Predicting PTSD, Depression, and Fatigue after Military Deployment:

Identification of Biological Vulnerability Factors

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Het voorspellen van PTSS, depressie en vermoeidheid na een militaire uitzending: Identificatie van biologische kwetsbaarheidsfactoren

(met een samenvatting in het Nederlands)

Proefschrift

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Voor mijn ouders

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General Introduction

INTRODUCTION

Psychological stress can be defined as the presence of a specific situation in which individuals perceive that they are not able to adequately deal with the demands or threats to their well-being that the situation imposes (1). When an individual experiences, witnesses, or is confronted with an event that involves actual or threatened death or serious injury, or a threat to the physical integrity of self and others, the experienced stress may be traumatic. For an event to be defined as traumatic, the individuals' response to the event must have involved intense fear, helplessness, or horror (2). Thus, the interpretation of a situation or event as being stressful or traumatic will differ between individuals. Nevertheless, exposure to severe stress and trauma is very common: a recent study reported that approximately 80% of the Dutch general population between the age of 18 and 65 has experienced at least one potentially traumatic event during their life (3).

A minority of individuals who are exposed to severe, chronic or traumatic stress subsequently develops ongoing mental or physical health problems, which impair emotional, occupational and social functioning. Conditions that may arise in response to stress- and trauma-exposure include posttraumatic stress disorder (PTSD), major depressive disorder (MDD) and prolonged severe fatigue.

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that may develop in response to a traumatic event. An individual can be diagnosed with PTSD when symptoms from three clusters have developed after a traumatic event: re-experiencing of the traumatic event, avoidance of stimuli associated with the traumatic event and numbing of general responsiveness, and symptoms of increased arousal. For a diagnosis of PTSD, these symptoms have to be present for more than one month and cause significant distress (2). In the Netherlands, the 12-month prevalence of PTSD is estimated at 2.6% (4), and the lifetime prevalence is estimated at 7.4% (3).

Another psychiatric disorder that may develop in response to stressful or traumatic events is Major Depressive Disorder (MDD). Individuals with MDD suffer from depressed mood, or from loss of interest or pleasure in activities that they used to enjoy (i.e. anhedonia) for most of the day. Accompanying symptoms include feelings of worthlessness or guilt, sleeping disturbances, fatigue and cognitive disturbances. When symptoms persist for at least two weeks and cause significant distress, MDD may be diagnosed (2). Although exposure to stress or trauma is no prerequisite for a diagnosis of MDD, it is generally accepted that exposure to severe, chronic or traumatic stress is an important risk factor for the subsequent development of MDD (5). In the Netherlands, the 12-month prevalence of MDD is estimated at 5.8%, and the lifetime prevalence of MDD is estimated at 15.4% (6). These estimates also include cases of MDD that are not specifically induced by stressful or traumatic events.

Exposure to severely stressful or traumatic events is a well-known risk factor for the subsequent development of prolonged severe fatigue without an identifiable medical cause

(7, 8). We are not aware of any studies on the incidence of severe prolonged fatigue in the Dutch adult general population, but in the adult general population of Great Britain the point prevalence of severe prolonged fatigue was estimated to be 9% (9). The presence of severe prolonged fatigue without an identifiable medical cause is a well-known risk factor for Chronic Fatigue Syndrome (CFS) (10, 11). This condition is characterized by the presence of persistent and debilitating fatigue, accompanied by at least four specified symptoms, which may include sleeping- and cognitive disturbances (12). For a diagnosis of CFS, symptoms have to be present for at least six months, and cannot be explained by the presence of medical or psychiatric disorders. Underlying factors of fatigue have mostly been investigated in relation to CFS. The point prevalence of CFS in the Netherlands was estimated at 0.11% (13), although this estimate was only based on data collected from general practitioners, which may have resulted in an underestimation. Individuals with CFS typically report more stressful life events and more subjective stress before symptom onset compared to individuals without CFS (14).

PTSD, MDD and severe fatigue often do not occur in isolation. Co-morbidity between PTSD and MDD is very common, and may be as high as 50% (15). In addition, co-morbidity between fatigue and psychiatric disorders, including PTSD and MDD, is also very common (16). It was previously estimated that 15% of individuals with CFS in the US general population also fulfilled the diagnostic criteria for PTSD and that 22.1% also fulfilled diagnostic criteria for MDD (17).

IDENTIFYING BIOLOGICAL VULNERABILITY FACTORS FOR PTSD, MDD AND SEVERE FATIGUE

Not all individuals exposed to severe, chronic or traumatic stress subsequently develop significant mental or physical health problems, such as PTSD, MDD and severe fatigue. Therefore, many researchers have focused on biological and psychological correlates associated with the development of these conditions. We hypothesize that the development of these stress-related conditions is associated with vulnerability factors, which are already present prior to the exposure to the stressful or traumatic event that eventually results in symptom development. However, most of the current knowledge on biological and psychological correlates of PTSD, MDD and severe fatigue has been established using cross-sectional designs, or prospective designs with the first data collection shortly after the stressful or traumatic event (18). Therefore, these studies do not inform us as to whether these observed correlates were already present before the development of symptoms. Identification of pre-existing vulnerability factors for the development of PTSD, MDD and severe fatigue is important, since they can be used to detect individuals at risk for development of these conditions after exposure to severe, chronic or traumatic stress.

To reliably identify pre-existing vulnerability factors for the development of stress-related conditions, the collection of data has to begin before the occurrence of the event that eventually results in symptom development. Therefore, in practice this type of research can only be

performed in populations with increased risk for stress- and trauma exposure. For example, one can think of individuals working in occupations with a high risk for stress- and trauma exposure, such as the military, police or fire-fighters. However, gaining access to these populations prior to stress- or trauma-exposure is difficult and therefore literature on pre-existing vulnerability factors for the development of PTSD and stress-related MDD and severe fatigue remains scarce.

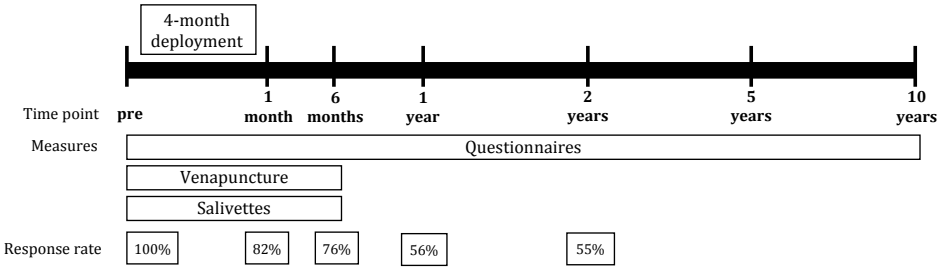
THE STUDY POPULATION: PRISMO

Military deployment provides a unique opportunity to investigate vulnerability factors associated with the development of stress-related conditions. Military personnel deployed to a combat-zone are at risk to experience severely stressful and traumatic events during their deployment. In addition, being away from family and friends for a long period is stressful for the majority of deployed individuals. However, only a minority of deployed military personnel will develop stress-related conditions such as PTSD, MDD and severe fatigue in response to their deployment. For example, a relatively recent study on the prevalence of PTSD in Dutch military personnel deployed to Iraq showed that less than 5% of deployed individuals fulfilled the DSM-IV diagnostic criteria for PTSD after return from their deployment (19).

The chapters in this PhD-thesis have been written using data from the ongoing 'Prospective Research In Stress-related Military Operations' (PRISMO) study. This study is carried out by the Research Centre for Military Mental Health of the Dutch Ministry of Defence, and the Laboratory of NeuroImmunology and Developmental Origins of Disease of the UMC Utrecht. All participants of the study were Dutch military personnel who were deployed to Afghanistan between 2005 and 2008 as part of the International Security Assistance Force (ISAF) of the NATO. The Dutch troops were first stationed in North-Afghanistan. From 2006 the Dutch deployed troops were stationed in the province of Uruzgan, in South-Afghanistan. During their presence in Afghanistan, Dutch military personnel frequently encountered combat situations and improvised explosive devices (IEDs). A total of 25 Dutch military personnel died during the ISAF-mission between 2006 and 2010, of which 19 died in combat or due to IEDs. In addition, a substantial number of deployed military personnel were injured during deployment to Afghanistan. The Dutch presence in Uruzgan ended in 2010.

In the PRISMO study, biological and psychological aspects of the development of stress-related (mental) health problems are investigated in a prospective, longitudinal design. The first assessment of the study was performed before deployment to Afghanistan. In addition, 6 consecutive assessments took place after return (Figure 1). A total of 1032 military personnel were included prior to their deployment. During all assessments participants filled out questionnaires. Additionally, during the assessments before deployment and one and six months after deployment, blood was drawn and salivary samples were collected. Within this PhD-thesis, data obtained during the first three assessments are described.

FIGURE 1. Design of the PRISMO-study.



The figure represents the time-points at which the assessments took place, which outcome measures were collected, and the percentage of included participants that completed the assessments.

BIOLOGICAL CORRELATES OF PTSD, MDD AND SEVERE FATIGUE

In this section, an overview of previously identified biological correlates of PTSD, MDD and severe fatigue will be presented. Many bodily systems are associated with the development of these conditions. However, for this brief overview of the existing literature, we exclusively focus on the hypothalamic-pituitary-adrenal (HPA) axis, cytokine production and glucocorticoid signalling in the immune system.

The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a major part of the neuroendocrine system, which regulates the body's response to external stressors. The HPA axis is controlled by the hippocampus, which induces the release of corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) from the hypothalamus. Subsequently, CRH stimulates the release of adrenocorticotropic hormone (ACTH) by the anterior pituitary. Then, ACTH induces the production and release of glucocorticoids (GCs, in humans: cortisol) by the adrenal cortex. GCs have numerous functions and thereby influence almost every tissue of the human body, for example by regulation of energy metabolism, appetite, reproductive function and memory formation. In addition, GCs enable the body to prepare for, respond to and cope with stress. GCs provide negative feedback on the hypothalamus and anterior pituitary, and thereby inhibit the release of CRH and ACTH and the subsequent secretion of GCs. Thereby GCs terminate the stress response and restore homeostasis (i.e. balance) in the body.

The experience of chronic or traumatic stress can result in changes in the functioning of the HPA axis. In response to chronic stress, decreased cortisol levels and increased negative feedback of GCs on the HPA axis, has been described. Contrastingly, increased cortisol levels and decreased negative feedback of GCs has also been described repeatedly (20). Altered functioning of the HPA axis has repeatedly been observed in adults who experienced

childhood trauma or adversity (21, 22). In addition, increased negative feedback regulation of GCs on the HPA axis was observed in military personnel who had experienced traumatic stress during their deployment more than a decade ago (23). These results indicate that the effects of chronic or traumatic stress on the functioning of the HPA axis can be long-lasting.

Since PTSD, MDD and severe fatigue may develop in response to (traumatic) stress, many studies investigating the biological correlates of these conditions have focused on the functioning of the HPA axis. Although not all data in the literature provide consistent results, increased sensitivity of the HPA axis to negative feedback by GCs is considered as one of the hallmark biological correlates of PTSD (18, 24). In addition, altered cortisol levels have frequently been observed in individuals with PTSD, but these results have been mixed with respect to the direction of the changes (25, 26). It was previously observed that cortisol levels might be associated with the development of PTSD: low cortisol levels assessed directly after trauma exposure were associated with increased risk for subsequent development of PTSD in adults (27-30). With regard to MDD, the existing data in the literature indicates that individuals with MDD or depressive symptomatology have decreased negative feedback of GCs on the HPA axis (20, 24, 31) and increased cortisol levels (32) compared to non-depressed controls. In contrast, the presence of low cortisol levels immediately after trauma exposure was found to be associated with subsequent development of depressive symptomatology (28). For severe fatigue, the functioning of the HPA axis has predominantly been assessed in individuals with CFS (33, 34). A recent meta-analysis showed that low cortisol levels are a consistent finding in individuals with CFS (35). In addition, data in the literature suggests that adults with CFS may have increased negative feedback of GCs on the HPA axis (36).

Cytokine production

When the immune system is challenged, immune mediators such as cytokines and chemokines are released by immune cells and organs, including the liver, spleen, endothelial cells, macrophages, monocytes and lymphocytes, but also microglia in the brain. Cytokines and chemokines are signalling molecules that facilitate communication between these immunocompetent cells and organs. Cytokines can be classified in various ways according to their function, structure, or genetics. One classification that is frequently used is the distinction between pro-inflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, and anti-inflammatory cytokines, including IL-4, IL-5 and IL-10. Chemokines, including IL-8/CXCL8, MCP1/CCL3 and Rantes/CCL5, are immune mediators that attract immune cells to a specific location by using a chemical attractant (i.e. chemotaxis). The message of peripheral cytokines and chemokines is transmitted to the brain via neural and humoral pathways. This induces the production of cytokines and chemokines in the brain. Vice versa, central cytokines and chemokines can relay their signal to the periphery, and thereby induce peripheral immune responses (37, 38).

An abundance of data in the literature supports the notion that individuals exposed to chronic stress are more susceptible to disease (39). In addition, the presence of PTSD, MDD and severe fatigue is associated with increased risk for the development of medical conditions, including cardiovascular disease, rheumatoid arthritis, asthma and dementia (40-45). The association between chronic stress, PTSD, MDD and fatigue and the subsequent development of medical conditions is allegedly mediated by the presence of a (low grade) inflammatory state in chronically stressed individuals and in individuals with PTSD, MDD and fatigue. It has indeed been described that experiencing chronic stress may result in increased levels of circulating pro-inflammatory cytokines (46). Just as for the effects in the HPA axis, these effects of chronic stress can be long-lasting: healthy adults who had experienced childhood maltreatment had greater stress-induced increases in IL-6 during adulthood than non-maltreated individuals (47).

Injecting rodents with lipopolysaccharide (LPS) or IL-1β induces an increase in pro-inflammatory cytokines, both in the periphery and in the CNS. This pro-inflammatory state causes sickness behavior, which is characterized by fatigue, depressed mood, pain and reduced appetite. After these initial symptoms have subsided, a depressive-like state may remain present for a prolonged period in part of the rodents (37). Administration of cytokines to humans also leads to development of severe fatigue and MDD. The treatment of hepatitis C and cancer with intravenous administration of pro-inflammatory cytokines induces fatigue in almost all treated individuals. In addition, up to 60% of individuals who develop cytokine-induced fatigue also develop a depressive episode in response to the cytokine administration (38, 48, 49).

An extensive body of data in the literature indicates that increased levels of serum cytokines are also associated with PTSD, MDD and severe fatigue in individuals without major medical conditions. Although not all data in the literature are consistent, circulating peripheral levels of C-reactive protein (CRP) and serum pro-inflammatory cytokines appear to be increased in individuals with PTSD (50-52), MDD (53, 54) and severe fatigue (primarily investigated in CFS) (36, 55). In addition, increased levels of chemokines and anti-inflammatory cytokines have been reported in PTSD (56). The potential role of inflammatory mediators in PTSD and MDD is underscored by the fact that epigenetic changes in methylation of inflammation-associated genes have been observed in individuals with lifetime PTSD and MDD (57, 58). In addition, a recent study reported that breast cancer survivors with severe fatigue had increased expression of genes with NF-κB response elements, the most important inflammatory transcription factor (59). Since it was previously shown that high concentrations of serum IL-6, as assessed within 24 hours after an accident, predicted the presence of PTSD six months later (60), it may well be that the presence of a pro-inflammatory state precedes the actual development of symptoms.

It is possible to investigate the capacity of peripheral blood mononuclear cells (PBMCs) to produce cytokines after stimulation with a mitogen or cytokine *in vitro*. Mitogen-induced

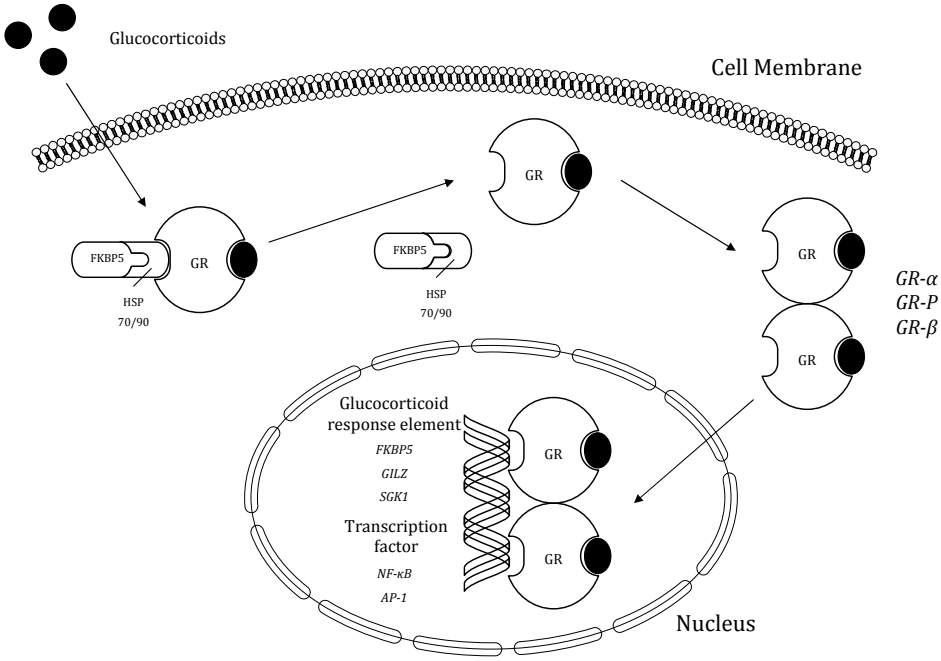
cytokine production has been studied repeatedly in individuals with PTSD, depressive symptomatology and fatigue. However, these studies did not lead to unequivocal conclusions. Increased mitogen-induced pro-inflammatory cytokine production by PBMCs has been observed in female and mixed-gender samples with PTSD, but not in males with PTSD, compared to healthy traumatized and non-traumatized controls (61-65). In addition, increased (66-71), unaltered (72-76) and decreased (66, 77, 78) mitogen-induced pro-inflammatory cytokine production by PBMCs from individuals with MDD or depressive symptoms compared to non-depressed controls have been reported. Associations between mitogen-induced cytokine production and fatigue have mainly been investigated in patients with CFS. Most studies reported increased mitogen-induced anti-inflammatory cytokine production by PMBCs from fatigued individuals compared to non-fatigued individuals: this was previously observed in adults with CFS (79, 80) and in adolescent girls with CFS (81), although not in another study in adolescent girls with CFS (82). In addition, LPS-induced IL-6 and TNF-α production positively correlated with fatigue levels in patients with CFS and in non-severely fatigued individuals (83).

Glucocorticoid signalling in the immune system

Although the HPA axis and immune system have been frequently studied as isolated systems, they actually function in close interaction. When the immune system is triggered, the HPA axis is activated by pro-inflammatory cytokines. In turn, after activation of the HPA axis, GCs regulate the functioning of the immune system. Two important mechanisms by which GCs regulate the immune system are by inhibition of cell proliferation and inhibition of production of pro-inflammatory cytokines (84). When a stressor becomes chronic or when an individual is repeatedly stressed, the sensitivity of the immune system to regulation by GCs may change (20, 85, 86). This change in GC-sensitivity of the immune system can be long-lasting: it was observed that a decade after combat exposure, veterans without psychiatric disorders had decreased GC-sensitivity of immune cells compared to non-trauma exposed (64). The association between PTSD, MDD, and fatigue and the GC-sensitivity of peripheral immune cells has been investigated multiple times. Overall, results indicate increased GC-sensitivity of immune cells of individuals with PTSD (61, 87), and decreased GC-sensitivity of immune cells of individuals with MDD (73, 88-90). In addition, non-clinical levels of severe fatigue (91) and the presence of CFS in adolescents (82, 92) have been associated with decreased GC-sensitivity of immune cells (91), while CFS in adults has been associated with increased GC sensitivity of immune cells (79, 93, 94).

Central and peripheral actions of GCs are mediated via mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). MRs have high affinity for GCs, and therefore are almost completely saturated at basal cortisol levels. The affinity of GR to bind GCs is lower than the binding affinity of the MR, and therefore GR play a key regulatory role during periods of increased cortisol levels, i.e. during stressful conditions (95). GRs reside in the cytosol and

FIGURE 2. Schematic representation of the glucocorticoid receptor (GR) pathway.



upon GC-binding translocate to the nucleus to regulate gene transcription (Figure 2). Gene transcription is regulated by the binding of GRs to glucocorticoid response elements of target genes, or by interaction with transcription factors, such as NF-κB and AP-1 (96). The functioning of the GR is influenced by heat-shock proteins chaperoning the GR (HSP70/HSP90), which influence proper folding and maturation of the GR and subsequent translocation to the nucleus (97). One of the proteins directly induced by GR activation is FK506 binding protein 5 (FKBP5). FKBP5 acts as a co-chaperone of the GR-HSP70/90 heterocomplex, lowering GR binding affinity and thereby thus diminishing GR signalling (98). GR signalling is also influenced by the number of GRs and by GR subtypes. Various GR subtypes have been identified, which differ in overall expression levels and potential to induce gene transcription (99-102).

In rodents, psychological stress induces both short- and long-term downregulation of GR expression (103-107). In addition, it has been shown that childhood trauma or adversity affects hippocampal GR expression in rodents (108) and humans (109). This long-lasting effect of childhood adversity is associated with epigenetic changes in methylation status of the promoter of the GR gene (109). As of yet, it is not possible to investigate the number and functioning of the GR in the brain *in vivo* (110). Therefore, clinical studies investigating GR expression have to rely on investigating GR expression in PBMCs. Preclinical studies in rodents indicate that investigating peripheral GR expression in PBMCs represents a reliable model for GR number and signalling in the brain (111-113).

Studies on the association between GR number in PBMCs and PTSD have yielded mixed results about the presence and direction of differences in GR number between PTSD patients and controls. Higher (114, 115), lower (116), and similar (64, 117-120) peripheral GR numbers in PTSD patients compared to controls have been reported. In addition, studies on the peripheral GR number in individuals with MDD have also yielded mixed results (121-123). Only a single study on GR number in individuals with severe fatigue has been performed previously. In this study no significant differences in the number of GR in PBMCs between patients with CFS and healthy controls were found (79).

In recent studies, other aspects of the GR pathway were also investigated. Results suggest that FKBP5 mRNA expression differs between HIV-positive individuals with MDD and HIV-positive individuals without MDD (124). FKBP5 mRNA expression also differed between survivors of the 9/11 World Trade Center attacks with and without subsequent PTSD development (125, 126). Moreover, the FKBP5 mRNA expression in trauma survivors in the emergency room was found to predict PTSD status four months later (127).

AIMS AND OUTLINE

The aim of this PhD-thesis was to identify vulnerability factors for the development of PTSD, depressive symptoms and severe fatigue in response to military deployment. Our predominant focus was on identifying biological predictors ('biomarkers') relating to GR pathway components within PBMCs. In addition, we focused on cytokine production by peripheral blood cells *in vitro* after stimulation with a mitogen or pro-inflammatory mediator. Furthermore, promising psychological vulnerability factors were investigated as well. An additional aim of this thesis was to investigate whether military deployment and development of stress-related conditions in response to military deployment were associated with changes in cytokine production over time.

Outline

This thesis can be divided into two parts. The first part consists of chapters 2-6 and focuses on the predictive value of the GR pathway for the development of PTSD, depressive, and fatigue symptoms in response to military deployment.

The second part includes chapters 7 and 8. In these chapters, the association between peripheral cytokine production and the development of stress-related conditions in response to military deployment is investigated.

In **Chapter 2** we aimed at investigating whether the number of GR in PBMCs prior to deployment differed between military personnel with and without a high level of PTSD symptoms six months after deployment. In addition, we tested whether the GR number prior to

deployment was a significant predictor for the development of PTSD symptoms in response to deployment within these participants.

In **Chapter 3** the predictive value of other components of the GR pathway in PBMCs for the development of PTSD symptoms was assessed. In addition, we validated the predictive value of the pre-deployment GR number for the presence of PTSD symptoms six months after deployment within a larger, more heterogeneous group of military personnel. Furthermore, we investigated whether single nucleotide polymorphisms (SNPs) in the GR and FKBP5 gene were associated with the observed vulnerability factors for PTSD development in response to deployment.

In **Chapter 4** we investigated whether the peripheral GR number was associated with the development of high levels of fatigue and depressive symptoms in response to deployment. For this purpose we tested whether the peripheral GR number differed between severely fatigued military personnel with depressive symptoms after deployment, severely fatigued military personnel without depressive symptoms after deployment and military personnel who did not report fatigue or depressive symptoms after deployment. The GR number was investigated before, one and six months after deployment.

Chapter 5 reports on the predictive value of the pre-deployment cortisol awakening response, the rapid increase in cortisol levels after awakening, for the presence of PTSD symptoms six months after return from deployment. In addition, pre-existing psychological vulnerability factors for the presence of PTSD symptoms were also investigated. The predictive value of several personality traits for the presence of PTSD symptoms six month after deployment was investigated.

Chapter 6 describes the association between sensitivity of peripheral immune cells for regulation by GCs, and the subsequent development of fatigue, depressive, and PTSD symptoms. For this purpose, GC-sensitivity of T-cells and monocytes assessed prior to deployment was used to predict the presence of high levels of fatigue, depressive and PTSD symptoms six months after deployment.

In **Chapter 7** we aimed to investigate the association between the capacity of T-cells and monocytes to produce cytokines and the development of depressive symptoms. Therefore, we assessed whether the longitudinal course of stimulated T-cell and monocyte cytokine production *in vitro* differed between military personnel with and without high levels of depressive symptoms after deployment.

In **Chapter 8** we investigated differences in the longitudinal course of the reactivity of peripheral blood cells to IL-1β between military personnel with and without severe fatigue after return from deployment. For this purpose, IL-1β-induced IL-8 production was assessed before, and one and six months after deployment.

Chapter 9 contains the general discussion, in which the most important findings of this thesis are addressed.

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2

Pre-existing high glucocorticoid receptor number predicting development of posttraumatic stress symptoms after military deployment.

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ABSTRACT

The development of posttraumatic stress disorder (PTSD) is influenced by pre-existing vulnerability factors. We aimed at identifying a pre-existing biomarker representing a vulnerability factor for the development of PTSD. To that end, we determined whether the dexamethasone binding capacity of leukocytes, as a measure of glucocorticoid receptor (GR) number, before exposure to trauma was a predictor of development of PTSD symptoms. In addition, we analyzed mRNA expression for GR subtypes and GR target genes.

Participants were selected from a large prospective study on deployment-related disorders, in which peripheral blood mononuclear cells (PBMCs) were obtained prior to, and one and six months after military deployment. We included participants with high levels of PTSD symptoms six months after deployment (n=34) and comparison subjects without high levels of PTSD or depressive symptoms (n=34) matched for age, rank, previous deployments, educational level and function during deployment.

Before military deployment, the GR number in PBMCs was significantly higher in participants who developed high levels of PTSD symptoms after deployment relative to matched comparison subjects. Logistic regression analysis showed that the risk for inclusion in the PTSD group after deployment increased 7.5-fold with each GR increase of 1000. No group differences were observed in mRNA expression of GR-α, GR-P, GR-β, glucocorticoidinduced leucine zipper (GILZ), serum and glucocorticoid-inducible kinase-1 (SGK1) and FKBP5. The higher number of GR in the PTSD group was maintained at one and six months after deployment.

These results demonstrate that a pre-existing high GR number in PBMCs is a vulnerability factor for subsequent development of PTSD symptoms.

INTRODUCTION

Deployed military personnel are at risk for development of posttraumatic stress disorder (PTSD). Among US marines and soldiers, PTSD prevalence after deployment to Iraq and Afghanistan is estimated at 6%-13% (1). Development of PTSD is hypothesized to be influenced by pre-existing vulnerability factors. Pole et al. (2) observed in police academy cadets that increased skin conductance and decreased skin conductance habituation in response to startling sounds under experimental threat were prospectively related to PTSD symptoms one year later. No blood-derived biomarkers representing vulnerability factors for development of PTSD have been identified yet.

PTSD appears to be associated with altered activity of the hypothalamic–pituitary–adrenal (HPA) axis (3). However, a meta-analysis revealed that hypocortisolism is not a consistent finding in PTSD (4). In addition, there is evidence for higher glucocorticoid sensitivity of the HPA axis in PTSD (5, 6), but this may be induced by military deployment independently of the disorder (7, 8). Regulation of immune responses by glucocorticoids also seems to be altered in PTSD patients. Our group previously reported lower glucocorticoid sensitivity of T-cell proliferation and higher glucocorticoid sensitivity of lipopolysaccharide (LPS)-stimulated cytokine production in PTSD patients (9). Accordingly, higher in vitro inhibition of LPS-stimulated production of cytokines (10) and lysozymes (11) by glucocorticoids was observed in PTSD patients as well. However, at least part of the relation between PTSD and glucocorticoid regulation of the immune response could be attributed to trauma exposure, since trauma-exposed individuals without PTSD also displayed alterations in glucocorticoid sensitivity of the immune system in comparison to healthy subjects (9, 11).

Glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) mediate glucocorticoid actions. MRs are saturated at low basal cortisol levels, while GRs play a key regulatory role during periods of increased cortisol (e.g. in stressful conditions) (12). GRs reside in the cytosol and upon ligand-binding translocate to the nucleus to regulate gene transcription (13). GR sensitivity is influenced by heat-shock proteins (HSP70/HSP90) chaperoning GRs, which influence proper folding and maturation of the GR and subsequent translocation and gene transcription (14). FKBP5, both target gene and co-chaperone of the GR-HSP70/90 heterocomplex, can lower GR affinity and thereby alter glucocorticoid binding (15).

Cross-sectional studies on the GR number in peripheral mononuclear cells (PBMCs) in individuals with PTSD have yielded mixed results. Higher (16, 17), lower (18), and similar (5, 6, 8, 9, 11) GR number in PTSD patients relative to comparison subjects have been reported. Differences in duration of PTSD, type of trauma, reference groups (e.g. trauma-exposed comparison subjects or healthy comparison subjects) or techniques used to determine the GR number may all contribute to the reported variability in changes in GR number. In addition, it remains unknown whether pre-existing differences in GR number are present in individuals prone to developing PTSD after a traumatic experience.

We previously reported a higher GR number in PBMCs before deployment in military personnel who had developed depressive symptoms and severe fatigue after deployment. This higher GR number was not caused by pre-existing differences in mental health status (19). We hypothesized therefore that a high GR number in PBMCs before deployment may represent a vulnerability factor for development of mental health problems after deployment.

In the current study we investigated before deployment whether military personnel with and without deployment-related onset of PTSD symptoms differed in dexamethasone (DEX) binding capacity of PBMCs. Since the GR:MR ratio in human PBMCs is approximately 10:1 (20) we refer to the results of the binding assay as GR number or binding. Moreover, the predictive value of the pre-existing GR number for development of PTSD symptoms after deployment was investigated. Subsequently, we assessed whether group differences existed in mRNA expression for subtypes of GR (GR-α, GR-β and GR-P), GR target genes (glucocorticoid induced leucine zipper (GILZ) and serum and glucocorticoid inducible kinase-1 (SGK1)) and the GR target gene and co-chaperone FKBP5.

METHOD AND MATERIALS

General procedure

This study is part of a prospective cohort study on deployment-related disorders in the Dutch Armed Forces. Individuals volunteered to participate prior to a 4-month deployment to Afghanistan. Duties during deployment included combat patrols, clearing or searching buildings, participation in de-mining operations, and transportation across enemy territory. The combat group was exposed to typical war-zone stressors including exposure to enemy fire, armed combat, and seriously injured and dead fellow soldiers and civilians. The study was approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, the Netherlands). Written consent was also obtained. One to two months prior to deployment and approximately one and six months after deployment, participants completed questionnaires and a heparinized blood sample was drawn between 8.00 and 11.30 a.m.

Participant selection

Participants were selected from 455 males who completed all assessments. Analysis of GR binding and associated measures was performed by individuals blind to the participants' PTSD status. Participants were assigned to the PTSD group when their score on the Self-Rating Inventory for PTSD (SRIP) (21) six months after deployment was ≥ 38. This cut-off score corresponds to the mean plus two standard deviations, which coincides with the 95th percentile of SRIP scores before deployment within a population of 704 soldiers from the

Dutch Armed Forces (mean (SD): 26.91 (5.34)). The validity of this cut-off score is supported by van Zelst et al. (22) who compared SRIP scores and ratings from a diagnostic interview within a community population of older adults.

Since we investigated PTSD onset, 8 participants with SRIP scores ≥ 38 before deployment were excluded. All other participants fulfilling the selection criteria for the PTSD group were included (n=34). Subsequently, a comparison group (n=34) was identified by matching to participants in the PTSD group for age, period of deployment, function and rank during deployment, education, and number of previous deployments. SRIP scores had to be below the cut-off score at all time points. Since we previously found that depressive symptoms are related to high GR binding in PBMCs, participants with high levels of depressive symptoms before or after deployment (SCL-90 depression scale score ≥ 24, (19)) were excluded from the comparison group.

Matched comparison subjects were compared with non-selected participants without high levels of PTSD or depressive symptoms before or six months after deployment. Matched comparison subjects were somewhat younger (matched comparison subjects: mean (SD): 26.68 (9.28), non-selected comparison subjects: mean (SD): 29.42 (9.06); $t_{(380)}$= −1.679, p=.094) and had experienced fewer previous deployments (matched comparison subjects: mean (SD): 0.65 (0.85), non-selected comparison subjects: mean (SD): 1.06 (1.34), $t_{(50,71)}$= −2.547, p=.014) than non-selected comparison subjects. There were no group differences in year of deployment, rank and function during deployment, and education.

Measures

Questionnaires

PTSD symptom level over the past 4 weeks was assessed with the 22-item Self-Report Inventory for PTSD (SRIP). A higher score indicates more PTSD symptoms (range 22-88). This inventory has good concurrent validity with other PTSD measures, such as the Clinician Administered PTSD Scale (CAPS) and the Mississippi scale for PTSD (21). Level of depressive symptoms was assessed using the Dutch version of the 16-item symptom checklist (SCL-90) depression subscale. A higher score on this measure indicates more depressive symptoms (range 16-80) (23). Exposure to potentially traumatic experiences before the age of 18 was assessed using the Dutch version of the short form Early Trauma Inventory-Self-Report (24). The questionnaire consists of 27 dichotomous items. The total score represents the number of experienced events. Exposure to potentially traumatic deployment stressors was assessed with a 13-item checklist specifically developed for the present study.

Dexamethasone binding

For determination of the capacity of PBMCs to bind glucocorticoids, a validated whole cell single-point binding assay was used, as described previously (19). This method provides

a reliable estimate of Bmax as determined using a classical binding assay with 3-200 nM ^{3}H-dexamethasone (r^2=0.92) (19). Briefly, PBMCs were isolated from whole blood using Ficoll-Paque (Pharmacia and Upjohn, Uppsala, Sweden) and 10^7 cells were frozen in DMSO. After thawing and 60 minutes of equilibration in a culture medium, cells were washed twice, resuspended in assay buffer (RPMI-1640 with 5% FCS) and incubated in duplicate with 100 nM ^{3}H-dexamethasone (Amersham, Buckinghamshire, UK) in the presence or absence of excess unlabeled dexamethasone (Sigma-Aldrich, Steinheim, Germany) for 1 hour at 37°C. Cell bound radioactivity was quantified by liquid scintillation analysis.

mRNA expression

Total RNA was isolated from PBMCs with Trizol (Invitrogen, Carlsbad, Calif.). One µg of total RNA was used to synthesize cDNA with SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, Calif.). Real-time PCR reactions were performed with an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, Calif.) (see Table 1 for primer sequences). Data were normalized for GAPDH and β-actin expression.

TABLE 1. Forward (Fwd) and reverse (Rev) primer sequences used in quantitative real-time PCR.

Target	Primer sequences
GR-α	Fwd: 5'-TCAACTgACAAAACTCTTgg-3'
	Rev: 5'-TgATTggTgATgATTTCAgC-3'
GR-β	Fwd: 5'-AgCggTTTTATCAACTgAC-3'
	Rev: 5'-TgAgTTCTATTTTTTgAgCg-3'
GR-P	Fwd: 5'-gCTgTgTTTTgCTCCTgATCTgA-3'
	Rev: 5'-TgACATAAggTgAAAAggTgTTCTACC-3'
GILZ	Fwd: 5'-ACCgAAATgTATCAgACCCCCA-3'
	Rev: 5'-CgATCTTgTTgTCTATggCCACC-3'
SGK1	Fwd: 5'-gAgATTgTgTTAgCTCCAAAGC-3'
	Rev: 5'-CTgTgATCAgGCATAgCACACT-3'
FKBP5	Fwd: 5'-AAATCCAAACgAAggAgCAA-3'
	Rev: 5'-gCCACATCTCTgCAgTCAAA-3'
GAPDH	Fwd: 5'-gAAggTgAAggTCggAgTC-3'
	Rev: 5'-gAAgATggTgATgggATTTC-3'
β-actin	Fwd: 5'-CCTggCACCCAgCACAAT-3'
	Rev: 5'-gggCCggACTCgTCATACT-3'

Cortisol

Plasma cortisol levels were measured by electrochemiluminescence immunoassay on the Modular E170 (Roche Diagnostics, Mannheim, Germany). Lower limit of detection was 3 nmol/l; interassay variation was <3%; and reference values (7-10 a.m.) were 170 - 540 nmol/l.

Leukocyte subset analysis

Leukocyte subsets were assessed by dual-color fluorescence analysis with a Becton Dickinson Calibur flowcytometer to quantify cluster of differentiation (CD) 14+ (monocytes), CD3+ (T cells), CD4+ (T helper/inducer), CD8+ (T suppressor/cytotoxic) and CD19+ (B cells) as described previously (19).

Data analyses

Analyses were performed using SPSS, Version 15.0 (SPSS, Inc., Chicago). A p-value < 0.05 (two-tailed) was considered significant. Variables were tested for normality and log-transformed when necessary. Outliers were removed if z-values fell outside the range of ± 3.29 (GR-α: comparison group: n=1; GR-β: PTSD: n=1; GR-P: comparison group: n=1; FKBP5: PTSD: n=2; GILZ: PTSD: n=1; SGK1: PTSD: n=2). Group differences were tested with t-tests and ANCOVA for continuous parametric variables and Chi-square tests for categorical variables. Mann-Whitney tests were used for nonparametric log-transformed variables. Logistic regression analysis with group as a dependent variable, controlling for potentially traumatic childhood experiences and pre-deployment questionnaire scores for PTSD and depressive symptoms, was performed to test the predictive value of pre-existing GR number for PTSD status after deployment. This analysis provided the odds ratio for inclusion in the PTSD group with each GR increase of 1000. Linear regression analysis was performed with the pre-existing GR number as a dependent variable to test whether the GR number was associated with pre-deployment participant characteristics. For regression analyses missing variables for pre-deployment SCL-90 depression scores (PTSD: n=1), the number of previous deployments (PTSD: n=1) and BMI (PTSD: n=3) were imputed in SPSS using the option 'linear trend at point'.

RESULTS

Participant characteristics

Participant characteristics are depicted in Table 2. Exposure to potentially traumatic events during deployment was similar for both groups ($t_{(62)}$= −0.751, p=.456). After deployment the PTSD group had more depressive symptoms than the comparison group ($t_{(66)}$= −7.572, p<.001). There were no significant group differences for the matching variables. Participants in the PTSD group already reported somewhat more PTSD symptoms before deployment than matched comparison subjects ($t_{(66)}$= −4.284, p<.001). Participants in the PTSD group also had more depressive symptoms before deployment ($t_{(65)}$= −4.463, p<.001). However, the level of these symptoms was within the normal range. On average, participants in the PTSD group had experienced more potentially traumatic experiences during childhood ($t_{(66)}$=

TABLE 2. Pre- and post-deployment questionnaire scores, pre-deployment matching variables, and pre-deployment characteristics of participants in the PTSD group (n = 34) and matched comparison group (n = 34). Data are presented as mean (SD) for continuous variables and N (%) for categorical variables. Group differences were analyzed by t-tests for continuous variables and Chi-Square tests for categorical variables.

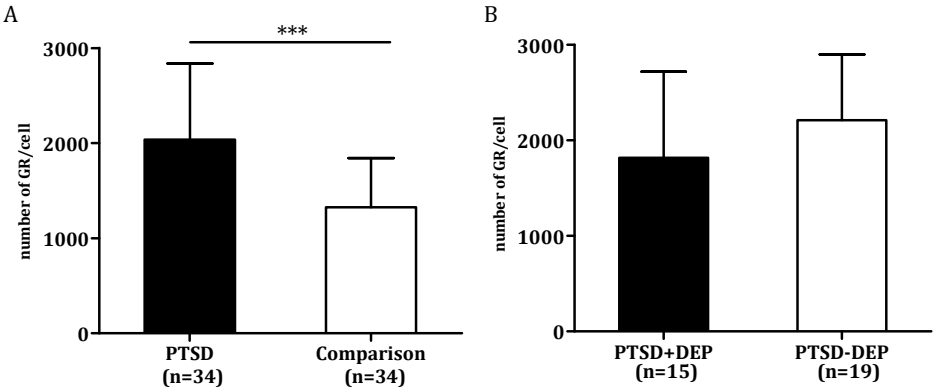
	PTSD group (n=34)	Comparison group (n=34)	p
SRIP total score after deployment	44.18 (5.59)	25.15 (4.05)	<.001
SCL-90 depression score after deployment	24.03 (5.83)	16.94 (1.67)	<.001
SRIP total score before deployment	28.35 (4.18)	24.65 (2.99)	<.001
SCL-90 depression score before deployment	19.82 (4.49)	16.56 (1.13)	<.001
Exposure to potentially traumatic deployment stressors	5.97 (2.48)	5.50 (2.51)	.456
Age during deployment	27.35 (10.09)	26.68 (9.28)	.774
Number of previous deployments	0.70 (1.16)	0.65 (0.85)	.869
Early Trauma Inventory, nr of experiences	4.62 (3.12)	2.62 (2.81)	.002
BMI before deployment	25.45 (3.66)	24.59 (2.53)	.613
Smoking before deployment (yes)	20 (60.6%)	16 (47.1%)	.330
Period of deployment			1.000
2006	3 (8.8%)	3 (8.8%)	
2007	11 (32.4%)	11 (32.4%)	
2008	20 (58.8%)	20 (58.8%)	
Rank during deployment			.724
Officers	4 (11.8%)	2 (5.9%)	
Non-commissioned officers	5 (14.7%)	7 (20.6%)	
Corporals	5 (14.7%)	7 (20.6%)	
Soldiers	20 (58.8%)	18 (52.9%)	
Function during deployment			.788
Base	9 (26.5%)	8 (23.5%)	
Off-base	19 (55.9%)	22 (64.7%)	
Both equally	6 (17.6%)	4 (11.8%)	
Education			.933
Lower	14 (42.4%)	16 (48.5%)	
Middle	16 (48.5%)	14 (42.4%)	
Higher	3 (9.1%)	3 (9.1%)	
Alcohol/week before deployment			.117
No alcohol	4 (12.1%)	2 (6.1%)	
1-20 units/week	26 (78.8%)	31 (93.9%)	
> 20/week	3 (9.1%)	0 (0.0%)	

−3.161, p=.002). The two groups did not differ in number of smokers, alcohol use and BMI before deployment. Medication use was very limited (ACE inhibitors and diuretics (n=1), glucocorticoids, local use (n=1) and antibiotics (n=1)). Excluding these participants from the analyses did not change the results.

Pre-deployment glucocorticoid receptor number in PBMCs

We quantified the pre-deployment DEX binding capacity of PBMCs, reflecting the GR number in these cells, by determining the specific binding of 100 nM ^{3}H-dexamethasone in a whole cell binding assay. The data demonstrate a higher GR number in PBMCs before deployment in participants who developed a high level of PTSD symptoms after deployment ($t_{(66)}$ = −4.343, p<.001) (Figure 1). Moreover, logistic regression analysis revealed that the pre-deployment GR number significantly predicted inclusion in the PTSD group after deployment, controlling for potentially traumatic childhood experiences and pre-deployment questionnaire scores for PTSD and depressive symptoms (W= 10.077, p=.002). The logistic regression showed that the risk for inclusion in the PTSD group after deployment increased 7.5-fold with each GR increase of 1000 (unadjusted odds ratio: 7.53; 95% Confidence interval: 2.16-26.20). We examined whether the GR number before deployment was related to pre-deployment age, rank, number of previous deployments, smoking, alcohol use, BMI, potentially traumatic childhood experiences, and questionnaire scores for PTSD and depressive symptoms (Table 3). None of these variables were associated with the GR number.

FIGURE 1. Pre-deployment group differences in number of GR binding sites in PBMCs.



All analyses were performed in duplicate in one pre-deployment sample per individual. Data are presented as mean (SD). Group differences were tested with t-tests. *** p<.001. (a) number of GR binding sites in PBMCs of participants with PTSD symptoms six months after deployment (PTSD, n=34, range: 519-3816) and matched comparison group (comparison group, n=34, range: 471-2939), (b) number of GR binding sites in PBMCs of participants in the PTSD group with (PTSD+DEP, n=15, range: 519-3816) and without (PTSD-DEP, n=19, range: 946-3255) co-morbid depressive symptoms six months after deployment.

TABLE 3. Predictive value of pre-deployment participant characteristics for pre-deployment number of GR binding sites in PBMCs within all participants (n=68), analyzed with linear regression analysis.

