miller, 2004; Stam, 2007; Wessa and Rohleder, 2007), could not be comprehensively controlled. Finally, further work is warranted to examine how our findings, especially on the impairment of NKCC, may be related to the health status of PTSD patients.

5. Conclusions

Our data does not support the hypothesis that compensation-seeking is an additional stressor capable of modifying biological changes in PTSD patients. An increased level of GCR (suggesting an increased sensitivity to cortisol) and decreased NKCC (suggesting immune suppression) were found in both groups of PTSD patients. Impairment of NKCC could not be attributed to perforin insufficiency in PTSD subjects. Other mechanisms, possibly related to sympathetic nervous system activation, should be investigated.

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