	Beta	t	p
Age	.141	0.575	.568
Rank	−.211	−1.044	.301
Number of previous deployments	−.006	−0.031	.975
Smoking	.196	1.478	.145
Alcohol use	−.112	−0.860	.393
BMI	.024	0.160	.874
Early Trauma Inventory, nr. of experiences	.025	0.166	.869
SRIP total score	.006	0.035	.972
SCL-90 depression score	.179	1.124	.266

Co-morbid depressive symptoms

Previously we reported a higher pre-existing GR number in PBMCs among deployed military personnel who had developed fatigue and depressive symptoms six months after deployment (19). Therefore, we investigated whether the observed group difference in the GR number in the current study was related to development of co-morbid depressive symptoms within the PTSD group. However, the pre-deployment GR number did not differ between participants of the PTSD group with (n=15) and without (n=19) co-morbid high levels of depressive symptoms after deployment ($t_{(32)}$= 1.447, p=.158) (Figure 1).

Five participants of the PTSD group and five comparison subjects were also included in our previous study (19). Therefore, we investigated whether the observed group difference in the GR number in PBMCs was influenced by the inclusion of these participants. The group difference in GR number remained highly significant after removal of these participants ($t_{(56)}$= −3.215, p=.002).

Glucocorticoid receptor subtypes, GILZ, SGK1 and FKBP5 mRNA expression

Using quantitative RT-PCR analysis we assessed whether the observed group differences in the pre-deployment GR number in PBMCs were associated with group differences in pre-deployment GR mRNA expression. There were no significant group differences in the level of pre-deployment mRNA expression for GR-α ($t_{(62)}$= −0.507, p=.614), GR-β ($t_{(62)}$= 0.115, p=.909) and GR-P ($t_{(62)}$= −0.426, p=.672). Furthermore, there were no significant group differences in the level of pre-deployment GILZ ($t_{(63)}$= −0.235, p=.815) and SGK1 (U= 415.00, p=.271) mRNA expression. In addition, there was no group difference in pre-deployment FKBP5 mRNA expression level in PBMCs ($t_{(61)}$= 0.939, p=.351).

Cortisol and composition of PBMC population

The observed group difference in the GR number in PBMCs was not associated with group differences in plasma cortisol or the subset composition of PBMCs (Table 4). However, in the PTSD group a higher percentage of CD19+ B-cells as well as a lower percentage of CD8+ T-cells nearly reached significance. The group difference in DEX binding capacity of PBMCs remained significant after controlling for these variables ($F_{(1,64)}$= 15.138, p<.001)

TABLE 4. Pre-deployment plasma cortisol levels, absolute leukocyte numbers and relative distribution of PBMC population of participants in the PTSD group (n = 34) and matched comparison group (n = 34). Data are presented as mean (SD). Group differences were analyzed by t-tests.

	PTSD group (n = 34)	Comparison group (n = 34)	p
Cortisol (nmol/l)	436.50 (139.14)	439.21 (120.91)	.932
Leukocytes (10^9/ l)	6.90 (1.41)	6.55 (1.60)	.344
% lymphocytes	34.80 (7.65)	35.42 (11.37)	.795
% monocytes	4.67 (1.72)	4.77 (1.73)	.808
% CD19+ B-lymphocytes	12.01 (3.90)	10.16 (3.88)	.054
% CD3+ T-lymphocytes	68.70 (8.08)	69.60 (8.22)	.653
% CD8+ T-lymphocytes	33.83 (6.49)	37.48 (8.52)	.051
% CD4+ T-lymphocytes	40.74 (8.23)	38.57 (8.66)	.294

Post-deployment glucocorticoid receptor number

We investigated whether the observed pre-deployment group difference in the GR number in PBMCs was still present after deployment. The PTSD group had a higher GR number at one ($t_{(66)}$= −2.117, p=.038) and six months ($t_{(66)}$= −2.366, p=.021) after deployment relative to the comparison group.

DISCUSSION

This study reveals that high pre-existing DEX binding capacity of PBMCs, reflecting a high GR number in these cells, represents a vulnerability factor for development of PTSD symptoms after military deployment. Before deployment, a higher GR number in PBMCs was present in military personnel who developed a high level of PTSD symptoms six months after deployment. The pre-deployment GR number was a strong predictor of inclusion in the PTSD group after deployment: with each increase of 1000 GRs, the odds ratio for the presence of PTSD symptoms after deployment increased 7.5-fold. The observed group difference was still present after deployment.

Although the PTSD group reported more pre-deployment symptoms of PTSD and depression, the pre-deployment GR number was not influenced by these pre-deployment symptoms. Furthermore, group differences in the PBMC composition also did not influence the results. In addition, group differences in the GR occupancy by endogenous glucocorticoids most likely did not influence the observed group differences, since plasma cortisol levels did not differ between groups. Moreover, cells were equilibrated in a culture medium and extensively washed before addition of ^{3}H-DEX to prevent possible effects of binding of endogenous glucocorticoids.

We previously reported a higher pre-existing GR number in PBMCs among military personnel with depressive symptoms and severe fatigue after deployment (19). Within the current study, almost one-half of the participants of the PTSD group reported high levels of depressive symptoms after deployment, which is consistent with co-morbidity estimates for PTSD and major depressive disorder (MDD) in war veterans (25). However, there was no difference in pre-deployment GR number between participants with and without depressive symptoms in the PTSD group. Therefore, the present finding is related to development of PTSD symptoms after deployment and not to development of depressive symptoms in a subgroup of participants.

Although changes in GR functioning in PTSD and major depressive disorder (MDD) are supposed to be different (3, 26), a high pre-existing GR number in PBMCs is related to both PTSD and depressive symptoms after deployment (19). Thus, high pre-existing GR number in PBMCs may be a predictor of mental health problems specifically induced by stress and/or trauma.

A limitation of the current study is that mental health status was not assessed with structured clinical interviews. Therefore, it is uncertain whether participants in the PTSD group fulfilled the DSM-IV diagnostic criteria for PTSD. Individuals with a sub-clinical level of PTSD symptoms therefore may also have been included in the PTSD group. However, it is reasonable to assume that the questionnaires are a reliable reflection of the presence of PTSD symptoms (21).

Currently, various subtypes of the GR have been identified. GR-α is the most abundant variant and is transcriptionally active (27), while GR-β is less ubiquitously expressed, does not bind glucocorticoids and has limited transcriptional capability (28). Additionally, GR-P is widely expressed and increases GR-α activity in cell lines (29, 30). To our knowledge, there are no published data on GR mRNA expression levels in PTSD. However, within MDD the peripheral GR number (for review see 26) and peripheral GR-α mRNA expression (31) both appear to be decreased. Our results show that the observed group difference in the GR number in PBMCs was not accompanied by group differences in GR mRNA expression level. This result implies that the higher GR number in the PTSD group is mediated by post-transcriptional mechanisms. Additionally, we cannot completely exclude that group differences in MR level influenced the observed higher DEX binding capacity of PBMCs in the PTSD group.

Alterations in FKBP5 expression influence glucocorticoid binding capacity of PBMCs (15). Segman et al. reported that the amount of up-regulation of FKBP5 mRNA expression after trauma predicted the presence of PTSD four months later (32). In addition, Yehuda et al. found decreased FKBP5 mRNA expression levels in PTSD patients (33). In our study, however, there was no group difference in FKBP5 mRNA expression before deployment. We also did not observe group differences in GR target genes GILZ and SGK1. It may well be possible, however, that compensation by other pathways that regulate these genes compensate for a putative difference in GR activity.

The observed higher GR binding in the PTSD group may be associated with the presence of specific GR polymorphisms, which could for example alter post-transcriptional mechanisms influencing GR protein expression or directly affect the binding capacity of the GR. Bachman et al. reported no association between PTSD and presence of GR polymorphisms BclI and N363S (34). However, currently more polymorphisms in the GR gene have been identified (for review see 35), which were not yet investigated in relation to development of PTSD at the time of the study. Whether genetic factors are involved in the higher GR number in PBMCs observed in the PTSD group will be investigated in a larger sample.

In our study, participants in the PTSD group experienced almost twice as many potentially traumatic events during childhood than comparison subjects. Although the number of traumatic experiences during childhood was not related to the GR number in PBMCs, it is possible that a higher GR number in PBMCs and exposure to childhood trauma together increase the vulnerability for PTSD development after deployment. In this context, Bet et al. reported that the co-occurrence of both the GR polymorphism 22/23EK and 9β with childhood trauma is associated with increased risk for development of a MDD (36).

Peripheral GRs are often considered to be an accessible model for GR expression in the brain. Nonetheless, whether the mechanisms of regulation of central and peripheral GRs are similar has not been studied extensively. In rats, neuronal and lymphoid cytosolic GRs are similar in glucocorticoid affinity and specificity (37), and cytosolic GRs in the brain and peripheral immune tissues are down-regulated after chronic corticosterone administration following adrenalectomy (38, 39). It remains unknown whether and how the observed higher pre-deployment GR number in PBMCs is involved in the pathophysiology of PTSD. We speculate that our finding of a higher GR number in PBMCs may be paralleled in the brain. If a suitable radiotracer could be found, PET-neuroreceptor mapping of GRs could provide valuable information about GR-mediated abnormalities in the brain (40).

The present study is the first, to our knowledge, to show that a high GR number in PBMCs before military deployment was a strong predictor of the presence of PTSD symptoms after military deployment and was maintained at least until six months after deployment. We propose that a high GR number in PBMCs may be a bio-marker of increased risk for development of PTSD symptoms after trauma exposure.

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3

Glucocorticoid receptor pathway components predict posttraumatic stress disorder symptom development: a prospective study.

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ABSTRACT

Biological correlates of posttraumatic stress disorder (PTSD) have mostly been studied using cross-sectional or post-trauma prospective designs. Therefore, it remains largely unknown whether previously observed biological correlates of PTSD precede trauma exposure. We investigated whether glucocorticoid receptor (GR) pathway components assessed in leukocytes before military deployment represent pre-existing vulnerability factors for development of PTSD symptoms.

448 male soldiers were assessed before and six months after deployment to a combat-zone. Participants were assigned to the PTSD or comparison group based on Self-Rating Inventory for PTSD scores after deployment. Logistic regression analysis was applied to predict development of a high level of PTSD symptoms based on pre-deployment GR number, messenger (m)RNA expression of GR target genes FKBP5, GILZ and SGK1, plasma cortisol, and childhood trauma. We also investigated whether pre-deployment GR number and FKBP5 mRNA expression were associated with single nucleotide polymorphisms in the GR- and FKBP5 genes, either alone or in interaction with childhood trauma.

Several GR pathway components predicted subsequent development of a high level of PTSD symptoms: pre-deployment high GR number, low FKBP5 mRNA expression and high GILZ mRNA expression were independently associated with increased risk for a high level of PTSD symptoms. Childhood trauma also independently predicted development of a high level of PTSD symptoms. Additionally, we observed a significant interaction-effect of GR haplotype B*cl*I and childhood trauma on GR number.

Collectively, our results indicate that pre-deployment GR pathway components are vulnerability factors for subsequent development of a high level of PTSD symptoms.

INTRODUCTION

Posttraumatic stress disorder (PTSD) is a common consequence of exposure to trauma, with life-time prevalence estimated at 7% in general populations from the US (1) and the Netherlands (2). Biological correlates of PTSD have been studied before, but most of these studies used a cross-sectional or post-trauma prospective design (3). Therefore, it remains to be determined whether biological differences between individuals with and without PTSD are already present before the traumatic event leading to PTSD. Identification of pre-existing vulnerability factors for PTSD development would contribute to the identification of vulnerable individuals working in professions with high risk of trauma exposure, such as the military and police. This identification could eventually lead to improved preventive care.

PTSD is associated with altered functioning of the hypothalamic–pituitary–adrenal axis (HPA axis) (3) although there is ongoing dispute with regard to the direction of these alterations. A meta-analysis showed that hypocortisolism is present in specific subgroups of PTSD patients (4). On the other hand, hypercortisolism has also been described in PTSD (5-8), and may be associated with (fear for) ongoing or repeated traumatization (5, 7). Increased negative feedback of glucocorticoids (GCs) on the HPA axis is often considered as one of the hallmark biological correlates of PTSD (3, 9), but this is not a consistent finding (6, 8). It has been reported that increased sensitivity of the HPA axis for negative feedback by GCs can also be induced by adulthood trauma exposure independently of PTSD (10-12). We propose that the mixed results are most likely caused by differences in populations with regard to gender, type and timing of trauma, presence of co-morbid disorders and time since trauma.

The regulation of the immune system by GCs in PTSD has also been studied. An increased sensitivity of immune cells to regulation by GCs has repeatedly been described for PTSD (13, 14), although decreased GC-sensitivity of immune cells has also been observed (15). GCs are important regulators of the immune system by inhibiting cell proliferation, regulating cytokine production and stimulating apoptosis (16). The actions of GCs are mediated by glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). GC-regulation of the immune system, especially under stressful conditions, is predominantly mediated via GR (17). The number of GR may contribute to the level of GR signalling, and there is evidence that the relative expression of various GR subtypes contributes to GR binding capacity and functional effects of GCs (18, 19). Several cross-sectional studies have indicated an increased GR number in peripheral blood mononuclear cells (PBMCs) of individuals with PTSD (20, 21). FK506 binding protein 5 (FKBP5) is a target gene of GR which is upregulated by activation of the receptor. Moreover, FKBP5 functions as a co-chaperone molecule of the GR and lowers the affinity of GR which reduces GC binding, leading to decreased GR signalling capacity (22). It has been shown that FKBP5 messenger (m)RNA expression is decreased in PTSD (23, 24). In addition, FKBP5 mRNA expression immediately post-trauma predicted subsequent PTSD status (25). We recently described that the GR number in PBMCs was higher before military

deployment in 34 soldiers with a high level of PTSD symptoms after deployment compared to a sample of 34 matched controls without a high level of PTSD symptoms after deployment (26).

The working model for the current study was that military personnel who develop a high level of PTSD symptoms in response to deployment have a dysregulation at various levels of the GR pathway prior to deployment. In the first part of this study we investigated which components of the GR pathway contribute to prediction of the development of PTSD symptoms in response to deployment. For this purpose, we investigated whether pre-deployment mRNA expression levels of genes directly regulated by the GR (i.e. genes with a glucocorticoid response element (27)), predicted the presence of a high level of PTSD symptoms six months after deployment. We selected three GR target genes; glucocorticoid-induced leucine zipper (GILZ), a mediator of the anti-inflammatory and immunosuppressive effects of GCs (28); serum/glucocorticoid regulated kinase 1 (SGK1), which is involved in modulating apoptosis (29); and FKBP5. Furthermore, we aimed at confirming our previous finding on the predictive value of the pre-deployment GR number for development of a high level of PTSD symptoms within this large sample of 448 male soldiers. We also investigated whether pre-deployment plasma cortisol, as an important outcome of the HPA-axis, predicted a high level of PTSD symptoms. In addition, because childhood trauma is a well-known risk factor for adult PTSD (30), we also investigated whether childhood trauma predicted a high level of PTSD symptoms.

GR number, GILZ mRNA expression and FKBP5 mRNA expression turned out to independently predict development of PTSD symptoms. Various single nucleotide polymorphisms (SNPs) in the GR and FKBP5 gene have been described. In the second part of the study, we investigated whether SNPs in these genes were related to the number of GR and the level of FKBP5 mRNA expression in our sample. In addition, we also investigated whether interactions between childhood trauma and SNPs in GR and FKBP5 genes were related to GR number and to FKBP5 mRNA levels. We selected five GR SNPs that are associated with sensitivity of PBMCs and the HPA axis for regulation by GCs and with cortisol and ACTH responses to stress in Caucasians (31, 32). In addition, one of these SNPs (B*cl*I) has previously been found to be associated with increased risk for development of PTSD (33). Furthermore, we selected two FKBP5 SNPs associated with peri- and posttraumatic dissociation within a population of Caucasian children (34). These SNPs have also been shown to be associated with increased risk for PTSD development in African American samples with high levels of childhood trauma (35, 36).

METHODS AND MATERIALS

General procedure

Military personnel of the Dutch Armed Forces assigned to a 4-month deployment to Afghanistan were included on a voluntary basis after oral and written informed consent. Their duties during deployment included combat patrols, clearing or searching buildings, participation in de-mining operations, and transportation across enemy territory. Participants were exposed to typical combat-zone stressors including enemy fire, armed combat and combat casualties. We included participants from 11 sequential rotations deployed from 2005 to 2009. Several weeks prior to deployment and approximately six months after deployment, participants filled out questionnaires and a blood sample was drawn. The study was approved by the Institutional Review Board of the University Medical Center Utrecht.

Participants

Since over 90% of the total participant population was male, we included only males in the current study. 448 male participants completed the assessments before and six months after deployment (for procedure: 26). The sample had a predominantly Caucasian background (>95%). Participants were assigned to the PTSD group (n=35) when their score on the Self-Rating Inventory for PTSD (SRIP) (37, 38) was above cut-off (≥38) six months after deployment and their SRIP score before deployment was below cut-off. This cut-off equals the mean plus two standard deviations, and corresponds with the 95th percentile of SRIP scores before deployment within a population of 704 soldiers from the Dutch Armed Forces (mean (SD): 26.91(5.34)). All remaining participants with SRIP scores below cut-off level both before and six months after deployment were included in the comparison group (n=413). Thirty-four participants of the PTSD group and 34 participants of the comparison group were included in our previous study (26). Before deployment, medication use was very limited (local use of corticosteroids (n=5), antihypertensives (n=3), antidepressants (n=2), antihistamines (n=11) and cholesterol lowering medications (n=5)) and did not differ between groups (p=1.000). Analysis of GR pathway-components was performed by investigators blind to the PTSD status of the participants.

Measures

Questionnaires

PTSD symptoms over the past 4 weeks were assessed with the 22-item Self-Report Inventory for PTSD (SRIP) (37, 38). The SRIP has good concurrent validity with other PTSD measures such as the Clinician Administered PTSD Scale and Mississippi scale for PTSD. The validity of

our cut-off score as representing a high level of PTSD symptoms is supported by van Zelst et al. (39), who tested the sensitivity and specificity of various cut-offs on the SRIP for a diagnosis of PTSD according to the DSM-IV. A cut-off in our range provided the highest sensitivity and specificity for a PTSD diagnosis.

Levels of depressive symptoms, anxiety symptoms and sleep disturbances were assessed using subscales of the Dutch version of the 90-item symptom checklist (SCL-90) (40). This questionnaire has good reliability and is frequently used within research and clinical settings. The validity of the depression subscale as a screening instrument for depression has been shown in various patient samples (41-43). Exposure to potential traumatic experiences during childhood was assessed using the Dutch version of the 27-item self-report version of the Early Trauma Inventory (44). Exposure to potentially traumatic deployment stressors was assessed with a 13-item checklist (26).

Dexamethasone binding

For determination of the capacity of PBMCs to bind GCs, a validated whole-cell single-point binding assay was used as described previously (45). This method provides a reliable estimate of Bmax as determined using a classical binding assay with 3-200 nmol/mL ^{3}H-dexamethasone (r^2=0.92) (45). Briefly, PBMCs were isolated from whole blood using Ficoll-Paque (Pharmacia, Uppsala, Sweden) and 10^7 cells were frozen in DMSO. After thawing and 60 minutes equilibration in culture medium, cells were washed twice, resuspended in assay buffer (RPMI-1640 with 5% FCS) and incubated in duplicate with 100 nmol/mL ^{3}H-dexamethasone (Amersham, Buckinghamshire, UK) in the presence or absence of excess unlabeled dexamethasone (Sigma-Aldrich, Steinheim, Germany) for 1 hour at 37°C. Cell bound radioactivity was quantified by liquid scintillation analysis. We refer to the results of the binding assay as GR number, since the GR/MR ratio in human PBMCs is approximately 10:1 and the ratio of dexamethasone binding affinity is 4:1 (17).

GR target gene mRNA expression

We investigated pre-deployment mRNA expression of GR target genes (FKBP5, GILZ and SGK1). Total RNA was isolated from PBMCs with Trizol (Invitrogen). One µg of total RNA was used to synthesize cDNA with SuperScript Reverse Transcriptase (Invitrogen). Real-time PCR reactions were performed with an iQ5 Real-Time PCR Detection System (Bio-Rad) (see 26 for primer sequences). Data were normalized for GAPDH and β-actin expression.

GR and FKBP5 SNPs

DNA was extracted from whole blood samples by using the Puregene DNA purification kit (Qiagen, Valencia, CA, USA). Five common polymorphisms of the GR gene (single nucleotide polymorphisms (SNPs T*th111*l (rs10052957), ER22/23EK (rs6189/90), N363S (rs6195), B*cl*l (rs41423247) and A3669G 9β (rs6198)) and two common polymorphisms of the FKBP5

gene (rs3800373, rs1360780) were selected (31, 34). SNPs were genotyped using Taqman Assay-by-design (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Assays were performed according to the manufacturer's instructions. The genotypes were analyzed using an ABI 7900HT instrument (Applied Biosystems).

Cortisol

A venous blood sample was collected between 8.00-11.30 a.m. in EDTA vacutainers. Plasma was collected after centrifugation and stored at -80°C. Cortisol levels were measured using electrochemiluminescence (ECL) immunoassay on the Modular E170 (Roche Diagnostics, Mannheim, Germany). Lower detection limit: 3 nmol/l. Interassay variation: <3%. Reference values (7-10 a.m.): 170-540 nmol/l.

Data analysis

Analyses were performed using SPSS 15.0. $P<0.05$ (two-tailed) was considered significant. Variables were tested for normality and ^{10}log-transformed when necessary. Non-transformed values are reported in figures and tables. Due to technical problems, missing values were present for a number of participants (GR number: 4; mRNA expression: 42; cortisol: 16; GR SNPs: 7, FKBP5 SNPs: 5). Outliers were removed if their values exceeded SD±3.29 from the mean (FKBP5: 4; GILZ: 3; SGK1: 3; cortisol: 2). Removal of outliers did not alter our results. In case of missing values, participants were deleted list-wise from the analyses for which the values were missing.

Group differences were tested with t-tests for continuous parametric variables, χ^2-tests for categorical variables and repeated measures ANOVA for variables measured longitudinally. Deviations from Hardy-Weinberg-equilibrium in genotype data were assessed with χ2-tests. Linkage disequilibrium among the SNPs was estimated with D' using HaploView (46). Haplotypes were assigned using PHASE, which uses a Bayesian estimation method to reconstruct haplotypes from population genotype data (47). Only haplotypes with a frequency ≥1% were included in the analyses. Haplotypes could be inferred with ≥95% certainty for both alleles in 95% of participants for GR haplotypes and in 89% of participants for FKBP5 haplotypes.

The predictive value of GR number, mRNA expression levels, plasma cortisol levels and childhood trauma for the presence of a high level of PTSD symptoms was investigated with logistic regression analysis. By standardizing the continuous variables we were able to compare the predictive value of the variables. We subsequently controlled for possibly confounding effects of deployment stressors, age, number of previous deployments, pre-deployment PTSD and depression questionnaire scores, and pre-deployment BMI, smoking, alcohol, and medication use. All variables in the model were forced into entry.

Additionally, we performed a median split on the number of traumatic childhood experiences (low childhood trauma: ≤2 reported events, high childhood trauma: ≥3 reported

events). We included childhood trauma, dichotomous haplotype carrier status and an interaction-term between haplotype carrier status and childhood trauma in linear regression analyses to predict pre-deployment GR number and FKBP5 mRNA expression. Bonferonni correction was applied.

RESULTS

Participant characteristics

We included 448 male soldiers, of which 35 reported a high level of PTSD symptoms after return from deployment (Table 1). Participants in the PTSD group reported more PTSD symptoms than the comparison group before and after deployment ($F_{(1,446)}$= 227.245, p<.001). More importantly, the PTSD group reported a strong increase in PTSD symptoms in response to deployment, while PTSD symptoms in the comparison group did not increase ($F_{(1,446)}$= 281.715, p<.001) (Table 1). The self-reported longitudinal course of depressive symptoms, general anxiety and sleep disturbances followed the same pattern (depression: group: $F_{(1,444)}$= 102.098, p<.001, interaction: $F_{(1,444)}$= 53.769, p<.001; anxiety: group: $F_{(1,442)}$= 64.005, p<.001, interaction: $F_{(1,442)}$= 31.757, p<.001; sleep problems: group: $F_{(1,444)}$= 45.013, p<.001,

TABLE 1. Participant characteristics and pre- and post-deployment questionnaires scores of the posttraumatic stress disorder (PTSD) symptoms group and comparison group.

	PTSD group (n = 35)		Comparison group (n = 413)		
	Before deployment	After deployment	Before deployment	After deployment	
PTSD (SRIP) total score	28.23 (4.19)	44.20 (5.51)	25.86 (3.63)	26.05 (4.03)	
SCL-90 depression score	19.76 (4.43)	23.97 (5.76)	17.50 (2.22)	17.67 (3.04)	
SCL-90 anxiety score	11.74 (1.87)	13.46 (3.97)	10.76 (1.36)	10.69 (1.46)	
SCL-90 sleep disturbances score	4.34 (1.68)	6.17 (2.55)	3.84 (1.37)	3.84 (1.48)	
					P-value
Nr of deployment stressors experienced		5.94 (2.44)		5.02 (2.60)	.055
Age during deployment	27.29 (9.95)		29.07 (8.98)		.265
Number of previous deployments	0.68 (1.15)		1.00 (1.28)		.098
Early Trauma Inventory, nr of experiences	4.45 (3.11)		2.96 (2.65)**		.001
BMI before deployment	25.39 (3.62)		24.84 (2.69)*		.324
Smoking before deployment (yes)	20 (58.8%)		169 (40.9%)		.042
Alcohol/week before deployment					.679
No alcohol	4 (11.8%)		37 (9.1%)		
1-20 units/week	27 (79.4%)		344 (84.9%)		
> 20/week	3 (8.8%)		24 (5.9%)		

interaction: $F_{(1,444)}$= 35.619, p<.001). Furthermore, participants in the PTSD group had experienced more potentially traumatic experiences during childhood ($t_{(444)}$= −3.217, p= .001). In addition, a higher percentage of participants in the PTSD group smoked before deployment (p=.042). The two groups did not differ in age, BMI and alcohol use before deployment.

Prospective analyses

Predictive value of GR number, GR target genes, plasma cortisol and childhood trauma for PTSD symptoms after deployment

We included GR number and mRNA expression of GR target genes in PBMCs, plasma cortisol and childhood trauma measured prior to deployment in the logistic regression to predict the presence of a high level of PTSD symptoms after deployment (Table 2). A high pre-deployment number of GR in PBMCs was independently associated with increased risk for a high level of PTSD symptoms after deployment (W= 13.631 p<.001); the odds for a high level of PTSD symptoms increased 2.6-fold with each SD increase in GR number. Furthermore, high pre-deployment GILZ mRNA expression was independently associated with increased risk for a high level of PTSD symptoms after deployment (W= 25.616, p<.001); the odds increased 5.0-fold with each SD increase in GILZ mRNA expression. Additionally, low pre-deployment FKBP5 mRNA expression was independently associated with increased risk for a high level of PTSD symptoms after deployment (W= 25.584, p<.001): the odds decreased 14.5-fold with each SD increase in FKBP5 mRNA expression. Furthermore, childhood trauma was independently associated with increased risk for a high level of PTSD symptoms (W= 4.748, p=.029): the odds increased 1.8-fold with each SD increase in the number of reported childhood traumatic experiences. SGK1 mRNA expression and plasma cortisol did not significantly predict PTSD symptom status.

The predictive value of the GR pathway components remained significant after controlling for deployment stressors, age, number of previous deployments, pre-deployment PTSD and

TABLE 2. Predictive value of pre-deployment glucocorticoid receptor number in PBMCs, GR target genes mRNA expression levels in PBMCs, plasma cortisol and childhood traumatic experiences for the development of a high level of PTSD symptoms six months after deployment within 388 male soldiers.

	B	S.E.	Wald	df	p	OR	95% CI
GR number in PBMCs	0.965	0.261	13.631	1	<.001	2.625	1.573-4.382
FKBP5 mRNA expression	−2.680	0.530	25.584	1	<.001	0.069	0.024-0.194
GILZ mRNA expression	1.605	0.317	25.616	1	<.001	4.978	2.674-9.268
SGK1 mRNA expression	−0.378	0.315	1.440	1	.230	0.685	0.369-1.271
Plasma cortisol levels	−0.062	0.272	0.052	1	.819	0.940	0.552-1.601
Childhood traumatic experiences	0.587	0.269	4.748	1	.029	1.798	1.061-3.048

All variables are standardized, mean (SD) = 0(1). CI: confidence interval; OR: odds ratio.

depression questionnaire scores, and pre-deployment BMI, smoking, alcohol and medication use. Childhood trauma no longer significantly predicted the development of a high level of PTSD symptoms after controlling for these variables.

Cross-sectional analyses

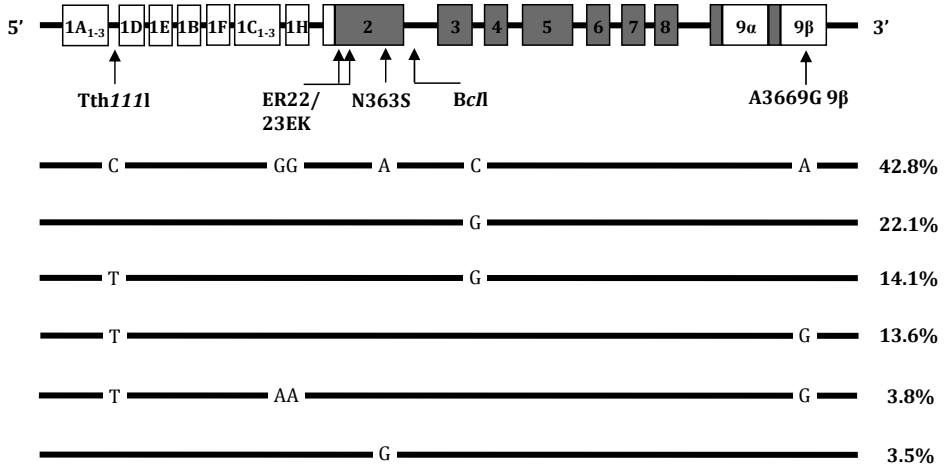
GR and FKBP5 genotypes

We determined carrier status for five common GR SNPs and two common FKBP5 SNPs. SNPs in the GILZ gene have not been identified yet and were therefore not investigated. All SNPs, except for N363S, were in Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) between the GR SNPs indicated high LD between a substantial proportion of SNPs. Additionally there was high LD between the two FKBP5 SNPs. Haplotype analysis indicated the presence of 6 GR haplotypes (Figure 1) and three FKBP5 haplotypes (Figure 2) with a frequency of ≥.01.

Predictive value of genotypes and childhood trauma for GR number and FKBP5 mRNA expression

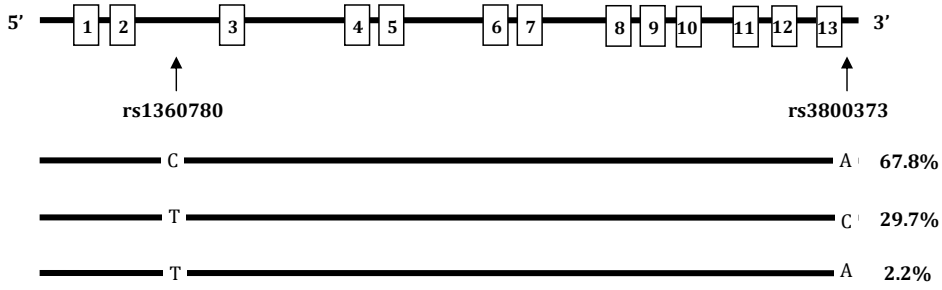
We investigated the predictive value of SNP haplotype carrier status, alone and in interaction with childhood trauma, for pre-deployment GR number and FKBP5 mRNA expression. This approach was selected because our PTSD group was not large enough to reliably predict PTSD group status based on the SNPs. Main effects of GR haplotype carrier status did not significantly predict pre-deployment GR number. In addition, main effects of childhood

FIGURE 1. Schematic overview of the glucocorticoid receptor single nucleotide polymorphisms (SNPs) and associated haplotypes.



Localization of the SNPs in the GR (NR3C1) gene is indicated. Estimated allele frequencies of the haplotypes are depicted at the right when expected to be greater than 1%.

FIGURE 2. Schematic overview of the FKBP5 single nucleotide polymorphisms (SNPs) and associated haplotypes.



Localization of the SNPs in the FKBP5 gene is indicated. Estimated allele frequencies of the haplotypes are depicted at the right when expected to be greater than 1%.

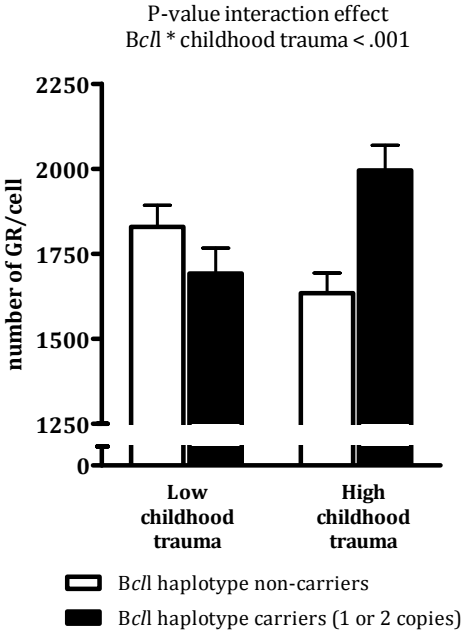
trauma did not significantly predict pre-deployment GR number after applying Bonferonni correction. We observed a significant interaction-effect between childhood trauma and GR B*cl*I haplotype carrier status: individuals with high childhood trauma who carried the B*cl*I haplotype had an increased pre-deployment GR number (Figure 3, Table 3). Within the group B*cl*I carriers with high childhood trauma, the percentage of individuals with PTSD symptomatology was 13.9%. Of the B*cl*I non-carriers with high childhood trauma, 9.4% had PTSD symptoms after deployment. In contrast, only 4.8% of the B*cl*I carriers, and 5.6% of the B*cl*I non-carriers with low childhood trauma had PTSD symptoms after deployment. However, this difference between groups in the percentage of individuals with a high levels of PTSD symptoms did not reach statistical significance ($\chi^2_{(3)}$= 6.122, p=.124).

TABLE 3. Predictive value of glucocorticoid receptor (GR) haplotype carrier status, childhood trauma and haplotype carrier status x childhood trauma for pre-deployment GR number within 412 male soldiers.

	Haplotype carrier status		Childhood trauma		Haplotype x childhood trauma	
	Beta	p	Beta	p	Beta	p
GR most common haplotype	−.006	.931	.001	.991	−.002	.984
GR B*cl*I	−.097	.155	−.141	.024	.284	<.001
GR T*th111*I + B*cl*I	.034	.625	.040	.486	−.103	.175
GR T*th111*I + A3669G 9β	−.008	.906	.005	.925	−.014	.856
GR N363S	.054	.409	.016	.748	−.093	.159
GR T*th111*I + ER22/23EK + A3669G 9β	.031	.647	.012	.812	−.065	.350

Haplotype carrier status was included as a dichotomous variable: 1 or 2 copies carried versus no copies carried. See figure 1 for a schematic overview of the observed haplotypes and estimated allele frequencies. Childhood trauma was included as dichotomous variable based on a median split: low childhood trauma versus high childhood trauma. Bonferonni correction was applied: α: .05/6 = .0083 for significance.

FIGURE 3. Significant interaction effect of glucocorticoid receptor (GR) gene haplotype B*cl*I carrier status and childhood trauma on pre-deployment GR number.



N B*cl*I non-carriers with low childhood trauma = 83, N B*cl*I carriers with low childhood trauma = 128, N B*cl*I non-carriers with high childhood trauma = 126, N B*cl*I carriers with high childhood trauma = 79. The interaction effect depicted is corrected for main effects of B*cl*I haplotype carrier status and childhood trauma.

FKBP5 mRNA expression was not significantly associated with FKBP5 haplotype carrier status, childhood trauma or interactions between FKBP5 haplotype carrier status and childhood trauma (all p-values >.05).

DISCUSSION

This study reveals that multiple GR pathway components measured prior to deployment are vulnerability factors for development of a high level of PTSD symptoms in response to military deployment. We identified three independent predictors of a high level of PTSD symptoms in the GR pathway, i.e. low FKBP5 mRNA expression, high GILZ mRNA expression and high GR number, measured in PBMCs obtained from a group of 448 male soldiers before their deployment to a combat-zone in Afghanistan.

We show that high levels of GILZ and low levels of FKBP5 mRNA expression prior to deployment were independently associated with increased risk for a high level of PTSD symptoms after deployment. Within a small, carefully matched subgroup of the population studied here, we recently reported a higher number of pre-deployment GR in the PTSD group

compared to the matched controls (26). Our present results validate this initial observation within a larger, heterogeneous group. High GR number, high GILZ mRNA expression and low FKBP5 mRNA expression in PBMCs suggest elevated signalling in the peripheral GR pathway in individuals vulnerable for development of PTSD symptomatology. It remains to be determined whether the observed vulnerability factors mediate the repeatedly observed increased GC-sensitivity of the immune system in PTSD (48, 49).

Interestingly, a recent study showed that a higher cortisol awakening response (CAR) prior to trauma exposure predicted peritraumatic dissociation (50), a risk factor for subsequent development of PTSD (51). In our study, morning plasma cortisol levels prior to deployment did not predict PTSD symptomatology after deployment. Moreover, the predictive effect of GR, FKBP5 and GILZ for a high level of PTSD symptoms was independent of plasma cortisol levels. These observations fit with data in the literature showing that the CAR prior to trauma exposure was not directly associated with subsequent PTSD symptoms (52, 53)

Experiencing traumatic events during childhood is one of the most consistently observed risk factors for adult PTSD (30). As expected, our PTSD group on average reported a higher number of childhood traumatic experiences. Childhood trauma significantly contributed to the prediction of a high level of PTSD symptoms independently of the contribution of the identified predictors in the GR pathway.

The peripheral GR pathway is often considered to be an accessible model for GR signalling in the brain. However, it has not been studied extensively whether regulatory mechanisms of central and peripheral GR signalling are similar. Rodent studies have shown that neuronal and lymphoid cytosolic GRs are similar in GC-affinity and specificity (54). In addition, cytosolic GRs in the brain and peripheral immune tissues are both downregulated after chronic corticosterone administration following adrenalectomy (55). We speculate that the observed peripheral GR pathway components may be paralleled in the brain, and that an imbalance within the GR signalling cascade in the brain may be involved in the pathophysiology of PTSD. In individuals with PTSD, central GC-sensitivity has been investigated by assessing the effects of GC administration on learning and memory (56), and on glucose metabolic rate of brain regions with FDG-PET studies (57). Overall results suggest increased GC-sensitivity in the brain of PTSD patients. We hypothesize that this increased GC-sensitivity may be a pre-existing characteristic in individuals vulnerable for PTSD development. To further elucidate GR-mediated abnormalities in the brain, PET neuroreceptor mapping of GRs would be a useful method. Unfortunately a suitable GR radiotracer is not yet available (58).

We sought to identify causal factors associated with the pre-deployment high GR number, and low FKBP5 mRNA expression predictive for subsequent PTSD symptom development. Therefore we investigated whether GR number and FKBP5 mRNA expression were associated with 5 common GR SNPs (*Tth111*I, ER22/23EK, N363S, B*cl*I and A3669G 9β) and 2 common FKBP5 SNPs (rs3800373, rs1360780), either alone or in interaction with childhood trauma. We did not observe an association between the haplotypes of the selected SNPs and

GR number or FKBP5 mRNA expression, indicating that these SNPs are not the major determinants of GR number and FKBP5 mRNA expression. However, we did observe a significant interaction-effect between haplotype carrier status and childhood trauma on GR number: high levels of GR before deployment were present in individuals with the minor allele of GR SNP B*cl*I who had also experienced a high number of childhood traumatic events. These results indicate that these individuals may be at greater risk for developing PTSD symptoms after a traumatic event in adulthood.

The two FKBP5 SNPs analyzed in our study (rs3800373, rs1360780), were associated with higher peri- and posttraumatic dissociation in Caucasian children with an acute medical injury (34), which are risk factors for subsequent PTSD development (51). Additionally, a significant interaction-effect between these SNPs and childhood trauma on PTSD risk was established in African-American samples previously (35, 36). However, we did not observe a significant association between FKBP5 SNPs and childhood trauma on FKBP5 mRNA expression in our predominantly Caucasian sample. Mehta et al. (59) recently described a reversal of the association between FKBP5 SNP rs9296158 and FKBP5 mRNA expression in individuals with PTSD. Within healthy individuals, the FKBP5 SNP was associated with increased FKBP5 mRNA expression; while in PTSD patients this SNP was associated with decreased FKBP5 mRNA expression. It is well possible that a similar mechanism is operative in our sample. However, due to our limited number of participants with a high level of PTSD symptoms, we were not able to perform these analyses with sufficient statistical power.

A limitation of the current study is that participants in the PTSD group were not diagnosed with PTSD according to the DSM-IV criteria. However, the validity of our questionnaire and the cut-off we used for identifying high levels of PTSD symptoms score is supported by van Zelst et al. (39), who showed that the cut-off we used is sensitive and specific for a DSM-IV PTSD diagnosis.

In conclusion, our results demonstrate that GR pathway components FKBP5 mRNA expression, GILZ mRNA expression and GR number measured in PMBCs prior to deployment, are vulnerability factors for development of a high level of PTSD symptoms in response to deployment to a combat-zone. One of these vulnerability factors is influenced by a gene-environment interaction: a high GR number may develop as a consequence of the presence of GR haplotype B*cl*I combined with a high number of childhood traumatic events. Future research should aim at elucidating the causal relation between the observed vulnerability factors and subsequent development of PTSD symptoms.

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4

Deployment-related severe fatigue with depressive symptoms is associated with increased glucocorticoid binding to peripheral blood mononuclear cells.

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ABSTRACT

Severe fatigue and co-morbid depressive symptoms are frequently reported by recently deployed military personnel. Stress can induce lasting changes in the negative feedback regulation of the hypothalamic-pituitary-adrenal axis (HPA axis) and the regulation of the immune system by cortisol. Since these actions of cortisol are modulated via glucocorticoid receptors (GR), we investigated the effect of deployment and of deployment-related fatigue on glucocorticoid binding to peripheral blood mononuclear cells (PBMCs) in a prospective design.

Psychological assessments and blood sample collection took place before and one and six months after deployment. Participants were selected from a larger group and assigned to three groups based on their level of fatigue and depressive symptoms six months after deployment. We compared fatigued participants without depressive symptoms (n=21), fatigued participants with depressive symptoms (n=14) and non-fatigued participants without depressive symptoms (n=21).

Before deployment, levels of fatigue and depressive symptoms were already higher in both fatigued groups than in the non-fatigued group. Fatigued participants with depressive symptoms at six months after deployment had higher glucocorticoid binding to PMBCs than the other two groups at all three time points. Notably, this difference was already present before deployment. There was no effect of deployment on glucocorticoid binding to PBMCs. No group differences were observed in the composition of the PBMC population and plasma cortisol levels.

These results indicate that high glucocorticoid binding to PBMCs might represent a vulnerability factor for the development of severe fatigue with depressive symptoms after a sustained period of stress, such as deployment.

INTRODUCTION

Military personnel who return from deployment frequently suffer from severe fatigue and psychiatric disorders such as posttraumatic stress disorder (PTSD) and major depressive disorder (MDD) (1, 2). Prevalence of severe fatigue within recently deployed populations can be as high as 28% (3). Co-morbidity between fatigue and psychiatric disorders, in particular MDD, is common (4-6). Adverse experiences during childhood are also associated with the development of both fatigue and MDD during adulthood (7-10).

Changes in the activity of the hypothalamic-pituitary-adrenal axis (HPA axis) are thought to be involved in the pathophysiology of stress-related disorders. Cortisol is the end product of HPA axis activation and its release is controlled by a balance between activation of the HPA axis and negative feedback pathways. It has been shown that stress can cause lasting alterations in the negative feedback of cortisol on different levels of the HPA axis (10-12). In addition, stress can cause lasting changes in the inhibitory effect of cortisol on the functioning of the immune system (12-14). Since central and peripheral effects of cortisol are both mediated via glucocorticoid receptors (GR), the number and affinity of GR and molecular interactions downstream of GR activation are of utmost importance to investigate (15-17).

It has been shown that genetically altered expression of GR in mice provokes alterations in HPA axis functioning and behavior after exposure to a stressor (18, 19). In addition, preclinical studies on central expression of GR have shown that psychological stress induces both short- and long-term down regulation of the expression of GR (20-24). Studies on mother-offspring interactions in rodents (25) and childhood abuse in humans (26) have reported lasting changes in hippocampal GR expression that are associated with epigenetic changes in the GR promoter.

Clinical studies of expression of GR have predominantly reported expression of GR in peripheral blood mononuclear cells (PBMCs) *in vitro*. In a study with patients with chronic fatigue syndrome (CFS) no significant differences in the number of GR between patients and healthy controls were found (27). Studies have also been performed on expression of GR in PBMC from patients with MDD (for review see 17) and PTSD (12, 14, 28-30). However, these studies have yielded conflicting results about the presence and direction of differences in GR binding between affected participants and controls. The inconsistent results of studies on GR binding in stress-related disorders may be caused by differences in reference groups used (e.g. stressed controls or healthy controls) or in the technique used to determine GR expression. In addition, and perhaps more importantly, all studies differ with respect to the time past since the stressful event or onset of symptoms.

To our knowledge, no prospective studies on the effects of stressful events and onset of stress-related symptoms on peripheral expression of GR in humans have been performed. Therefore, we investigated the binding of dexamethasone to PBMCs in a prospective design within a population of military personnel who were deployed to Afghanistan. Our aim was

to determine whether there was an effect of deployment and of deployment-related fatigue on glucocorticoid binding to PBMCs. Assessment took place before deployment, and one and six months after return from deployment. We compared 3 groups of participants: participants with increased fatigue after deployment without co-morbid depressive symptoms, participants with increased fatigue with a co-morbid high level of depressive symptoms and participants with stable low levels of fatigue without depressive symptoms. We examined whether these three groups differed in glucocorticoid binding capacity of PBMCs by determining specific binding of 100 nM ^{3}H-dexamethasone (^{3}H-DEX). In addition, the composition of the PMBC population and plasma cortisol levels were assessed.

METHODS

General procedure

For the current study we selected groups of male participants from a large prospective cohort study on biological and psychological aspects of the development of deployment-related disorders in the Dutch Armed Forces. Participants volunteered for the study prior to a 4-month deployment to Afghanistan. Recruitment of participants took place at military bases in the Netherlands. Written consent was obtained from all participants after a complete written and verbal description of the study. The study was approved by the Institutional Review Board of the University Medical Centre of Utrecht, the Netherlands. The first assessment took place between two and four months prior to deployment. Participants were also assessed approximately one and six months after their return. All assessments took place during the morning at military bases or at the Central Military Hospital in Utrecht. At each assessment, participants filled out several 'paper-and-pencil' questionnaires on demographic variables, previous and current deployments, adverse experiences during childhood, personality and current psychological and physical symptoms. In addition, a venous blood sample was drawn. The samples were collected between March 2005 and September 2008.

Participant selection

Participants were selected from a group of 240 males who completed both psychological and biological assessment at the three time points. We selected participants and divided them into groups based on their level of fatigue six months after return from deployment. The cut-off criteria for increased and stable levels of fatigue were based on the distribution of the change in CIS-20R scores between assessment before and six months after deployment within the total participant group of 240. Stable scores were change scores clustering around 0, with a maximum change of 8 points on the CIS-20R. The right tail of this distribution represents a large increase in CIS-20R scores (18 points or more). Consequently, participants

were classified as non-fatigued when their total score on the Checklist Individual Strength (CIS-20R) was below the 30th percentile of the Dutch norm scores for healthy controls of the CIS-20R (31) six months after deployment, with a maximum change of 8 points in CIS-20R total score between assessment before and six months after deployment. Six persons reported more than two symptoms of PTSD as assessed with the Self-Rating Inventory for posttraumatic stress disorder (32) and were excluded from the non-fatigued group. This resulted in a non-fatigued group of 22 participants. Participants were classified as experiencing increased fatigue after deployment when their total score on the CIS-20R was above the 90th percentile of the Dutch norm scores for healthy controls six months after deployment (31), with a concurrent increase of 18 points or more in CIS-20R total score between assessment before and six months after deployment. The 36 fatigued participants we identified in our sample were divided into two groups based on their level of depressive symptoms six months after return from deployment, as assessed with the SCL-90 depression subscale. As cut-off score to identify individuals with high levels of depressive symptoms a cut-off score of 24 was used. This cut-off score was based on the Dutch norm scores for the general population. These norm scores are divided into 7 categories of symptom levels. The cut-off of 24 corresponds with the highest 3 categories of symptom levels (above average, high, extremely high) (33). Moreover, this cut-off corresponds to the 95th percentile on the SCL-90 depression subscale within a population of 840 Dutch military personnel (mean (SD): 18.06 (3.15)). The fatigued group without depressive symptoms was composed of 21 participants. The fatigued group with depressive symptoms was composed of 15 participants. In our cohort of 240 individuals, we could not identify participants with a high level of depressive symptoms six months after deployment and a concurrent score below the 90th percentile on the CIS-20R.

Measures

Questionnaires

Level of fatigue was assessed using the Dutch version of the 20-item fatigue questionnaire Checklist Individual Strength (CIS-20R). Item responses range from 1 (correct) to 7 (incorrect). Calculation of a total fatigue score is based on the sum score of all items. A higher score indicates a higher level of fatigue (31). Level of depressive symptoms was assessed using the Dutch version of the symptom checklist (SCL-90) depression subscale (33). This subscale consists of 16 items ranging from 1 (not at all) to 5 (very much). A higher score indicates more depressive symptoms. Both questionnaires are well validated and have good reliability. Exposure to adverse experiences during childhood was assessed using the Dutch version of the short form self-report version of the Early Trauma Inventory (ETISR-SF) (34, 35). The questionnaire is designed to assess exposure to potential traumatic experiences before the age of 18 years. The questionnaire consists of 27 dichotomous items. Calculation of a total score is based on the sum of four subscales: general trauma, physical punishment, emotional

abuse and sexual abuse. The total score represents the number of experienced events. Other demographics that were collected included age, body height, weight, smoking, alcohol use, rank and function during deployment, number of previous deployments, education, relationship status and use of medication. Medication use did not differ between groups and was very limited (ACE inhibitors (n=1); cholesterol lowering medications (n=3), beta blockers (n=1); glucocorticoids, local use (n=1)). Participants using medication did not show manifestly lower or higher values on the biological variables. These participants were therefore not excluded from analysis. Body Mass Index (BMI) was calculated by dividing body weight by the square of body height (kg/m^2).

Blood sampling

At all assessments, a venous blood sample was collected between 8.00 and 11.30 a.m. in heparinized vacutainers for determining glucocorticoid binding capacity of PBMC and FACS analysis and in EDTA vacutainers for plasma cortisol measurements. Blood for cortisol measurement was immediately put on ice and centrifuged at 4°C. Plasma was stored at -80°C.

Glucocorticoid binding to PBMCs

For determination of glucocorticoid binding a modification of the whole cell single point binding assay described by Yehuda et al. (36) was used. Briefly, PBMC were isolated from heparinized whole blood using Ficoll Hypaque (Pharmacia, Uppsala, Sweden) and 10^7 cells were frozen in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY) and 20% DMSO. The three samples of each individual were always analyzed simultaneously. Cells were thawed carefully, washed twice in RPMI-1640 and incubated at 37°C for 30 minutes. Cells were washed twice, resuspended in assay buffer (RPMI-1640 with 10% FCS) and incubated in duplicate with 100 nM ^{3}H-dexamethasone (Amersham, Buckinghamshire, UK) in the presence or absence of excess unlabeled dexamethasone (Sigma-Aldrich, Steinheim, Germany). After 1h incubation at 37°C, cell-bound label was separated from free label by washing the cells twice in ice cold assay buffer followed by density centrifugation over Ficoll hypaque. Cell bound radioactivity was analyzed by liquid scintillation analysis. The number of cells was counted in two samples processed in parallel. In preliminary studies, we determined that specific binding at 100nM ^{3}H-dexamethasone, as analyzed using this protocol gives a reliable estimate of Bmax as determined using a classical binding assay with 3-200 nM ^{3}H-dexamethasone in the presence or absence of excess unlabeled dexamethasone and analyzed by Graph Pad PRISM software (r^2=0.92, $p<0.001$, n=14).

FACS analysis

Leukocyte subsets in peripheral blood were assessed using dual color fluorescence analysis with a Becton Dickinson Calibur flowcytometer. Whole blood was stained using monoclonal antibodies labelled with either fluorescine isothiocyanate or phyco-erythrin to quantify

CD14+ (monocytes), CD3+ (total T-cells), CD4+ (T-helper/inducer), CD8+ (T-suppressor/cytotoxic) and CD19+ (B-cells).

Plasma cortisol

Cortisol levels were measured using an electrochemiluminescence (ECL) immunoassay on the Modular E170 (Roche Diagnostics, Mannheim, Germany). The lower limit of detection was 3 nM/l and interassay variation was <3%. Reference values (7-10 a.m): 170 - 540 nM/l.

Statistics

Analyses were performed using the Statistical Package for the Social Sciences version 15.0 (SPSS 15.0). Differences between groups were considered significant at $p<0.05$. To detect outliers, distributions were visually inspected. Two participants had obvious outliers in number of glucocorticoid binding sites and were removed from all analyses. This resulted in group sizes of n=21 for the non-fatigued group and n=14 for the fatigued group with depressive symptoms. In addition, variables were classified as outliers and removed if standardized z-values fell outside the range of ± 3.29. The number of missing values due to handling and omission errors differed per variable. Demographic characteristics were assessed before deployment. Continuous demographic characteristics did not follow a normal distribution and differences between groups were therefore tested by Kruskal Wallis tests, followed up by post hoc Mann-Whitney tests with Bonferroni correction (p-value at which significance is reached: 0.05/3 = 0.0167). Differences in non-continuous demographic variables between groups were tested by Chi-square tests. All longitudinal variables were tested for normality and a log or square root transformation was performed when necessary. Repeated measures analysis was performed to analyze the longitudinal courses of CIS-20R total scores, SCL-90 depression subscale scores, glucocorticoid binding capacity of PBMCs, leukocyte population composition and cortisol levels. Time was used as within-subjects factor and group as between-subjects factor. The potentially confounding variables age, education, smoking, alcohol use and number of adverse experiences during childhood were included as covariates in all analyses. A Greenhouse-Geisser correction was applied when sphericity was violated. Bonferroni post hoc analysis was applied when significant effects were present. Non-transformed data are used in all tables and figures.

RESULTS

Demographic characteristics before deployment

Male participants were selected out of a group of 240 participants from a larger prospective study on biological and psychological aspects of the development of deployment-related

disorders after deployment to Afghanistan. Participants were selected based on their level of fatigue six months after return from deployment. Initially two groups of participants were selected: a group of 21 participants with low stable levels of fatigue between assessment before and six months after deployment and a group of 35 participants with high levels of fatigue six months after deployment with a concomitant increase in level of fatigue between assessment before and six months after deployment. Fatigued participants were divided into two groups based on their level of depressive symptoms six months after return from deployment as described in detail in the section methods. This resulted in a group of 21 fatigued participants without depressive symptoms and a group of 14 fatigued participants with depressive symptoms. Demographic characteristics before deployment of the three groups are presented in Table 1. Participants within the non-fatigued group were approximately ten

TABLE 1. Demographic characteristics before deployment of the non-fatigued group, fatigued group without depressive symptoms and fatigued group with depressive symptoms. Data represent mean, standard deviation (SD) and range, or percentages.

	Non-fatigued group	Fatigued group without depressive symptoms	Fatigued group with depressive symptoms	P
Age [a]	39.05 (9.07) 20-55	30.10 (10.02) 18-49	28.07 (8.17) 21-43	.005
Early Trauma Inventory, nr of experiences [a]	2.90 (3.05) 0-13	3.00 (2.10) 0-8	5.71 (4.27) 0-16	.032
Number of previous deployments [b]	1.71 (1.68) 0-5	1.10 (1.37) 0-5	1.23 (1.24) 0-4	.505
Smoking (yes) [b]	23.8%	52.4%	50.0%	.149
Alcohol/week [b]				.110
No alcohol	5.0%	11.1%	7.1%	
1-20 units/week	95.0%	77.8%	64.3%	
> 20 units/week	0.0%	11.1%	28.6%	
Rank [b]				.201
Officers	19.0%	19.0%	7.1%	
Non-com. officers	52.4%	38.1%	28.6%	
Corporals	19.0%	4.8%	21.4%	
Soldiers	9.5%	38.1%	42.9%	
Function during deployment [b]				.336
Base	52.4%	33.3%	21.4%	
Off-base	38.1%	61.9%	71.4%	
Both equally	9.5%	4.8%	7.1%	
Education [b]				.074
Lower	47.6%	28.6%	42.9%	
Middle	33.3%	23.8%	50.0%	
Higher	19.0%	47.6%	7.1%	
Longterm relationship (yes) [b]	85.7%	65.0%	64.3%	.238

[a] Kruskal–Wallis test; [b] Chi-square test.

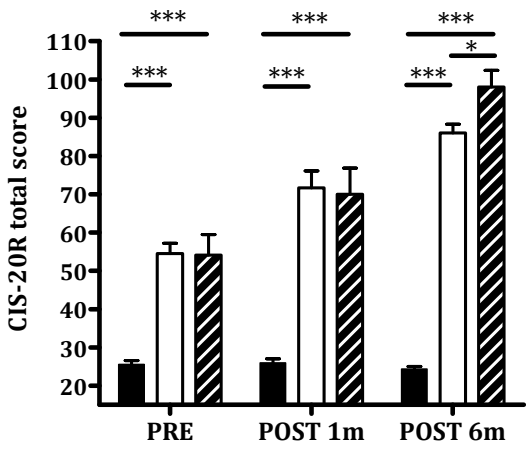
years older than participants in the other two groups ($H^2_{(2)}$= 10.629, p=.005). Participants within the fatigued group with depressive symptoms had experienced more potentially traumatic experiences during childhood than participants in the other two groups ($H^2_{(2)}$= 6.885, p=.032). Participants within the fatigued group without depressive symptoms tended to be slightly higher educated than participants in the other two groups ($H^2_{(2)}$= 8.621, p=.074). No additional group differences in demographic characteristics were observed.

Longitudinal course of level of fatigue and depressive symptoms

Participant selection was based on level of fatigue and depressive symptoms six months after deployment (POST 6m). Interestingly, in the two groups with a high level of fatigue at six months after deployment, reported fatigue was already higher than in the non-fatigued group one month after deployment and before deployment as well (Figure 1). Fatigue levels of the fatigued group without depressive symptoms and the fatigued group with depressive symptoms did not differ at any time point tested (group effect: $F_{(2,48)}$= 181.045, p=.000; time effect: $F_{(2,96)}$= 0.073, p=.929; interaction effect: $F_{(4,96)}$= 9.530, p=.000). No significant effects of the covariates age, smoking, alcohol use, education and adverse experiences during childhood were observed.

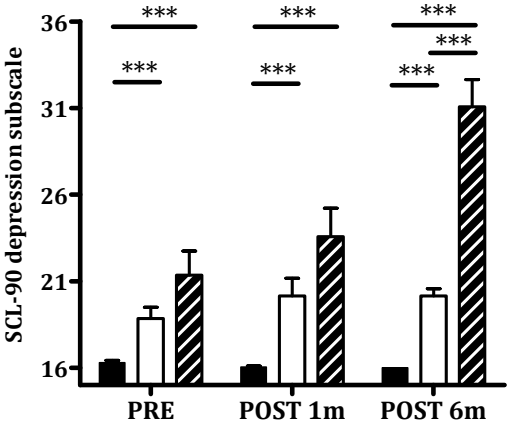
Analysis of the course of depressive symptoms over time revealed that the non-fatigued group had lower levels of depressive symptoms than the fatigued group with depressive symptoms at all time points tested (Figure 2). In addition, the non-fatigued group had lower levels of depressive symptoms than the fatigued group without depressive symptoms one

FIGURE 1: Longitudinal course of self-reported fatigue of non-fatigued participants (black, n = 21), fatigued participants without depressive symptoms (white, n = 21) and fatigued participants with depressive symptoms (hatched, n = 14).



Means and SEMs are presented. *** p < .001, * p < .05.

FIGURE 2: Longitudinal course of self-reported levels of depressive symptoms of non-fatigued participants (black, n = 21), fatigued participants without depressive symptoms (white, n = 21) and fatigued participants with depressive symptoms (hatched, n = 14).



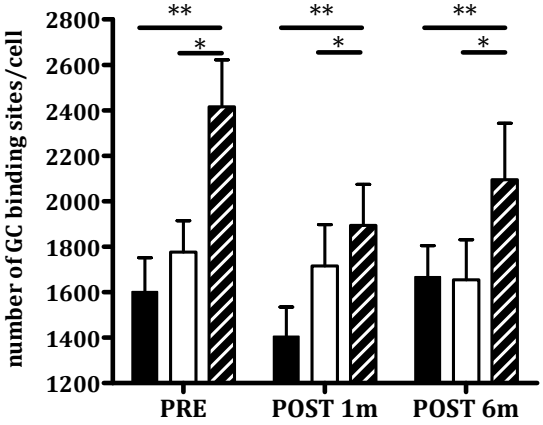
Means and SEMs are presented. *** p < .001.

and six months after deployment. Since selection of the two fatigued subgroups was based on level of depressive symptoms six months after deployment, level of depressive symptoms differed between the fatigued groups six months after deployment. However, before and one month after deployment, the level of depressive symptoms between the fatigued group without depressive symptoms and the fatigued group with depressive symptoms was not statistically different (group effect: $F_{(2,46)}$ = 26.583, p=.000; time effect: $F_{(1.73,79.72)}$= 1.819, p=.174; interaction effect: $F_{(3.47,79.72)}$= 14.841, p=.000). No significant effects of the covariates age, smoking, alcohol use, education and number of adverse experiences during childhood were observed.

Longitudinal course of glucocorticoid binding to peripheral blood mononuclear cells

Specific binding of 100 nM ^{3}H-DEX to PBMCs was determined to estimate the number of glucocorticoid binding sites on these cells (modification of assay described in 36). The possible confounders age, smoking, alcohol use, education and number of adverse experiences during childhood were included as covariates in the analysis. The number of glucocorticoid binding sites differed between groups at all time points tested (group effect: $F_{(2,48)}$= 7.879, p=.001). Glucocorticoid binding was higher in the fatigued group with depressive symptoms compared to the non-fatigued group (p=.003) and the fatigued group without depressive symptoms (p=.002) (Figure 3). The non-fatigued participants and fatigued participants without depressive symptoms did not differ in number of glucocorticoid binding sites on PBMCs (p=1.00). There were no significant effects of deployment on the number of glucocorticoid binding sites on PBMCs (time effect: $F_{(2,96)}$= 1.986, p=.143, interaction effect time

FIGURE 3: Longitudinal course of estimated number of glucocorticoid binding sites/cell of non-fatigued participants (black, n = 21), fatigued participants without depressive symptoms (white, n = 21) and fatigued participants with depressive symptoms (hatched, n = 14).



Means and SEMs are presented. ** p < .01, * p < .05.

x group: $F_{(4,96)}$= 0.656, p=.624). The number of adverse experiences during childhood did have an effect on the overall number of glucocorticoid binding sites ($F_{(1,48)}$= 5.382, p=.025). Furthermore, we observed a trend significant interaction effect between number of adverse experiences during childhood and deployment on glucocorticoid binding sites (interaction effect time x early trauma: $F_{(2,96)}$= 2.723, p=.071). In addition, smoking had a significant effect ($F_{(1,48)}$= 6.690, p=.013) and education had a trend significant effect ($F_{(1,48)}$= 3.565, p=.065) on the overall number of glucocorticoid binding sites. There were no significant effects of age and alcohol use.

Composition of PBMC population

To determine whether the observed difference in glucocorticoid binding sites on PBMCs between groups was caused by group differences in subset composition of PBMCs, we used FACS analysis. There were no group differences in percentage of monocytes, CD19+ B-lymphocytes and T-lymphocytes (total CD3+, CD8+ cytotoxic/suppressor and CD4+ helper/inducer). We did not observe effects of deployment on any of the variables (Table 2).

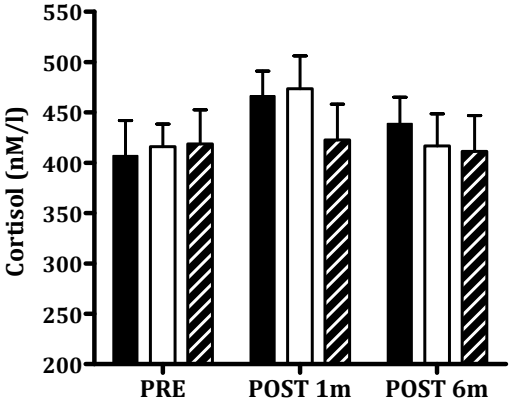
Plasma cortisol levels

Subsequently, we assessed whether the observed group differences in glucocorticoid binding sites on PBMCs were associated with group differences in plasma cortisol. As shown in Figure 4, no differences were observed in cortisol levels between groups (group effect: $F_{(2,46)}$= 1.056, p=.356). Furthermore, cortisol levels were not significantly altered between

TABLE 2. Longitudinal course of total number of leukocytes and relative subset distributions within PBMCs of the non-fatigued group, fatigued group without depressive symptoms and fatigued group with depressive symptoms. Data represent mean (SD).

	Non-fatigued group			Fatigued group without depressive symptoms			Fatigued group with depressive symptoms			P-values		
	PRE	POST 1m	POST 6m	PRE	POST 1m	POST 6m	PRE	POST 1m	POST 6m	Group effect	Time effect	Inter-action
% monocytes	5.4 (1.6)	5.2 (1.4)	4.8 (1.7)	6.0 (1.2)	5.5 (1.5)	5.2 (2.1)	5.80 (1.8)	5.4 (1.0)	4.5 (2.0)	.579	.940	.907
% CD19+ B-lymphocytes	10.7 (4.6)	10.6 (3.6)	10.4 (3.3)	12.2 (5.8)	12.6 (4.8)	11.9 (4.4)	10.4 (2.7)	11.0 (2.4)	10.5 (2.7)	.976	.485	.349
% CD3+ T-lymphocytes	70.9 (7.6)	71.4 (9.3)	73.0 (6.6)	66.2 (9.3)	68.1 (7.2)	69.5 (6.6)	71.2 (8.2)	72.9 (5.4)	72.7 (5.3)	.307	.712	.968
% CD8+ T-lymphocytes	34.6 (7.3)	34.9 (7.8)	34.6 (11.1)	33.2 (6.4)	32.8 (6.2)	34.1 (6.0)	34.9 (8.3)	33.2 (7.8)	34.0 (8.9)	.639	.054	.568
% CD4 + T-lymphocytes	39.0 (9.7)	41.0 (7.3)	41.7 (9.6)	32.9 (6.2)	40.4 (7.3)	41.6 (6.5)	40.1 (6.1)	42.7 (7.5)	41.6 (8.8)	.548	.291	.767

FIGURE 4: Longitudinal course of plasma cortisol levels (nM/l) of non-fatigued participants (black, n = 21), fatigued participants without depressive symptoms (white, n = 19) and fatigued participants with depressive symptoms (hatched, n = 14).



Means and SEMs are presented.

the assessment before and after deployment (time effect: $F_{(2,92)}$= 0.435, p=.649, interaction effect time x group: $F_{(4,92)}$= 0.062, p=.993).

DISCUSSION

To the best of our knowledge, this study is the first to prospectively investigate the association between glucocorticoid binding to PBMCs and deployment-related fatigue. In addition, we

prospectively examined the influence of the presence of a high level of co-morbid depressive symptoms after deployment on GR binding. At all time points assessed, GR binding was higher in participants who experienced deployment-related fatigue with depressive symptoms at six months after deployment in comparison with participants with deployment-related fatigue without depressive symptoms and participants who were not fatigued after deployment. Interestingly, this increased binding of GR was already present before deployment.

Glucocorticoid binding to PBMCs did not differ between fatigued participants without depressive symptoms and non-fatigued participants at any time point. Since plasma cortisol levels and leukocyte composition did not differ between groups during the assessments, the observed group differences in GR binding can not be explained by reduced occupancy of GR by endogenous cortisol or differences in the subset composition of the PBMC population. Subsequently, group differences can also not be explained by confounding effects of age, smoking, alcohol use, education and number of adverse experiences during childhood. In addition, the observed group differences in GR binding are not related to group differences in sleep problems six months after deployment, as assessed with the SCL-90 sleep subscale (33). Although both the fatigued groups reported more sleep problems than the non-fatigued group six months after return from deployment, there were no differences in self-reported sleep problems between the fatigued groups (data not shown).

Symptoms of fatigue and depression often occur as a cluster, but the causation of this co-occurrence remains unresolved (4). As a result, it is difficult to establish whether findings pertaining to underlying pathophysiological mechanisms are related to fatigue or depression. For that reason, in the current study fatigued participants were divided into two groups based on having high or low levels of depressive symptoms. Since there was an evident difference in GR expression between the fatigued groups with and without co-morbid depressive symptoms, we suggest that the observed high GR expression within the fatigued group with depressive symptoms is predominantly related to the risk of developing symptoms of depression. However, we were unable to directly test this hypothesis by including a group of participants with depressive symptoms without high levels of fatigue, since there were no participants with this profile. In addition, it should be noted that a high level of depressive symptoms does not necessarily imply the presence of clinical depression.

There is ongoing debate whether results on GR function in PBMCs *in vitro* can be used as a model for GR binding in the HPA-axis (12, 37, 38). Preclinical studies on the effects of adrenalectomy (39) and administration of corticosterone (40) on GR expression in the brain and peripheral lymphoid tissue i.e. spleen, have reported similarities in the responses of central and peripheral GR to changes in glucocorticoid availability. Therefore, the observed increased PBMC GR binding within the fatigued group with depressive symptoms may possibly reflect similar changes in GR expression in the brain.

We observed that participants with severe fatigue after deployment already had higher levels of fatigue and depressive symptoms before deployment than participants who were

not fatigued after deployment. However, before deployment there was no difference in fatigue or depressive symptoms between fatigued participants with or without depressive symptoms six months after deployment. Therefore, pre-existing psychological symptoms in this sample do not seem to predict development of deployment related symptoms. Notably, we did observe that before deployment GR binding was already increased in the group reporting high levels of fatigue with depressive symptoms after deployment compared to the group with high levels of fatigue without depressive symptoms. Therefore, we propose that increased expression of GR on PBMCs might represent a vulnerability factor for the development of psychiatric disorders after a prolonged stressful period, such as deployment.

We analyzed GR binding in fatigued participants and in a subgroup of fatigued participants who also reported a high level of depressive symptoms. Therefore, our finding of increased GR expression within fatigued participants with depressive symptoms cannot be directly compared to the results of studies assessing leukocyte GR expression in patients with a diagnosis of MDD. Nevertheless, it is interesting to note that in most of these studies GR expression was similar in MDD compared to controls, except for three studies in which decreased GR expression was found (for review see 17). In addition, one recent study reported increased GR binding to B-lymphoblastoids that had been in culture for 12 weeks when comparing cells from patients with MDD to healthy individuals (41). However, these studies were performed after the onset of MDD, whereas we examined GR binding to PBMCs before the onset of the increase in depressive symptoms. In addition, in these studies the onset of MDD in participants was not related to a common evoking stressful experience, such as the deployment in our study.

This study is not the first to suggest a neuroendocrine vulnerability factor for the development of depressive symptoms. Reduced negative feedback regulation of cortisol production, as measured with the DEX/CRH test, has been observed within first-degree family members of MDD patients. However, the reduced negative feedback of the HPA axis did not predict whether the family members actually developed MDD (42). In addition, increased waking (43) and evening (44) salivary cortisol levels have been found within first-degree family members of patients with MDD.

From the available data in the literature, we hypothesize that the high GR expression is associated with the presence of specific GR polymorphisms, although conflicting evidence has been described for an association between vulnerability to mental health problems and specific GR polymorphisms. With respect to depressive disorders, some studies point towards an increased frequency of polymorphisms associated with increased or decreased glucocorticoid sensitivity, although others report no associations of depressive disorders with GR polymorphisms (45-48).

An alternative explanation might be that the high binding capacity of GR may have developed as a compensatory mechanism due to (early) life stress, possibly because of epigenetic changes in the GR promoter (25). Exposure to adverse experiences during childhood

is a risk factor for the later development of mental health problems (7-9). As stated above, experiencing adversity during childhood can cause epigenetic alterations in various genes including the gene encoding the GR with possible consequences for receptor expression, and neuroendocrine responses to stressful experiences (26, 49). In this study, the fatigued participants with depressive symptoms had experienced on average approximately twice as many adverse experiences during childhood than the other participants. Therefore, we included the number of adverse experiences during childhood as a covariate in our analyses. We found that GR expression was related to the number of adverse experiences during childhood but that this variable did not explain the group difference in GR binding between fatigued participants with and without depressive symptoms. It is possible that these early adverse experiences have long-lasting effects on GR binding via epigenetic programming as has been described before in rodent models of neonatal stress induced changes in GR binding (49). In addition, it may well be possible that the presence of both increased GR expression and exposure to adverse events early in life together increase vulnerability to develop severe fatigue with depressive symptoms after deployment. Similarly, it has previously been suggested that the presence of GR polymorphisms ER22/23EK and A3669G 9β and childhood adversity constitutes a phenotype vulnerable to the development of MDD at old age (50). Whether such an interaction is also involved in the development of deployment-related fatigue with depressive symptoms remains to be investigated.

Preclinical studies on central GR expression in rodents have shown short- and long-term down regulation of GR expression after chronic and severe stress (20, 22-24). In the current study we did not observe effects of deployment on GR binding to PBMCs as determined at two time points after deployment. However, we cannot exclude that the assessment one month after return from deployment was too late to detect transient changes in glucocorticoid binding to PBMCs.

A limitation of the current study is that the presence of physical disorders, which may have influenced the level of fatigue, was not investigated in detail. However, since participants were found to be fit for deployment, the presence of major medical conditions before deployment can be excluded. One fatigued participant without depressive symptoms and one fatigued participant with depressive symptoms reported the presence of medical conditions after deployment. Excluding these participants from the analyses did not alter the results (data not shown).

Symptoms of PTSD are expected to occur within recently deployed military populations. It has been shown multiple times that PTSD is associated with changes in GR expression, although the direction of the change in GR is still unclear (12, 14, 28-30). Unfortunately, the sample size of the current study was not large enough to analyze the influence of possible co-morbid symptoms of PTSD on GR expression.

In conclusion, severe fatigue with depressive symptoms in military personnel after deployment was found to be associated with an increased GR binding to PBMCs. This increased GR

binding was already present before deployment, but was not associated with symptoms of fatigue and depression before deployment. Therefore, we suggest that increased GR binding to PBMCs is a vulnerability factor for the development of mental health problems after a sustained period of stress such as deployment. Although this finding should be replicated in prospective studies within large populations affected by stressful experiences, it may be wise to implement increased screening for mental health problems after return from deployment within military personnel with increased GR binding to PBMCs.

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5

A prospective study on personality and the cortisol awakening response to predict posttraumatic stress symptoms in response to military deployment.

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ABSTRACT

Few prospective studies on pre-trauma predictors for subsequent development of post-traumatic stress disorder (PTSD) have been conducted. In this study we prospectively investigated whether pre-deployment personality and the cortisol awakening response (CAR) predicted development of PTSD symptoms in response to military deployment. Furthermore, we hypothesized that potential effects of age, childhood trauma and previous deployment on development of PTSD symptoms were mediated via pre-deployment personality, CAR and PTSD symptoms.

Path analysis was performed on data from 470 male soldiers collected before and six months after a 4-month deployment to Afghanistan. Before deployment, personality was assessed with the short-form Temperament-Character Inventory and the Cook-Medley Hostility scale. In addition, pre-deployment saliva sampling for assessment of the CAR was performed immediately after awakening and 15, 30 and 60 minutes thereafter.

Pre-deployment high hostility and low self-directedness represented intrinsic vulnerabilities for development of PTSD after deployment. The CAR assessed before deployment did not predict PTSD symptoms after deployment. Pre-deployment low-to-moderate PTSD symptoms were associated with PTSD symptoms after deployment. As hypothesized, the effects of age and childhood trauma on PTSD symptoms after deployment were mediated via personality and pre-deployment PTSD symptoms. However, the number of previous deployments was not related to development of PTSD symptoms. The total model explained 24% of variance in PTSD symptoms after military deployment.

INTRODUCTION

A significant proportion of trauma-exposed individuals develop psychiatric disorders such as posttraumatic stress disorder (PTSD) in the aftermath of a traumatic event. PTSD is characterized by re-experiencing of the traumatic event, avoidance of trauma-related stimuli and numbing of affect accompanied by symptoms of hyper-arousal. Deployed military personnel are a population at risk for development of PTSD. One year after return from deployment to Iraq, the prevalence of PTSD in combat exposed U.S. soldiers has been estimated to range from 23.7 to 30.5% (1).

Many possible psychological and biological vulnerability factors for the development of PTSD have been proposed (2, 3), including personality (4), exposure to childhood trauma (5), and smaller hippocampal size (6). However, predictors of PTSD have predominantly been studied using cross-sectional designs in individuals who already had developed PTSD or using post-trauma prospective designs. Therefore, it remains uncertain whether the factors identified in these earlier studies indeed represent pre-existing risk factors for development of PTSD. It is possible that these supposed predictors have actually changed by the presence of PTSD. For example, a meta-analysis of cross-sectional studies on hostility and PTSD showed a strong association between high hostility and PTSD, with a stronger effect size for studies with increased time since the traumatic event (7). This increasing effect size over time indicates that hostility may increase as a consequence of or in conjunction with development of PTSD.

The first prospective studies on the predictive value of pre-trauma personality for subsequent PTSD development predominantly relied on personality data collected for other purposes, such as recruitment of military personnel (4, 8-11). More recently the focus of studies on predictors of PTSD shifted to prospective follow-up of populations specifically at-risk for trauma exposure and subsequent development of PTSD, such as fire-fighters, police personnel and pregnant women. In these studies the predictive value of a limited number of personality traits for PTSD development was investigated. These studies showed that hostility and anger, self-efficacy and neuroticism are significant predictors of later development of PTSD symptoms (12-14).

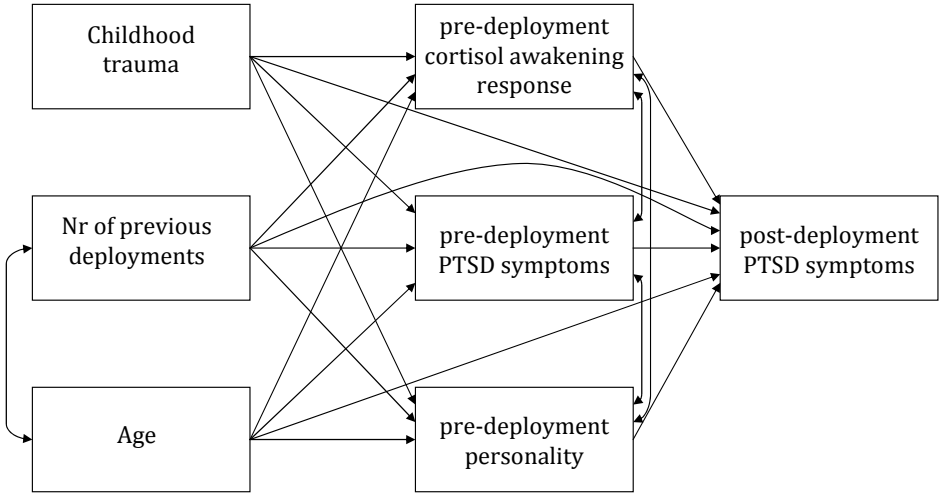
PTSD has been associated with altered functioning of the hypothalamic-pituitary-adrenal axis (HPA axis) (for review: 2, 15). We previously observed that a high number of glucocorticoid receptors in leukocytes before deployment is a vulnerability factor for development of a high level of PTSD symptoms in response to deployment (16). We hypothesize that in this respect it may well be that basal functioning of the HPA axis also is a predictor for subsequent development of PTSD symptoms. Cortisol levels increase immediately after awakening, with a 50-75% increase in the first 30 minutes after awakening, and remain elevated for at least 60 minutes. This rapid increase in cortisol levels after awakening is called the cortisol awakening response (CAR), and the CAR is thought to be a good indicator of adrenocortical activity (17). Multiple studies indicated that the CAR is lower in adult PTSD patients than in

healthy controls (18-21). However, whether an attenuated CAR before deployment is a pre-existing vulnerability factor for the development of PTSD remains unknown. Thus far one pre-trauma prospective study has examined the predictive value of the CAR for development of PTSD symptoms after trauma exposure (12). Within 43 male fire-fighters, the CAR was assessed immediately after basic training. The pre-trauma CAR was not related with PTSD symptoms after two years of service, but the small number of participants may have resulted in insufficient power to detect group differences.

Within military populations there is evidence that being deployed at a younger age (22-24), previous deployments (23, 25) and traumatic experience(s) during childhood (5, 22, 24) are associated with higher rates of PTSD. Associations between age, previous deployment, traumatic experience(s) during childhood and personality (26, 27) and the CAR (28-31) have been previously reported. In addition, the presence of PTSD symptoms before deployment predicts the presence of PTSD after military deployment (32).

In the current study we prospectively investigated within a group of 470 male soldiers whether pre-deployment hostility measured with the Cook-Medley Hostility scale (33), temperament and character traits measured with the short-form Temperament-Character Inventory (TCI-SF; (34)) and the pre-deployment CAR predicted the development of PTSD symptoms in response to deployment to Afghanistan, as measured six months after return. In addition, we investigated whether the previously reported associations between development of PTSD symptoms after military deployment and age, traumatic experiences during childhood and previous deployments were mediated via pre-deployment personality traits,

FIGURE 1. Hypothesized relationships between the level of PTSD symptoms after deployment and all pre-deployment variables.



Single-headed arrows represent causal relationships. Double-headed arrows connect variables that co-vary but for which causality of the relation could not be determined.

CAR and PTSD symptoms (Figure 1). Path analysis, which represents a form of structural equation modelling, allowed us to investigate both direct and indirect effects of predictors on the outcome variable.

MATERIALS AND METHODS

Participants

The current study is part of a prospective cohort study on biological and psychological aspects of development of deployment-related disorders in the Dutch Armed Forces. Participants volunteered for the study prior to a 4-month deployment to Afghanistan. Duties during deployment included combat patrols, clearing or searching homes and buildings, participation in de-mining operations, and transportation across enemy territory. Written informed consent was obtained after a written and verbal description of the study. The study was carried out in accordance with the latest version of the Declaration of Helsinki and was approved by the Institutional Review Board of the University Medical Center Utrecht, the Netherlands. Participants filled out questionnaires before deployment, including demographic variables, previous and current deployments, traumatic experiences during childhood, personality and current psychological and physical symptoms, including PTSD symptoms.

Initially, 508 male participants were included. Since we were interested in the development of PTSD symptoms, we excluded 20 participants who already had high levels of PTSD before deployment. For determination of high levels of PTSD we used a cut-off score of ≥ 38 on the Self-Report Inventory for PTSD (SRIP) (35, 36) before deployment (16). In addition, we excluded 38 participants with incomplete test scores on the Self-Report Inventory for PTSD (SRIP) before or after deployment, resulting in a final sample size of 470 male participants. Of these participants, 322 participants completed saliva collection for assessment of the cortisol awakening response. The pre-deployment measurements were used to predict PTSD symptoms assessed six months after return from deployment. Data were collected between April 2006 and September 2009.

Questionnaires

Level of PTSD symptoms over the past 4 weeks was assessed with the Dutch 22-item Self-Report Inventory for PTSD (SRIP). The questionnaire consists of three subscales representing the PTSD symptom clusters re-experiencing, avoidance and hyper-arousal. The items are measured on a 4-point scale, ranging from 1 (not at all) to 4 (very much). The total PTSD score is the sum score for all items (range: 22-88). The SRIP has good concurrent validity with other PTSD measures such as the Clinician Administered PTSD Scale (CAPS) and Mississippi scale for PTSD (35, 36).

Hostility was assessed with the MMPI-based Cook-Medley Hostility Scale (33), consisting of 50 true/false items. The total hostility score is the sum of all items. Additional personality characteristics were assessed with the Dutch short-form version of the Temperament-Character Inventory (TCI-SF) (34, 37, 38) that distinguishes between four temperament traits: Novelty Seeking, Harm Avoidance, Reward Dependence and Persistence and three character traits: Self-Directedness, Cooperativeness and Self-Transcendence. The questionnaire consists of 105 true/false items.

Exposure to traumatic experiences during childhood was assessed using the Dutch version of the short form self-report version of the Early Trauma Inventory (27, 39). The questionnaire is designed to assess exposure to potential traumatic experiences before the age of 18 years (general trauma, physical abuse, emotional abuse and sexual abuse) and consists of 27 dichotomous items. The total score represents the number of different traumatic events experienced. Exposure to deployment stressors was assessed with a 13-item checklist specifically developed for this study.

Cortisol sampling procedure

Salivettes (Sarstedt Inc. Newton, NC, USA) were used for sampling of salivary free cortisol. Each participant received a set of salivettes along with verbal and written instructions about the sampling procedure. Participants were instructed to collect the first sample directly after awakening ("The moment you can open your eyes"). The next samples were taken at 15, 30 and 60 minutes after awakening. Participants were instructed to chew gently on the cotton swab for approximately one minute and then return it to the salivette. Participants were free to follow their normal routines, but reported their time of awakening, alcohol consumption during the previous day and their caffeine (coffee and tea) and nicotine consumption during the sampling procedure. The participants were asked to return their samples directly by mail. Returned samples were encoded and frozen (−20°C), before they were shipped to the Biological Psychology Laboratory at Dresden, Germany for analysis. The samples were analyzed using a luminescence immunoassay (IBL International, Germany). Intra- and inter-assay variations are < 4.5% and < 4.3% respectively.

The 'area under the curve with respect to the ground' (AUGg) was used as a measure for the overall cortisol production in the first hour after awakening (40). In addition, the 'Mean Increase' (MeanInc) was used as a measure for the increase in cortisol production the first hour after awakening (41). The MeanInc includes both the increasing and the decreasing phase of the CAR. Since we included an additional sampling point at 15 minutes after awakening, we investigated whether the maximum increase (MaxInc) in cortisol levels in the first 30 minutes after awakening (e.g. either at 15 or 30 minutes) could be used as an additional measure to assess the increasing phase of the CAR. However, we observed a very strong cor-

relation between the MeanInc and the MaxInc in cortisol levels (r = .82), and therefore did not include the MaxInc as an additional measure of the CAR.

Data analyses

Basic statistical analyses were conducted using SPSS 15.0. Continuous variables were tested for univariate normality and log transformation was performed when necessary. For the CAR, multiple imputation (Amalia for R statistical software) was used to impute cortisol values for individuals with one missing sample point (n=12). All sampling points were excluded for participants with outliers (z ≥ 3.29) in the individual sample points (n=4). Analysis of multicollinearity showed that the variance inflation factor (VIF) of all variables was well below the tolerable limit of 10 and therefore was not considered to be problematic. Using Mahalonobis distance analysis (p<.001) one multivariate outlier was detected and removed from the analysis (42).

Path analysis, using AMOS version 16.0, was used to examine relationships between variables. First, the 8 pre-deployment personality traits and the 2 CAR measures were included in two separate models with covarying predictors to identify whether personality and the CAR were significantly associated with PTSD total scores six months after deployment. Subsequently, the significant variables were included in the hypothesized model (Figure 1) to predict PTSD total scores six months after deployment based on direct and indirect effects of the included pre-deployment variables.

Since 12.2% of the participants had some missing values on the questionnaires and the CAR was not available for all participants, full information maximum likelihood parameter estimation was used. Standardized parameters are presented to facilitate interpretation. The model chi-square (χ^2), comparative fit index (CFI) and the root mean square error of approximation (RMSEA) were used to test the goodness-of-fit of the overall model. Acceptable fit was defined as χ^2: p≥.05, CFI >.9, NFI >.9 and RMSEA <.05 (Kline, 2005). The change of the model χ^2 ($\Delta\chi^2$) was used to compare the full hypothesized model and the final trimmed model. The fit of the trimmed model was defined to be as good as the fit of the full model when p ≥ .05.

RESULTS

Group characteristics

The mean level of PTSD symptoms was increased six months after deployment (SRIP total score before deployment: 26.10 (SD: 3.80), SRIP total score six months after deployment: 27.44 (SD: 6.34), $F_{(1, 468)}$= 18.392, p<.001). Moreover, six months after deployment 36 participants (7.7%) had developed high levels of PTSD. On average participants experienced 5.11 (SD: 2.58) potentially traumatic events during deployment. The combat group was exposed

to typical war-zone stressors such as exposure to enemy fire, armed combat, and seeing seriously injured and dead fellow soldiers and civilians (including women and children). The mean age during deployment was 29.02 years (SD: 9.17). Participants had experienced on average 0.98 previous deployments (SD: 1.29). They reported a mean of 3.11 (SD: 2.72) potentially traumatic experiences in their childhood.

Predictive value of personality

First, we tested whether the 8 personality traits as determined by the Cook-Medley Hostility Scale and short-form Temperament-Character Inventory were significantly associated with PTSD total scores six months after deployment. The pre-deployment distribution of the personality traits and the predictive value of the traits for PTSD total scores six months after deployment are presented in Table 1. Only hostility ($\beta = .254$, $p<.001$) and self-directedness ($\beta = -.178$, $p<.001$) were significantly associated with PTSD total scores after deployment.

Since aspects of hostility, e.g. irritability and outbursts of anger, are also symptoms of PTSD, we investigated whether the association between hostility and PTSD was still present when these hostility-related items were removed from the PTSD score. The direct effect of hostility on PTSD total scores after deployment remained approximately the same ($\beta = .20$, $p < .001$).

TABLE 1. Pre-deployment distribution and predictive value of personality traits assessed with the Cook Medley Hostility Scale and short-form Temperament and Character Inventory (n = 469).

	Mean	SD	β	p
Cook-Medley Hostility total score	16.98	6.98	.254	<.001
TCI Novelty Seeking	7.93	2.78	−.010	.821
TCI Harm Avoidance	3.01	2.73	.029	.562
TCI Reward Dependence	8.21	2.91	−.062	.164
TCI Persistence	10.58	2.59	−.005	.919
TCI Self-Directedness	13.62	1.86	−.178	<.001
TCI Cooperativeness	11.76	2.89	−.058	.267
TCI Self-Transcendence	2.63	2.68	.071	.126

Predictive value of cortisol awakening response

Subsequently, we tested whether the pre-deployment CAR measures (Table 2) were significantly associated with PTSD total scores six months after deployment. The AUCg ($\beta = .008$, $p=.904$) and MeanInc ($\beta = .023$, $p=.708$) were not associated with PTSD total scores six months after deployment. A moderate correlation between the AUCg and MeanInc was observed ($r= .45$, $p<.001$). Medication use was very limited (non-systemic glucocorticoid use

TABLE 2. Pre-deployment distribution of salivary cortisol levels during the cortisol awakening response (n = 318).

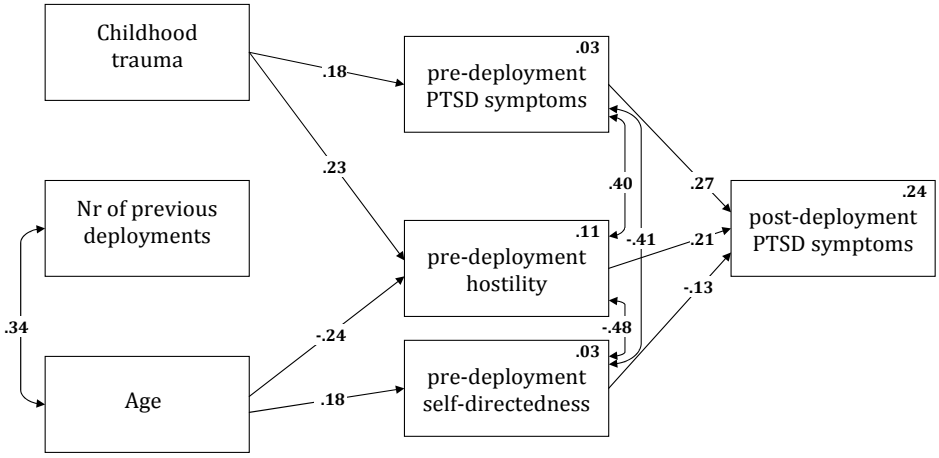
	Mean	SD
Awakening (nmol/l)	11.19	7.02
15 min. after awakening (nmol/l)	14.28	8.86
30 min. after awakening (nmol/l)	15.17	9.13
60 min. after awakening (nmol/l)	12.24	8.56
Area under the curve with respect to ground (AUCg) (nmol/l)	827.35	404.48
Mean Increase (MeanInc) (nmol/l)	2.79	7.70

(nasal spray or crème), n=5; antihistamines, n=8; and anti-hypertensives, n=5). Removing the CAR measures of these participants from the analysis did not alter the results (AUCg: β = .011, p=.859; MeanInc: β = .021, p=.741).

Fit of full hypothesized model

Personality traits hostility and self-directedness were included in the hypothesized model. The CAR measures did not significantly predict PTSD total scores six months after deployment and were therefore not included. The hypothesized model had an excellent fit to the data, $\chi 2_{(2,\ n=469)} = 0.531$, p=.767, CFI= 1.000, NFI= .998, RMSEA= .000. All pathways that did not reach statistical significance (all effects of previous deployments and direct effects of age and childhood trauma on post-deployment PTSD total scores) were removed from the

FIGURE 2. Relationships between the level of PTSD symptoms after deployment and pre-deployment variables that were significant in the initial analyses.



Standardized regression weights and correlations are presented for each significant pathway. The total variance explained is presented in the right upper corner of all dependent variables in the model.

hypothesized model. The final trimmed model depicted in Figure 2 fitted the data as good as the full hypothesized model ($\Delta \chi 2_{(8, n=469)} = 11.045$, p=.199) and had an excellent fit, $\chi 2_{(10, n=469)} = 11.576$, p=.314, CFI= .997, NFI= .983, RMSEA= .018.

Direct and indirect effects in hypothesized model

In the final model high hostility (β = .211, p<.001) and low self-directedness (β = −.132, p=.006) before deployment had significant direct effects on PTSD total scores after deployment. In addition, a moderate negative correlation between hostility and self-directedness was observed (r = −.48, p<.001). PTSD total scores before deployment were positively associated with PTSD total scores after deployment (β = .27, p<.001). Furthermore, significant correlations between PTSD total scores before deployment and hostility (r = .40, p<.001) as well as self-directedness (r =−.41, p<.001) were observed.

The effects of age and childhood trauma on PTSD total scores after deployment were only indirect. Reporting more childhood trauma was associated with higher hostility (β = .230, p<.001) and a higher PTSD score before deployment (β = .183, p<.001). Being younger was associated with higher hostility (β = −.236, p < .001) and lower self-directedness (β =. 181, p<.001). Number of previous deployments was positively related to age (r = .34, p<.001).

Childhood trauma and age explained 11% of the variance in hostility before deployment. In addition, 3% of the variance in self-directedness before deployment was explained by age. Three percent of the variance in PTSD total scores before deployment was accounted for by childhood trauma. In the final model 24% of the variance in PTSD total scores six months after deployment was explained by hostility, self-directedness and PTSD symptoms before deployment.

DISCUSSION

Our path analysis revealed that higher hostility and lower self-directedness before deployment predicted the presence of PTSD symptoms after military deployment. Pre-deployment PTSD symptoms also significantly predicted the presence of PTSD symptoms after deployment. The cortisol awakening response (CAR) before deployment was not associated with PTSD total scores after deployment. Together, the predictors explained 24% of the variance in PTSD symptomatology after deployment.

Our results show that high hostility and low self-directedness represent intrinsic vulnerabilities for development of PTSD after deployment. Heinrichs et al. (12) also observed in a small sample that pre-trauma personality traits hostility and self-efficacy, which is linked to self-directedness, were significant predictors of PTSD symptom development in the following two years. Only two additional pre-trauma prospective studies on concepts related to hostility have been performed. High trait anger measured in 180 police recruits at the start of basic

police training was a significant predictor of PTSD symptoms after one year of police service (13). Furthermore, PTSD symptoms one to six years after return from peacekeeping missions could be predicted by pre-deployment assessment of the Dutch short-form MMPI negativism scale, which measures the presence of a negative, hostile attitude towards others and life in general (8). Irritability and outbursts of anger are both signs of hostility and symptoms of the hyper-arousal cluster of PTSD. However, it has been reported previously that the association between hostility and PTSD does not constitute an artefact of the overlap in symptomatology (7, 13). The current results confirm these previous findings, since hostility was also related to the level of PTSD symptoms when the overlapping symptoms were removed.

Individuals scoring low on the TCI-SF subscale self-directedness have been described as being immature and irresponsible: they generally have difficulties accepting responsibilities and setting long-term goals (34). The predictive value of self-directedness has not been investigated previously within prospective studies. However, cross-sectional studies that assessed self-directedness after development of PTSD, have reported lower self-directedness within PTSD patients compared to controls without psychopathology (43, 44).

It has been previously hypothesized that both high hostility and low self-directedness are related to low social support and ineffective coping behavior (8, 12, 45), which are associated with increased prevalence of PTSD in post-trauma studies (22, 46). Furthermore, high hostility and low self-directedness might be associated with altered emotion processing, leading to greater peri-traumatic distress or altered peri-traumatic information processing, which are both risk factors for development of PTSD symptoms (47, 48).

Aside from associations between personality and psychological processes involved in PTSD, there are indications that personality traits are related to various biological systems involved in the pathophysiology of PTSD. The pre-trauma CAR was not associated with PTSD symptoms after deployment, and hence does not mediate the relationship between personality and PTSD. Changes in the functioning of the immune system also have been associated with PTSD (49-51). Our group previously reported that hostility was related to increased levels of T-cell mitogen-stimulated pro-inflammatory cytokines, accompanied by increased levels of anti-inflammatory cytokines and decreased levels of IL-6 and chemokines (52). Therefore, we propose that the functioning of the immune system could possibly be mediating the effects of hostility and self-directedness on PTSD development. Further research is needed to elucidate the psychological and biological pathways contributing to the relation between self-directedness, hostility and PTSD.

Since we previously observed that a pre-existing high number of glucocorticoid receptors in leukocytes is a vulnerability factor for development of a high level of PTSD symptoms in response to deployment (16), we hypothesized that basal functioning of the HPA axis might also be different in individuals vulnerable for PTSD symptom development. However, our results indicate that the CAR is not a pre-existing vulnerability factor for the development of PTSD symptoms. This confirms the negative findings of Markus et al. (12), which was

performed in a much smaller sample. Since assessment of cortisol directly after trauma has been previously found to predict PTSD development (53, 54), it might be that HPA axis reactivity to a traumatic event is involved in the pathophysiology of PTSD, instead of basal HPA axis functioning. We propose that an attenuated CAR, which was repeatedly observed in individuals with PTSD (18-21), develops as a consequence of the presence of PTSD. In addition, some studies have also reported an attenuated CAR in trauma-exposed individuals without PTSD (21, 55), indicating that the CAR may also be altered by trauma exposure as such.

Non-compliance in saliva sampling times may confound assessment of the CAR. Kupper et al. (56) reported that a decrease in cortisol levels in the first 30 minutes after awakening was related to non-compliance in sampling times in 80% of participants. In the current study a negative mean increase within the first 30 minutes of the CAR was observed in a substantial proportion of participants (n=94; 29.6%). Removing these participants from the analyses did not alter the results of our initial path analysis however (data not shown), and therefore we did not exclude these participants from the analysis. Comparison of pre-deployment morning plasma cortisol levels (collected between 8.00-11.30 a.m.) between participants with positive and negative mean increases in the first 30 minutes after awakening, revealed that participants with a negative mean increase had lower plasma cortisol levels (Positive MeanInc 0-30, mean (SD): 451.71 (156.04) nmol/l; negative MeanInc 0-30, mean (SD): 409.54 (118.59) nmol/l, $t_{(305)}$= 2.027, p=.044). This indicates that the observed negative mean increase in these participants may not be associated with non-compliance only, but may represent genuine alterations in HPA axis functioning.

Other factors influencing the CAR may also have confounded the association between the CAR and other observed variables in the path analysis (57). We analyzed the influence of body mass index, alcohol consumption on the day before saliva sampling, caffeine (coffee/tea) and nicotine consumption during saliva sampling and awakening time during the sampling day on the CAR. These variables did not significantly predict the AUCg and MeanInc (data not shown) and therefore we can exclude that these factors confounded the current results.

Rona et al. (32) previously reported that pre-deployment PTSD increased the risk for PTSD after deployment. The results from the current study, in which participants with a pre-deployment abnormal level of PTSD symptoms were excluded, indicate that even a low-to-moderate pre-deployment PTSD symptom level is a predictor for later PTSD symptoms.

It was previously reported that the impact of combat is increased for individuals with adverse experiences during childhood (22, 24) and for younger individuals (22-24). The results of the path analysis confirmed our hypotheses that the effects of childhood trauma and age on development of PTSD symptoms after deployment are indirect. Childhood trauma was related to higher hostility and the presence PTSD symptoms before deployment, which in turn influenced the development of PTSD symptoms after deployment. The relationship between childhood trauma and higher hostility may be mediated by attachment style.

Childhood maltreatment is associated with increased risk for developing insecure attachment styles (58). Insecure attachment is associated with higher levels of reported hostile emotions and aggressive behaviours during adolescence, and also with a smaller decline in these emotions and behaviours during adolescence (59). Younger age was related to higher hostility and lower self-directedness, which in turn influenced the development of PTSD symptoms after deployment. This effect of age on personality implies that levels of self-directedness and hostility may change over time. However, since effects of aging on hostility and self-directedness have not been studied within longitudinal designs with a substantial period between assessments, birth-cohort-effects caused by for example differences in societal influences cannot be ruled out.

In our study the number of previous deployments was not related to PTSD symptoms before and after deployment. Thus our data do not support a dose-response relationship between previous deployments and subsequent development of PTSD symptoms. This is contradictory to findings of previous studies in US and Canadian troops (23, 25), but not to findings of a recent study in UK troops (60). In the studies cited above PTSD symptomatology was only assessed after deployment. Thus the conflicting results may have been influenced by the inclusion of individuals with high pre-deployment PTSD symptoms caused by previous deployments in the cited studies, while participants with high levels of pre-deployment PTSD samples were excluded in the current study.

A limitation of the current study is that PTSD was not assessed with a structured clinical interview. However, it is known that the SRIP has good concurrent validity with diagnostic clinical interviews for PTSD (35, 36). Therefore, the PTSD symptomatology as assessed with the SRIP probably is a reliable reflection of actual PTSD symptomatology and clinical impairment. Co-morbid major depressive disorder (MDD) is usually present in approximately 50% of combat veterans with PTSD (61). However, potential co-morbid MDD and other DSM-IV axis-I disorders were not assessed. We cannot completely exclude that our results are influenced by the development of co-morbid disorders in a subgroup of participants.

The current study provides valuable information about vulnerability factors for development of PTSD symptoms after trauma in male soldiers. Our results indicate that pre-deployment high hostility, low self-directedness and the presence of low to moderate levels of PTSD symptoms are vulnerability factors for the development of PTSD symptoms after deployment. These vulnerability factors are influenced by the age during deployment and traumatic experience(s) during childhood. Identification of vulnerability factors for PTSD, such as described in this study, provides an opportunity to target primary or secondary interventions towards individuals at risk and to tailor these interventions to the specific needs of these individuals. Our findings indicate that post-deployment screening for early detection of PTSD symptoms might be specifically valuable for individuals with low self-directedness and high hostility, and for those with pre-deployment low-to-moderate symptoms of PTSD. In addition, pre-deployment psycho-education aimed at fostering resilience might be

specifically directed towards these individuals. However, future research should investigate which underlying mechanisms cause the increased vulnerability for PTSD in individuals presenting with the identified vulnerability factors, so interventions may be tailored to modify these underlying mechanisms. In addition, it has previously been established that the personality profile of military personnel differs from the profile of the general population (27). Therefore further research is warranted to investigate whether our findings can be generalized to other trauma populations.

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6

Glucocorticoid sensitivity of leukocytes predicts fatigue, depressive, and PTSD symptoms after military deployment: a prospective study.

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ABSTRACT

Severe fatigue, major depressive disorder (MDD), posttraumatic stress disorder (PTSD), may develop in response to severe stress and trauma. These conditions have all been shown to be associated with altered sensitivity of leukocytes for regulation by glucocorticoids (GCs). However, it remains unknown whether sensitivity of leukocytes for GC is a pre-existing vulnerability factor, or whether GC-sensitivity of immune cells alters as a consequence of stress and stress-related conditions. Our aim was to investigate whether sensitivity of T-cells and monocytes for regulation by GCs assessed before military deployment predicts high levels of fatigue, depressive, and/or PTSD symptoms six months after return from deployment.

We included 523 male military personnel before deployment to Afghanistan. Logistic regression analysis was performed to predict fatigue, depressive, and PTSD symptoms six months after deployment based on sensitivity of LPS-induced TNF-α production and PHA-induced T-cell proliferation to dexamethasone (DEX)-inhibition before deployment.

Severe fatigue six months after deployment was independently associated with low DEX-sensitivity of monocyte TNF-α production before deployment. A high level of depressive symptoms six months after deployment was independently associated with a low DEX-sensitivity of T-cell proliferation. In contrast, a high level of PTSD symptoms after deployment was independently associated with a high DEX-sensitivity of T-cell proliferation before deployment, but only in individuals who reported PTSD symptoms without depressive symptoms. DEX-sensitivity was not associated with childhood trauma, a known risk factor for development of fatigue, depressive and PTSD symptoms.

We present here for the first time that the sensitivity of leukocytes for GCs prior to deployment is a predictive factor for fatigue, depressive, and PTSD symptomatology in response to deployment. Notably, fatigue, depressive, and PTSD symptoms were differentially associated with GC-sensitivity of monocytes and T-cells and therefore may have different biological underpinnings.

INTRODUCTION

Exposure to severe stress and traumatic events frequently occurs during military deployment to a combat-zone. As a consequence, military deployment may serve as a risk factor for the development of mental and physical health problems. Among the mental and physical health problems regularly reported by military personnel returning from the combat field are major depressive disorder (MDD) (1) and severe fatigue (2). Furthermore, deployed military personnel are at risk to develop posttraumatic stress disorder (PTSD) (1). Nevertheless, only a minority of deployed military personnel ultimately develops MDD, severe fatigue, and/or PTSD after homecoming. We suggested that development of these conditions might be influenced by biological vulnerability factors that are already present prior to exposure to stress or trauma (3-5). The identification of these pre-existing biological vulnerability factors could eventually lead to identification of vulnerable individuals within groups at risk for stress- or trauma-exposure, and could thereby improve selection of individuals who will benefit from preventive care (6).

It is now generally accepted that both MDD and PTSD are associated with a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (7-9). In addition, there are indications that the presence of severe fatigue is also associated with changes in the functioning of the HPA axis (10, 11). Glucocorticoids (GCs), the end-product of the HPA axis, are important regulators of the immune system. Two important effects of GCs on the immune system are inhibition of T-cell proliferation and inhibition of the production of pro-inflammatory cytokines (12). The glucocorticoid receptor (GR) signalling pathway is complex, and GC-sensitivity of immune cells is not only determined by the number and affinity of GR, but at many levels of the signalling route downstream of the receptor (13, 14). One way to quantify the net effect of GC signalling is by measuring the GC-sensitivity of leukocytes (15). The GC-sensitivity of leukocytes is usually investigated by assessing the regulatory effect of increasing doses of the synthetic GR agonist dexamethasone (DEX) on *in vitro* activated leukocytes.

Enhanced GC-sensitivity of monocytes (16) and T-cells (17, 18) has been observed in adults diagnosed with chronic fatigue syndrome (CFS) when compared to healthy controls. In contrast, within a community sample, high levels of vital exhaustion were associated with decreased GC-sensitivity of monocytes (19). Furthermore, in adolescents girls diagnosed with CFS (20) and with persistent severe fatigue (21) decreased GC-sensitivity of T-cells was observed compared to non-fatigued adolescents.

Decreased GC-sensitivity of T-cells has repeatedly been observed in blood samples from adults with MDD compared to healthy controls (22-25). Results on the GC-sensitivity of monocytes of individuals with MDD are less consistent. Decreased inhibition of innate pro-inflammatory cytokine production by GCs has been observed in females with MDD compared to non-depressed females (26). However, individuals with treatment-resistant depression did not show a different GC-sensitivity of monocytes compared to healthy controls (27).

Moreover, the presence of depressive symptoms is associated with increased GC-sensitivity of monocytes in adults with coronary heart disease (28).

The GC-sensitivity of immune cells from individuals with PTSD has been investigated predominantly in monocytes. The results of most studies indicate that monocytes of individuals with PTSD have enhanced GC-sensitivity compared to monocytes of individuals without PTSD (29-31). In contrast, our group showed that male combat veterans with PTSD did not have increased GC-sensitivity of monocyte cytokine production compared to combat veterans without PTSD (32). To the best of our knowledge, only our group has studied the GC-sensitivity of T-cells of individuals with PTSD thus far (32). Compared to combat veterans without PTSD and healthy controls, T-cells of veterans with PTSD showed a decreased GC-sensitivity.

These cross-sectional studies clearly indicate that severe fatigue, MDD, and PTSD are associated with changes in the regulation of the immune system by GCs. However, since all of these studies have been performed with individuals who already had developed MDD, fatigue or PTSD, it remains unknown whether altered GC-sensitivity of immune cells is already present prior to the development of these conditions.

We hypothesized that GC-sensitivity of leukocytes prior to stress- and trauma exposure is predictive for subsequent development of stress-related conditions. In the current study, we therefore investigated whether GC-sensitivity of monocytes and T-cells measured prior to deployment predicted the presence of a high level of fatigue, depressive, and/or PTSD symptoms six months after return from military deployment. For that purpose, we investigated DEX-regulation of LPS-induced TNF-α production as a measure of monocyte DEX-sensitivity. As a measure of T-cells GC-sensitivity we analyzed the effect of DEX on T-cell proliferation in response to a T-cell mitogen. We included 523 male Dutch soldiers who were subsequently deployed to a combat-zone in Afghanistan. Six months after their return, the level of fatigue, depressive, and PTSD symptoms was assessed.

METHODS

General procedure

Military personnel of the Dutch Armed Forces assigned to a 4-month deployment to Afghanistan were included on a voluntary basis after giving verbal and written informed consent. Their duties during deployment included combat patrols, clearing or searching buildings, participation in de-mining operations, and transportation across enemy territory. Participants were exposed to typical combat-zone stressors including enemy fire, armed combat and combat casualties. We included participants from 11 sequential rotations deployed from 2005 to 2009. Participants were assessed one to two months prior to deployment (T0), and approximately one (T1) and six months (T2) after their return from deployment. During

each assessment, participants filled out questionnaires and a heparinized blood sample was drawn between 8.00 and 11.30 a.m. Heparinized blood was kept at room temperature. The pre-deployment blood sample (T0) was used to predict the presence of a high level of PTSD, depressive and fatigue symptoms at T2, six months after return from deployment. The study was approved by the Institutional Review Board of the University Medical Center Utrecht.

Questionnaires

Level of fatigue over the past two weeks was assessed with the Dutch 20-item Checklist Individual Strength (CIS-20R) (33). This questionnaire consists of four subscales: severity of fatigue, concentration, motivation and physical activity. The total fatigue score is the sum score of all items (range 20-140). The questionnaire is well validated and has good reliability. The used cut-off for the total score on the CIS-20R was ≥81. This cut-off corresponds to the 95th percentile of scores before deployment within a population of 862 Dutch military personnel (mean (SD): 45.87 (17.69)).

Level of depressive symptoms over the past week was assessed with the Dutch 16-item symptom checklist (SCL-90) depression subscale (34). The total depressive symptom score is the sum score for all items (range 16-80). The questionnaire has good reliability and is frequently used within research and clinical settings (35). The used cut-off for the SCL-90 depression subscale was ≥24 (4). This cut-off corresponds to the 95th percentile of scores before deployment within a population of 840 Dutch military personnel (mean (SD): 18.06 (3.15)). In the studies of Aben et al. (36) and Strik et al. (37) the sensitivity and specificity of various cut-offs on the SCL-90 depression subscale for a diagnosis of MDD according to the Structured Clinical Interview for DSM-IV (SCID) was tested. The results of these studies support the validity of our cut-off score as representing a high level of depressive symptoms. Aben et al. (36) observed that a cut-off of ≥25 provided the highest sensitivity and specificity for a MDD diagnosis in a group of patients after myocardial infarction, and Strik et al. (37) observed that a cut-off of ≥24 provided the highest sensitivity and specificity for a MDD diagnosis in a group of patients after stroke.

Level of posttraumatic stress disorder (PTSD) symptoms over the past 4 weeks was assessed with the Dutch 22-item Self-Report Inventory for PTSD (SRIP) (38). The questionnaire consists of three subscales representing the PTSD symptom clusters re-experiencing, avoidance and hyper-arousal. The total PTSD score is the sum score for all items (range: 22-88). The SRIP has good concurrent validity with other PTSD measures such as the Clinician Administered PTSD Scale (CAPS) and Mississippi scale for PTSD (38, 39). The used cut-off for the total score on the SRIP (38) was ≥38 (3, 5). This cut-off corresponds to the 95th percentile of scores before deployment within a population of 704 soldiers from the Dutch Armed Forces (mean (SD): 26.91 (5.34)). The validity of our cut-off score as representing a high level of PTSD symptoms is supported by van Zelst et al. (40), who tested the sensitivity

and specificity of various cut-offs on the SRIP for a diagnosis of PTSD according to the DSM-IV. A cut-off in our range provided the highest sensitivity and specificity for a PTSD diagnosis.

Exposure to potentially traumatic events during childhood was assessed using the 27-item Dutch version of the short-form self-report version of the Early Trauma Inventory (ETI) (41). The questionnaire has been designed to assess exposure to potentially traumatic experiences before the age of 18 years (general trauma, physical abuse, emotional abuse and sexual abuse). The total score represents the number of different events experienced.

Collected demographics included age during deployment, body height, weight, smoking, alcohol use, and use of medication. Body Mass Index (BMI) was calculated by dividing body weight by the square of body height (kg/m^2). Exposure to deployment-stressors was assessed with a 13-item checklist during the T1 assessment (3).

Participants

A total of 725 male participants completed questionnaires before deployment (T0) and had pre-deployment data available for both measures of DEX sensitivity. Twelve participants (1.7%) were not available for follow-up (non-deployed (n=10); deceased during deployment (n=2)). Of the eligible 713 participants, 589 completed the questionnaires at one month after deployment (T1) (82.6%) and 546 completed the questionnaires at six months after deployment (T2) (76.6%). We were interested in the presence of fatigue, depressive, and PTSD symptoms at T2. Therefore, we excluded 20 participants who did not complete all three questionnaires under study at T2, resulting in a total sample of n=526 at T2 (73.8%). Compared to eligible individuals who completed the three questionnaires at T2, dropouts were younger during deployment (mean (SD): dropouts: 26.99 (8.16); completers: 29.67 (9.53), $t_{(709)}= 3.399$, p=.001). In addition, they had been deployed less often (mean (SD): dropouts: 0.64 (0.92); completers: 0.96 (1.25), $t_{(685)}= 2.880$, p=.004) and were lower ranked ($\chi^2_{(3)}= 14.641$, p<.001). There was no significant difference in educational level between completers and dropouts ($\chi^2_{(2)}= 4.909$, p=.086). In addition, at T0 there were no differences in questionnaire scores for PTSD ($t_{(583)}= -0.222$, p=.824), depression ($t_{(704)}= -0.634$, p=.526) and fatigue ($t_{(710)}= 0.263$, p=.793).

Participants' questionnaires scores for fatigue, depression and PTSD on were categorized into low and high symptom levels for each questionnaire separately. 66 participants reported severe fatigue at T2 (9.1%). In addition, 45 participants reported a high level of depressive symptoms at T2 (8.6%). Furthermore, 46 participants reported a high level of PTSD symptoms at T2 (8.7%). 423 participants (80.4%) did not report high levels of symptoms on any of the three questionnaires. Compared to participants who did not report high symptom levels on any questionnaire at T2, participants who reported high levels of depressive symptoms and PTSD symptoms had experienced a higher number of potentially traumatic childhood experiences (Table 1). In addition, compared to participants who did not report high symptom

TABLE 1. Participant and deployment characteristics.

	Low symptom levels at T2 (n = 423)	PTSD symptoms at T2 (n = 46)	Depressive symptoms at T2 (n = 45)	Severe fatigue at T2 (n = 66)
Mean (SD)				
Age during deployment	29.91 (9.61)	27.26 (8.71)	29.31 (8.80)	29.48 (8.88)
Previous deployments, nr.	0.98 (1.28)	0.72 (1.14)	0.91 (1.02)	1.08 (1.25)
BMI at T0	25.00 (2.85)	25.26 (3.59)	25.98 (3.68)*	25.14 (3.01)
Deployment stressors, nr	4.69 (2.58)	5.87 (2.41)**	5.66 (2.34)*	5.60 (2.57)*
Potentially traumatic childhood experiences, nr	3.19 (2.73)	5.24 (3.74)***	5.04 (3.46)***	3.91 (3.37)
N (%)				
Smoking at T0 (yes)	168 (40.7%)	24 (54.5%)	19 (43.2%)	28 (43.8%)
Alcohol use at T0				
none	33 (8.1%)	4 (9.1%)	6 (13.6%)	4 (6.5%)
1-20 units/week	350 (86.0%	36 (81.8%)	35 (79.5%)	53 (85.5%)
> 20 units week	24 (5.9%)	4 (9.1%)	3 (6.8%)	5 (8.1%)
Education				
Low	157 (37.6%)	18 (40.0%)	17 (37.8%)	23 (34.8%)
Middle	212 (50.7%)	22 (48.9%)	23 (51.1%)	34 (51.5%)
High	49 (11.7%)	5 (11.1%)	5 (11.1%)	9 (13.6%)
Rank during deployment				
Soldiers	153 (36.2%)	24 (52.2%)	19 (42.2%)	21 (31.8%)
Corporals	78 (18.4%)	9 (19.6%)	11 (24.4%)	13 (19.7%)
Non-commissioned officers	131 (31.0%)	8 (17.4%)	8 (17.8%)	21 (31.8%)
Officers	61 (14.4%)	5 (10.9%)	7 (15.6%)	11 (16.7%)
Use of medication at T0 (yes)	24 (5.7%)	2 (4.3%)	1 (2.2%)	5 (7.6%)

* p<.05, ** p<.01, ***p<.001

levels on any questionnaire at T2, participants who reported high levels of PTSD symptoms at T2 were somewhat younger during deployment and tended to smoke more often before deployment. Participants who reported high levels of depressive symptoms at T2 had a higher Body Mass Index before deployment than individuals without high symptom levels on any questionnaire. In addition, participants who reported high levels of PTSD, depressive or fatigue symptoms after deployment reported a higher number of deployment-stressors than participants without high symptom levels after deployment.

Biological measures

In vitro sensitivity of peripheral blood cells to DEX

The sensitivity of peripheral blood cells to DEX was measured in vitro, using methodology as described previously (21, 32). DEX-sensitivity of T-cell mitogen-induced proliferation was determined in whole blood (diluted 1:10 with RPMI-1641 (Gibco, Grand Island, NY), 100

U/ml penicillin, 100 mg/ml streptomycin and 2mM L-glutamine) cultured with phytohemagglutinin (PHA) (Remel Europe Ltd, final concentration 25 mg/ml) at 37°C/5% CO_2 in 96 wells round-bottom plates in the presence of increasing concentrations of DEX (0–1000 nM DEX). After 72 h of culture, 1 μCi/well [^{3}H]-thymidine was added (Amersham, Buckinghamshire, UK) and 16-18 hours later, [^{3}H]-thymidine incorporation was measured using a liquid scintillation betacounter.

The effect of DEX on LPS-induced TNF-α production was measured in whole-blood (1:10 diluted with RPMI-1641, supplemented with antibiotics) stimulated with lipopolysaccaride (LPS, Escherichia coli 0127: B8, Sigma, final concentration 2 ng/ml) in the presence of 0–1000 nM DEX at 37°C/5% CO_2 in 96 wells flat bottom plates for 24 hours. Supernatants were stored at -80C and subsequently TNF-α concentrations were determined using standard ELISA kits (Sanquin, Amsterdam, the Netherlands).

Statistics

Analyses were performed using PASW/SPSS version 17. As a measure of total DEX inhibition, the area under the curve (AUC) for relative T-cell proliferation and TNF-α production after administration of 0-1000nM DEX was calculated. Variables used for analysis of group differences were tested for normality and log-transformed when necessary. Missing values in questionnaire scores at T0 and T1 were present for a number of participants (missing values: SRIP T0: n=77; CIS-20R T0: n=1; SCL-90 depression T0: n=1; ETI: n=3; participant characteristics T0: n=12; deployment stressor questionnaire T1: n=68). Outliers were removed if their values exceeded the SD ± 3.29 from the mean. Within a normal distribution no values are expected to exceed this threshold (42). Removal of outliers did not alter our results (AUC DEX-inhibition of T-cell proliferation: n = 1; AUC DEX-inhibition of TNF-α production: n = 2; total n for subsequent analyses = 423). Group differences in participant characteristics were tested using χ^2-tests and t-tests. Repeated measures ANOVAs were used to test group differences in the longitudinal course of questionnaire scores.

We performed three logistic regression analyses, to investigate the predictive value of the AUC for DEX-inhibited T-cell proliferation and DEX-inhibited TNF-α production for the presence of a high level of fatigue, depressive and PTSD symptoms after deployment (T2). By standardizing the AUCs we were able to compare their predictive value (Odds ratios). The variables were included in two blocks: in the first block we entered both AUCs and the questionnaire score at T0 for the condition under investigation. In the second block we entered the scores for the other two questionnaires at T2. We subsequently controlled for the possibly confounding effect of participants characteristics that differed between groups at T0. ANOVA, followed up with Bonferonni-post hoc tests, was used to test differences between participants with PTSD symptoms without depressive symptoms, participants with PTSD and depressive symptoms and participants without PTSD with depressive symptoms.

Additionally, we examined correlations between the number of potentially traumatic childhood experiences and both AUCs. Subsequently, we included the number of potentially traumatic childhood experiences in a third block in the logistic regression analyses to investigate whether the predictive value of GC sensitivity for symptom development could be explained by the experience of childhood trauma

All variables in the logistic regression analyses were forced into entry. In case of missing values in the questionnaire scores at T0, participants were deleted list-wise from the logistic regression analysis for which the specific questionnaire was missing, but were included in the other two logistic regression analyses.

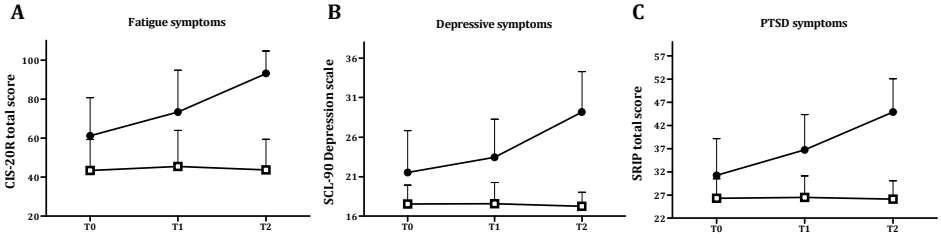
RESULTS

Longitudinal course of fatigue, depressive and PTSD symptoms

Participants with severe fatigue six months after deployment (T2) had higher fatigue questionnaire scores at all assessment points compared to participants with low levels of fatigue at T2 ($F_{(1,477)}$= 284.814, p<.001). As expected, the participants with severe fatigue at T2 showed a strong increase in questionnaire scores over time, while participants with low levels of fatigue did not show an increase in questionnaire scores over time ($F_{(2,954)}$= 97.127, p<.001) (Figure 1A).

Participants with high levels of depressive symptoms at T2 had higher depression questionnaire scores at all assessment points compared to participants with low levels of depressive symptoms at T2 ($F_{(1,468)}$= 407.244, p<.001). As was shown for fatigue, the participants with high levels of depressive symptoms at T2 showed a strong increase in questionnaire scores in response to deployment. Interestingly, questionnaire scores of the participants with

FIGURE 1. Longitudinal course of fatigue, depression, and PTSD questionnaire scores over time.



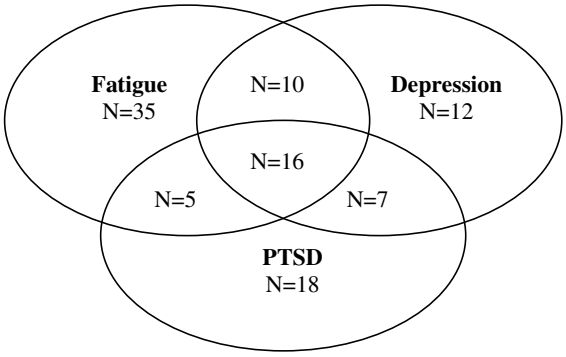
The graphs depict the questionnaire scores for A) fatigue: CIS-20R total score; B) depression: SCL-90 depression subscale; C) PTSD: SRIP total score, as assessed before deployment (T0) and one month (T1) and six (T2) months after return from deployment. The lines with black circles represent the questionnaire score of the participants who scored above the cut-off of the specific questionnaire at T2. The lines with white rectangles represent the questionnaire scores of the participants who scored below the cut-off of the specific questionnaire at T2. Means and SDs are presented.

low levels of depressive symptoms slightly decreased over time ($F_{(2,936)}$= 123.518, p<.001) (Figure 1B).

Participants with high levels of PTSD symptoms at T2 also had higher PTSD questionnaire scores at all assessment points compared to participants with low levels of PTSD symptoms at T2 ($F_{(1,399)}$= 215.579, p<.001). As expected, the participants with high levels of PTSD symptoms at T2 showed a strong increase in questionnaire scores in response to deployment, while questionnaire scores of participants with low levels of PTSD symptoms did not increase over time ($F_{(2,798)}$= 113.388, p<.001) (Figure 1C).

Co-morbidity between severe fatigue, MDD and PTSD, MDD is very common (43-45). Therefore, we investigated the correlations between the fatigue, depression and PTSD questionnaire scores in our sample. We observed strong correlations between fatigue and depression questionnaire scores (r =.673, p<.001), fatigue and PTSD questionnaire scores (r =.648, p<.001) and PTSD and depression questionnaire scores (r =.695, p<.001) at T2. Moreover, 36.9% of participants with a high level of PTSD, depressive or fatigue symptoms at T2 also scored above the cut-off of at least one of the other questionnaires at T2 (Figure 2). Given the strong correlations between the questionnaire scores, for each condition under investigation, we subsequently controlled for the scores on the other two questionnaires at T2 in the analysis.

FIGURE 2. Number of participants with a high level of fatigue, depressive, and/or PTSD symptoms six months after return from deployment to a combat-zone in Afghanistan.



The overlapping circles represent the participants scoring above the cut-off of more than one questionnaire at six months after return from deployment. Total N = 526.

Predictive value of DEX-sensitivity of monocytes and T-cells for severe fatigue after deployment

We first investigated whether DEX-sensitivity of monocytes and T-cells prior to deployment was predictive for the development of severe fatigue in response to military deployment. The area under the curves for DEX-inhibition of LPS-induced TNF-α production and

mitogen-induced T-cell proliferation did not correlate significantly (Spearman's rho = .083, p=.057). This indicates that DEX-inhibition of LPS-induced TNF-α production and mitogen-induced T-cell proliferation represent two independent measures of GC-sensitivity.

The area under the curve (AUC) for DEX-inhibition of LPS-induced TNF-α production and mitogen-induced T-cell proliferation were included in the logistic regression analysis to predict the presence of severe fatigue after deployment. In addition, the fatigue questionnaire score at T0 was included in the same block of the regression analysis to control for the level of fatigue before deployment. A high AUC for DEX-inhibition of TNF-α production, indicating low DEX-sensitivity of monocytes, was associated with increased risk for development of severe fatigue in response to deployment (W= 4.182, p=.041). The AUC for DEX-inhibition of T-cell proliferation was not a significant predictor of severe fatigue after deployment (W= 0.154, p=.695).

Since we observed strong correlations between the scores on the three questionnaires under investigation, depression and PTSD questionnaire scores at T2 were included in the second block of the regression analysis. The predictive value of monocyte DEX-sensitivity remained significant after inclusion of the questionnaire scores for depression and fatigue at T2, and even increased somewhat (W= 6.865, p=.009). In the final model the risk for severe fatigue increased 1.6-fold with every increase of 1 SD in the AUC of DEX-inhibited TNF-α production (Table 2). Inclusion of the number of reported deployment stressors, which differed between participants with and without severe fatigue at T2, did not alter the results.

TABLE 2. Predictive value of the sensitivity of monocytes and T-cells for regulation by dexamethasone (DEX) prior to deployment for the presence of a high level of fatigue symptoms six months after deployment.

	Block 1 $F_{(3)} = 59.416$***		Block 2 $F_{(2)} = 73.502$***	
	Wald	OR	Wald	OR
DEX-sensitivity TNF-α production (AUC)	4.182*	1.372 (1.013-1.857)	6.865**	1.578 (1.122-2.220)
DEX-sensitivity T-cell proliferation (AUC)	0.154	1.057 (0.801-1.395)	0.025	0.974 (0.701-1.352)
Fatigue symptoms at T0	49.870***	1.057 (1.041-1.073)	21.940***	1.044 (1.025-1.063)
Depressive symptoms at T2			20.322***	1.228 (1.013-1.120)
PTSD symptoms at T2			6.133*	1.065 (1.013-1.120)

Block 1 presents the predictive value of DEX-sensitivity after controlling for fatigue symptoms prior to deployment (T0). Block 2 presents the predictive value of DEX-sensitivity after controlling for fatigue symptoms prior to deployment (T0) and depressive and PTSD symptoms six months after deployment (T2). AUC: Area under the curve for T-cell proliferation and TNF-α production after administration of 0-1000nM DEX. *p<.05, **p<.01, ***p<.001.

Predictive value of DEX-sensitivity of monocytes and T-cells for depressive symptoms after deployment

We investigated whether DEX-sensitivity of monocytes and T-cells prior to military deployment was also predictive for the presence of a high level of depressive symptoms six months after deployment. The AUCs for DEX-inhibition of TNF-α production and T-cell proliferation were included in the logistic regression analysis, together with depression questionnaire scores at T0. In contrast to what was observed for fatigue, DEX-inhibition of TNF-α production at T0 did not predict the presence of depressive symptoms six months after deployment (W= 0.667, p=.414). However, a high AUC DEX-inhibition of T-cell proliferation at T0, indicating a low DEX-sensitivity of T-cells, was associated with increased risk for a high level of depressive symptoms six months after deployment (W= 5.081, p=.024).

The predictive value of T-cell DEX-sensitivity for depressive symptoms increased after PTSD and fatigue symptoms at T2 were also included in the model. The risk for a high level of depressive symptoms increased 1.9-fold with every increase of 1 SD in the AUC of DEX-inhibition of T-cell proliferation after controlling for PTSD and fatigue symptoms (W= 7.534, p=.006; Table 3).

The results did not change after controlling for number of deployment stressors and for BMI at T0, which significantly differed between participants with high levels of depressive symptoms and participants without high symptom levels after military deployment.

TABLE 3. Predictive value of the sensitivity of monocytes and T-cells for regulation by dexamethasone (DEX) prior to deployment for the presence of a high level of depressive symptoms six months after deployment.

	Block 1 $F_{(3)} = 52.078$***		Block 2 $F_{(2)} = 102.175$***	
	Wald	OR	Wald	OR
DEX-sensitivity TNF-α production (AUC)	0.667	0.859 (0.596-1.237)	0.612	0.833 (0.526-1.317)
DEX-sensitivity T-cell proliferation (AUC)	5.081*	1.498 (1.054-2.130)	7.534**	1.886 (1.199-2.968)
Depressive symptoms at T0	39.220***	1.324 (1.212-1.445)	7.161**	1.167(1.042-1.307)
Fatigue symptoms at T2			19.827***	1.051 (1.028-1.074)
PTSD symptoms at T2			25.893***	1.173 (1.103-1.247)

Block 1 presents the predictive value of DEX-sensitivity after controlling for depressive symptoms prior to deployment (T0). Block 2 presents the predictive value of DEX-sensitivity after controlling for depressive symptoms prior to deployment (T0) and fatigue and PTSD symptoms six months after deployment (T2). AUC: Area under the curve for T-cell proliferation and TNF-α production after administration of 0-1000nM DEX. *p<.05, **p<.01, ***p<.001.

Predictive value of DEX-sensitivity of monocytes and T-cells for PTSD symptoms after deployment

Finally, we investigated whether the DEX-sensitivity of monocytes and T-cells prior to military deployment was also predictive for a high level of PTSD symptoms six months after deployment. Both AUCs and PTSD questionnaire scores at T0 were included in the logistic regression analysis to predict the presence of a high level of PTSD symptoms at T2. In this first step of the analysis, DEX-inhibition of TNF-α production and T-cell proliferation were not significantly associated with the development of PTSD symptomatology in response to deployment (AUC TNF-α production: W= 0.521, p=.471; AUC T-cell proliferation: W= 1.199, p=.274). However, when we included the questionnaire scores for depression and fatigue at T2, the AUC for DEX-inhibition of T-cell proliferation became significantly associated with PTSD symptom development: the risk for a high level of PTSD symptoms decreased 0.7-fold with every increase of 1 SD in the AUC for DEX-inhibition of T-cell proliferation (W= 4.019, p=.045; Table 4). The results did not change after controlling for reported deployment stressors, age during deployment and smoking at T0.

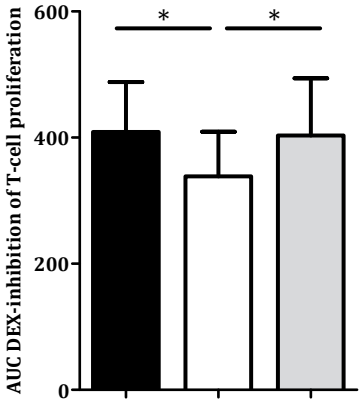
We performed two additional logistic regression analyses to investigate whether the significant result in the final step of the model was associated specifically with inclusion of either fatigue or depressive symptoms at T2. After inclusion of only depressive symptoms in the second block, DEX-sensitivity of T-cells remained significantly associated with the development of PTSD symptoms (W= 4.100, p=.043). However, when we included fatigue symptoms only, DEX-sensitivity of T-cells was not significantly associated with development of PTSD symptoms (W= 2.459, p=.117). Thus, it appears that increased DEX-sensitivity of T-cells before deployment is a biological correlate for a subgroup of participants with a high

TABLE 4. Predictive value of the sensitivity of monocytes and T-cells for regulation by dexamethasone (DEX) prior to deployment for the presence of a high level of PTSD symptoms six months after deployment.

	Block 1 $F_{(3)} = 30.279$***		Block 2 $F_{(2)} = 55.239$***	
	Wald	OR	Wald	OR
DEX-sensitivity TNF-α production (AUC)	0.521	1.146 (0.792-1.659)	0.607	1.185 (0.773-1.819)
DEX-sensitivity T-cell proliferation (AUC)	1.199	0.826 (0.587-1.163)	4.019*	0.665 (0.446-0.991)
PTSD symptoms at T0	26.497***	1.159 (1.096-1.227)	3.102#	1.065 (0.993-1.143)
Fatigue symptoms at T2			10.767**	1.034 (1.014-1.055)
Depressive symptoms at T2			11.280**	1.178 (1.071-1.296)

Block 1 presents the predictive value of DEX-sensitivity after controlling for PTSD symptoms prior to deployment (T0). Block 2 presents the predictive value of DEX-sensitivity after controlling for PTSD symptoms prior to deployment (T0) and fatigue and depressive symptoms six months after deployment (T2). AUC: Area under the curve for T-cell proliferation and TNF-α production after administration of 0-1000nM DEX. #p<.10, *p<.05, **p<.01, ***p<.001.

FIGURE 3. Dexamethasone (DEX) sensitivity of T-cell proliferation of participants with PTSD-DEP+ (black bars, n = 22), PTSD+DEP+ (grey bars, n = 23) and PTSD+DEP- (white bars, n = 23).



Means and SDs are presented. * p < .05.

level of PTSD symptoms after deployment. To further interpret the results of the logistic regression analysis, we divided participants scoring above the cut-off of the PTSD and/or depression questionnaires at T2 in three groups: participants with high levels of PTSD symptoms without high levels of depressive symptoms (PTSD+DEP-, n=23), participants with high levels of PTSD and depressive symptoms (PTSD+DEP+, n=23) and participants with high levels of depressive symptoms without high levels of PTSD symptoms (PTSD-DEP+, n=22).

DEX-sensitivity of T-cell proliferation at T0 differed between the three groups ($F_{(2,67)}$= 5.402, p=.007). The AUC of participants with high levels of PTSD symptoms without co-morbid depressive symptoms (PTSD+DEP-) was significantly lower than the AUC of the other two groups (PTSD+DEP+, p=.024; PTSD-DEP+, p=.014) (Figure 3). The AUC was not significantly different between participants with high levels of PTSD and depressive symptoms and participants with high levels of depressive symptoms without co-morbid PTSD symptoms (p=1.000). These results indicate that increased DEX-sensitivity of T-cells before deployment was present in individuals who developed a high level of PTSD symptoms without depressive symptoms in response to deployment.

Childhood trauma

The experience of adverse or traumatic events during childhood is an important risk factor for development of severe fatigue (46), MDD (47) and PTSD (48) during adulthood. In addition, childhood adversity may result in altered GC signalling in the immune system (49). Therefore, we investigated whether the predictive value of DEX-sensitivity for the development of a high level of fatigue, depressive and PTSD symptoms could be explained by the experience of childhood trauma. Inclusion of the number of potentially traumatic childhood

experiences as an additional variable in the logistic regression analyses did not change the significant predictive value of the AUC of DEX-inhibited TNF-α production for fatigue (W= 6.135, p=.013) and the AUC of DEX-inhibited T-cell proliferation for depression (W= 7.340, p=.007) and PTSD (W=3.918, p=.048), and was not independently associated with the development of fatigue (W= 2.992, p=.084), depression (W= 0.085, p=.771) or PTSD symptoms (W= 2.880, p=.090) in response to deployment.

DISCUSSION

Cross-sectional studies previously indicated that GC-sensitivity of leukocytes differs between individuals with severe fatigue, MDD, or PTSD and healthy individuals (16-32). However, from these cross-sectional studies it could not be concluded whether altered GC-sensitivity of leukocytes precedes the development of these conditions. To the best of our knowledge, we are the first to perform a prospective study on GC-sensitivity of leukocytes and subsequent development of high levels of fatigue, depressive and PTSD symptoms.

In the current study, we investigated whether dexamethasone- (DEX-) sensitivity of T-cells and monocytes measured prior to deployment to a combat-zone as a measure of GC-sensitivity predicted the presence of a high level of fatigue, depressive, and PTSD symptoms six months after return from deployment. Our results clearly show that GC-sensitivity of T-cells and monocytes as determined prior to the military deployment are related to development of high levels of fatigue, depressive and PTSD symptoms. Interestingly, despite the strong correlations between the questionnaire scores, the predictive value of the GC-sensitivity of T-cells and monocytes prior to deployment was condition-specific. Low GC-sensitivity of monocytes prior to deployment was associated with increased risk for severe fatigue in response to deployment. Low GC-sensitivity of T-cells was associated with increased risk for a high level of depressive symptoms. In contrast, high GC-sensitivity of T-cells prior to deployment was associated with increased risk for a high level of PTSD symptoms in response to deployment, but only if participants did not also report a high level of depressive symptoms.

Low GC-sensitivity of monocytes, but also T-cells, has previously been observed within younger (20, 21) or pre-clinical (19) samples with severe fatigue. We observed that low GC-sensitivity of monocytes predicts the presence of severe fatigue after exposure to the severe life stress of military deployment to a combat zone, also when controlling for the level of fatigue prior to deployment. Thus, our finding implies that low GC-sensitivity of monocytes most likely precedes the development of severe fatigue. This finding is in contrast to the repeatedly observed high GC-sensitivity of immune cells in adult CFS patients (16-18). It may well be possible that in these adult patients high GC-sensitivity developed as a consequence of the prolonged presence of CFS, since it has been suggested that some biological changes observed in CFS result from the behavior associated with the condition (11). On the other hand, the etiopathogenesis of CFS might be totally different from the severe fatigue as

diagnosed after subjection to stressful and traumatic events such as encountered during a military mission in Afghanistan.

It is plausible that the observed low GC-sensitivity of monocytes, as observed in our study, is paralleled by increased levels of circulating pro-inflammatory cytokines in individuals vulnerable for developing fatigue, especially under stressful conditions. Interestingly, higher plasma levels of pro-inflammatory cytokines have repeatedly been observed in medically healthy fatigued individuals compared to healthy non-fatigued individuals (50). This pro-inflammatory state could be involved in the development of fatigue symptoms, since administration of pro-inflammatory cytokines induces symptoms of fatigue and malaise (i.e. sickness behavior) in rodents and humans (51, 52).

Low GC-sensitivity of T-cells has been associated with the presence of MDD and depressive symptoms in cross-sectional studies (22-25). Our results indicate that this low GC-sensitivity of T-cells may already be present prior to the development of the depressive symptoms, and even prior to the event possibly triggering the depressive symptoms. Within the same participant sample as that of the current study, we also observed that a relatively high production of T-cell cytokines upon stimulation with a mitogen was predictive for the presence of a high level of depressive symptoms after deployment (53). Therefore, we are tempted to speculate that this high T-cell cytokine production may result from the low capacity of GCs to regulate T-cell activation as observed in the current study on the level of proliferation.

We previously reported that military personnel with PTSD symptoms after deployment already differed on several levels of the GR signalling pathway prior to the deployment, compared to military personnel without PTSD symptoms after deployment. A high GR number prior to deployment increased the risk for PTSD symptom development in response to deployment (3). In addition, low mRNA expression of FKBP5 was associated with increased risk for development of PTSD symptoms (5). FKBP5 is a target gene of the GR and a co-chaperone of the GR-HSP70/90 heterocomplex, which lowers GR affinity (54). Furthermore, high mRNA levels of GR target gene glucocorticoid-induced leucine zipper (GILZ), which is involved in inhibiting cell proliferation (55), were also associated with increased vulnerability for PTSD development (5). These data may be a reflection of a more effective GR signalling pathway. In the current study we observed that high GC-sensitivity of T-cells prior to deployment predicted the presence of PTSD symptoms after deployment in the subgroup without depressive symptoms. The current results add to our previous findings, since we now demonstrate an additional pre-existing vulnerability factor in the GR signalling pathway, which may have functional consequences on the level of T cell proliferation.

Increased negative feedback of GCs on the HPA axis is often considered as one of the hallmark biological correlates of PTSD (9, 56). Furthermore, recent data in the literature indicate that enhanced GC-sensitivity may also be present in other brain regions of individuals with PTSD (29-31). Additionally, decreased GC-sensitivity of the HPA-axis measured in vivo is also a robust finding in individuals with MDD compared to healthy controls (8, 56,

57). Although there is ongoing debate about the validity of the use of results on GC signalling in immune cells in vitro as a model for GC signalling in the central nervous system in vivo (58), on the basis of our results, we hypothesize that the observed pre-existing differences in GC-sensitivity of T-cells in participants who developed high levels of depressive and/or PTSD symptoms compared to participants who did not developed these symptoms may be paralleled in the HPA axis at the level of the hypothalamus and/or the pituitary.

Most of the studies in which the GC-sensitivity of PBMCs or the HPA axis in individuals with PTSD was investigated did not assess the influence of co-morbid MDD on GC-sensitivity. Co-morbid MDD in individuals diagnosed with PTSD is a frequent phenomenon and may be as high as 50 % in deployed military personnel (45). Given this high co-morbidity, it has been suggested that a co-morbid diagnosis of MDD may be an epiphenomenon of the presence of more severe PTSD symptoms, of which some overlap with symptoms of MDD (e.g. dysphoria and emotional numbing) (59). Our results on GC-sensitivity of T-cells prior to deployment suggest, however, that the presence of depressive symptoms in individuals with PTSD symptoms is not merely an epiphenomenon of more severe PTSD: the presence of a high level of PTSD symptoms without co-morbid depressive symptoms after deployment was associated with high GC-sensitivity of T-cells prior to deployment. In contrast, individuals who had developed high levels of depressive symptoms, either with or without PTSD symptoms, showed low GC-sensitivity of T-cells prior to deployment. A parallel distinction was previously observed by Gill et al. (60) for PTSD patients with and without MDD. In this study increased negative feedback of GCs on the level of the HPA axis and on serum IL6-production was present in PTSD patients without co-morbid MDD, but not in PTSD patients with co-morbid MDD. In addition, Gill et al. (60) previously pointed out that the most pronounced results regarding the presence of high GC- sensitivity of the HPA axis in PTSD have been observed in samples with low levels of co-morbid MDD. Our results suggest that the observed differences in central and peripheral GC-sensitivity between individuals with PTSD symptomatology with and without co-morbid MDD or depressive symptoms may be involved in the development into different symptom profiles. Moreover, our results suggest that some biological vulnerability factors for PTSD symptom development can only be identified if co-morbid depressive symptomatology is taken into consideration.

Childhood adversity has been described as one of the most important risk factors for the development of fatigue, MDD, and PTSD during adulthood (46-48), and it may result in long-lasting effects on GC signalling within the immune system (49). Therefore we hypothesized that the predictive value of GC-sensitivity for fatigue, depressive, and PTSD symptoms might be explained by childhood traumatic experiences. However, childhood traumatic experiences did not explain the predictive value of GC-sensitivity for the presence of high levels of fatigue, depressive and PTSD symptoms after deployment, indicating that these specific biological vulnerability factors in the participants who did develop stress-related symptomatology had not developed as a consequence of childhood trauma.

A limitation of the current study is that questionnaires were used to assess symptoms of MDD and PTSD, instead of structured diagnostic interviews. However, the validity of our questionnaires and the cut-offs used to identify high levels of PTSD and depressive symptoms in our sample are supported by several studies (36, 37, 40).

Another limitation is that we did not assess the presence of medical conditions that may have influenced levels of fatigue or GC-sensitivity. However, participants were physically fit for military deployment and therefore the presence of major medical conditions prior to deployment is highly unlikely. Furthermore, removing participants who used medication prior to deployment and participants who were injured during deployment did not alter the results (data not shown). Since only a relatively limited number of participants reported high levels of fatigue, depressive and PTSD symptoms, we were not able to reliably investigate GC sensitivity associated with all different combinations of symptomatology separately. However, in all analyses we did control for the presence of other symptoms in addition to the condition under investigation.

The current study provides novel information about biological vulnerability factors for development of stress-related fatigue, depressive, and PTSD symptoms in response to deployment to a combat-zone. We are the first to show that GC-sensitivity of T-cells and monocytes assessed prior to a period of acute stressors and potentially traumatic events significantly predicted the subsequent development of fatigue, depressive, and PTSD symptomatology. Interestingly, although we observed strong correlations between fatigue, depressive and PTSD symptom severity six months after deployment, the development of these different conditions was associated with different GC-sensitivity profiles prior to deployment. Therefore we propose that stress-related fatigue, MDD, and PTSD symptoms have different biological underpinnings, which are already present before development of these symptoms.

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7

Cytokine production by leukocytes of military personnel with depressive symptoms after deployment to a combat-zone: a prospective, longitudinal study.

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ABSTRACT

Major depressive disorder (MDD) is frequently diagnosed in military personnel returning from deployment. Literature suggests that MDD is associated with a pro-inflammatory state. To the best of our knowledge, no prospective, longitudinal studies on the association between development of depressive symptomatology and cytokine production by peripheral blood leukocytes have been published. The aim of this study was to investigate whether the presence of depressive symptomatology six months after military deployment is associated with the capacity to produce cytokines, as assessed before and after deployment.

1023 military personnel were included before deployment. Depressive symptoms and LPS- and T-cell mitogen-induced production of 16 cytokines and chemokines in whole blood cultures were measured before (T0), one (T1), and six (T2) months after return from deployment. Exploratory structural equation modeling (ESEM) was used for data reduction into cytokine patterns. Multiple group latent growth modeling was used to investigate differences in the longitudinal course of cytokine production between individuals with (n=68) and without (n=665) depressive symptoms at T2. Individuals with depressive symptoms after deployment showed higher T-cell cytokine production before deployment. Moreover, pre-deployment T-cell cytokine production significantly predicted the presence of depressive symptomatology six months after return. There was an increase in T-cell cytokine production over time, but this increase was significantly smaller in individuals developing depressive symptoms. T-cell chemokine and LPS-induced innate cytokine production decreased over time and were not associated with depressive symptoms.

These results indicate that increased T-cell mitogen-induced cytokine production before deployment may be a vulnerability factor for development of depressive symptomatology in response to deployment to a combat-zone. In addition, deployment to a combat-zone affects the capacity of T-cells and monocytes to produce cytokines and chemokines until at least six months after return.

INTRODUCTION

Mental health disorders frequently diagnosed in military personnel after deployment include major depressive disorder (MDD). Prevalence estimates for MDD range from 7.3% to 15.9% in US infantry soldiers 12 months after return from deployment to Iraq (1). A number of studies have investigated the capacity of peripheral leukocytes of individuals with MDD or depressive symptoms to produce cytokines after in vitro stimulation. Increased (2-7), unaltered (8-12) and decreased (2, 13, 14) mitogen-induced pro-inflammatory cytokine production by leukocytes from individuals with MDD or depressive symptoms compared to non-depressed controls have been reported. However, in the majority of these studies only a small number of predominantly innate pro-inflammatory cytokines has been investigated. In addition, all of these studies have been performed within a cross-sectional design, with a different 'time since onset' of the depressive symptoms. Therefore, it is as yet unknown whether a causal relation exists between the development of MDD and the capacity to produce cytokines.

The potential involvement of inflammatory mediators in depression is underscored by the observation that individuals with lifetime MDD have epigenetic changes in methylation of inflammation-associated genes (15). In addition, meta-analyses investigating the association between MDD and circulating levels of C-reactive protein (CRP), innate pro-inflammatory cytokines IL-6, TNF-α, IL-1, and the IL-1 receptor antagonist showed that these inflammatory markers are increased in MDD (16, 17). These effects were present within both clinical and community samples, and in studies using clinical interviews and studies using self-report measures (16). Furthermore, higher amounts of circulating IL-2 soluble receptors (s-IL2-r) (18-20) have been observed in individuals with MDD or depressive symptomatology.

In the current study our aim was to determine whether the level of mitogen-induced cytokine production before and/or after deployment was associated with the presence of a high level of depressive symptoms six months after return from military deployment. We used a prospective, longitudinal design, in which data were collected before, as well as one and six months after deployment to a combat-zone. We investigated the production of a broad range of innate and T-cell cytokines, including pro- and anti-inflammatory cytokines, as well as chemokines. There is functional overlap between cytokines, and chance capitalization for type-I errors will occur when testing 16 longitudinal models. Therefore, we performed data reduction by using exploratory structural equation modeling (ESEM), which is a recently developed statistical method in which exploratory factor analysis is performed within a structural equation modeling setting (21). Subsequently, differences in the longitudinal course of cytokine production between individuals with and without depressive symptomatology six months after deployment were investigated using multiple group latent growth modeling (LGM) (22). In addition, logistic regression analysis was performed to test the predictive value of cytokine production for the presence of depressive symptomatology after deployment.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Institutional Review Board of the University Medical Center Utrecht, the Netherlands. Written informed consent was obtained after participants got a written and verbal description of the study.

Participants

Military personnel of the Dutch Armed Forces assigned to a 4-month deployment to Afghanistan were included in this study. Duties during deployment included combat patrols, clearing or searching homes and buildings, participation in de-mining operations, and transportation across enemy territory. They were exposed to typical war-zone stressors such as exposure to enemy fire, armed combat, and seeing seriously injured and dead fellow soldiers and civilians (including women and children).

Participants were assessed 1 to 2 months prior to deployment (T0) and approximately one (T1) and six months (T2) after their return. At each assessment, participants filled out several 'paper-and-pencil' questionnaires. In addition, a heparinized blood sample was drawn between 8.00 and 11.30 a.m. Heparinized blood was kept at room temperature. Data were collected between April 2005 and September 2009.

We included 1023 participants before deployment. Twenty-eight participants (2.5%) were not available for follow-up (non-deployed (n=26); deceased during deployment (n=2)). Of the eligible 995 participants, 825 completed the assessment at T1 (82.9%) and 749 completed the assessment at T2 (75.3%). Compared to eligible individuals who completed the T2 assessment, dropouts were younger during deployment (mean (SD): dropouts: 26.03 (7.08); completers: 29.09 (9.24), $t_{(976)}= -4.604$, $p<.001$). Consequently they had been deployed less often (mean (SD): dropouts: 0.55 (0.88); completers: 0.93 (1.23), $t_{(886)}= -4.03$, $p<.001$) and were lower ranked ($\chi^2_{(3)}= 21.656$, $p<.001$). There were no significant differences in gender distribution ($\chi^2_{(1)}= 1.738$, $p=.187$) and educational level ($\chi^2_{(2)}= 5.922$,$p=.052$). In addition, there were no differences in pre-deployment questionnaire scores for depression ($t_{(821)}= 0.385$, $p=.701$) and PTSD ($t_{(682)}= 0.247$, $p=.805$).

Depression scores at T2 were missing for 16 participants (2.1%). The remaining 733 participants were divided into two groups based on their level of depressive symptoms at T2. Participants were assigned to the depressive symptoms group when their score on the Symptom Checklist (SCL-90) depression subscale was ≥ 24 at T2 (n=68) (23). This cut-off corresponds to the mean plus 2 standard deviations (95^{th} percentile) on the SCL-90 depression subscale within a population of 840 Dutch military personnel (mean (SD): 18.06 (3.15)).

Participants scoring below the cut-off on the SCL-90 depression subscale at T2 were assigned to the non-depressed group (n=665).

Questionnaires

Level of depressive symptoms over the past week was assessed with the Dutch version of the Symptom Checklist (SCL-90) depression subscale (24). This subscale consists of 16 items ranging from 1 (not at all) to 5 (very much). The total depressive symptom score is the sum score for all items (range: 16-80). A higher score indicates more depressive symptoms. The questionnaire has good reliability and is frequently used within research and clinical settings. The validity of the subscale as a screening instrument for MDD has been shown in primary care patients (25), and in the aftermath of stroke (26) and myocardial infarction (27).

Depression and PTSD are frequently co-morbid (28). Therefore, the level of posttraumatic stress disorder (PTSD) symptoms over the past 4 weeks was assessed with the Dutch 22-item Self-Report Inventory for PTSD (SRIP). The questionnaire consists of three subscales representing the PTSD symptom clusters re-experiencing, avoidance and hyper-arousal. The total PTSD score is the sum score for all items (range: 22-88). The SRIP is well validated and has good concurrent validity with other PTSD measures such as the Clinician Administered PTSD Scale (CAPS) and Mississippi scale for PTSD (29, 30). Exposure to deployment-stressors was assessed with a 13-item checklist (31).

Collected demographics included age during deployment, sex, body height, weight, smoking, alcohol use, and use of possibly interfering medication (non-systemic glucocorticoids (nasal spray or crème), antihistamines, cholesterol lowering medications and antihypertensive medication). Body Mass Index (BMI) was calculated by dividing body weight by the square of body height (kg/m^2).

Cytokine production

CD2/CD28-induced T-cell cytokine production

Whole blood, diluted 1:10 with RPMI-1640 (Gibco, Grand Island, NY), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µM L-glutamine was stimulated with the T-cell mitogen anti-CD2/CD28 monoclonal antibodies (CLB, Amsterdam, Netherlands, final concentration anti-CD2.1/anti-CD2.2 0.33 µg/ml and anti-CD28 1.33 µlg/ml) for 72 hours at 37°C/5% CO2 in 96-well round-bottomed plates (32, 33). T cell mitogen-induced secretion of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, TNF-α, monocyte chemoattractant protein (MCP)-1 (CCL2), interferon-gamma induced protein (IP)-10 and RANTES (CCL5) were measured in supernatants using multiplex cytokine assay as described before (34-37). IFN-γ was analyzed by ELISA (CLB, Amsterdam, the Netherlands).

Lipopolysaccharide-induced innate cytokine production

Whole blood, diluted 1:10 with RPMI-1640 (Gibco, Grand Island, NY), was stimulated with Lipopolysaccharide (LPS, Escherichia Coli 0127:B8, Sigma, final concentration 1 ng/ml) for 24 hours at 37°C/5% CO2 in 96-well flat-bottomed plates to activate cytokine production. Supernatants were analyzed by using multiplex assay for the presence of IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α, as described previously (37).

FACS analysis

Leukocyte subsets in peripheral blood were assessed using dual colour fluorescence analysis with a Becton Dickinson Calibur flowcytometer. Whole blood was stained using monoclonal antibodies labelled with either fluorescine isothiocyanate or phyco-erythrin to quantify CD14+ (monocytes), CD3+ (total T-cells), CD4+ (T-helper/inducer) and CD8+ (T-cytotoxic/suppressor-effector) cells. Absolute numbers of cells were calculated from a total leukocyte count.

Data analyses

Basic statistical analyses were conducted using SPSS 15.0. Exploratory structural equation modeling (ESEM), multiple group latent growth modeling and logistic regression analysis were performed using Mplus 6.1 (38). Immune parameters and questionnaire scores were tested for normality and transformed when necessary (see Table 2 for applied transformations). A limited number of missing values in the immune parameters were present due to technical and handling problems (cytokines: T0: 0.91%, T1: 1.46%, T2: 2.00%; cell subsets: T0: 1.68%, T1: 1.41%, T2: 2.93%). Outliers in the immune parameters were removed if z-values were outside the range of ± 3.29 (39) (cytokines: T0: 0.57%, T1: 0.80%, T2: 0.89%; cell subsets: T0: 1.17%, T1: 0.85%, T2: 0.45%)

Exploratory structural equation modeling

For data reduction of the cytokines, exploratory structural equation modeling (ESEM) was used (21). Within this recently developed statistical method, exploratory factor analysis (EFA) is performed within a structural equation modeling (SEM) setting. Combining EFA with SEM provides the opportunity to assess goodness-of-fit indices and measurement invariance of the factor solution across time or groups, which was previously not possibly within EFA. ESEM is more appropriate to model complex biological data than conventional confirmatory factor analysis (CFA): whereas CFA only provides adequate model fit with simple structure (i.e. each indicator loads on one pre-determined factor), in ESEM all indicator loadings on all factors are estimated by default (21).

ESEM was performed, using data from T0 of all participants with 1-5 specified factors, using maximum likelihood estimation. To select the best fitting model the Aikake information

criterion (AIC (40)) and Bayesian information criterion (BIC (41)) of the models were compared. The model with the smallest AIC and BIC was chosen. Additionally, the comparative fit index (CFI), Tucker-Lewis index (TLI), root mean square error of approximation (RMSEA) and standardized root mean square residual (SRMR) were used to test the goodness-of-fit of the models. Adequate fit was defined as CFI>0.9, TLI>0.9, RMSEA<0.08, and SRMR<0.08 (42).

Subsequently, it was investigated whether the chosen ESEM model for T0 was stable (i.e. measurement invariant) across time. For reliable estimation of differences in mean factor scores across time, the presence of scalar and metric measurement invariance (i.e. similar factor loadings and item means across time) is a prerequisite (42, 43). For this purpose ESEM with target rotation was performed, using data from T0-T2. Insignificant factor loadings at T0 were estimated at 0 in the target model to simplify the final model (21).

To deal with missing data, full information maximum likelihood estimation (FIML) was used, which includes all available data in the model. Thus, individuals with missing time points or missing values within time points were retained in the analyses. This provides more reliable estimates compared to other methods of handling missing data, such as listwise deletion or mean imputation (44).

Multiple group latent growth modeling

Within latent growth modeling, the average starting point (intercept) and average change over time (slope) are estimated in longitudinal data (22). In multiple group latent growth modeling (LGM), models are estimated simultaneously across groups. Using multiple group LGM we investigated whether the intercept and slope of questionnaire scores, cytokine production (factor scores) and cell subsets differed between participants with and without a high level of depressive symptoms at T2. A two-step model was used. First, means of the intercepts and slopes were freely estimated across groups. Then, a model was examined in which the intercept and slope were constrained to be equal for both groups. Chi-square difference testing ($\Delta \chi^2$) was used to formally test whether the more constrained model fitted the data as well as the non-constrained model: a significant $\Delta \chi^2$ indicates that the more constrained model fits the data significantly worse (38). When significant group differences in intercept and slope were found, the association between possibly confounding variables and the intercept and/or slope of both groups was tested.

Logistic regression analysis

Logistic regression analysis was performed to test the predictive value of cytokine factor scores for the presence of depressive symptomatology. In the regression analysis, predeployment SCL-90 depression score and BMI, which differed between groups, were included to determine that the observed association between cytokine factors scores and depression were not confounded by these variables. To be able to compare the odds ratios associated with the included variables, variables were standardized (mean (SD): 0(1)).

RESULTS

Participant characteristics

Participants were assessed 1 to 2 months before a 4-month military deployment to Afghanistan (T0), and one (T1) and six (T2) months after return from deployment. 68 (9.3%) participants reported a high level of depressive symptoms at T2. The intensity and frequency of depressive symptoms increased over time in the group with depressive symptoms at T2, while the mean depressive symptom score of the non-depressed group decreased slightly over time. The group with a high level of depressive symptoms at T2 already had more depressive symptoms before deployment than the non-depressed group (depressive symptoms group: intercept: 1.327(0.013), p<.001; slope: 0.010(0.001), p<.001. non-depressed group: intercept: 1.241 (0.002), p<.001; slope: −0.001 (0.000), p=.004; $\Delta \chi^2_{(3)}$= 130.939, p<.001).

Individuals with depressive symptoms at T2 reported higher levels of PTSD symptoms at T2 ($t_{(726)}$= −17.546, p<.001), although the variance in SRIP total scores was considerable. In addition, individuals with a high level of depressive symptoms at T2 reported a larger number of deployment stressors, including a larger number of combat experiences (Table 1). Furthermore, BMI at T0 was higher in individuals who had depressive symptoms at T2 ($t_{(675)}$= −2.203, p=.028).

Data reduction of cytokine parameters

We determined 10 CD2/CD28-induced and 6 LPS-induced cytokines before (T0), one (T1) and six months (T2) after deployment. Non-transformed values and applied transformations for the CD2/CD28- and LPS-induced cytokine production are reported in Table 2. We performed data reduction by exploratory structural equation modeling (ESEM) on the data from T0 of all participants. Correlation matrices of the immune parameters revealed that IL-8 was only weakly correlated with the other variables (strongest correlation −.13 with MCP1). IL-8 was therefore not included in the factor analyses. We analyzed consecutive ESEM models with 1-5 factors respectively. According to the AIC and BIC measures, the 5-factor model provided the best fit (Table 3). However, within this model the estimated residual variance for CD2/CD28-induced IL-4 was negative, and therefore this variable was removed from the model (45). We reran the 5-factor model without CD2/CD28-induced IL-4 and obtained a better fit compared to the 5-factor model including IL-4 (AIC= 34271, BIC= 34704). Within this model the estimated factor loading for LPS-induced IL-6 was larger than 1. This cytokine only loaded on one factor, with small factor loadings from the other variables on this factor (largest standardized loading: .044), and was removed from the model as suggested (45). We then analyzed a 4 factor model without CD2/CD28-induced IL-4 and LPS-induced IL-6. The model had good fit and lower AIC/BIC than the previous model (CFI= .962, TLI= .908,

TABLE 1. Questionnaire scores and deployment, demographic and pre-deployment characteristics of the total sample, and the sample divided based on depressive symptoms six months after return from deployment.

	Total sample (n = 1023)	Depressive symptoms (n = 68)	No depressive symptoms (n = 665)	P
Mean (SD)				
SCL-90 depression score at T0 (range: 16-80)	18.06 (3.15)	22.15 (5.47)	17.55 (2.33)	<.001
SCL-90 depression score at T1 (range: 16-80)	18.30 (3.83)	24.63 (5.87)	17.71 (3.10)	<.001
SCL-90 depression score at T2 (range: 16-80)	18.34 (4.16)	29.12 (5.39)	17.24 (1.75)	<.001
SRIP (PTSD) total score at T2 (range: 22-88)	27.79 (7.15)	40.44 (10.88)	26.54 (5.21)	<.001
Nr. of deployment stressors (range: 0-13)	4.77 (2.55)	5.63 (2.40)	4.75 (2.55)	.013
Age during deployment	28.44 (8.89)	29.54 (8.84)	28.98 (9.20)	.629
Nr. of previous deployments	0.83 (1.16)	1.08 (1.31)	0.92 (1.23)	.268
BMI at T0	24.71 (2.78)	25.54 (3.41)	24.70 (2.74)	.028
N (%)				
Gender				.729
Male	931 (91.0%)	61 (98.7%)	605 (91%)	
Female	92 (9.0%)	7 (10.3%)	60 (9%)	
Education				.675
Low	371 (40.0%)	29 (44.6%)	242 (39.2%)	
Middle	454 (48.9%)	28 (43.1%)	299 (48.5%)	
High	103 (11.1%)	8 (12.3%)	76 (12.3%)	
Rank during deployment				.844
Soldier	400 (40.2%)	24 (35.3%)	246 (37.1%)	
Corporal	206 (20.7%)	16 (23.5%)	134 (20.2%)	
Non-commissioned officer	255 (25.7%)	20 (29.4%)	185 (27.9%)	
Officer	133 (13.4%)	8 (11.8%)	98 (14.8%)	
Smoking at T0 (yes)	407 (44.5%)	28 (43.8%)	258 (42.2%)	.814
Alcohol use at T0				.786
None	93 (10.3%)	7 (11.3%)	63 (10.5%)	
0-20 units/week	754 (83.9%)	50 (80.6%)	502 (83.5%)	
> 20 units/week	52 (5.8%)	5 (8.1%)	36 (6.0%)	
Medication use at T0 (yes)	56 (5.5%)	3 (4.4%)	42 (6.3%)	.533
Season of assessment at T0				.181
Spring	343 (33.5%)	27 (39.7%)	204 (30.7%)	
Summer	102 (10.0%)	9 (13.2%)	67 (10.1%)	
Autumn	308 (30.1%)	13 (19.1%)	202 (30.4%)	
Winter	270 (26.4%)	19 (27.9%)	192 (28.9%)	

TABLE 2. CD2/CD28- and LPS- induced cytokine and chemokine production before (T0), one (T1) and six (T2) months after deployment.

	T0			T1			T2		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
CD2/CD28									
IL-2 [a]	1082.6	1369.1	995	1243.9	1591.0	819	1387.1	1654.1	743
IL-4 [a]	79.2	84.5	1002	115.2	129.4	814	125.2	135.0	742
IL-5 [a]	613.1	1008.1	983	642.4	1026.0	811	715.2	1088.4	736
IL-6 [a]	1229.5	1348.6	991	977.1	831.0	810	937.1	843.6	742
IL-10 [b]	432.7	356.8	999	543.3	454.4	817	589.1	440.7	744
TNF-α [a]	560.8	475.7	1000	837.0	853.1	802	1209.3	1089.8	733
IFN-γ [a]	20593.2	23593.4	998	21117.9	21951.8	764	28376.0	34652.1	708
MCP-1 [a]	18308.0	14514.0	998	14577.4	10102.1	817	12728.2	8818.9	741
IP-10 [b]	5977.5	3485.5	994	7355.7	4539.4	818	9099.8	5977.1	744
RANTES [a]	7142.0	6893.1	995	6298.5	4833.4	815	5069.5	4514.0	741
LPS									
IL1-α [a]	68.3	51.1	1002	60.1	47.0	793	57.0	41.4	721
IL1-β [b]	314.4	227.4	996	260.6	179.0	802	241.1	156.6	730
IL-6 [b]	2951.7	1812.7	1008	2492.8	1988.3	806	2201.1	1286.4	712
IL-8 [b]	1955.9	1144.3	1006	2048.0	1087.3	805	2069.7	1076.8	725
IL-10 [a]	33.8	35.6	1006	28.8	25.5	805	31.0	28.7	733
TNF-α [a]	851.5	677.9	1005	578.7	467.5	796	489.2	385.7	729

[a] log10-transformation applied; [b] square root-transformation applied.

TABLE 3. Fit and measurement parameters of the subsequent exploratory structural equation modeling (ESEM)-models.

	Model Fit							Model Comparison	
	χ^2	df	CFI	TLI	RMSEA	90% C.I. RMSEA	SRMR	AIC	BIC
1-factor ESEM model	5281.581	90	.427	.332	.239	.233-.244	.187	38895	39117
2-factor ESEM model	2267.782	53	.700	.559	.203	.196-.210	.090	20241	20492
3-factor ESEM model	1744.690	63	.814	.691	.162	.156-.169	.050	35412	35767
4-factor ESEM model	1037.845	51	.891	.776	.138	.131-.146	.040	34729	35143
5-factor ESEM model	577.735	40	.941	.844	.115	.107-.124	.027	34291	34759
5-factor ESEM model without CD2/CD28-induced IL-4	273.136	31	.968	.906	.088	.078-.097	.024	34271	34704
4-factor ESEM model without CD2/CD28-induced IL-4 and LPS-induced IL-6	270.840	32	.962	.908	.086	.077-.095	.026	27052	27406

χ^2: Pearson's Chi Square; df: degrees of freedom; CFI: comparative fit index; TLI: Tucker-Lewis Index; RMSEA: root mean square error of approximation; 90% CI: 90% confidence interval; SRMR: standardized root mean square residual; AIC: Aikake information criterion; BIC: Bayesian information criterion.

RMSEA= .086, SRMR= .026, AIC= 27052, BIC= 27406). The factor structure was similar at T1 and T2 compared to T0 (i.e. model with scalar and metric measurement invariance fitted the data better than the free model) (model fit: CFI= .862, TLI= .835, RMSEA= .072, SRMR= .074, AIC= 63043, BIC= 64015).

The first factor that emerged out of the ESEM including the data from T0-T2 contained CD2/CD28-induced cytokines. Given the evidence that CD2/CD28 is a strong activator of T-cells (33), we refer to Factor 1 as T-cell cytokine production. Since CD2 is also expressed on NK-cells, NK-cells may also contribute to the production of the cytokines in this factor. The second factor included all CD2/CD28-induced chemokines and CD2/CD28 induced IL-6 (referred to as T-cell-induced chemokine/IL-6 production). The third factor included all LPS-induced cytokines (referred to as innate cytokine production). The fourth factor contained CD2/CD28-induced IP-10 with lower loadings of CD2/CD28-induced TNF-α and RANTES and LPS-induced IL-10. This factor could not be interpreted in a functional way, and therefore we chose not to include it in the subsequent analyses. The final factor solution is depicted in Table 4. Most cytokines had significant cross-loadings, underscoring the appropriateness of using an ESEM model.

TABLE 4. Final exploratory structural equation modeling (ESEM) factor solution.

	Factor 1	Factor 2	Factor 3	Factor 4
	T-cell cytokines	T-cell-induced chemokines/IL-6	Innate cytokines	Residual factor
CD2/CD28				
IL-2	**.815**	−.048	.013	−.071
IL-5	**.663**	.044	−.036	.010
IL-6	**.462**	**.488**	.024	−.107
IL-10	**.662**	−.154	.000	.094
TNF-α	**.812**	.142	.054	.206
IFN-γ	**.867**	.001	.022	−.068
MCP-1	−.019	**.835**	.137	−.014
IP-10	**.491**	**.388**	.000	**.447**
RANTES	**.455**	**.518**	−.063	−.258
LPS				
IL-1α	−.061	**−.322**	**.837**	.173
IL-1β	.001	.001	**.700**	.001
IL-10	−.068	−.137	**.507**	.232
TNF-α	−.060	.054	**.715**	−.180

Indicator loadings > .3 are depicted in bold.

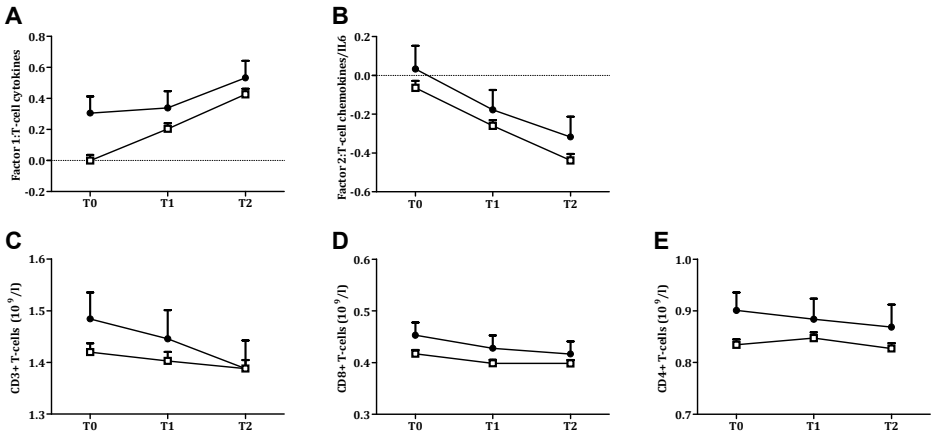
Longitudinal trajectory of mitogen-induced T-cell cytokine production

We investigated whether the longitudinal course of T-cell cytokine production (Factor 1) differed between the group with high levels of depressive symptoms at T2 and the non-depressed group. The multiple group latent growth model in which the intercept and slope of both groups were constrained to be equal, fitted the data less well (AIC= 4975, BIC= 5039) ($\Delta \chi^2_{(2)}$= 8.784, p=.012) than the model in which the intercept and slope were freely estimated for both groups (CFI= .990, TLI= .971, RMSEA= .101, SRMR=.020, AIC= 4970, BIC= 5043). Additional models constraining the intercept and slope separately confirmed that both intercept and slope differed between the two groups (intercept-model: $\Delta \chi^2_{(1)}$= 7.449, p=.006; slope-model: $\Delta \chi^2_{(1)}$= 4.395, p=.036).

Before deployment, the group with depressive symptoms at T2 already had higher T-cell cytokine production than the non-depressed group (depressive symptoms group: 0.296 (0.103), p=.004; non-depressed group: −0.016 (0.036), p=.661). T-cell cytokine production increased over time for both groups, but the magnitude of the increase was significantly smaller in the depressed group (depressive symptoms group: 0.018 (0.007), p=.003; non-depressed group: 0.033 (0.003), p<.001) (Figure 1A).

Since the group with depressive symptoms at T2 also had a higher level of PTSD symptoms at T2, we investigated whether this might have influenced the observed association between depressive symptoms and T-cell cytokine production. PTSD symptoms at T2 were

FIGURE 1. Longitudinal course of T-cell cytokine production and T-cell counts.



Longitudinal course of CD2/CD28-induced Factor 1: T-cell cytokine production (A); CD2/CD28-induced Factor 2: T-cell chemokine/IL-6 production (B) and CD3+ T-cell counts (C); CD8+ cytotoxic/suppressor-effector T-cell counts (D) and CD4+ T helper-cell counts (E), as assessed before deployment (T0), one month (T1) and six months (T2) after return from deployment. The course of the group with depressive symptoms at T2 (n=69) is depicted by the black circles (mean + SEM). The course of the group without depressive symptoms at T2 (n=664) is depicted by the white rectangles (mean + SEM).

not significantly associated with the intercept and slope of T-cell cytokine production in both groups (depressive symptoms group: intercept: 0.383 (0.871), p=.660; slope: 0.027 (0.053), p=.610; non-depressed group: intercept: −0.469 (0.483), p=.0332; slope: 0.036(0.036), p=.316).

In addition, since the group with depressive symptoms reported a higher number of deployment stressors, we investigated whether the group difference in experienced deployment stressors might explain the difference in change in cytokine production over time between the groups. However, the number of reported deployment-stressors was not significantly associated with the slope of T-cell cytokine production in both groups (depressive symptoms group: −0.003 (0.003), p=.254; non-depressed group: −0.001 (0.001), p=.257). These findings indicate that the group difference in the trajectory of cytokine production over time cannot be attributed to differences in the number of experienced deployment stressors.

We investigated whether the observed higher level of T-cell cytokine production in the depressed group was associated with group differences in the longitudinal course of total numbers of T-cells (CD3+), cytotoxic/suppressor-effector T-cells (CD8+), and T-helper cells (CD4+). The intercept and slope of the cell subsets did not differ between groups for CD3+ T-cells ($\Delta \chi^2_{(2)}$= 2.038, p=.361), CD8+ cytotoxic/suppressor-effector T-cells ($\Delta \chi^2_{(2)}$= 4.189, p=.123), and CD4+ T-helper cells ($\Delta \chi^2_{(2)}$= 2.088, p=.352) (Figure 1CDE). In addition, the increased T-cell cytokine production over time was not caused by an increase in the number of T-cells or T-cell subsets over time. The number of CD8+ cytotoxic/suppressor-effector T-cells decreased over time within both groups (depressive symptoms group: −0.001 (0.000), p=.018; non depressed group: 0.000 (0.000), p=.002. The total number of T-cells (depressive symptoms group: −0.007 (0.004), p=.115; non-depressed group: −0.002 (0.001), p=.132) and T-helper cells did not significantly change over time (depressive symptoms group: −0.001 (0.003), p=.819; non-depressed group: 0.000 (0.000), p=.899).

Longitudinal trajectory of mitogen-induced T-cell chemokine/IL6 production

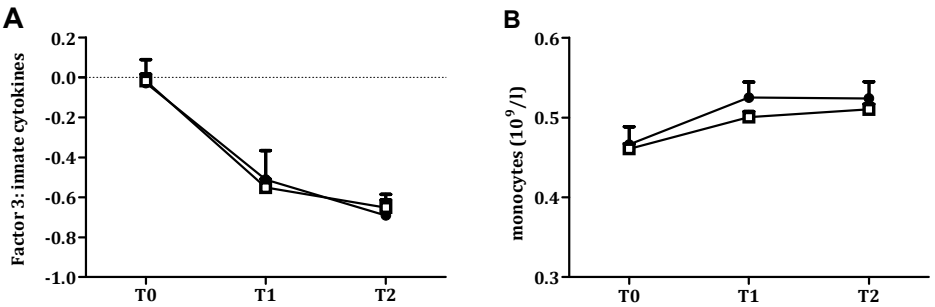
Next, we investigated whether the longitudinal course of T-cell-induced chemokine/IL-6 production (Factor 2) differed between the group with high levels of depressive symptoms at T2 and the non-depressed group. T-cell chemokine production decreased over time for both groups (depressive symptoms group: −0.027(0.008), p=.001; non-depressed group: −0.028 (0.003), p<.001) (Figure 1B). Comparison of the models with freely estimated (AIC= 4875, BIC= 4948) and constrained intercepts and slopes (CFI= 1.000, TLI=. 1.000, RMSEA= .000, SRMR= .022, AIC= 4872, BIC= 4936) showed that the intercept and slope did not significantly differ between the two groups ($\Delta \chi^2_{(2)}$= 1.258, p=.533).

Longitudinal trajectory of mitogen-induced innate cytokine production

Innate cytokine production (Factor 3) decreased over time for both groups (depressive symptoms group: −0.052 (0.010), p<.001; non-depressed group: −0.051 (0.003), p<.001) (Figure 2A). The model fit of the models with freely estimated intercepts and slopes (AIC= 5726, BIC= 5749) was not significantly better than the model fit of the model with constrained intercepts and slopes (CFI= .974, TLI= .961, RMSEA= .090, SRMR= .037, AIC= 5722, BIC= 5787) ($\Delta \chi^2_{(2)}$= 0.592, p=.745). Therefore we concluded that there were no differences in intercept and slope between groups.

The decrease in innate cytokine production over time was not due to a decrease in total numbers of monocytes over time. In fact, the total number of monocytes significantly increased over time within both groups (depressive symptoms group: 0.002 (0.000), p<.001; non-depressed group: 0.001 (0.000), p<.001) (Figure 2B). There were no differences in the intercept and slope between depressed and non-depressed individuals (fit unconstrained model: AIC= 6311, BIC= 6240; fit constrained model: CFI= .992, TLI= .988, RMSEA= .048, SRMR= .026, AIC= 6312, BIC= 6250; $\Delta \chi^2_{(2)}$= 2.980, p=.225).

FIGURE 2. Longitudinal course of innate cytokine production and monocytes.



Longitudinal course of LPS-induced Factor 3: innate cytokine production (A); and monocyte counts (B), as assessed before deployment (T0), one month (T1) and six months (T2) after return from deployment. The course of the group with depressive symptoms at T2 (n=69) is depicted by the black circles (mean + SEM). The course of the group without depressive symptoms at T2 (n=664) is depicted by the white rectangles (mean + SEM).

Predictive value of T-cell cytokine production at T0 for depressive symptomatology at T2

Multiple group LGM revealed that individuals with high levels of depressive symptoms had higher T-cell cytokine production at T0, compared to individuals who did not have depressive symptoms at T2. The predictive value of T-cell cytokine production at T0 for the presence of a high level of depressive symptoms at T2 was investigated using logistic regression analysis. We also included depressive symptoms and BMI at T0, to ascertain that these

pre-deployment group differences did not confound our results. T-cell cytokine production at T0 significantly and independently predicted the presence of depressive symptomatology at T2 (estimate (SE): 0.615 (0.191), p=.001): with each standard deviation increase in T-cell cytokine production at T0 the odds for the presence of a high level of depressive symptoms at T2 increased approximately 1.9-fold (Odds Ratio: 1.850). In addition, depressive symptoms at T0 also significantly predicted the presence of a high level of depressive symptoms at T2 (Odds ratio for 1 SD increase: 2.955; estimate (SE): 1.083 (0.141), p<.001). Pre-deployment BMI was not significantly associated with the presence of depressive symptoms at T2 (Odds ratio for 1 SD increase: 1.254; estimate (SE): 0.226 (0.141), p=.103).

DISCUSSION

This is the first prospective, longitudinal study in which associations between the capacity of T-cells and monocytes to produce cytokines and the development of depressive symptoms in response to a period of severe stress have been investigated. We aimed to investigate whether T-cell and monocyte cytokine production represent vulnerability factors for the development of a high level of depressive symptoms in response to a period of severe stress.

To the best of our knowledge, the association between depression and the capacity of monocytes and T-cells to produce cytokines has only been investigated using cross-sectional designs. Moreover, these cross-sectional studies predominantly determined innate cytokines and investigated only a limited range of pro-inflammatory cytokines. We used a unique design: within a large cohort of participants, three assessments were performed, spanning a time period from approximately one month before deployment to a combat-zone, until approximately six months after return. Moreover, we investigated the production of a broad range of LPS- and CD2/CD28-induced pro- and anti-inflammatory cytokines, and also included several chemokines in our analyses.

The majority of our participants appeared to be resilient and reported no depressive symptoms six months after deployment. However, 10% of the participants reported a high level of depressive symptoms six months after deployment. We aimed to investigate whether these participants with high levels of depressive symptoms had different longitudinal trajectories in cytokine production than the participants who did not become depressed. For this purpose, we decided to use a dichotomous approach in which participants were divided into groups with high and low levels of depressive symptoms six months after deployment (i.e. a score above or below cut-off on the SCL90 depression subscale).

Our results show that individuals with a high level of depressive symptoms six months after return from deployment already had higher T-cell mitogen-induced cytokine production before deployment compared to individuals without depressive symptoms six months after return. Moreover, T-cell cytokine production before deployment was a significant predictor of the presence of depressive symptoms after deployment. Although the group

with depressive symptoms after deployment already had a larger mean depression score before deployment, the observed predictive value of the T-cell cytokines was independent of the severity of depressive symptoms before deployment. Thus, our findings indicate that high mitogen-induced T-cell cytokine production before deployment is a pre-existing vulnerability factor for the development of depressive symptoms in response to a period of severe stress, in this case military deployment. In addition, the observed high T-cell cytokine production was not caused by the presence of posttraumatic stress symptoms in a subset of participants with depressive symptoms: the depressive group did report a higher level of posttraumatic stress symptoms six months after deployment, but the T-cell cytokine production was unrelated to the severity of these posttraumatic stress symptoms.

We did not observe differences in the number of T-cells (CD3+, CD4+ and CD8+) before deployment between individuals with and without depressive symptoms after deployment. Hence, the observed higher T-cell cytokine production before deployment in individuals with depressive symptoms after deployment is not caused by higher numbers of T-cells and T-cell subsets in the blood samples obtained from these individuals. Therefore, we propose that the higher T-cell cytokine production in individuals with depressive symptoms reflects a higher capacity of T-cells to produce cytokines that may be related to an increased activation status of these cells.

Higher circulating serum levels of soluble receptors for the T-cell cytokine IL-2 (sIL-2r) have been observed previously in individuals with MDD and depressive symptomatology compared to non-depressed individuals (18-20). In addition, in individuals with cancer, treatment with high doses of the T cell cytokine IL-2 induces a cluster of mood and cognitive symptoms, overlapping with MDD (46) and the depressive symptoms observed in our study, in up to 60% of treated individuals, depending on the dose and modality of treatment (47-49). Interestingly, higher levels of circulating T-cell cytokines in serum were found to be predictive for the development of MDD in response to cytokine administration: higher levels of circulating sIL-2r, and of the anti-inflammatory cytokine IL-10 (50) before the start of IFN-α treatment were associated with increased risk for development of MDD in response to the treatment. These previous findings support our observation of the involvement of both pro- and anti-inflammatory T-cell cytokines in the development of depressive symptomatology.

Our data may also add to the theory that increased cytokine production leads to increased signalling to brain structures involved in the development of depressive symptomatology (48). The pro-inflammatory cytokine IFN-γ had the highest loading on our T-cell cytokine factor, and therefore had the largest contribution to the score of each participant on the T-cell cytokine factor. Studies on inflammation-induced depressive-like behavior in mice have shown that IFN-γ may be a pivotal mediator in the development of depressive-like behavior, since the development of depressive-like behavior after immune activation by Bacille Calmette-Guerin (BCG) was completely attenuated in IFN-γ receptor knock-out mice (51).

Indoleamine 2,3-dixoygenase (IDO) is a likely intermediate in this relationship (for review see 47, 48, 52). This tryptophan metabolising enzyme is upregulated by pro-inflammatory cytokines like IFN-γ and inhibition of IDO activity as well as genetic ablation of IDO prevents development of depressive-like behavior in response to BCG (53). IDO-dependent degradation of tryptophan leads to production of its metabolite kynurenine, which is further metabolised to quinolinic acid and kynurenic acid, an NMDA-receptor agonist and NMDA-receptor antagonist respectively. Glutamergic dysfunction has been implicated in the development of MDD. In addition, trypophan/kynurenine and kynurenic acid/kynurenine ratios appear to be distorted in individuals with cytokine-induced MDD as well as in otherwise healthy individuals with MDD (48).

We observed pre-existing higher levels of T-cell cytokine production in individuals who subsequently developed depressive symptomatology after exposure to a period of severe stress, i.e. military deployment, suggesting increased T-cell functioning prior to the development of depressive symptoms. In apparent contrast to our data, decreased mitogen-induced T-cell proliferation in vitro and decreased virus-specific T-cell responses in vivo have been observed in individuals who already had developed MDD (54, 55), indicating decreased T-cell function within these individuals. Based on these previous studies, we speculate that depressive symptomatology may decrease T-cell function over time. In this respect it is of interest that IDO can inhibit T-cell function (52). It is possible that the observed higher capacity of T-cells to produce cytokines in individuals vulnerable to development of depressive symptoms eventually results in upregulation of IDO by peripheral antigen presenting cells. Therefore, one could suggest that in the long run, upregulation of IDO may contribute to the downregulation of T-cell function in depressed individuals.

Exploratory structural equation modeling, in which exploratory factor analysis is performed within a structural equation modeling setting (21), was used for reduction of the cytokine data. This advanced statistical method permitted us to reduce the complex dataset into factor scores and to investigate the longitudinal stability of our obtained factor solution. The raw data of T-cell-produced cytokines and monocyte-induced cytokines support our factor solution in which pro- and anti-inflammatory cytokines load on the same factors: we did not observe differences in the direction of the observed change over time between pro- and anti-inflammatory cytokines.

The longitudinal design of our study also allowed us to investigate the effect of military deployment on mitogen-induced cytokine production within the whole sample. Military deployment induced changes in mitogen-induced T-cell cytokine production, T-cell-induced chemokine/IL6 production and innate cytokine production. Interestingly, the direction of the observed change in cytokine production upon stimulation differed between monocytes and T-cells. Innate cytokine production decreased over time. In contrast, the total number of monocytes increased over time for both groups, indicating that the total capacity of a single monocyte to produce cytokines upon stimulation decreased even more strongly over time.

The production of T-cell-induced chemokines and IL-6 after stimulation also decreased over time, as well as the number of cytotoxic/suppressor-effector T-cells (CD8+). In contrast, the T-cell cytokine production in response to a mitogen was increased after return from deployment. The observed changes in cytokine production over time were not caused by differences in season during the subsequent assessments influenced our results.

Our group previously reported that mitogen-induced cytokine production was not a stable trait, but differed over time in healthy adolescents, depending on the season of assessment (35). Nevertheless, after correcting our latent growth models for the seasons in which the subsequent assessments took place, we still observed significant changes in cytokine production over time (data not shown). In addition, the changes in cytokine production over time were not caused by differences in the duration of sample storage in the freezer, since the participants were included in cohorts and therefore the samples from the first assessments have not all been stored longer than samples from the second and third assessments. In addition, if the changes had resulted from degradation of samples then we would have expected that the changes over time were all in the same direction.

It was previously reported that severely stressed individuals (56), including deployed military personnel (57, 58), are at increased risk for later development of medical conditions. It may well be that the observed decreased innate cytokine production after deployment is involved in this increased risk for development of medical conditions, such as bacterial infections. In addition, if the increased capacity of T-cells to produce cytokines lasts for a long period or becomes a stable feature, it may contribute to development of inflammatory conditions, such as atherosclerosis (59, 60).

The observed change in cytokine and chemokine production was long-lasting: six months after return the effect was still present. Future studies should investigate how long after return from deployment the observed changes in cytokine production last. This is important since we observed that high T-cell cytokine production is a risk factor for development of depressive symptoms. If a new stressor would occur when the observed T-cell cytokine production has not yet decreased, it may well be possible that the increased T-cell cytokine production facilitates development of depressive symptomatology in response to this new stressor. Indeed, there is evidence that soldiers who have been deployed previously are at increased risk for development of combat-related MDD (61). However, not all studies have found an association between previous deployment and development of depression in response to a new deployment (62). One possible confounding factor could be that the interval between deployments differs between studies and between participants within the studies. In future studies, it would be interesting to take the interval between the deployments into account while investigating this relationship.

In addition to the observed pre-existing difference in T-cell cytokine production, individuals with depressive symptoms after deployment also responded differently to the deployment: the group with depressive symptoms after deployment showed a smaller increase

in mitogen-induced T-cell cytokine production in response to deployment than the non-depressed group. This observed group difference in the trajectory of T-cell cytokine production over time could not be attributed to a difference in the number of experienced deployment stressors. However, we cannot exclude that potential differences in the experienced subjective severity of these stressors did affect the trajectory of T-cell cytokine production.

The development of depressive symptoms was not associated with the change in T-cell-induced chemokine/IL-6 production and innate cytokine production over time. In addition, innate cytokine production and chemokine production before deployment were also unrelated to development of depressive symptoms

In individuals who already had developed MDD or depressive symptoms, increases in innate cytokine production have repeatedly been reported (2-6). Based on our findings we hypothesize that an increase in mitogen-induced production of innate cytokines may develop only as a consequence of the presence of depressive symptomatology and does not represent a vulnerability factor for development of depressive symptoms.

A limitation of the current study is that depressive symptoms were assessed with self-report questionnaires, and that official MDD diagnoses could therefore not be made. However, the validity of the used SCL-90 depression subscale has repeatedly been investigated and the questionnaire has been found to be a valid and reliable screening tool for the presence of MDD (25-27). Another limitation is that we did not include a non-deployed control group. We interpret the observed changes in mitogen-induced cytokine production over time as consequences of the severe stress experienced during the deployment, but we cannot exclude that other factors contribute to the observed changes in mitogen-induced cytokine production.

The current study is the first to show that the capacity of monocytes and T-cells to produce cytokines and chemokines was altered after exposure to severe stress, i.e. deployment to a combat-zone, until at least six months after return. In addition, we found that pre-existing high mitogen-induced T-cell cytokine production is a predictor for the development of depressive symptoms in response to military deployment. We propose that high mitogen-induced T-cell cytokine production may be a vulnerability factor for increased risk for development of depressive symptomatology after exposure to a period of severe stress, such as experienced during military deployment.

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8

IL-1β reactivity and the development of severe fatigue: a longitudinal study.

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ABSTRACT

It has been suggested that pro-inflammatory cytokine signaling to the brain may contribute to severe fatigue. We propose that not only the level of circulating cytokines, but also increased reactivity of target cells to these cytokines contribute to the effect of cytokines on behavior. Based on this concept, we assessed the reactivity of peripheral blood cells to IL-1β *in vitro* as a novel approach to investigate whether severe fatigue is associated with increased pro-inflammatory signaling. We included 504 military personnel before deployment to a combat-zone. We examined fatigue severity and IL-8 production after *in vitro* stimulation with IL-1β one to two months prior to deployment (T0), and one month (T1) and six months (T2) after deployment.

At T2, the group who had developed severe fatigue (n=65) had significantly higher IL-1β-induced IL-8 production than the non-fatigued group (n=439). This group difference was not present at T0, but developed over time. Longitudinal analysis revealed that in the non-fatigued group, IL-1β-induced IL-8 production decreased over time, while IL-1β-induced IL-8 production in the fatigued group did not decrease in response to deployment. To determine whether the observed group difference was specific for IL-1β reactivity, we also analyzed LPS-induced IL-8 production. We did not observe group differences in LPS-induced IL-8 production at the three time points tested.

Collectively, our findings indicate that severe fatigue is associated with a higher reactivity to IL-1β signaling. We propose that assessment of the reactivity of the immune system to IL-1β may represent a promising novel method to investigate the association between behavioral abnormalities and pro-inflammatory cytokine signaling.

INTRODUCTION

The experience of prolonged severe fatigue after return from military deployment is a common phenomenon. The prevalence of severe fatigue in Dutch military personnel one to four years after return from deployment to Cambodia, Rwanda, and Bosnia has been estimated to be 7.6-12.4 times higher than in non-deployed military personnel (1). In addition, the prevalence of chronic fatigue syndrome (CFS)-like symptoms in US military personnel five years after return from deployment to the Gulf Region was 6.8 - 9.1-times higher compared to non-deployed military personnel (2).

It has been suggested that the development of severe fatigue may result from behavioral consequences associated with increased pro-inflammatory signaling (for review: 3-7). An increase in pro-inflammatory signaling may result from increased levels of circulating pro-inflammatory cytokines. Consistent with this notion, increased levels of serum pro-inflammatory cytokines have repeatedly been observed in individuals with severe fatigue or CFS compared to non-fatigued individuals (for review: 3-7), However, not all results of studies on cytokines levels in fatigue are consistent with increased levels of circulating pro-inflammatory cytokines: decreased or unaltered levels of pro-inflammatory serum cytokine levels also have been described in severely fatigued individuals compared to non-fatigued individuals (for review: 3-7).

The response of the body to an inflammatory mediator or other regulatory mediators does not only depend on the circulating levels of the specific mediator at a given moment, but also depends on the sensitivity or reactivity of the target system to regulation by the specific mediator. This reactivity of the target cells is determined at the level of the receptor, by receptor number, ligand binding affinity and coupling of the receptor to intracellular signaling pathways. In addition, intracellular processes downstream of the receptor determine the reactivity of a cell to regulation by specific mediators (8). Thus, an increase in pro-inflammatory signaling may also result from increased reactivity of target cells to pro-inflammatory cytokines.

Based on this concept, we assessed IL-1β-induced cytokine production by peripheral blood cells *in vitro* to determine whether severe fatigue is associated with altered reactivity of immune cells to pro-inflammatory cytokines. We selected IL-1β because of the existing evidence for a pivotal role of IL-1β signaling in the behavioral consequences of inflammation. For example, systemic or central administration of IL-1β triggers the development of sickness behavior in rodents (for review: 9). Moreover, the development of peripheral inflammation-induced sickness behavior in rodents can be completely prevented when IL-1 action is blocked (for review: 10). Furthermore, the fatigue symptoms of patients with the chronic inflammatory disease rheumatoid arthritis were significantly reduced after administration of an IL-1-receptor antagonist (11). In peripheral blood mononuclear cells, IL-1β induces the production of pro-inflammatory cytokines, including IL-8 (12). Therefore altered

IL-8 production by peripheral blood mononuclear cells in response to exposure of these cells to IL-1β is an indicator of altered reactivity of IL-1receptors and/or downstream signaling pathways.

We assessed whether military personnel with and without severe fatigue six months after return from deployment to a combat-zone differed in IL-1β-induced IL-8 production by peripheral blood cells, as assessed *in vitro* at six months (T2) after return from deployment. We also investigated the longitudinal course of IL-1β-induced IL-8 production in samples obtained prior to (T0), one month (T1) and six months after deployment (T2).

METHODS

General procedure

Military personnel of the Dutch Armed Forces assigned to a 4-month deployment were included on a voluntary basis. Their duties during deployment included combat patrols, clearing or searching buildings, participation in de-mining operations, and transportation across enemy territory. Typical combat-zone stressors included enemy fire, armed combat and combat casualties. We included participants deployed from 2006 to 2009. Participants were assessed one to two months prior to deployment (T0), and approximately one (T1) and six months (T2) after their return from deployment. During each assessment, participants filled out questionnaires. In addition, a heparinized blood sample was drawn between 8.00 and 11.30 a.m. Heparinized blood was kept at room temperature. The study was approved by the Institutional Review Board of the University Medical Center Utrecht. Written informed consent was obtained after a written and verbal description of the study.

Participants

721 participants completed questionnaires and blood sampling for measurement of IL-1β sensitivity before deployment (T0). Since we were interested in the development of severe fatigue in response to deployment, we excluded 32 (4.4%) participants who already reported high levels of fatigue before deployment, resulting in 689 participants at T0. 12 participants (1.7%) were not available for follow-up (non-deployed (n=10); deceased during deployment (n=2)). Of the eligible 677 participants after deployment, 504 participants (74.4%) completed the assessments at T1 and T2. Participants were divided into groups based on their level of fatigue at T2. A total of 65 participants (12.9%) reported severe fatigue at T2 and were therefore included in the fatigued group. The remaining 439 participants (87.1%) were included in the non-fatigued group.

Compared to eligible individuals who did complete the assessments after deployment, dropouts were younger during deployment (mean (SD): dropouts: 25.89 (6.08); completers:

28.57 (8.98), $t_{(673)=}$−3.635, p<.001). As a result they had been deployed less often (mean (SD): dropouts: 0.64 (0.93); completers: 0.91 (1.22), $t_{(650)=}$−2.329, p=.020) and were lower ranked ($\chi^2_{(3)}$= 13.908, p=.003). There was no significant difference in educational level between completers and dropouts ($\chi^2_{(2)}$= 4.500,p=.105). In addition, there was no significant difference in fatigue severity at T0 (mean (SD): dropouts: 43.54 (14.42); completers: 44.25 (15.33); $t_{(675)}$= −0.534, p=.593).

Questionnaires

Level of fatigue over the past two weeks was assessed with the Dutch 20-item Checklist Individual Strength (CIS-20R) (13). The questionnaire consists of four subscales: severity of fatigue, concentration, motivation and physical activity. The total fatigue score is the sum score of all items (range 20-140). The questionnaire is well validated and has good reliability. The used cut-off for the total score on the Checklist Individual Strength (CIS-20R) was ≥81. This cut-off corresponds to the 95th percentile of scores before deployment within a population of 862 Dutch military personnel (mean (SD): 45.87 (17.69)).

Collected demographics and deployment characteristics included age and rank during deployment, gender, educational level, number of previous deployments and use of medication (non-systemic glucocorticoids (nasal spray or crème), antihistamines, cholesterol lowering medications and antihypertensives). Exposure to deployment-stressors was assessed with a 13-item checklist during the T1 assessment (14).

IL-1β-reactivity

Whole blood, diluted 1:10 with RPMI-1640 (Gibco, Grand Island, NY), was stimulated with human interleukin (IL)-1β (Pepro Tech Inc, Rocky Hill, New Jersey, final concentrations (0, 1, 3, 10, 30 ng/ml)) for 24 hours at 37°C/5% CO2 in 96-well flat-bottomed plates. Supernatants were stored at -80°C. In a pilot analysis, the level of IL-8, IL-6, IL-10 and TNF-α were determined by ELISA (Sanquin, the Netherlands). In samples from 37 individuals it already became apparent that IL-1β did not induce IL-10 production. In addition, in approximately 750 samples it became apparent that IL-8 was most constantly induced by IL-1β. Based on these results we selected IL-8 as read out parameter for IL-1β sensitivity. Absolute numbers of monocytes, granulocytes, lymphocytes and CD3+ T-cells were calculated from a total leukocyte count. To determine the response to LPS, *w*hole blood was diluted 1:10 with RPMI-1640 (Gibco, Grand Island, NY), and stimulated with lipopolysaccharide (LPS, Escherichia Coli 0127:B8, Sigma, final concentrations 1 ng/ml) for 24 hours at 37°C/5% CO2 in 96-well flat-bottomed plates. Supernatants were stored at -80C and IL-8 concentrations were determined. We used a dose of 1ng/ml LPS, since preliminary analysis of a dose-response curve (0, 0.01, 0.1 and 1 ng/ml LPS) revealed that a plateau in IL-8 production was reached at a dose of 1 ng/ml LPS.

Statistics

Statistical analyses were conducted using PASW/SPSS 18.0. Differences between groups were considered significant at $p<0.05$. All continuous variables were tested for normality and log-transformed when necessary. A limited number of missing values were present due to technical and handling problems (<7.5% for each variable). Outliers were removed if z-values were outside the range of ± 3.29 (15) (<2% for each variable).

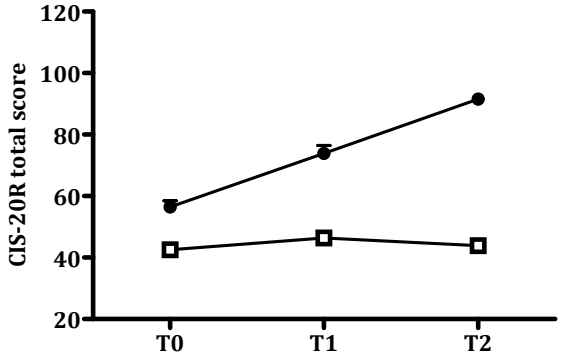
Differences between groups in continuous demographic and deployment characteristics were assessed with t-tests. Differences in non-continuous demographic variables between groups were tested with Chi-square (χ^2) tests. Repeated measures ANOVA was used to analyze the dose-response of IL-8 production after stimulation with increasing doses of IL-1β at T2. In addition, repeated measures ANOVA was used to analyze the longitudinal course of CIS-20R total scores, IL-1β-induced IL-8 production, non-stimulated IL-8 production, cell subsets and LPS-induced IL-8 production. Time was used as within-subjects factor and group as between-subjects factor. A Greenhouse-Geisser correction was applied when sphericity was violated and $\varepsilon \leq .75$. A Huyn-Feldt correction was applied if sphericity was violated and $\varepsilon > .75$ (15). Posthoc t-tests with Bonferroni correction were used for follow-up of significant effects. In addition, significant group x time interactions differences were followed by simple effects analyses (15). Pearson's r correlations were used to investigate associations between IL-1β-induced IL-8 production and demographic and deployment characteristics for each assessment point. Demographic and deployment characteristics that significantly correlated with IL-8 production were included as covariates in the repeated measures ANOVA. Non-transformed data are presented in all tables and figures.

RESULTS

Participant characteristics and longitudinal course of fatigue symptoms

Participants were divided into two groups based on their level of fatigue symptoms at six months after return from deployment (T2). Although participants with severe fatigue before deployment (T0) were excluded from the analyses, participants with severe fatigue at T2 had higher fatigue questionnaire scores than the non-fatigued group at all assessment points ($F_{(1,491)}= 283.73$, $p<.001$; Figure 1). The participants with severe fatigue at T2 showed a strong increase in fatigue severity after deployment compared to fatigue levels at T0. Moreover, the fatigue severity at T2 had continued to increase compared one month after deployment (T1). The non-fatigued group had slightly increased fatigue questionnaire scores at T1 compared to T0. However, their questionnaire scores had returned to baseline level at T2 (time: $F_{(1.98, 970.51)}= 138.17$ $p<.001$; interaction effect time x group: $F_{(1.98, 970.51)}= 118.403$, $p<.001$).

FIGURE 1. Longitudinal course of fatigue questionnaire scores for the fatigued (black circles, n= 65) and non-fatigued (white rectangles, n= 428) group.



Longitudinal course of fatigue questionnaire scores, as assessed before deployment (T0) and one month (T1) and six months (T2) after return from deployment. Repeated measures ANOVA: time: $F_{(1.98, 970.51)}$= 138.17 p<.001; group: $F_{(1,491)}$= 283.73, p<.001; time x group: $F_{(1.98, 970.51)}$= 118.403, p<.001. Data are presented as mean ± SEM.

TABLE 1. Characteristics of the fatigued (n = 65) and non-fatigued group (n = 439). Data represent mean (SD) or frequency (%).

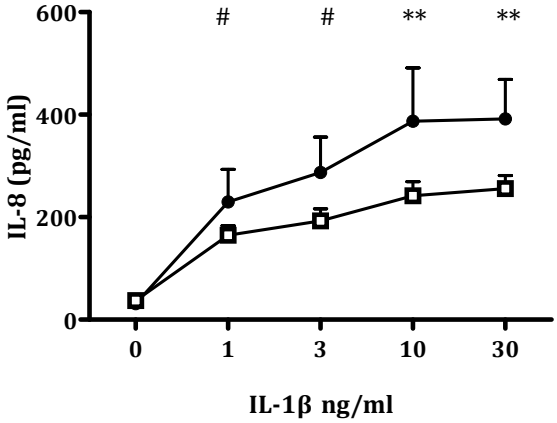
	Fatigued group	Non-fatigued group	P
Age during deployment	29.29 (9.19)	28.47 (8.96)	.490
Number of previous deployments	1.05 (1.31)	0.89 (1.20)	.320
Number of deployment stressors	5.53 (2.46)	4.98 (2.92)	.106
Gender			.667
Male	60 (92.3%)	398 (90.7%)	
Female	5 (7.7%)	41 (9.3%)	
Rank			.454
Soldiers	20 (30.8%)	179 (40.8%)	
Corporals	13 (20.0%)	84 (19.1%)	
Non-commissioned officers	20 (30.8%)	112 (25.2%)	
Officers	12 (18.5%)	64 (14.6%)	
Education			.744
Low	23 (35.9%)	170 (39.3%)	
Middle	32 (50.0%)	215 (49.7%)	
Higher	9 (14.1%)	48 (11.1%)	

We did not observe any significant group differences in demographic and deployment characteristics between the fatigued and non-fatigued participants (Table 1).

IL-1β-induced IL-8 production six months after deployment

We first investigated whether the fatigued and non-fatigued group differed in IL-1β-induced IL-8 production six months after return from deployment. For this purpose, we analyzed the dose-response relationship for IL-1β-induced IL-8 production in cultures of whole blood collected at the assessment six months after deployment (T2). Repeated measures ANOVA showed that IL-1β induced a dose-dependent increase in IL-8 production (dose: $F_{(2.004, 924.018)}$= 602.822, p<.001). Interestingly, IL-1β-induced IL-8 production was increased in the fatigued group as compared to the non-fatigued group (group: $F_{(1,461)}$= 4.553, p=.003; dose x group: $F_{(2.004, 924.018)}$= 7.941, p=<.001) (Figure 2). Posthoc tests revealed that the fatigued group had significantly higher IL-8 production than the non-fatigued group after administration of 1 ng/ml (p=.018), 3 ng/ml (p=.022), 10 ng/ml (p=.003) and 30 ng/ml IL1β (p=.001). The group difference at 10ng/nl and 30 ng/ml IL-1β remained significant after applying a Bonferonni correction (sig α = 0.05/5=.01).

FIGURE 2. Dose-response curve for IL-1β-induced IL-8 production measured six months after return from deployment.



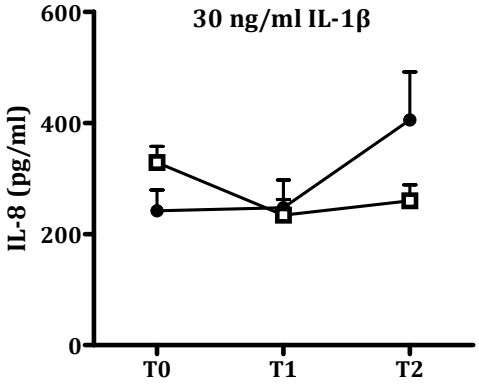
Whole blood samples obtained six months after deployment from participants assigned to the fatigued (black circles, n= 62) and non-fatigued (white rectangles, n= 401) group were stimulated for 24 hours with increasing concentrations of IL-1β. The amount of IL-8 in the culture supernatant was measured by Elisa. Repeated measures ANOVA: dose: $F_{(2.004, 924.018)}$= 602.822, p<.001; group: $F_{(1,461)}$= 4.553, p=.003; dose x group: $F_{(2.004, 924.018)}$= 7.941, p<.001. Data are presented as mean ± SEM. #p<.05, significant before Bonferonni correction; ** p<.01, also significant after Bonferonni correction.

The observed difference in the longitudinal course of IL-1β-induced IL-8 production between the fatigued and non-fatigued group was not paralleled by significant group differences in the number of monocytes ($t_{(470)}$= 0.296, p=.767), granulocytes ($t_{(468)}$= −1.479, p=.140), lymphocytes ($t_{(468)}$= −0.654, p=.514) or CD3+ T-cells ($t_{(462)}$= −0.636, p=.525) over time.

Longitudinal course of IL-1β-induced IL-8 production

We investigated whether the higher IL-1β -induced IL-8 production in the fatigued group was already present prior to deployment or whether the observed group difference developed over time. At T2, we observed the largest group difference in IL-1β-induced IL-8 production between the fatigued and non-fatigued group after stimulation with 30 ng/ml IL-1β. Therefore, we selected this dose for our subsequent analyses in which we compared the longitudinal course of IL-1β-induced IL-8 production in the fatigued and non-fatigued group. We observed a significant difference between the fatigued and non-fatigued group in IL-1β-induced IL-8 production over time (Figure 3; time: $F_{(1.957,\ 794.676)}$= 2.599, p=.076; group: $F_{(1,406)}$= 5.273, p=.022; time x group: $F_{(1.957,\ 794.676)}$= 4.301, p=.014).

FIGURE 3. Longitudinal course of IL-1β-induced IL8-production for the fatigued and non-fatigued group.



Whole blood samples obtained from participants assigned to the fatigued (black circles, n=55) and non-fatigued (white rectangles, n=353) group were stimulated for 24 hours with 30 ng/ml IL-1β. The amount of IL-8 in the culture supernatant was measured by Elisa, at the assessments before deployment (T0), and one month (T1) and six months (T2) after return from deployment. Repeated measures ANOVA: time: $F_{(1.957,\ 794.676)}$= 2.599, p=.076; group: $F_{(1,406)}$= 5.273, p=.022; time x group: $F_{(1.957,\ 794.676)}$= 4.301, p=.014. Data are presented as mean ± SEM.

Simple effects analysis revealed that IL-1β-induced IL-8 production of the non-fatigued group decreased after deployment ($F_{(1.962;\ 690.656)}$= 13.566, p<.001). Both at T1 and T2, Il-1β -induced IL-8 production of the non-fatigued group was significantly lower compared to T0 (T0-T1: p<.001; T0-T2: p<.001; T1-T2: p=1.000). In contrast, the IL-1β-induced IL-8 production of the fatigued group did not significantly change over time, and if anything tended to increase ($F_{(1.800;\ 97.185)}$= 2.297, p=.111). Posthoc t-tests with Bonferonni correction (significant $\alpha = .05/3 = .016$) revealed that the IL-8 production of the fatigued and non-fatigued group did not differ at T0 (p=.867) or T1 (p=.062).

TABLE 2. Differences in the longitudinal course various cell subsets between the fatigued and non-fatigued group.

	Time effect	Group effect	Group x time effect
Monocytes	F= 16.913, p<.001	F= 0.832, p=.362	F= 0.942, p=.388
Granulocytes	F= 5.878, p=.003	F= 0.226, p=.635	F= 2.671, p=.070
Lymphocytes	F= 5.929, p=.003	F= 2.372, p=.095	F= 2.146, p=.144
T-cells (CD3+)	F= 2.737, p=.067	F= 2.032, p=.133	F= 0.461, p=.497

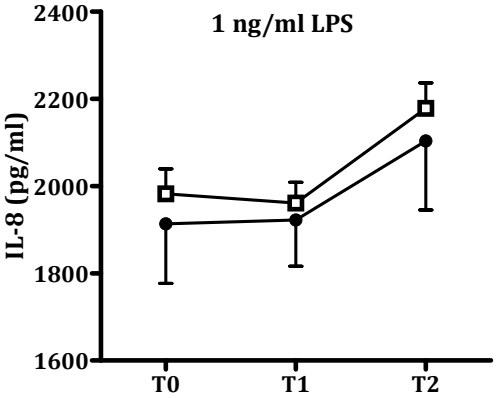
The observed difference in the longitudinal course of IL-1β-induced IL-8 production between the fatigued and non-fatigued group was not paralleled by significant group differences in the number of monocytes, granulocytes, lymphocytes or CD3+ T-cells (Table 2) over time.

Longitudinal course of LPS-induced IL-8 production

Next, we addressed the question whether the observed difference in IL-8 production between fatigued and non-fatigued individuals was specific for IL-1β-signaling or represents a general group difference in the capacity to produce IL-8.

To that end, we compared the longitudinal course of LPS-induced IL-8 production between the fatigued and non-fatigued group. The data presented in Figure 4 demonstrate

FIGURE 4. Longitudinal course of LPS-induced IL8-production for fatigued and non-fatigued group after deployment.



Whole blood samples obtained from participants assigned to the fatigued (black circles, n = 53) and non-fatigued (white rectangles, n = 388) group were stimulated for 24 hours with 1 ng/ml LPS and IL-8 levels in the culture supernatant were quantified. Samples were collected before deployment (T0), and one month (T1) and six months (T2) after return from deployment. Repeated measures ANOVA: time: $F_{(1.901, 934.511)}$= 1.868, p=.157; group: $F_{(1,439)}$= 0.357, p=.551; time x group: $F_{(1.901, 934.511)}$= 0.096, p=.900. Data are presented as mean ± SEM.

that LPS-induced IL-8 production did not change after deployment ($F_{(1.901, 934.511)}$= 1.868, p=.157). Moreover, there were no differences between the fatigued and non-fatigued group (group: $F_{(1,439)}$= 0.357, p=.551; interaction group x time: $F_{(1.901, 934.511)}$= 0.096, p=.900).

Influence of demographic and deployment characteristics

Since we are the first to examine IL-1β-induced cytokine production, it is not yet known which demographic and deployment characteristics may have influenced IL-1β-induced IL-8 production. There were no differences in demographic and deployment characteristics between the fatigued and non-fatigued group (Table 1). Nevertheless, to ascertain that our results were not caused by confounding factors, we examined correlations between demographic and deployment characteristics and IL-1β-induced IL-8 production for each assessment point separately (Table 3). Demographic and deployment characteristics that correlated significantly with IL-8 production for at least one assessment point were subsequently included as covariates in our analyses.

TABLE 3. Pearson's correlations between IL-8 production after stimulation with 30 ng/ml IL-1β and demographic and deployment characteristics one month before deployment (T0), one month after deployment (T1) and six months after deployment (T2).

	IL-8 T0	IL-8 T1	IL-8 T2
Age	−.132*	−.045	−.208***
Gender	.078	−.064	.021
Previous deployments, nr	−.035	−.016	−.036
Rank	−.167***	−.002	−.160**
Educational level	−.123**	.021	−.080
Medication use (y/n)	.001	.026	.001
Deployment stressors, nr		.149**	.063
Injury during deployment (y/n)		−.065	.032

* p<.05; ** p<.01; *** p<.001

After inclusion of age, rank, educational level and the number of reported deployment stressors in the analysis, the change in IL-8 production over time remained significantly different between fatigued and non-fatigued individuals (time: $F_{(1.983, 745.474)}$= 9.594, p<. 001 time x group interaction: $F_{(1.938, 745.474)}$= 3.890, p=.022; group: $F_{(1,376)}$= 5.649, p=.018).

DISCUSSION

This study was designed based on the concept that the response of the body to a regulatory mediator is not only determined by the concentration of the mediator, but also by the reactivity of the target cells to regulation by a particular mediator (8). Our findings indicate

that assessment of the reactivity of immune cells to IL-1β *in vitro* may represent a promising novel approach to investigate the relation between severe fatigue and pro-inflammatory cytokine signaling. Fatigue and IL-1β-induced IL-8 production by peripheral blood cells *in vitro* were assessed in a unique longitudinal prospective design within a large cohort of military personnel measured before, and at two time points after deployment (n=504) to a combat zone in Afghanistan. None of the included participants reported severe fatigue prior to the deployment, and therefore the observed effects are most likely associated with the development of severe fatigue in response to the deployment.

At six months after return from military deployment, the fatigued group had higher IL-1β-induced IL-8 production than the non-fatigued participants, indicating that the peripheral blood cells of fatigued participants had a higher reactivity to IL-1β than those of the non-fatigued group. The observed group-difference in IL-1β-induced IL-8 production was specifically associated with a group difference in the reactivity of peripheral blood cells to stimulation with IL-1β because we did not observe a group difference in LPS-induced IL-8 production. In addition, the increased IL-1β-induced IL-8 production in the fatigued group could not be attributed to group differences in the cellular composition of the peripheral blood.

Investigation of a potential group difference in the longitudinal course of IL-1β-induced IL-8 production revealed that the group difference in IL-1β reactivity between participants with and without severe fatigue after return from deployment was not a pre-existing characteristic, but had developed over time. The IL-1β-induced IL-8 production of non-fatigued participants had decreased one and six months after deployment compared to the assessment before deployment. These findings indicate that the leukocytes of non-fatigued participants had become less reactive to stimulation with IL-1β over time. In the group of participants with severe fatigued six months after deployment, we did not observe this decrease in IL-1β-induced IL-8 production over time.

During deployment to Afghanistan the participants in this study encountered a variety of stressors, such as armed combat, improvised explosive devices (IEDs), mortar attacks, and witnessing colleagues or civilians being injured or killed as a result. Given the severity of these deployment stressors, we interpret the 4-month deployment as prolonged stress. We did not include a non-deployed control group and therefore, we cannot conclude that the observed changes in fatigue and IL-1β-induced cytokine production result from the stress of the deployment. However, it is unlikely that the observed effects can be attributed to aspecific time-effects such as the year or season of assessment, since we included military personnel in several subsequent cohorts between 2006 and 2009.

It has been reported previously that severe or chronic stress, such as expected to occur during deployment, results in increased levels of circulating pro-inflammatory cytokines (16), upregulated expression of genes with NFκB response elements and downregulated expression of genes with GR response elements in leukocytes (17, 18). In the current study,

the participants who did not develop fatigue six months after deployment showed a decrease in their reactivity to IL-1β *in vitro* after return from deployment. Interestingly, in a previous study the upregulation of gene expression with NFκB response elements in chronically stressed individuals was paralleled by increased serum IL-1RA, which could decrease IL-1β capacity(18). These data indicate that in periods of severe or chronic stress, adaptive mechanisms may develop to reduce IL-1 reactivity. Our finding that the IL-1β-induced IL-8 production in the participants who had become fatigued after deployment did not decrease over time could indicate that they have adapted less well to the stress experienced during the deployment.

At present, it remains unknown what the underlying mechanism is for the observed higher IL-1β-induced IL-8 production by cells from participants with severe fatigue after deployment as compared to the non-fatigued group. It is known that activation of the transcription factor NFκB, in combination met NF-IL6, is essential and sufficient to induce upregulation of IL-8 expression after stimulation with IL-1β (19). LPS-induced Il-8 production is also dependent on activation of NFκB, but in this case in combination with AP-1 (19). Thus, IL-1β and LPS both induce IL-8 via transcription factor NFκB, but in addition use separate other transcription factors. Therefore it is possible that the group difference in IL-1β-induced IL-8 production and not LPS-induced IL-8 production is mediated by a preferential activation of NF-IL6 in the fatigued group after stimulation with IL-1 β. It is also possible that the mechanism(s) involved in the development of the group difference in IL-1β reactivity is located upstream of transcription factor activation, i.e. at the level of IL-1 receptor expression and/or signaling. The type I IL-1 receptor (IL-1RI) mediates the biological effects of IL-1α and IL-1β (12). The type II IL-1 receptor (IL-1RII) binds IL-1α and IL-1β with high affinity, but does not signal: it functions as a 'decoy' receptor, which prevents signal transduction via IL-1RI and thereby negatively regulates IL-1 signaling (12). The higher response to IL-1β in the fatigued group compared to the non-fatigued group may hypothetically have resulted from a higher IL-RI number, lower IL-1RII levels or higher IL-1RI signaling to downstream targets such as the transcription factors mentioned above.

In addition, it is possible that IL-1 receptor antagonist (IL-1RA) adds to the observed group difference in IL-1β reactivity six months after deployment. IL-1RA can negatively regulate IL-1β signaling, since it's binding to IL-1RI does not elicit signal transduction, but inhibits activation of the receptor by IL-1β (20). It is possible that the fatigued group has lower levels of IL-1RA than the non-fatigued group at six months after deployment. Miller et al. (18) observed that serum IL-1RA levels in individuals with chronic caregiving stress were on average 450 pg/ml, while serum IL-1RA levels of non-stressed healthy controls were on average 200 pg/ml. On the basis of these data, we expect that serum IL-1RA levels in our participants are likely to be in the 200-450 pg/ml range. The final concentration of IL-1RA in our culture supernatants, which were used in a dilution of 1:20, is therefore expected to be 10-22,5 pg/ml. However, a 10 to 100-fold excess of IL-1RA is necessary to block the binding

of IL-1β to the IL-1R (21). Therefore, the expected IL-1RA levels in our *in vitro* cultures are probably too low to block the effects the ng concentrations of IL-1β. In addition, if IL-1RA was responsible for the observed group difference in IL-1β-induced IL-8 production, the largest group differences would be expected at the lower doses of IL-1β, instead of the highest doses of IL-1β.

In rodents it has been shown cytokine levels in the brain are the mirror image of cytokine levels in the periphery (9). For example, peritoneal administration of IL-1β in rats upregulate mRNA expression of various pro-inflammatory cytokines in the brain (22). Given these results, it may well be that the observed higher IL-1β reactivity, which was present in the fatigued group compared to the non-fatigued group during the assessment six months after deployment, is paralleled in the brain. A high IL-1β reactivity in the brain could have two possible downstream consequences. On the one hand it could mean that in the fatigued group even a normal level of IL-1β, which may not have behavioral effects in individuals with normal IL-1β receptor reactivity, influences brain functioning (23) and induces behavioral changes directly (24). On the other hand, high IL-1β reactivity could also lead to increased production of pro-inflammatory cytokines in the brain in response to normal levels of IL-1β. Such an increase in pro-inflammatory cytokine levels could then lead to increased behavioral effects (24). High IL-1β reactivity in the brain could thereby be involved in the development of the fatigue, and also in the maintenance or aggravation of the fatigue severity.

A limitation of the current study is that we did not formally investigate the presence of medical conditions that may have influenced the IL-1β-sensitivity of peripheral blood cells or the experienced levels of fatigue. However, participants were physically fit for military deployment and therefore the presence of major medical conditions prior to deployment can be excluded. In addition, the presence of injuries after deployment and medication use during the three assessments was very limited. Moreover, medication use and sustained injuries did not significantly correlate with IL-1β-induced IL-8 production. It thus seems highly unlikely that the presence of medical conditions influenced our results.

We are the first to report that the response of peripheral blood cells *in vitro* to IL-1β differs between military personnel with and without severe fatigue six months after return from deployment. Six months after return from deployment, the group who had become severely fatigued had higher IL-1β-induced IL-8 production than the non-fatigued group. When analyzing the longitudinal course of IL-1β reactivity we observed that this group difference had developed in response to the deployment, since only in the non-fatigued group the IL-1β-induced IL-8 production had decreased after deployment. These findings indicate that investigating the reactivity of the immune system to stimulation with IL-1β is a promising novel method to study the association between behavioral abnormalities and pro-inflammatory cytokine signaling.

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9

Summary and General Discussion

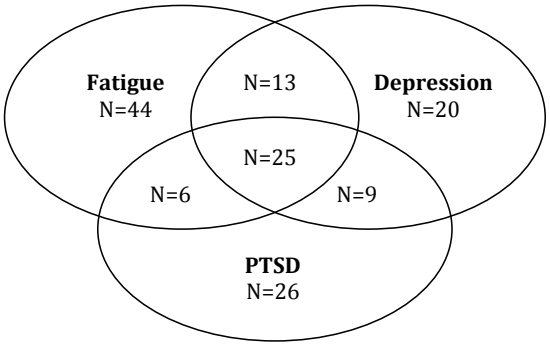
BIOLOGICAL VULNERABILITY FACTORS FOR PTSD, DEPRESSIVE AND FATIGUE SYMPTOMS

A substantial minority of individuals exposed to severe, chronic or traumatic stress subsequently develops long-lasting mental or physical health problems, which may severely impair emotional, occupational or social functioning. These stress-related conditions include posttraumatic stress disorder (PTSD), major depressive disorder (MDD) and severe fatigue. We hypothesized that the development of these stress-related conditions is associated with vulnerability or risk factors, which are by definition present prior to the stressful or traumatic event. To investigate our hypothesis, we predominantly focused on identifying biological vulnerability factors, i.e. biomarkers, which were associated with increased risk for development of these conditions. We assessed whether glucocorticoid receptor (GR) pathway components within peripheral blood mononuclear cells (PBMCs) predicted the subsequent development of PTSD, depressive and/or fatigue symptoms in response to military deployment.

In addition, we investigated whether the capacity of peripheral blood cells to produce cytokines upon stimulation, and the reactivity of peripheral blood cells to regulation by pro-inflammatory mediator IL-1β, predicted the development of stress-related conditions in response to military deployment. We also examined whether military deployment and development of stress-related conditions in response to military deployment were associated with longitudinal changes in cytokine production. In this chapter, the identified biological vulnerability factors will be discussed and placed into a broader perspective.

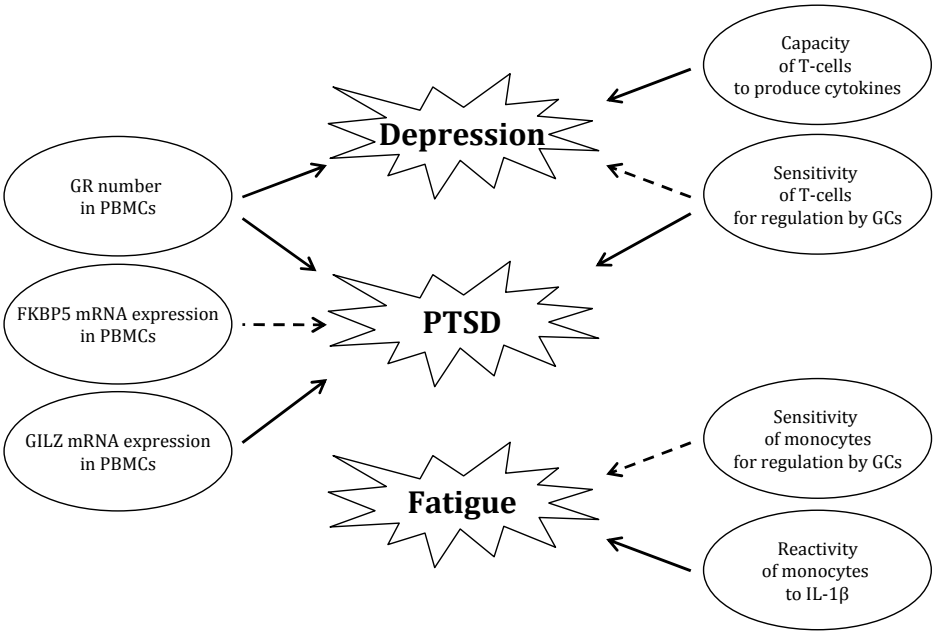
We determined whether our participants had high levels of PTSD, depressive or fatigue symptoms six months after return from their deployment. 19.8% of the PRISMO-participants who completed the questionnaires six months after deployment scored above our cut-off on the PTSD, depression, or fatigue questionnaire (Figure 1) (internal communication Ministry

FIGURE 1. Number of PRISMO-participants with a high level of PTSD, depressive, and/or fatigue symptoms six months after return from deployment to a combat-zone in Afghanistan.



Numbers in the overlapping circles represent the participants scoring above the cut-off of more than one questionnaire at six months after return from deployment. Total N = 722.

FIGURE 2. Overview of all identified biological vulnerability factors for the development of a high level of PTSD, depressive, and/or fatigue symptoms in response to deployment to a combat-zone in Afghanistan.



Solid arrows indicate that high levels of the vulnerability factors are associated with development of a high level of symptoms. Dotted arrows indicate that low levels of the vulnerability factors are associated with development of a high level of symptoms.

of Defence). Of these participants, 37.1% scored above the cut-off of more than one questionnaire. In addition, within the whole sample we observed strong correlations between total scores on the PTSD, depression, and fatigue questionnaire six months after deployment (all r>.6, p<.001). These results are consistent with an abundance of literature showing that co-morbidity between diagnoses of PTSD, MDD and severe fatigue is common (1-3). Interestingly, we were able to identify different vulnerability factors for the development of a high level of PTSD, depressive, and fatigue symptoms in response to deployment, which indicates that the symptoms of PTSD, depression and fatigue have different biological mechanisms (see figure 2 for an overview).

PTSD

9.1% of the PRISMO-participants reported a high level of depressive symptoms six months after return from deployment (Figure 1). Our research revealed that the presence of a high level of PTSD symptoms six months after return from deployment could be predicted by several components of the glucocorticoid receptor (GR) signalling pathway (Chapter 2 (4), Chapter 3 (5), Chapter 6).

GCs are important regulators of the immune system by inhibiting cell proliferation, regulating cytokine production and stimulating apoptosis (6). The regulation of the immune system by GCs, especially under stressful conditions, is predominantly mediated via GRs (7). An increased sensitivity to regulation by GCs has been observed in peripheral blood cells of individuals with clinically diagnosed PTSD (8, 9). In addition, some studies have indicated an increased GR number in PBMCs of individuals with PTSD when compared with healthy controls (10) and psychiatric patients with mood, psychotic or other anxiety disorders (11). On the other hand, lower (12), and similar (9, 13-16) GR number in individuals with PTSD compared to controls have also been reported. Since previous studies used a cross-sectional design, it remains unknown whether the observed biological correlates of PTSD are already present before the traumatic event leading to PTSD or whether they are induced by the traumatic exposure or the psychological symptoms developed in response to the trauma.

We are the first to observe that the mean number of GR in PBMCs *prior* to deployment was higher within a group of participants who developed a high level of PTSD symptoms after deployment compared to a group of carefully matched participants without a high level of PTSD symptoms after deployment (Chapter 2 (4)). Moreover, the number of GR was a highly significant predictor for the development of a high level of PTSD symptoms within this sample of participants. Interestingly, one and six months after return from deployment the GR number in the PTSD group was still higher than in the comparison group. In a second study with a larger number of participants, we validated the predictive value of the GR number prior to deployment for the presence of a high level of PTSD symptoms (Chapter 3 (5)). Due to the limited number of participants who had developed a high level of PTSD symptoms after deployment, only one additional participant could be added to the PTSD group. But, we included a more than 10-fold larger and more heterogeneous comparison group, which also included participants with high levels of fatigue and depressive symptoms. Within this more heterogeneous participant sample, a high GR number remained associated with increased risk for the presence of a high level of PTSD symptoms after deployment.

In this second study (Chapter 3 (5)), we also investigated whether additional components in the GR pathway prior to deployment contributed to the prediction of the presence of a high level of PTSD symptoms in response to deployment. For this purpose, we investigated the predictive value of pre-deployment mRNA expression of three genes directly regulated by the GR (i.e. genes with a glucocorticoid response element (17) in PBMCs for the presence of a high level of PTSD symptoms after deployment. We selected glucocorticoid-induced leucine zipper (GILZ), a mediator of the anti-inflammatory and immunosuppressive effects of GCs (18), and serum/glucocorticoid regulated kinase 1 (SGK1), which is involved in modulating apoptosis (19). In addition, we selected FK506 binding protein 5 (FKBP5), which also functions as a co-chaperone molecule of the GR and can lower the affinity of GR and thereby reduce GC binding to the GR (20). We observed that FKBP5 and GILZ mRNA expression significantly contributed to the prediction of a high level of PTSD, above and beyond the number of GR:

low FKBP5 and high GILZ mRNA expression prior to deployment were independently associated with increased risk for the presence of a high level of PTSD symptoms after deployment.

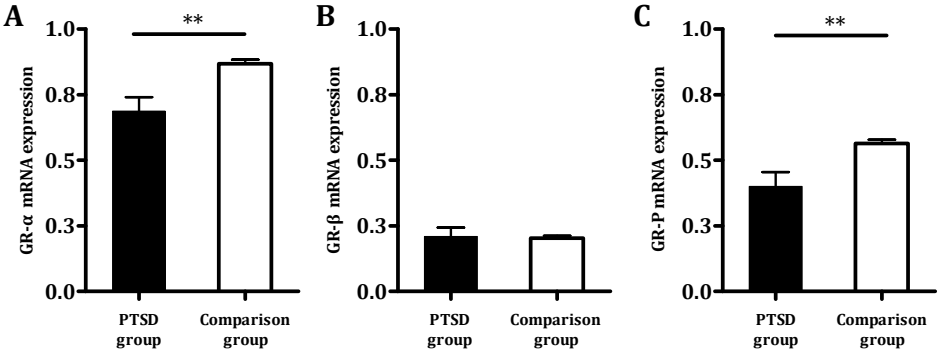
Two recent cross-sectional studies on survivors of the 9/11 World Trade Center attacks found lower FKBP5 mRNA expression in survivors with PTSD compared to survivors without PTSD (21, 22). In addition, a prospective study with the first assessment shortly after trauma exposure showed that FKBP5 mRNA expression measured after admission to the emergency room predicted the subsequent development of PTSD (23). Our results indicate that the predictive value of FKBP5 mRNA expression observed in the study of Segman et al. most likely was not associated with the immediate response to trauma, but with a pre-existing vulnerability of a low FKBP5 mRNA expression.

A very recent study performed in mice shows that deletion of FKBP5 causes less downregulation of hippocampal GR expression in response to acute stress, which results in increased sensitivity of the HPA axis for negative feedback regulation by GCs and lower cortisol levels in response to acute stress (24). These results indicate that FKBP5 plays a pivotal role in regulation of the stress response, and indicates that a lower FKBP5 leads to a more pronounced negative feedback regulation of the cortisol response due to an inefficient downregulation of central GR. This study underscores our observation that FKBP5 may be involved in the development of PTSD, since genetic deletion of the FKBP5 gene resulted in a comparable biological profile as was repeatedly observed in individuals with PTSD.

Various subtypes of the GR have been identified that play a differential role in the overall binding capacity and transcriptional activity of GR. GR-α is the most abundant variant and is transcriptionally active (25). GR-P is also widely expressed and increases GR-α activity (26, 27). In contrast, GR-β is less ubiquitously expressed, does not bind GCs and has limited transcriptional capacity (28). We propose that differences in GR subtypes may underlie our observed predictive value of the GR number and FKBP5 and GILZ mRNA expression for the presence of PTSD symptoms. However, within our group of individuals with PTSD symptoms and matched controls, we did not observe significant group differences in mRNA expression of GR subtypes GR-α, GR-β and GR-P prior to deployment (Chapter 2 (4)). We have now also analyzed the data on the pre-deployment GR-α, GR-β and GR-P mRNA expression within the sample of our larger study, as described in Chapter 3. Interestingly, the PTSD group had significantly lower GR-α ($t_{(400)}$= 3.291, p=.001) en GR-P mRNA expression than the comparison group ($t_{(397)}$= 3.120, p=.002) (Figure 3), while the amount of GR-β did not significantly differ between groups ($t_{(400)}$= −0.205, p=.838). These observations suggest that the GR signalling pathway in individuals vulnerable for development of PTSD symptoms may be dysregulated at more levels than we have currently identified.

The net result of signalling in the GR pathway can be quantified by measuring the sensitivity of activated leukocytes to regulation by the synthetic GC dexamethasone (DEX) *in vitro* (29). We investigated the predictive value of DEX-inhibition of T-cell proliferation and DEX-inhibition of monocyte TNF-α production, as assessed prior to deployment, for

FIGURE 3. messenger (m)RNA expression levels of GR-subtypes GR-α, GR-β and GR-P in peripheral blood mononuclear cells prior to deployment in participants with (black bars, n = 31) and without (white bars, n = 372) high levels of PTSD symptoms six months after return from deployment to a combat-zone.



Group differences for A) GR-α; B) GR-β; C) GR-P. Data are presented as mean (SEM). mRNA expression of GR-subtypes was normalized for GAPDH/actin mRNA expression. Group differences were tested using t-tests. ** p<.01.

the presence of a high level of PTSD symptoms after deployment (Chapter 6). Our analyses revealed that a high sensitivity of T-cell proliferation for regulation by GCs prior to deployment was significantly associated with increased risk for the presence of a high level of PTSD symptoms in response to deployment, but only if participants did not report co-morbid high levels of depressive symptoms. These results added to our previous findings, since we now had identified a vulnerability factor for a high level of PTSD symptoms in the GR pathway that had functional consequences for the level of T-cell proliferation.

It is of particular interest that high GC-sensitivity of T-cells only predicted the development of a high level of PTSD symptoms without a high level of depressive symptoms, especially since the predictive value of GR number, FKBP5 and GILZ mRNA expression did emerge without controlling for the presence of depressive symptoms. We are the first to identify different biological profiles associated with the presence of PTSD symptoms with and without depressive symptoms in a pre-trauma prospective study. However, others have already identified different biological profiles for PTSD patients with and without co-morbid MDD in cross-sectional studies (30). Interestingly, Gill et al. (30) pointed out that in PTSD, the most pronounced results regarding the presence of increased sensitivity of the HPA axis for negative feedback by GCs have been observed in samples with low levels of co-morbid MDD.

Our results show that it is important to take co-morbidity into consideration when investigating vulnerability factors for the development of stress-related conditions The mean GC-sensitivity of T-cells within our whole group of participants with PTSD symptoms did not differ from the GC-sensitivity of participants without PTSD symptoms, because the two profiles of GC-sensitivity within the PTSD group (increased sensitivity for PTSD-DEP, decreased sensitivity for PTSD+DEP) resulted in a mean net effect of 'no change'. This shows

that some vulnerability factors may not be identified if co-morbidity between conditions is not investigated.

Collectively, the findings of these three studies demonstrate that individuals vulnerable for development of PTSD in response to trauma exposure have a dysregulation on various levels of the GR signalling pathway, which is already present prior to the traumatic event that caused the development of symptoms. All together, these results indicate increased signalling of the GR pathway prior to development of PTSD symptoms. These predictors in the GR signalling pathway are associated with the actual *development* of a high level of PTSD symptoms, since participants with a high level of PTSD symptoms prior to deployment were excluded from the analyses on the predictive value of the GR number and mRNA expression for subsequent PTSD symptoms. In addition, the association between the presence of a high level of PTSD symptoms and all significant predictors in the GR signalling pathway remained significant after inclusion of pre-deployment PTSD symptoms in the analyses.

We also investigated whether cortisol levels prior to deployment were associated with the development of a high level of PTSD symptoms in response to deployment (Chapter 2 (4), Chapter 3 (5)). Basal cortisol levels, measured in plasma collected during the morning of the pre-deployment baseline assessment, did not significantly predict the development of a high level of PTSD symptoms. Importantly, the predictive values of GR number, and FKBP5 and GILZ mRNA expression for the development of PTSD symptoms remained significant after controlling for cortisol levels. The latter implies that the observed vulnerability factors in the GR pathway for a high level of PTSD symptoms were not associated with changes in cortisol levels.

Immediately after awakening, cortisol levels rise sharply: cortisol levels increase by 50-70% in the first 30 minutes after awakening, and remain elevated for at least 60 minutes. This rapid increase in cortisol level is defined as the cortisol awakening response (CAR), and is assumed to be a good indicator of adrenocortical activity (31). We observed that the CAR prior to deployment did not significantly predict continuous PTSD symptoms six months after deployment (Chapter 5 (32)). This observation is consistent with two other prospective studies (33, 34), which also investigated the predictive value of the CAR for subsequent PTSD symptoms). Multiple cross-sectional studies have observed that the CAR is lower in PTSD patients compared to healthy controls (8, 35-37). Our results suggest that an attenuated CAR, such as repeatedly found within PTSD patients, may not represent a vulnerability factor but may develop as a consequence of PTSD. However, it may also be that in our study and the studies of Heinrichs et al (33). and Inslicht et al (34) no significant association between the CAR and subsequent PTSD symptoms was detected due to the use of continuous PTSD symptoms scores as outcome measure, while there was low variability in PTSD symptoms. Within our participant sample 70% of our participants reported no or minimal PTSD symptoms six months after deployment. Inslicht et al. (34) also reported low variability in PTSD symptoms in their sample of police officers, but also observed that a high CAR was associated with

higher peri-traumatic distress and higher symptoms of acute stress disorder after trauma exposure, which are both well-known risk factors for PTSD (38).

Compared to healthy controls, increased levels of serum pro- and anti-inflammatory cytokines and chemokines have been observed in individuals with PTSD (39-41). In addition, an increased capacity of PBMCs to produce pro-inflammatory cytokines upon stimulation with a mitogen has been observed within female and mixed-gender samples of PTSD patients, but not in male samples of PTSD patients, compared to healthy traumatized and non-traumatized controls (8, 13, 42-44). For this discussion, we performed additional analyses to investigate whether *in vitro* cytokine production was associated with the development of PTSD symptoms in response to deployment. The level of CD2/CD28-induced T-cell cytokines and IL6/chemokines, reflecting the adaptive immune response, prior to deployment did not significantly predict the presence of a high level of PTSD symptoms after deployment (cytokines: W= 0.324, p=.569; IL6/chemokines: W= 0.000, p=.999). Furthermore, the level of LPS-induced monocyte cytokines, reflecting the response of the innate immune system, also did not significantly predict the presence of a high level of PTSD symptoms after deployment (W= 0.194, p=.660).

Given these results, we suggest that the changes in cytokine production previously observed for PTSD may evolve as a consequence of the PTSD and do not represent a vulnerability factor for development of PTSD. Possibly the imbalance in GR signalling within individuals vulnerable for PTSD symptoms in combination with behavioural and physical changes associated with the presence of PTSD, such as increased body weight (45) may direct the enhanced capacity of cytokine secretion in PTSD.

The response of an individual is not only determined by the level of hormones or cytokines produced. Another important factor for target responsivity is the reactivity or sensitivity of the specific receptor for the mediator (29), e.g. of the GR for GCs (as discussed above), and of cytokine receptors e.g. the IL-1 receptor for IL-1β. We investigated the reactivity of (the Il-1 receptor of) peripheral blood leukocytes to the pro-inflammatory mediator IL-1β. After controlling for the level of fatigue symptoms after deployment, the longitudinal course of IL-1β-induced cytokine production, measured from before deployment until six months after deployment, did not differ between individuals with and without a high level of PSTD symptoms after deployment (group effect: $F_{(1,389)}$= 2.313, p=.129; interaction-effect time x group: $F_{(2, 778)}$= 1.200, p=.302). The analysis of receptor sensitivity for immune and hormonal mediators has only just begun to be researched, but it may contribute to the understanding of the mechanism of dysregulations associated with the development of PTSD or other stress-related conditions.

Depression

9.3% of the PRISMO-participants reported a high level of depressive symptoms six months after return from their deployment to Afghanistan (Figure 1). The presence of a high level of depressive symptoms six months after return from deployment could be predicted by several components of the GR signalling pathway (Chapter 4 (46), Chapter 6). In addition, a high level of depressive symptoms after deployment was significantly predicted by a high capacity of T-cells to produce cytokines upon stimulation prior to deployment (Chapter 7).

Cross-sectional studies have shown that altered regulation of the peripheral immune system by GCs appears to be associated with the presence of MDD or depressive symptomatology. In contrast to the findings for PTSD, a decreased sensitivity of T-cells to regulation by GCs was observed in individuals with MDD compared to healthy controls (47-50). Studies investigating the GR number in PBMCs in individuals with MDD have yielded mixed results regarding the presence and direction of alterations (51-53). None of these studies had a prospective design with the first assessment before the onset of depressive symptoms. Therefore, it remains unknown whether changes in the GR signalling pathway in PBMCs represent biological vulnerability factors for the development of depressive symptoms.

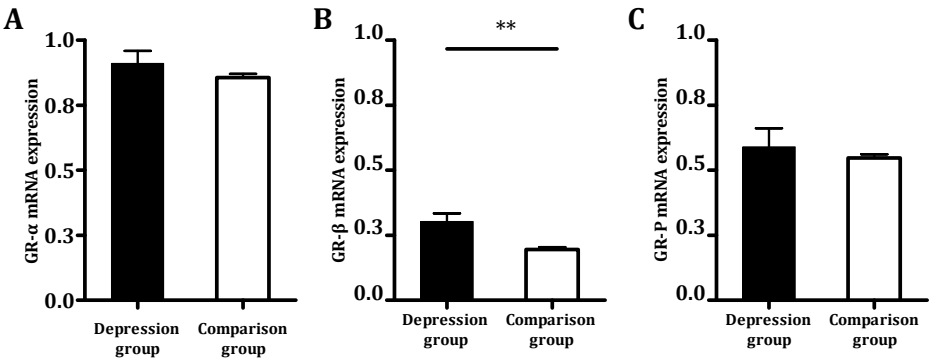
Our first prospective study on the association between development of mental and physical health problems in response to deployment and GR number in PBMCs was performed when approximately half of our total participant sample had completed the assessment six months after deployment (Chapter 4 (46)). In this study, we assessed the GR number in PBMCs before and one and six months after deployment. We investigated whether the GR number differed between participants who had high levels of depressive symptoms and fatigue after deployment, participants who had high levels of fatigue only, and participants who did not have high levels of depressive and fatigue symptoms after deployment. We observed that participants who reported both depressive and fatigue symptoms after deployment had higher GR numbers than the other two groups at all time points. These differences in GR number were not caused by pre-deployment depressive and fatigue symptoms or by differences in plasma cortisol levels. Since we did not observe a difference for the fatigue-only group, we attributed the observed increased GR number in the group with depressive and fatigue symptoms to the presence of depressive symptoms. Based on these results we suggested that a high GR number in PBMCs may represent a pre-existing vulnerability factor for the development of depressive symptoms in response to severe stress, such as may happen during military deployment. However, an important limitation of this study was that the number of individuals with a high level of PTSD symptoms was too small to investigate the effect of a co-morbid high level of PTSD symptoms with sufficient power. In our larger, more heterogeneous sample which was described in Chapter 3, the GR number did no longer significantly predict the presence of a high level of depressive symptoms ($W = 1.331$, $p = .249$).

In a subsequent study, we investigated the predictive value of pre-deployment DEX-inhibited T-cell proliferation and DEX-inhibited monocyte TNF-α production for the presence of a high level of depressive symptoms after deployment (Chapter 6). The sensitivity of T-cells to regulation by GCs prior to deployment significantly predicted the presence of depressive symptoms after deployment: low sensitivity of T-cell proliferation to inhibition by GCs was associated with increased risk for the presence of a high level of depressive symptoms. The predictive value of GC-sensitivity of T-cells was independent of the level of depressive symptoms prior to deployment. Thus, we observed an additional vulnerability factor for the development of depressive symptoms in the GR signalling pathway in PBMCs, which had functional consequences on the level of T-cell proliferation. This vulnerability factor was already present prior to the deployment. Interestingly, this is the opposite direction as the increased GC-sensitivity of T-cells which was observed for participants who developed a high level of PTSD symptoms without depressive symptoms. Our data indicate that individuals vulnerable for development of a high level of depressive symptoms in response to severe stress, such as may happen during military deployment, have pre-existing decreased signalling in the GR pathways in PBMCs. These results support the hypothesis that the development of depression has different biological underpinnings than the development of PTSD.

For the purpose of this discussion, we investigated whether the GR pathway components for which we investigated the predictive value for PTSD symptom development, were associated with the development of a high level of depressive symptoms. Our hypothesis was that these GR pathway components would also point towards decreased GR signalling in the GR pathway in individuals vulnerable for development of depressive symptoms in response to severe stress. We analyzed within 448 male soldiers whether mRNA expression of GR target genes GILZ, SGK1 and FKBP5 prior to deployment was associated with the presence of a high level of depressive symptoms after deployment. After correcting for the presence of a high level of PTSD symptoms, none of the included GR target genes significantly predicted a high level of depressive symptoms (all p-values >.50).

In addition, we investigated whether mRNA expression of GR subtypes GR-α, GR-β and GR-P prior to deployment was associated with the presence of a high level of depressive symptoms after deployment. The participants with high levels of depression after deployment had significantly higher GR-β mRNA expression than the participants without high levels of depression ($t_{(384)}$= −3.030, p=.003) (Figure 4), while the GR-α en GR-P mRNA expression did not significantly differ between groups (p-values >.35). The GR-β does not bind GCs and has limited transcriptional capacity (28). Increased mRNA expression of GR-β has repeatedly been found to be associated with decreased GC-sensitivity of various target cells (28). Therefore, the observed increase in GR-β mRNA expression supports our hypothesis that decreased GR pathway signalling in PBMCs is associated with the development of depressive symptoms in response to military deployment, i.e. a period of severe stress.

FIGURE 4. messenger (m)RNA expression levels of GR-subtypes GR-α, GR-β and GR-P in peripheral blood mononuclear cells prior to deployment in participants with (black bars, n=26) and without (white bars, n=362) high levels of depressive symptoms six months after return from deployment to a combat-zone.



Group differences for A) GR-α; B) GR-β; C) GR-P. Data are presented as mean (SEM). mRNA expression of GR-subtypes was normalized for GAPDH/actin mRNA expression. Group differences were tested using t-tests. ** p<.01.

There are strong indications that the presence of depressive symptomatology is associated with a low-grade pro-inflammatory state. Increased levels of C-reactive protein (CRP), serum pro-inflammatory cytokines, and soluble IL-2-receptors have been observed in individuals with MDD and non-clinical levels of depressive symptoms (54-58). In addition, epigenetic changes in the methylation status of inflammation-associated genes have been observed in individuals with MDD (59). The capacity of PBMCs to produce cytokines upon stimulation has also been investigated in individuals with MDD and depressive symptoms. However, from these studies no clear picture with regard to the presence and direction of changes in cytokine production in depressed individuals emerged (49, 60-71). These mixed results are probably influenced by the fact that all studies differed in the time past since the onset of depressive symptoms.

To the best of our knowledge, no prospective, longitudinal studies have been performed on the association between development of depressive symptoms and the capacity of PBMCs to produce cytokines upon stimulation, and therefore it remains unknown whether a causal relationship exists between these two processes.

We are the first to investigate the association between the development of depressive symptoms and mitogen-induced cytokine production within a prospective, longitudinal design (Chapter 7). We investigate a broad range of T-cell (CD2/CD28-induced) and innate (LPS-induced) pro- and anti-inflammatory cytokines and chemokines. These cytokines were reduced into three functional clusters using exploratory structural equation modeling, which resulted in separate factor scores for T-cell cytokines, IL6/T-cell chemokines and innate cytokines. We observed that a high capacity of T-cells to produce cytokines was associated with increased risk for the presence of a high level of depressive symptoms six months after

deployment. Interestingly, the T-cell cytokine production of all participants had gradually increased over time and was still elevated at six months after deployment. If this increased capacity to produce T-cells would become a stable trait in these stress-exposed participants, this may hypothetically lead to increased risk for development of inflammatory conditions such as atherosclerosis (72, 73). In addition, as long as the increase in T-cell cytokine production in participants who did not develop depressive symptoms persists, these participants may hypothetically be at increased risk for development of depressive symptoms after the occurrence of a new stressor (i.e. stress sensitization).

In the group who developed depressive symptoms after deployment, the increase in T-cell cytokine production over time was smaller than in the non-depressed group. The latter is in apparent contrast with the selective pro-inflammatory state in depressed patients. Possibly the time of attenuation of the increased cytokine production may differ between depressed patients and non-depressed individuals in a way that stress-induced cytokine responses may eventually be downregulated in non-depressed individuals while they may persist in depressed individuals.

The observed effects were independent of the level of depressive symptoms prior to deployment and the level of PTSD symptoms after deployment. Thus, we identified another vulnerability factor associated with the development of a high level of depressive symptoms in response to severe stress.

Previous studies on cancer patients treated with pro-inflammatory cytokines already indicated that T-cell cytokines might be involved in the development of depressive symptoms. Administration of the T-cell cytokine IL-2 for cancer-treatment led to depressive symptomatology in up to 60% of cancer patients (74-76). In addition, high levels of circulating sIL-2R and anti-inflammatory T-cell cytokine IL-10 prior to interferon-α treatment were associated with increased risk for subsequent development of MDD (77).

We are the first to show that a similar association exists between the intrinsic capacity to produce and secrete T-cell cytokines, such as IFN-γ, and the development of depressive symptoms in medically healthy individuals. The observed increased capacity of T-cells to produce cytokines upon stimulation in individuals vulnerable for development of depression may be a consequence of the observed lower sensitivity of T-cells to be inhibited by glucocorticoids, as analyzed prior to deployment, which was observed within the same participant sample. The other way around, it may also be that the increased capacity of T-cells to produce cytokines may have resulted in a lower sensitivity of GR to regulate T-cell function (78).

Interestingly, for both depressed and non-depressed individuals, the (LPS-induced) monocyte cytokine production and IL6/chemokine production by T-cells decreased after deployment. It is conceivable, that these long-term decreases in cytokine production may add to the increased development of medical morbidities, such as bacterial infections. The monocyte cytokine production and T-cell IL6/chemokine production were not associated with the development of a high level of depressive symptoms. In addition, the longitudinal course of

the reactivity of monocytes to regulation by pro-inflammatory mediator IL-1β did not differ between individuals with and without a high level of depressive symptoms after deployment (group effect: $F_{(1,384)}$= 0.059, p=.808; interaction-effect time x group: $F_{(2, 768)}$= 0.942, p=.390). Thus, the development of depressive symptoms in response to severe stress appears to be specifically associated with pre-existing alterations in T-cell cytokine production.

Fatigue

12.2% of the PRISMO-participants reported severe fatigue six months after return from their deployment (Figure 1). The presence of severe fatigue six months after return from deployment could be predicted by the sensitivity of (GRs in) monocytes to regulation by GCs prior to deployment (Chapter 6). In addition, the longitudinal course of sensitivity of the IL-1β receptor in peripheral immune cells for regulation by pro-inflammatory mediator IL-1β differed between participants with and without severe fatigue after deployment (Chapter 8).

Participants of previous cross-sectional studies on the association between severe fatigue or chronic fatigue syndrome (CFS) and sensitivity of peripheral blood cells for regulation by GCs differed in age and fatigue severity. A decreased GC-sensitivity of peripheral blood cells has been observed for monocytes of adults with non-clinical levels of severe fatigue (79) and for T-cells of adolescent girls with CFS (80, 81). In contrast, increased GC-sensitivity of T-cells and monocytes has been observed in adults with CFS (82). However, the association between sensitivity of peripheral blood cells for regulation by GCs and the actual development of severe fatigue had never been investigated. Based on the data in the literature, we hypothesized that the development of severe fatigue in response to severe stress may be associated with decreased GC-sensitivity of peripheral blood cells.

Just as was done for PTSD and depressive symptoms, we investigated the predictive value of pre-deployment DEX-inhibition of T-cell proliferation and DEX-inhibition of monocyte TNF-α production for the presence of severe fatigue after deployment (Chapter 6). We observed that the GC-sensitivity of monocytes prior to deployment significantly predicted the presence of severe fatigue after deployment: low sensitivity of monocyte TNF-α production to inhibition by GCs was associated with increased risk for the presence of severe fatigue. The predictive value of GC-sensitivity of monocytes was independent of the level of fatigue symptoms prior to deployment, indicating that low GC-sensitivity of monocytes is a predictor for the development of severe fatigue in response to severe stress, i.e. military deployment. This observation fits with our hypothesis that development of severe fatigue in response to severe stress is associated with decreased GC-sensitivity of peripheral blood cells, which was previously observed in adolescents with CFS (80, 81) and adults with severe fatigue but without CFS (79).

DEX-inhibition of monocyte cytokine production *in vitro* reflects the net effect of the total GC signalling via the GR pathway in monocytes. Therefore, our results suggest that decreased

GC signalling in peripheral blood cells precedes the development of severe fatigue in response to a period of severe stress. However, we did not identify any other components within the GR pathway specifically associated with the development of severe fatigue in response to deployment. We had already observed that the GR number in PBMCs and plasma cortisol levels did not differ between participants with severe fatigue without depressive symptoms and non-fatigued participants before or after deployment (Chapter 4 (46)). In addition, after correcting the analyses for the presence of a high level of PTSD symptoms, the pre-deployment mRNA expression of GR target genes FKBP5, GILZ and SGK1 did not significantly predict the presence of severe fatigue after deployment (all p-values >.66, unpublished data). In addition, mRNA expression levels of GR-α, GR-β and GR-P prior to deployment did not significantly differ between participants with and without severe fatigue six months after deployment (all p-values >.079, unpublished data). We should keep in mind that although we observed that low GC-sensitivity of monocytes was associated with subsequent development of severe fatigue, the GR pathway components were studied in the total subset of PBMCs. Since less than 10% of PBMCs consist of monocytes, a change in monocytes only may not be detected while investigating PBMCs. The T-cell compartment represents more than 40% of PBMCs which may explain why we could find differences in GR signalling components when T cell responses were involved. Studying the expression levels of GR pathway components specifically in monocytes may be a promising future approach for elucidating the intracellular mechanisms associated with the decreased GC-sensitivity within individuals vulnerable for development of severe fatigue.

An abundance of literature has investigated the association between severe fatigue and the functioning of the peripheral immune cells. Although results have been mixed (83), data in the literature point towards the presence of increased pro-inflammatory signaling in individuals with severe fatigue (84-86). The association between fatigue and increased pro-inflammatory signalling has predominantly been investigated by measuring circulating levels of pro-inflammatory cytokines. But, the response of the body to an inflammatory mediator or other regulatory mediators is not only determined by the circulating levels of the specific mediator at a given moment. The response of the body also depends on the reactivity of the target cells to a specific mediator (87). This reactivity of the target cells is determined by receptor number, ligand binding affinity and coupling of the receptor to intracellular signaling pathways. In addition, intracellular processes downstream of the receptor determine the reactivity of a cell to regulation by specific mediators (87). Thus, an increase in pro-inflammatory signaling may also result from increased reactivity of target cells to pro-inflammatory cytokines.

We investigated whether the development of severe fatigue was associated with altered reactivity of peripheral immune cells to pro-inflammatory mediator IL-1β (Chapter 8). Studies investigating the behavioral effects of cytokine administration have shown that the development of cytokine-induced sickness behavior in rodents is completely abrogated when

IL-1 action is blocked (88). Furthermore, it was previously shown that fatigue symptoms in patients with rheumatoid arthritis were attenuated after administration of an IL-1-receptor antagonist (IL-1RA) (89). Based on these data, we hypothesized that severe fatigue may be associated with increased reactivity to pro-inflammatory mediator IL-1β. Six months after deployment, the fatigued participants had higher IL-1β-induced IL-8 production than the non-fatigued participants. We observed that this group difference in IL-1β reactivity had developed over time, in response to the deployment. Our results showed that participants without severe fatigue showed a decrease in IL-1β-induced IL-8 production at one and six months after return from deployment compared to before deployment, indicating decreased reactivity to stimulation with IL-1β. We hypothesized that this decreased reactivity to IL-1β may have developed as a compensatory mechanism, to adapt to increased levels of pro-inflammatory cytokines during the deployment. In contrast, the peripheral immune cells of participants with severe fatigue did not show a change in IL-1β reactivity over time, which may indicate that participants who developed severe fatigue adapted less adequately to the stress experienced during the deployment.

Since fatigued and non-fatigued participants did not show different longitudinal courses of LPS-induced IL-8 production, the observed group difference in the IL-1β-induced IL-8 production was specifically associated with group differences in reactivity of peripheral blood cells to stimulation with IL-1β. Therefore, the mechanisms underlying the group difference in the IL-1β reactivity appear to be located at the level of the IL-1 receptor or signaling (90) or at the level of transcription factor NF-IL6 (91), which is specifically associated with IL-1β-induced IL-8 production (and not with LPS-induced IL-8 production).

These results show that assessing the reactivity of immune cells for regulation by inflammatory mediators *in vitro* is a novel and promising method to investigate whether increased pro-inflammatory signaling is involved in the pathophysiology underlying severe fatigue.

Peripheral IL-8 is predominantly produced by monocytes (92). Therefore, the pre-existing decreased GC-sensitivity of monocytes in participants who subsequently developed severe fatigue may be causally involved in the inadequate adaptation of IL-1β-sensitivity to stress within these individuals. Furthermore, our group previously observed decreased sensitivity of monocytes for β2-adrenergic regulation in adolescents with CFS (80). If this decreased β2-adrenergic-sensitivity of monocytes also precedes the development of severe fatigue, this process may also underlie the inadequate adaptation of IL-1β signalling to stress in the participants who subsequently developed fatigue.

In conclusion, both our identified biological correlates of severe fatigue suggest that the development of severe fatigue is associated with a net increase in pro-inflammatory signaling in monocytes, due to less efficient regulation of IL-1 and GR receptor function. We first observed that participants who developed severe fatigue in response to deployment had decreased sensitivity of monocytes for regulation by an anti-inflammatory mediator, ie GCs, prior to deployment. Additionally, we observed that those individuals showed increased

reactivity of monocytes to a pro-inflammatory mediator, i.e. IL-1β, after deployment. Bower et al. previously observed that female breast cancer survivors with severe fatigue had increased expression of pro-inflammatory genes and decreased expression of anti-inflammatory genes (85). Our findings of a less efficient receptor regulation indicate that these biological correlates of fatigue may be part of the underlying mechanism of the development of severe fatigue.

For the purpose of this discussion, we also investigated whether the capacity of T-cells to produce cytokines was associated with development of fatigue. The T-cell cytokine production prior to deployment did not predict subsequent development of severe fatigue (W= 0.406, p=.524). Thus, the development of severe fatigue in response to severe stress appears to be specifically associated with monocyte cytokine production, or more specifically: the sensitivity of receptors of monocytes to regulation by (anti-)inflammatory mediators.

THE PERIPHERY AS A MODEL FOR THE BRAIN

GC signalling and the GR pathway

Since it is not yet possible to directly assess expression of GRs and other GR pathway components in the brain *in vivo* (93), comparing the expression of GR pathway components in peripheral blood cells *in vitro* with HPA axis function is currently the only way to approach GR signalling centrally. However, whether the functioning of the GR pathway in peripheral blood cells *in vitro* actually is a reliable model for the GR pathway in the brain has not been studied extensively. Preclinical studies in rodents have shown that the affinity and specificity for GCs of neuronal and lymphoid cytosolic GRs are similar (94), and that cytosolic GRs in the brain and peripheral immune tissues are both downregulated after chronic corticosterone administration following adrenalectomy (95). These studies suggest that the peripheral GR pathway may indeed be a reliable model for GR pathway signalling in the brain. However, one has to keep in mind that the local cellular environment may also be an important factor for establishing the sensitivity of receptors such as GR. For example, the amount of circulating GCs during exposure to stress will also influence the sensitivity of the GR.

Although expression levels of the GR pathway components in the brain cannot be assessed, the net outcome of the total GR signalling pathway can be investigated by investigating the GC-sensitivity of the HPA axis. An abundance of studies have investigated the sensitivity of the GRs in the hypothalamus and pituitary for negative feedback by GCs, by administering DEX, DEX combined with CRH, or hydrocortisone, and subsequently measuring cortisol levels. Although not all data in the literature provide consistent results, currently increased sensitivity of (the GR in the hypothalamus and pituitary in) the HPA axis to negative feedback by GCs is considered as one of the hallmark biological correlates of PTSD (96, 97). Contrastingly, decreased sensitivity of the HPA axis to negative feedback by GCs is currently thought to be

one of the hallmark biological correlates of MDD (97-99). Studies on GC-sensitivity of the HPA axis in individuals with severe fatigue have been less consistent, but the overall data in the literature suggest that the presence of sever fatigue (CFS) may be associated with increased negative feedback of GCs on the HPA axis, implying more sensitive GRs centrally (84).

Interestingly, we observed that a high sensitivity of T-cells for GCs prior to deployment was associated with increased risk for development of PTSD symptoms, and that low GC-sensitivity of T-cells prior to deployment was associated with increased risk for development of depressive symptoms (Chapter 6). Thus, the sensitivity of the GRs in PBMCs for regulation by GCs observed prior to the development of PTSD and depressive symptoms in our prospective study are similar to the results of previous cross-sectional studies investigating the sensitivity of the GRs in the hypothalamus and pituitary for negative feedback regulations by GCs in individuals with PTSD and MDD. Since the observed directions in GC-sensitivity are the same within PBMCs and HPA-axis, we hypothesize that the vulnerability factors identified in the GR pathway in PBMCs may be paralleled in the HPA axis. Thus, we suggest that the observed changes in the GC-sensitivity of the HPA axis in individuals with PTSD and MDD is already present prior to the development of these symptoms, and represents a vulnerability factor for the development of these conditions.

We do not know whether an altered sensitivity of GRs for regulation by GCs in other brain regions than the hypothalamus and pituitary is also present prior to development of PTSD, depressive and fatigue symptoms. In individuals with PTSD and depressive symptomatology, the sensitivity of several other brain regions than the hypothalamus and pituitary for regulation by GCs has been investigated by measuring the effects of GC administration on memory and the effects of GCs on glucose metabolic rate and blood oxygenation. The overall results of these studies suggest that altered GC-sensitivity may indeed be present in various brain regions of individuals with PTSD or depressive symptomatology (97, 100-102) compared to healthy controls. A very recent study showed that mice with high trait anxiety, associated with more pronounced HPA axis activation and behavioural responses after exposure to an acute stressor, had increased levels of hippocampal GR protein and mRNA expression under non-stressed conditions (103), indicating that a central GR pathway dysregulation may be associated with increased vulnerability for the development of stress-related symptomatology.

In a spin-off of the PRISMO study, changes in brain activity were investigated before and after deployment with functional MRI (fMRI) in first-time deployers (104). A small subset of our participants also participated in this spin-off study. We observed that the number of GR in PBMCs prior to deployment correlated negatively with brain activity in the amygdala in response to biologically salient stimuli. We previously observed that a high GR number prior to deployment predicted the development of PTSD symptoms. Interestingly, a higher number of GR prior to deployment was also associated with a larger increase in amygdala activity in response to deployment, assessed approximately 1.5 months after return from deployment (Geuze et al, submitted). The amygdala plays an important role in threat detection and fear

regulation (105). It was repeatedly observed that amygdala hyperactivity is present in individuals with PTSD (106, 107). Furthermore, individuals with PTSD appear to have difficulties with fear extinction, including delayed fear extinction (108) and impaired fear extinction recall (109). Notably, a recent study in deployed military personnel showed that delayed fear extinction prior to deployment was associated with higher PTSD symptoms levels approximately two months after return from deployment (Lommen, presented at VGCT conference, the Netherlands, 2010).

Based on the results described above and on the data in the literature, we are tempted to speculate that the observed vulnerability factors (i.e. peripheral GR pathway components) for PTSD, depressive and fatigue symptoms may also be paralleled in the brain, and that an imbalance within the GR signalling cascade in the brain may be involved in the pathophysiology of PTSD, MDD, and perhaps also severe fatigue.

Cytokines

In rodents it has been shown that cytokine levels in the brain are the mirror image of cytokine levels in the periphery (110). Injecting rodents with a pro-inflammatory mediator induces an increase in pro-inflammatory cytokines in the periphery, but also in the CNS. For example, peritoneal administration of IL-1β in rats upregulated mRNA expression of various pro-inflammatory cytokines throughout the brain (111). In addition, the development of cytokine-induced sickness behavior in rodents is completely prevented when IL-1 action is blocked, either in the periphery or in the brain (88).

Given the fact that peripheral and central cytokine profiles are mirror images, we suggest that the inadequate adaptation to stress in the IL-1β signaling pathway, which we observed for participants with severe fatigue after deployment, is also paralleled in the brain. Six months after return from deployment, fatigued participants showed a higher reactivity of peripheral immune cells to stimulation with IL-1β, as assessed by measuring IL-1β-cytokine production, compared to non-fatigued participants. Hypothetically, an increased reactivity to pro-inflammatory mediators such as IL-1β could directly or indirectly (via upregulation of other pro-inflammatory cytokines) influence brain functioning (112) and induce behavioural changes (113). If increased reactivity of immunocompetent cells in the brain to IL-1β would indeed be present, then it may well be that the IL-1β-reactivity is involved in the development of the fatigue, or in maintenance or aggravation of the fatigue severity.

We observed that a high capacity of T-cells to produce cytokines prior to deployment predicted the development of depressive symptoms in response to deployment. The association between the presence of peripheral or central pro-inflammatory cytokines and the subsequent development of depressive symptomatology may be mediated by increased signalling to brain structures in response to upregulation of pro-inflammatory cytokines in vulnerable individuals (75). One likely candidate involved in the development of cytokine-induced

depression is indoleamine 2,3-dixoygenase (IDO) (74, 75, 114). IDO is upregulated by pro-inflammatory cytokines and blocking IDO activity and genetic ablation of IDO both prevents the development of depressive-like behavior in response to immune stimuli such as LPS or Bacille Calmé-Guerrin (115). The upregulation of IDO leads to the degradation of tryptophan, a precursor of 5HT-serotonin. The degradation of tryptophan subsequently leads to upregulation of the production of the tryptophan-metabolite kynurenine. The upregulated kynurenine is then metabolised into two important acids: the NMDA receptor agonist quinolinic acid and the NMDA receptor anatagonist kynurenic acid. Notably, glutamergic dysfunction has been implicated in the development of MDD. Furthermore, it was previously described that individuals with MDD have distorted trypophan/kynurenine and kynurenic acid/kynurenine ratios (115).

POSSIBLE CAUSAL MECHANISMS

We identified several vulnerability factors which appear to put individuals at increased risk for development of high levels of PTSD, depressive and fatigue symptoms after exposure to severe stress. The presence of these vulnerability factors may have evolved due to several causal mechanisms. We investigated several possible causal mechanisms for the observed vulnerability factors in the GR signalling pathway.

First of all, we hypothesized that the presence of specific single nucleotide polymorphisms (SNPs) in GR-associated genes may be related to the presence of these vulnerability factors. We investigated whether a high GR number and FKBP5 expression in PBMCs prior to deployment, which we identified to be vulnerability factors for development of PTSD symptomatology, were associated with the presence of specific SNPs in the GR and FKBP5 gene (Chapter 3 (5)). The presence of specific SNPs in the GR gene has been associated with increased risk for development of PTSD (116), MDD (117) and severe fatigue (118) during adulthood. More important with respect to our identified vulnerability factors, several SNPs in the GR gene appear to be associated with sensitivity of PBMCs and the HPA axis for regulation by GCs (119) and with HPA axis responsivity to stress (120) in healthy individuals. In addition, several SNPs in the FKBP5 gene have been associated with increased risk for development of PTSD and MDD during adulthood (121-125). These SNPs have also been found to be associated with the sensitivity of the HPA axis to negative feedback regulation by GCs (124, 125).

However, we did not observe significant associations between these SNPs and the expression of GR and FKBP5 prior to deployment. However, for these analyses we combined participants with and without high levels of PTSD symptoms after deployment. Mehta et al. (125) investigated associations between a common SNP in the FKBP5 gene and the FKBP5 mRNA expression separately in individuals with and without PTSD. It was observed that the association between the FKBP5 SNP and FKBP5 mRNA expression was reversed in individuals with PTSD. Within healthy individuals, the FKBP5 SNP was associated with increased FKBP5

mRNA expression; while in PTSD patients this SNP was associated with decreased FKBP5 mRNA expression. We hypothesize that a similar mechanism may be operative in our sample. Unfortunately, due to our limited number of participants with a high level of PTSD symptoms, we were not able to perform these analyses with sufficient statistical power.

Our identified vulnerability factors may also have developed due to previous exposure to severe or chronic stress during childhood or adulthood. The experience of childhood trauma or adversity is one of the most consistently identified risk factors for the development of PTSD (126), MDD (127) and severe fatigue (128) throughout adulthood. Therefore, it has repeatedly been suggested that childhood trauma may be causally associated with the biological correlates of PTSD, MDD and severe fatigue. Within the PRISMO-study, participants who had developed a high level of PTSD or depressive symptoms in response to their deployment reported a higher level of childhood traumatic experiences than participants who did not develop these symptoms. However, the number of childhood traumatic experiences was not significantly associated with GR pathway components in PBMCs within the whole participant group (Chapters 2 (4), 3 (5), 6). However, it has been repeatedly observed that only the experience of childhood trauma is associated with increased risk for development of PTSD and MDD only in individuals who also carry specific SNPs in the GR or FKBP5 gene (124, 129, 130). Likewise, we investigated whether the presence of a high GR number and low FKBP5 mRNA expression prior to deployment could be predicted by the experience of childhood trauma in combination with the presence of specific SNPs in the GR and FKBP5 genes (Chapter 3 (5)). We did observe that a high GR number in PBMCs prior to deployment was associated with the presence of GR SNP B*cl*I in combination with a high level of childhood trauma. Participants who carried the B*cl*I SNP and who had experienced a high level of childhood trauma also appeared to be at increased risk for the development of PTSD symptoms in response to deployment, although this effect failed to reach statistical significance. These results suggest that (some of) our observed vulnerability factors may indeed have developed as a result from exposure to childhood trauma or adversity.

Furthermore, the vulnerability factors may also have developed in response to previous exposure to severe or chronic stress during adulthood. It has repeatedly been observed that the GR pathway components in PBMCs may change in response to chronic or repeated stress during adulthood (98, 131, 132). For example, previous results of our group have shown that GR expression and GC-sensitivity of PBMCs decreased in response to military deployment without subsequent development of PTSD (13).

We did not investigate causal mechanisms associated with the presence of a high T-cell cytokine production capacity in participants who developed depressive symptoms, or with the presence of the inadequate adaptation of the IL-1β-reactivity of monocytes to the deployment in participants who developed severe fatigue after deployment. Clearly, these vulnerability factors may also be associated with the presence of specific SNPs, or changes due to exposure to severe stress during childhood or adulthood. Notably, our finding that the

T-cell cytokine production of participants who did not develop depressive symptoms had increased after deployment (Chapter 7), clearly demonstrates that the high T-cell cytokine production capacity, which was predictive for depression, may also have emerged in result to previous stress during adulthood.

The exposure to severe or chronic stress, either during childhood of adulthood, may have resulted in epigenetic changes in the methylation status of genes which are associated with our vulnerability factors (133), thereby leading to changes in the GR signalling pathway. In addition, these epigenetic changes as a result of severe stress may also have been transmitted trans-generationally (134). Therefore, the presence of the vulnerability factors may also have resulted from exposure to severe stress by the parents of the vulnerable individuals. Moreover, the stress of the military deployment may also cause epigenetic changes on the level of the capacity to produce cytokines, which may also be transferred trans-generationally.

CONCLUSIONS, CLINICAL PERSPECTIVES AND FUTURE RESEARCH

We were able to identify several biological vulnerability factors for the development of PTSD, depressive and fatigue symptoms in response to deployment, i.e. a period of severe and possibly traumatic stress. These vulnerability factors were identified prior to the deployment preceding the onset of symptoms. Intriguingly, these identified vulnerability factors were condition specific (Figure 2). Increased risk for development of a high level of PTSD symptoms was shown to be associated with increased signalling GC signalling in PBMCs, as assessed on various levels of the GR signalling pathway (Chapter 2 (4), Chapter 3 (5), Chapter 6). In contrast, increased risk for development of a high level of depressive symptoms appears to be associated with decreased GC signalling in PBMCs, and specifically in T-cells (Chapter 6). In addition, participants with a high T-cell cytokine production capacity prior to deployment were also at increased risk for development of depressive symptoms (Chapter 7). Furthermore, these participants also showed a smaller increase in T-cell cytokine production capacity after deployment, compared to non-depressed participants. Increased risk for development of severe fatigue appears to be associated with pre-existing high pro-inflammatory signaling in monocytes: we observed that low GC-sensitivity of monocytes prior to deployment in participants who developed severe fatigue (Chapter 6). In addition, these participants showed an increased reactivity of monocytes to IL-1β after deployment (Chapter 8).

Collectively, our results suggest that the biological profile prior to exposure to severe stress or trauma is involved in the development of mental and physical health problems in response to the exposure. Moreover, our results indicate that the biological profile may not only determine *if* an individual will develop any stress-related condition, but also *which* specific stress-related condition the individual will develop.

By identifying several condition-specific vulnerability factors we convincingly showed that performing prospective, longitudinal studies in groups at risk for stress- and trauma

exposure is a promising and fruitful approach for identifying biological vulnerability factors for development of stress-related conditions. Ideally, future prospective studies in populations at risk for trauma exposure should investigate the sensitivity and specificity of various cut-offs for our identified vulnerability factors for the actual development of PTSD, MDD and severe fatigue in response to stress- and trauma exposure. Thereby, optimal cut-offs may eventually be determined, which could ultimately result in condition-specific risk-assessments, in which several biological and psychological vulnerability factors are combined. Preferably, these future studies would investigate multiple outcomes of exposure to stress and also take co-morbidity between stress-related conditions into account. Furthermore, these studies may define the stability of the identified vulnerability factors. Additionally, causal mechanisms associated with the presence of these vulnerability factors may also be investigated, for example by investigating recent exposure to life events and severe stress.

Determining vulnerability for stress-related conditions is likely to be valuable for individuals in jobs with a high risk for stress- and trauma exposure, such as the military, but also police personnel, fire-fighters and emergency workers. If optimal cut-offs for our vulnerability factors can be identified, vulnerable individuals may in the future be identified via screening prior to stress- and trauma-exposure e.g. in case of military deployment, or via screening at regular intervals in jobs with more continuous stress exposure e.g. fire-fighters. Vulnerable individuals could then be assigned to interventions to boost their resilience to stress- and trauma exposure. In addition, they could be assigned to early interventions immediately after the occurrence of a severely stressful or traumatic event. This could potentially prevent the development or aggravation of symptoms. It has previously been described that directing a single psychological intervention directly after a traumatic event to all trauma-exposed individuals does not diminish prevalence rates of PTSD, and may sometimes even be detrimental (135). Selecting individuals who are most likely to benefit from interventions directly after stress- or trauma exposure seems to be a more promising approach (136).

From a clinical perspective, our findings are also important. Our results underscore that individuals who developed various conditions in response to comparable exposure to stress and trauma may not necessarily benefit from a similar pharmaceutical approach, since the underlying biological causal mechanisms are different. Perhaps, our results are a starting point for new directions for potential targets for pharmaceutical interventions for prevention or treatment of stress-related conditions. With respect to pharmaceutical interventions, the observed difference in the directions of sensitivity of peripheral blood cells for regulation by GCs between PTSD on the one hand and depressive symptomatology and severe fatigue on the other hand may be a very important guide.

It has repeatedly been investigated whether GR-antagonists can be used as a pharmaceutical treatment for MDD or depressive symptomatology (137). This treatment approach was initiated after it was observed that successful treatment of MDD with anti-depressants modulates GR function (138). It has been suggested that prolonged administration of a

GR-antagonist results in a long-lasting increase in GR function, thereby restoring the impaired negative feedback of GCs on the HPA axis associated with MDD (137). Pre-clinical studies in rodents have shown that administration of GR-antagonist mifepristone/RU486 reduced the development of depressive-like behavior and stress-induced changes in the HPA axis and limbic brain regions in response to an acute stressor (139). However, clinical studies have reported mixed results with respect to the effectivity of GR-antagonist treatment in diminishing symptoms of MDD (137). If the prolonged administration of a GR antagonist would indeed result in increased GR function and GC-sensitivity, then it is conceivable that treated individuals become more vulnerable for development of PTSD after exposure to trauma.

The other way around, administration of synthetic GCs has been suggested to be an effective treatment for PTSD (140). It may be that prolonged administration of GCs results in decreased GR function and a subsequent decrease in sensitivity of the HPA axis for negative feedback by GCs. If this would indeed happen, then treated individuals may become more vulnerable for development of depressive symptoms and severe fatigue in response to severe stress. Perhaps another pharmaceutical approach, such as blockade of the GR immediately after recall of a traumatic memory during a therapeutic session (as suggested by 141), might be a safer approach in the treatment of PTSD.

We propose that future studies investigating the efficacy of treatment of MDD with GR-antagonists or treatment of PTSD with high doses of GCs should also include long-term follow-up measures, investigating exposure to stressful events after treatment in relation to a broad variety of mental and physical health outcomes. In addition, a very recent study suggested that blocking FKBP5 might be an effective treatment for MDD (142). Since we observed that low FKBP5 expression predicted the development of PTSD symptoms in response to trauma exposure, it must be very carefully studied whether such an approach would perhaps induce an increased vulnerability for PTSD development.

For this thesis we also investigated the longitudinal course of cytokine production. Interestingly, we observed that military deployment induced changes in the cytokine production by peripheral immune cells upon stimulation in all participants, also in participants who did not develop stress-related conditions. These changes were long-lasting: six months after return from deployment they were still present. We think that it is important to investigate whether these observed changes in cytokine production after deployment will indeed result in increased risk for development of medical conditions and increased vulnerability for development of stress-related conditions in response to new stress exposure, as we previously hypothesized. It should be investigated how long after return from military deployment, or comparable periods of severe stress, these observed changes in cytokine production remain present. These remarkable observations demonstrate that using prospective, longitudinal designs is very valuable for investigating the effects of exposure to severe stress in individuals who do not develop stress-related conditions in the immediate aftermath.

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10

Nederlandse Samenvatting

(Dutch Summary)

INLEIDING

Een deel van de mensen die stressvolle gebeurtenissen meemaken ontwikkelt hierna psychische en/of lichamelijke klachten. Wanneer een gebeurtenis als traumatisch werd ervaren, kan er daarna sprake zijn van een posttraumatische stress stoornis (PTSS). Een PTSS-diagnose kan gesteld worden wanneer iemand na blootstelling aan een traumatische ervaring drie verschillende soorten symptomen ontwikkeld heeft:

1. Het voortdurend herbeleven van de traumatische gebeurtenis (o.a. opdringende onaangename herinneringen, nachtmerries en flashbacks).
2. Het vermijden van prikkels die doen denken aan het trauma of afstomping van de algemene reactiviteit (o.a. vermijden van gesprekken, plaatsen of mensen die herinneringen oproepen, verminderde belangstelling voor activiteiten, gevoelens van vervreemding en beperkte toekomst).
3. Voortdurende verhoogde prikkelbaarheid (o.a. slaapproblemen, concentratieproblemen en voortdurende waakzaamheid).

Voor het officieel stellen van de diagnose PTSS moeten deze symptomen meer dan een maand aanwezig zijn en ernstig lijden veroorzaken.

Naast PTSS kunnen ook andere symptomen ontstaan na blootstelling aan stressvolle of traumatische gebeurtenissen, waaronder depressie en ernstige vermoeidheid. PTSS, depressie en ernstige vermoeidheid kunnen leiden tot een ernstige verstoring van het dagelijks functioneren, zowel in het contact met familie en vrienden, als in het beroepsmatig functioneren. Bij deze stressgerelateerde aandoeningen is er vaak sprake van comorbiditeit: dit houdt in dat er op een zeker moment verschillende klachten en/of stoornissen tegelijk aanwezig zijn.

Omdat niet alle mensen die een stressvolle of traumatische gebeurtenis meemaken vervolgens stressgerelateerde aandoeningen ontwikkelen, is het van belang om te weten welke factoren een rol spelen bij het ontwikkelen van de klachten. Daarom wordt er veel onderzoek gedaan naar de biologische en psychologische oorsprong van deze aandoeningen.

Onze hypothese is dat de ontwikkeling van de stressgerelateerde aandoeningen PTSS, depressie en vermoeidheid samenhangt met (biologische en psychologische) kwetsbaarheidsfactoren, die al aanwezig zijn voor de stressvolle of traumatische gebeurtenissen die uiteindelijk tot de klachten leiden. De identificatie van kwetsbaarheidsfactoren is belangrijk, omdat deze kwetsbaarheidsfactoren vervolgens gebruikt kunnen worden om te bepalen wie er risico loopt op het ontwikkelen van deze aandoeningen na blootstelling aan stressvolle of traumatische gebeurtenissen.

Om kwetsbaarheidsfactoren te kunnen identificeren, zijn prospectieve studies nodig, waarbij het verzamelen van gegevens al begint voor blootstelling aan de gebeurtenis die uiteindelijk leidt tot het ontwikkelen van de stressgerelateerde klachten. In de praktijk kan dit soort onderzoek alleen maar worden uitgevoerd in mensen die een verhoogd risico

hebben op blootstelling aan stressvolle en traumatische gebeurtenissen, bijvoorbeeld vanwege hun beroep. Omdat het moeilijk is om op het juiste moment toegang te krijgen tot zulke populaties, is er nog maar weinig onderzoek gedaan naar kwetsbaarheidsfactoren voor stressgerelateerde aandoeningen.

DIT PROEFSCHRIFT

Onderzoekspopulatie

Militairen die worden uitgezonden hebben een hoog risico om stressvolle, en mogelijk traumatische, gebeurtenissen mee te maken. Enkele voorbeelden hiervan zijn: betrokken raken in gevechtshandelingen, tegenkomen van bermbommen, en getuige zijn van het gewond raken of overlijden van collega's. Verder ervaart het merendeel van de uitgezonden militairen het als stressvol om langdurig van hun familie en vrienden gescheiden te zijn. Ondanks de hoge kans op blootstelling aan stress- en trauma tijdens uitzending, ontwikkelt slechts een minderheid van de uitgezonden militairen stressgerelateerde aandoeningen zoals PTSS, depressie en ernstige vermoeidheid in reactie op de uitzending.

De hoofdstukken van dit proefschrift zijn geschreven aan de hand van data verzameld in het kader van het PRISMO-onderzoek ('Prospectie In Stressgerelateerd Militair Onderzoek'). Dit onderzoek is in 2005 gestart en zal in 2018 worden afgerond. In het onderzoek worden biologische en psychologische aspecten van de ontwikkeling van stressgerelateerde (geestelijke) gezondheidsklachten onderzocht. De opzet van de studie is prospectief (eerste meting voorafgaand aan de uitzending) en longitudinaal (deelnemers worden tot tien jaar na de uitzending gevolgd).

Het onderzoek wordt uitgevoerd door het onderzoekscentrum van de Militaire Geestelijke Gezondheidszorg en het NIDOD-Laboratorium ('Neuroimmunology and Developmental Origins of Disease') van het UMC Utrecht. Alle deelnemers van de studie zijn Nederlandse militairen die tussen 2005 en 2008 werden uitgezonden naar Afghanistan (ISAF-missie). In totaal hebben 1032 Nederlandse militairen zich voor de studie aangemeld. Het onderzoek werd goedgekeurd door de Medisch Ethische Toetsingscommissie (METC) van het UMC Utrecht.

Voor dit proefschrift werd gebruik gemaakt van de data die werd verzameld tijdens de eerste drie meetmomenten: voorafgaand aan de uitzending, en één en zes maanden na terugkeer van de uitzending. Tijdens deze metingen vulden de deelnemers vragenlijsten in en stonden zij bloed- en speekselmonsters af.

Doel

Het doel van dit proefschrift was het identificeren van kwetsbaarheidsfactoren voor het ontwikkelen van PTSS symptomen, depressieve symptomen en vermoeidheid in reactie op uitzending. In dit proefschrift lag de focus voornamelijk op het identificeren van biologische kwetsbaarheidsfactoren, gemeten voorafgaand aan de uitzending. Ook onderzochten wij de voorspellende waarde van enkele veelbelovende psychologische variabelen, gemeten voorafgaand aan de uitzending.

Achtergrond

Cortisol

De hypothalamus-hypofyse-bijnier as (Engelse afkorting: HPA as) is een belangrijk deel van het stressregulerende systeem. Tijdens stress wordt in de hypothalamus 'corticotrophin-releasing hormone' (CRH) en vasopressine (AVP) aangemaakt. Hierdoor wordt 'adrenocorticotropic hormone' (ACTH) uitgescheiden door de hypofyse. Daarna zorgt ACTH ervoor dat de bijnieren glucocorticoïden (in mensen: cortisol) maken en vervolgens uitscheiden in het bloed. Cortisol heeft verschillende functies en beïnvloedt vrijwel elke cel in het menselijk lichaam. Eén van de functies is het voorbereiden en adequaat reageren van het lichaam op stress. Een andere functie van cortisol is het beëindigen van de stressreactie en het herstellen van de balans (homeostase) in het lichaam: nadat cortisol in de bloedstroom uitgescheiden is, geeft cortisol negatieve feedback op de hypothalamus en hypofyse. Dit zorgt ervoor dat de uitscheiding van CRH en ACTH geremd wordt, en vervolgens dus ook de uitscheiding van cortisol.

Cytokinen

Activatie van het immuunsysteem door bijvoorbeeld een infectie leidt ertoe dat het immuunsysteem en diverse andere orgaansystemen (o.a. de lever, milt) cytokinen gaan produceren. Dit zijn eiwitten die functioneren als boodschappers tussen de verschillende organen. Een veelgebruikte manier om cytokinen te classificeren is door onderscheid te maken tussen pro-inflammatoire (ontstekingsbevorderende) en anti-inflammatoire (ontstekkingsremmende) cytokinen. Vervolgens zijn er ook nog chemokinen; deze cytokinen kunnen cellen naar een specifieke locatie, bijvoorbeeld een ontsteking of een wond, aantrekken (chemotaxis). De reactie van immuuncellen en organen op cytokinen wordt niet alleen bepaald door de hoeveelheid cytokinen die op dat moment aanwezig is. De responsiviteit of gevoeligheid van cellen voor een specifieke cytokine speelt ook een belangrijke rol. De responsiviteit wordt onder andere bepaald door hoeveel receptoren voor een specifieke cytokine er aanwezig zijn in een cel.

Cytokinen geproduceerd door het immuunsysteem kunnen ons gedrag beïnvloeden door dat ze naar de hersenen kunnen signaleren. Cytokinen reguleren niet alleen het functioneren van het immuunsysteem, maar ze zijn ook betrokken bij het ontstaan van niet-specifieke ziektesymptomen tijdens ziekte. Dit wordt ziekte-gedrag ("sickness behavior") genoemd. De belangrijkste symptomen hiervan zijn vermoeidheid, malaise, depressieve klachten en spierpijn. Deze symptomen vertonen veel overeenkomst met symptomen behorend bij stressgerelateerde aandoeningen, met in het bijzonder vermoeidheid en depressie.

Het aantal cytokinen en chemokinen kan worden gemeten in perifeer bloed, dit geeft een indicatie van de activatiestatus van het immuunsysteem. Eerdere studies lieten zien dat de hoeveelheid cytokinen in het bloed verhoogd kan worden door stress. Verder lijkt het zo te zijn dat mensen met PTSS, depressie en ernstige vermoeidheid onder basale omstandigheden ook een hogere hoeveelheid cytokinen in hun bloed hebben. Het is ook mogelijk om in een laboratorium de capaciteit van immuuncellen om cytokinen te produceren te meten (*in vitro* stimulatie). Dit kan onder andere worden gedaan door immuuncellen te stimuleren met een stof waarvan bekend is dat deze het immuunsysteem activeert. Verder kan de gevoeligheid van een specifieke cytokine-receptor worden gemeten door immuuncellen te stimuleren met een cytokine.

Uit eerdere studies bleek dat mensen met ernstige vermoeidheid, depressie en PTSS een verhoogde activatiestatus van het immuunsysteem lijken te hebben. Ook werd eerder al aangetoond dat de mensen met ernstige vermoeidheid, depressie en PTSS mogelijk een andere capaciteit hebben om cytokinen te produceren dan mensen zonder deze klachten. Het is echter nog onbekend of deze veranderingen al aanwezig zijn voorafgaand aan het ontstaan van de klachten.

De invloed van cortisol op het immuunsysteem

De HPA as staat in nauwe verbinding met alle organen in het lichaam, en dus ook met het immuunsysteem. Als het immuunsysteem geactiveerd wordt, wordt vervolgens ook de HPA as geactiveerd door pro-inflammatoire cytokinen. Cortisol kan op zijn beurt ook het functioneren van het immuunsysteem reguleren. Twee belangrijke mechanismen hierbij zijn remming (inhibitie) van de deling van immuuncellen en van de productie van pro-inflammatoire cytokinen.

Tijdens stressvolle omstandigheden worden de effecten van het stresshormoon cortisol op het lichaam doorgegeven door glucocorticoid receptoren (GR). Dit is een complex proces. Ten eerst hangt de hoeveelheid doorgegeven signaal af van de hoeveelheid cortisol die wordt uitgescheiden door de bijnieren. De mate van de signaaloverdracht van cortisol wordt ook bepaald door het aantal GR dat aanwezig is in een cel. De signaaloverdracht door de GR wordt ook bepaald door de hoeveelheid FKBP5; dit is een eiwit dat de binding van cortisol op de GR remt. Wanneer er meer FKBP5 aanwezig is in een cel, zal er dus minder signaaloverdracht van cortisol door de GR plaatsvinden. Zo zijn er nog een aantal andere componenten in de cel

die het uiteindelijke effect van cortisol op de cel beïnvloeden. Het totale effect van de cortisol op een cel kan worden bepaald door te meten hoe gevoelig de cellen zijn voor regulatie door cortisol. Dit kan bijvoorbeeld door te meten hoe sterk de productie van immuuncellen *in vitro* wordt geremd als dexamethasone (een synthetische vorm van cortisol) aan de cellen wordt toegevoegd.

In eerdere studies werd beschreven dat de leukocyten van mensen met ernstige vermoeidheid, depressie en PTSS een andere gevoeligheid voor regulatie door cortisol hebben dan de leukocyten van mensen zonder deze klachten. Ook werden in de leukocyten van mensen met ernstige vermoeidheid, depressie en PTSS veranderingen gemeten in de componenten betrokken bij het doorgeven van het signaal van cortisol in een cel, waaronder het aantal GR en de hoeveelheid FKBP5 aanwezig per cel. Het is echter nog onbekend of deze veranderingen al aanwezig zijn voorafgaand aan de gebeurtenissen waarna de klachten ontstaan. Het kan ook zo zijn dat deze veranderingen ontstaan als gevolg van het meemaken ingrijpende gebeurtenissen, of als gevolg van de aanwezigheid van vermoeidheid, depressie en PTSS.

Bevindingen

Deel 1: Cortisol en de invloed van cortisol op het immuunsysteem

In **hoofdstuk 2** (1) onderzochten wij of militairen die na uitzending veel PTSS symptomen rapporteerden voorafgaande aan de uitzending een hoger aantal GR in leukocyten hadden dan militairen zonder PTSS symptomen na uitzending. Verder onderzochten wij of het aantal GR voor uitzending een voorspeller was voor de aanwezigheid van een hoog aantal PTSS symptomen na uitzending.

Voor dit hoofdstuk werden 34 mannelijke militairen geïncludeerd die 6 maanden na uitzending een hoog aantal PTSS symptomen hadden (gemeten met de vragenlijst ZIL) zonder dat ze voor uitzending een hoog aantal PTSS symptomen hadden. Ook includeerden wij 34 mannelijke controles waarvan de leeftijd, rang, aantal eerdere uitzendingen, opleidingsniveau en functie tijdens de uitzending gelijk was aan die van de militairen in de PTSS groep. De controles hadden op geen van de tijdstippen een hoog aantal PTSS of depressieve symptomen. Het aantal GR in leukocyten werd bepaald in bloed verkregen voor de uitzending, en 1 en 6 maanden na terugkeer van de uitzending.

Militairen met veel PTSS symptomen na de uitzending hadden voorafgaand aan de uitzending significant meer GR in leukocyten dan de controle groep. Bij elke extra 1000 GR aanwezig voor uitzending werd het risico op een hoog aantal PTSS symptomen na uitzending 7.5 maal zo groot. Eén en zes maanden na uitzending hadden de militairen in de PTSS groep nog steeds significant meer GR dan de controlegroep. Het gevonden groepsverschil hing niet samen met het feit dat ongeveer de helft van de militairen in de PTSS groep ook depressieve klachten had. Deze resultaten tonen aan dat een hoog aantal GR in leukocyten voorafgaand

aan een militaire uitzending een kwetsbaarheidsfactor is voor het ontwikkelen van PTSS symptomen na uitzending.

Op basis van hoofdstuk 2 vormden wij de hypothese dat bij militairen die veel PTSS symptomen ontwikkelden in reactie op de uitzending, voorafgaand aan de uitzending de signaaloverdracht door de GR in leukocyten anders was dan bij militairen die geen PTSS symptomen ontwikkelden. In **hoofdstuk 3** (2) werd daarom de voorspellende waarde van verschillende componenten die te maken hebben met signaaloverdracht na stimulatie van de GR door cortisol voor het ontwikkelen van een hoog aantal PTSS symptomen onderzocht. Wij onderzochten ten eerste de voorspellende waarde van de hoeveelheid expressie van de intracellulaire eiwitten FKBP5, GILZ en SGK1 in leukocyten. Dit zijn eiwitten waarvan de expressie direct geïnduceerd wordt door de stimulatie van de GR door cortisol. Verder wilden wij de voorspellende waarde van het aantal GR voor het ontwikkelen van PTSS symptomen repliceren in deze grotere en meer heterogene groep deelnemers. Ook onderzochten wij de voorspellende waarde van andere factoren zoals de hoeveelheid cortisol in het bloed, en van trauma in de kindertijd voor het ontwikkelen van PTSS symptomen.

Dit werd onderzocht in een groep van 448 mannelijke militairen, waarvan 35 militairen zes maanden na uitzending een hoog aantal PTSS symptomen hadden ontwikkeld. Geen van deze militairen had voor uitzending een hoog aantal PTSS symptomen. Alle voorspellers werden gemeten voorafgaand aan de uitzending. Deze voorspellers werden gebruikt om de aanwezigheid van veel PTSS symptomen zes maanden na uitzending te voorspellen.

Wij toonden aan dat verschillende componenten die te maken hebben met de signaaloverdracht van de GR in leukocyten significante voorspellers waren voor het ontwikkelen van een hoog aantal PTSS symptomen. Een hoog aantal GR, lage FKBP5 expressie en/of hoge GILZ expressie voorafgaand aan de uitzending hangt samen met een verhoogd risico op het ontwikkelen van een hoog aantal PTSS symptomen in reactie op de uitzending. Afgezien van de biologische factoren was ook het meemaken van potentieel traumatische ervaringen in de kindertijd was ook een voorspeller van het ontwikkelen van PTSS symptomen in reactie op uitzending. Deze variabelen zijn dus kwetsbaarheidsfactoren voor het ontwikkelen van veel PTSS symptomen in reactie op een uitzending.

In het tweede deel van dit hoofdstuk onderzochten wij of 2 van de gevonden kwetsbaarheidsfactoren (hoog aantal GR en een lage FKBP5 expressie) terug te voeren waren op genetische variatie. Hiervoor onderzochten we polymorfismen in het GR gen en FKBP5 gen (dit zijn kleine verschillen op niveau van het DNA dat codeert voor deze genen). We vonden dat deelnemers die veel traumatische ervaringen in de kindertijd hadden meegemaakt en die ook een specifiek polymorfisme in het GR gen hadden, gemiddeld een hoger aantal GR hadden voorafgaand aan de uitzending. Mensen die veel trauma in de kindertijd hebben meegemaakt en die ook deze specifieke polymorfisme in het GR-gen hebben lijken als volwassene dus een verhoogd risico te hebben voor het ontwikkelen van PTSS symptomen.

Het meemaken van stressvolle gebeurtenissen, zoals een militaire uitzending, kan leiden tot ernstige vermoeidheid en depressieve klachten. In **hoofdstuk 4** (3) onderzochten we of het aantal GR in leukocyten samenhing met het ontwikkelen van ernstige vermoeidheid met of zonder depressieve symptomen in reactie op de uitzending. Hiervoor vergeleken we het aantal GR tussen 3 groepen: militairen met ernstige vermoeidheid zonder depressieve symptomen, militairen met ernstige vermoeidheid en depressieve symptomen en militairen zonder ernstige vermoeidheid en depressieve symptomen.

Voor dit hoofdstuk werden 35 mannelijke militairen geïncludeerd die 6 maanden na uitzending ernstige vermoeidheid rapporteerden (gemeten met de vragenlijst CIS), zonder dat ze voor uitzending ernstig vermoeid waren. Van deze vermoeide militairen rapporteerden 14 ook veel depressieve symptomen na de uitzending (gemeten met de subschaal depressie van de SCL90). Wij includeerden verder 21 militairen, die geen ernstige vermoeidheid of depressieve symptomen hadden. Het aantal GR in leukocyten werd bepaald in bloed verkregen voorafgaand aan de uitzending, en één en zes maanden na terugkeer van de uitzending.

Vermoeide deelnemers met depressieve symptomen zes maanden na uitzending hadden zowel voor als na uitzending meer GR in leukocyten dan de andere twee groepen. Net als in hoofdstuk 2 en 3, was het verschil in het aantal GR tussen de groepen dus al aanwezig voorafgaande aan de uitzending. Het groepsverschil in de GR werd niet veroorzaakt door verschillen tussen de groepen in vermoeidheid en depressieve symptomen voorafgaand aan de uitzending. Deze resultaten tonen aan dat een hoog aantal GR in leukocyten ook een kwetsbaarheids factor zou kunnen zijn voor het ontwikkelen van ernstige vermoeidheid met depressieve symptomen na een langdurige stressvolle periode, zoals een uitzending.

Cortisol speelt een belangrijke rol in de reactie van het lichaam op stress. De 'cortisol awakening response' (CAR) is een snelle stijging in cortisol spiegels onmiddellijk na het ontwaken. Het meten van de CAR geeft een indicatie van het vermogen van de bijnieren om cortisol aan te maken. Eerder werd beschreven dat PTSS-patiënten een lagere CAR hebben dan gezonde controles. In **hoofdstuk 5** (4) beschrijven we de voorspellende waarde van de CAR voorafgaand aan de uitzending voor de aanwezigheid van PTSS symptomen zes maanden na terugkeer van de uitzending.

Ook psychologische factoren spelen een belangrijke rol in stressreactiviteit en in de ontwikkeling van PTSS. Persoonlijkheidstrekken bepalen voor een groot deel hoe iemand reageert op ingrijpende gebeurtenissen. Daarom onderzochten wij ook of de aanwezigheid van bepaalde persoonlijkheidstrekken voorafgaand aan de uitzending de aanwezigheid van PTSS symptomen zes maanden na de uitzending voorspelden. Ook onderzochten we of persoonlijkheid, CAR en PTSS symptomen voor uitzending een causale rol hadden in de eerdere gevonden relaties tussen PTSS na uitzending en leeftijd, trauma in de kindertijd en eerdere uitzendingen.

We gebruikten data van 470 mannelijke militairen. Alle voorspellers werden gemeten voorafgaand aan de uitzending. Voor het meten van persoonlijkheid gebruikten we 8

persoonlijkheidskenmerken (gemeten met de Cook-Medley Hostility scale en de Temperament-Character Inventory). De CAR werd bepaald met behulp van speeksel verzameld direct na het ontwaken en 15, 30 en 60 minuten later. Deze voorspellers werden gebruikt om de hoeveelheid PTSS symptomen 6 maanden na uitzending te voorspellen (gemeten met de ZIL). Militairen met een hoog aantal PTSS symptomen voor de uitzending werden niet meegenomen in de analyses.

Wel voorspelden persoonlijkheidstrekken voorafgaand aan de uitzending het aantal PTSS symptomen na uitzending: militairen die voor uitzending hoog scoren op hostiliteit (vijandig, cynisch, wantrouwend, slechte relaties met anderen) en/of laag scoren op zelfsturendheid (onvolwassen, moeite met de inrichting van hun eigen leven), en militairen die voor uitzending al een zekere mate van PTSS symptomen hebben, kwetsbaar zijn voor de ontwikkeling van PTSS symptomen na uitzending. De aanwezigheid van deze persoonlijkheidstrekken wordt onder andere beïnvloed door een jongere leeftijd tijdens uitzending en traumatische ervaringen in de kindertijd. De CAR voorafgaand aan de uitzending was niet gerelateerd aan PTSS symptomen na uitzending.

In **hoofdstuk 6** werd onderzocht of de gevoeligheid van leukocyten voor regulatie door cortisol ook een kwetsbaarheidsfactor is voor het ontwikkelen van stressgerelateerde symptomen. Hiervoor onderzochten we of de gevoeligheid van leukocyten voor regulatie door cortisol voorafgaand aan de uitzending een voorspeller was voor het ontwikkelen van ernstige vermoeidheid, veel depressieve symptomen en/of veel PTSS symptomen in reactie op de uitzending.

Dit werd gedaan in een groep van 523 mannelijke militairen. We bepaalden de gevoeligheid van twee soorten perifere leukocyten (T-cellen en monocyten) voor regulatie door cortisol voorafgaand aan de uitzending. Dit werd gedaan door het remmende effect van dexamethasone (synthetisch cortisol) op cytokine productie door geactiveerde T-cellen en monocyten te meten. Deze voorspellers werden gebruikt om de aanwezigheid van veel vermoeidheid (gemeten met de CIS), depressieve (gemeten met de SCL90 depressie subschaal) en PTSS symptomen (gemeten met de ZIL) 6 maanden na terugkeer van uitzending te voorspellen.

Onze resultaten tonen aan dat de aanwezigheid van ernstige vermoeidheid zes maanden na terugkeer van uitzending samenhangt met een lage gevoeligheid van monocyten voor remming door cortisol voorafgaand aan de uitzending. De aanwezigheid van veel depressieve symptomen zes maanden na uitzending hangt samen met een lage gevoeligheid van T-cellen voor remming door cortisol voorafgaand aan de uitzending. De aanwezigheid van veel PTSS symptomen zes maanden na uitzending hangt juist samen met een hoge gevoeligheid van T-cellen voor remming door cortisol voorafgaand aan de uitzending. Dit is echter alleen het geval in militairen die na uitzending veel PTSS symptomen rapporteerden, zonder dat ze ook depressieve symptomen hadden. Deze resultaten werden niet veroorzaakt door

de aanwezigheid van vermoeidheid, depressieve en/of PTSS symptomen voorafgaand aan de uitzending in de mensen die na de uitzending veel symptomen rapporteerden.

De gevoeligheid van leukocyten voor remming door cortisol is dus een kwetsbaarheidsfactor voor het ontwikkelen van ernstige vermoeidheid, depressieve en PTSS symptomen in reactie op uitzending. Onze resultaten tonen aan dat stressgerelateerde symptomen van vermoeidheid, depressie en PTSS alle drie een verschillende biologische oorsprong hebben. Deze biologische verschillen lijken al aanwezig te zijn voordat de stressgerelateerde symptomen zich ontwikkelen.

Deel 2: de productie van cytokinen

In **hoofdstuk 7** (5) onderzochten we of de aanwezigheid van veel depressieve symptomen 6 maanden na uitzending samenhangt met de capaciteit van leukocyten om cytokinen te produceren. Hiervoor bepaalden we de capaciteit van T-cellen en monocyten om cytokinen en chemokinen te produceren.

Voor dit hoofdstuk includeerden we alle deelnemers van wie er bloed beschikbaar was; dit waren er 1023. Van 733 deelnemers waren er ook gegevens beschikbaar van de meting 6 maanden na terugkeer van de uitzending. 68 van deze deelnemers hadden 6 maanden na uitzending veel depressieve symptomen (gemeten met de SCL-90 depressie subschaal). We gebruikten cellen uit het bloed dat werd verzameld voorafgaand aan de uitzending, en 1 en 6 maanden na terugkeer van uitzending. Op elk tijdstip werden 16 cytokinen en chemokinen gemeten. Deze 16 cytokinen zijn vervolgens in 3 clusters geanalyseerd: T-cel cytokinen, T-cel chemokinen en monocyte cytokinen.

De T-cellen van deelnemers met veel depressieve symptomen na uitzending hadden voorafgaand aan de uitzending een hogere capaciteit om cytokinen te produceren. Bovendien was de mate van T-cel cytokinen productie voorafgaand aan de uitzending een significante voorspeller voor de aanwezigheid van veel depressieve symptomen na de uitzending. Deze voorspellende waarde was onafhankelijk van het aantal depressieve symptomen voorafgaand aan de uitzending, wat erop duidt dat de cytokinen productie door T-cellen een voorspeller is voor de ontwikkeling van depressieve symptomen in reactie op de uitzending. Na de uitzending was de gemiddelde productie van cytokinen door T-cellen voor de hele groep deelnemers toegenomen. Deze stijging was minder uitgesproken in de deelnemers die depressieve symptomen ontwikkelden dan in de deelnemers zonder depressieve klachten. De productie van chemokinen door T-cellen en de productie van cytokinen door monocyten was na uitzending lager dan voorafgaand aan de uitzending. Dit hield echter geen verband met de ontwikkeling van depressieve symptomen in reactie op de uitzending.

Onze resultaten duiden erop dat een hoge capaciteit van T-cellen om cytokinen te produceren een kwetsbaarheidsfactor is voor het ontwikkelen van depressieve klachten in reactie op een stressvolle periode, zoals een uitzending. Ook tonen onze resultaten aan dat een militaire uitzending een langdurig effect heeft op de capaciteit van T-cellen en monocyten om

cytokinen te produceren. Het is nog onbekend of en hoe lang na terugkeer deze veranderde capaciteit om cytokinen te produceren weer op het niveau van voor de uitzending komt. Ook is nog onbekend of deze veranderde capaciteit om cytokinen te produceren ook gevolgen heeft voor de gezondheid van de militairen.

In **hoofdstuk 8** onderzochten we of de ontwikkeling van ernstige vermoeidheid in reactie op uitzending samenhangt met een andere respons van perifere bloedcellen op de pro-inflammatoire cytokine interleukine (IL)-1β. Het is bekend dat IL-1β kan leiden tot de productie van andere pro-inflammatoire cytokinen door leukocyten. De respons van leukocyten op IL-1β werd daarom bepaald door te meten hoeveel IL-8 er geproduceerd werd na toevoeging van IL-1β.

Voor deze studie gebruikten we data van 504 militairen. Van deze deelnemers waren alle gegevens van ernst van vermoeidheid en IL-1β-reactiviteit beschikbaar, zowel voor uitzending als één en zes maanden na terugkeer. 65 van de deelnemers hadden zes maanden na uitzending ernstige vermoeidheid (gemeten met de vragenlijst CIS).

Onze resultaten laten zien dat de deelnemers met ernstige vermoeidheid zes maanden na terugkeer van uitzending een hogere IL-1β-geinduceerde IL-8 productie hadden dan de deelnemers die niet vermoeid waren op dat moment. Dit groepsverschil in IL-1β reactiviteit was nog niet aanwezig voorafgaand aan de uitzending. Het lijkt zich dus ontwikkeld te hebben in reactie op de uitzending. Verdere analyses toonden aan dat in de niet-vermoeide groep de IL-1β geïnduceerde IL-8 na de uitzending lager was dan voor de uitzending, wat duidt op een afname in IL-1β reactiviteit. Dit is mogelijk een compensatoir mechanisme, dat kan zijn ontstaan als gevolg van verhoogde cytokinen niveaus tijdens uitzending. In de vermoeide groep vonden we geen verandering in de IL-1β geïnduceerde IL-8 productie over tijd. Dit zou erop kunnen duiden dat het immuunsysteem van de deelnemers die ernstig vermoeid werden zich minder goed kon aanpassen aan de stress van de uitzending.

Onze resultaten duiden erop dat de aanwezigheid van ernstige vermoeidheid na een stressvolle periode, zoals militaire uitzending, samenhangt met een verhoogde reactiviteit van immuuncellen op de pro-inflammatoire cytokine IL-1β na afloop van de stressvolle periode.

Conclusie

Voor dit proefschrift onderzochten we of onze deelnemers zes maanden na terugkeer van de uitzending een hoog aantal PTSS, depressieve of vermoeidheidssymptomen hadden ontwikkeld. Vervolgens werd onderzocht of we de ontwikkeling van deze symptomen konden voorspellen aan de hand van biologische en psychologische factoren. Dit leidde ertoe dat we verschillende, voornamelijk biologische, kwetsbaarheidsfactoren voor het ontwikkelen van PTSS, depressieve, en vermoeidheidssymptomen hebben geïdentificeerd, die al aanwezig waren voorafgaand aan de uitzending en de ontwikkeling van de symptomen.

Het is algemeen bekend dat symptomen van PTSS, depressie en vermoeidheid vaak samen voorkomen. Ook in het PRISMO-onderzoek was er sprake van een hoge comorbiditeit tussen de verschillende symptomen. Toch hebben we verschillende biologische kwetsbaarheidsfactoren gevonden voor de ontwikkeling van PTSS, depressieve en vermoeidheidssymptomen (voor de psychologische kwetsbaarheidsfactoren werd de specificiteit voor de verschillende aandoeningen niet onderzocht).

Gezamenlijk wijzen onze resultaten erop dat het biologische profiel voorafgaand aan blootstelling aan stress en trauma gepaard gaat met de ontwikkeling van psychische en lichamelijke klachten die in reactie daarop kunnen ontstaan. Verder tonen onze resultaten aan dat het biologische profiel niet alleen betrokken is bij het feit *of* iemand een stressgerelateerde aandoening zal ontwikkelen, maar ook bij *welke* specifieke stressgerelateerde aandoening die persoon zal ontwikkelen.

Onze resultaten laten duidelijk zien dat het uitvoeren van prospectieve, longitudinale onderzoeken in groepen met een verhoogd risico op blootstelling aan stress en trauma een veelbelovende benadering is om kwetsbaarheidsfactoren voor de ontwikkeling van stressgerelateerde aandoeningen te identificeren. De identificatie van kwetsbaarheidsfactoren voor stressgerelateerde aandoeningen zou ons iets kunnen leren over het mogelijke onderliggende mechanisme in het ontstaan van deze klachten. Verder kan het identificeren van kwetsbaarheidsfactoren waardevol zijn voor mensen met beroepen met een verhoogd risico op blootstelling aan stress en trauma. Hieronder vallen militairen, maar bijvoorbeeld ook politie- en brandweerpersoneel. Als in toekomstig onderzoek goede grenswaarden (cut-offs) voor de door ons gevonden kwetsbaarheidsfactoren bepaald worden, dan zouden kwetsbare mensen in de toekomst mogelijk al geïdentificeerd kunnen worden voorafgaand aan eventuele blootstelling aan stress en trauma. Het is denkbaar dat deze mensen dan een interventie zouden kunnen krijgen om hun weerbaarheid te verhogen. Daarnaast zouden kwetsbare mensen gelijk na blootstelling aan stress of trauma een vroege interventie kunnen krijgen, die mogelijk het ontstaan of verergeren van symptomen zou kunnen voorkomen.

In dit proefschrift werd het effect van uitzending op de productie van cytokinen onderzocht. Wij lieten zien dat de uitzending naar Afghanistan veranderingen veroorzaakte in de productie van cytokinen door gestimuleerde immuuncellen. Deze veranderingen waren tot minimaal zes maanden na terugkeer van de uitzending aanwezig, ook in deelnemers die geen stressgerelateerde klachten ontwikkeld hadden. Deze opvallende resultaten tonen aan dat het gebruik van een prospectieve, longitudinale opzet ook zeer waardevol is voor onderzoek naar de gevolgen van blootstelling aan stress en trauma in mensen die geen stressgerelateerde aandoeningen ontwikkelen.

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Smid GE, Kleber R, Rademaker AR, van Zuiden M & Vermetten E (submitted): The role of stress sensitization in progression of posttraumatic distress following deployment.

van Liempt S, van Zuiden M, Westenberg H, Super A & Vermetten E (submitted): Impact of impaired sleep as predictor of PTSD symptoms in combat veterans; a prospective longitudinal cohort study.

Curriculum Vitae

Mirjam van Zuiden was born on March 17th, 1984 in Leiderdorp, the Netherlands. In 2002 she finished secondary school (VWO) at the Scala College in Alphen aan den Rijn, and started studying psychology at Leiden University. In 2005, she obtained her Bachelor's degree cum laude. She subsequently followed the Master Clinical Neuropsychology at Leiden University. During her Master's, she undertook a clinical internship at the Department of Neuropsychology at the Leiden University Medical Hospital (LUMC). For her Master's thesis, she assisted in a longitudinal study on the hereditary small-vessel disease CADASIL, carried out by the Department of Neuropsychology and the Department of Radiology (LUMC). In the summer of 2007, Mirjam obtained her Master's title cum laude. Thereafter, she worked as a neuropsychologist at the Department of Neuropsychology at the LUMC for a few months.

In November 2007, Mirjam started working as a PhD-student at the Laboratory of Neuroimmunology and Developmental Origins of Disease (NIDOD) at the University Medical Center Utrecht, and at the Research Centre for Military Mental Health (MGGZ) of the Dutch Ministry of Defence, under supervision of prof. dr. Cobi Heijnen (NIDOD), dr. Annemieke Kavelaars (NIDOD) and dr. Elbert Geuze (MGGZ). For the past 4 years, she was involved in the ongoing 'Prospective Research In Stress-related Military Operations' (PRISMO)-study. She investigated biological and psychological vulnerability factors for the development of posttraumatic stress disorder (PTSD), depressive, and fatigue symptoms. The results of her PhD-project are described in this PhD-thesis and have been published in various international scientific journals.

Currently, Mirjam is working as a postdoctoral researcher at the Research Center for Psychotrauma (Department of Anxiety Disorders) at the Academic Medical Center in Amsterdam, where she investigates the effects of stimulation of the oxytocin system in acutely traumatized individuals and in patients with PTSD.