Psychoneuroendocrinology (2010) 35, 663—673

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Decrease of CD4$^+$FOXP3$^+$ T regulatory cells in the peripheral blood of human subjects undergoing a mental stressor

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Received 19 February 2009; received in revised form 5 October 2009; accepted 7 October 2009

KEYWORDS
T cells;
T regulatory cells;
Cell surface molecules;
Acute psychological stress;
Psychoneuroimmunology;
Autoimmunity

Summary We have previously shown that acute psychological stress alerts the adaptive immune response causing an increase in antigen-experienced effector T cells in the peripheral blood. T regulatory cells (Tregs) play a central role in maintaining self-tolerance and controlling autoimmune responses. Here, we analyzed for the first time the behaviour of Tregs in the context of a stress-induced activation of the adaptive immune response.

31 healthy young males underwent a brief laboratory stressor and, in a crossover design, served as their own unstressed controls. We quantified effects of acute stress on CD4$^+$FOXP3$^+$ T regulatory cells and other T cell subpopulations using flow cytometry. In addition, the expression of Treg-related effector molecules and stress hormone receptors were analyzed in the subjects' peripheral T cells.

We confirmed our previous observation of a stress-induced decrease in CD45RA$^+$CCR7$^+$ "naïve" and CD45RA$^-$CCR7+ "central memory" T cells while CD45RA$^-$—CCR7$^-$ "memory effector" and CD45RA$^+$CCR7$^-$ "terminally differentiated" effector T cells remained stable or increased.

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0306-4530/$ — see front matter
doi:10.1016/j.psyneuen.2009.10.005

Importantly, we found acute psychological stress to cause a concomitant decrease in $CD4^+FOXP3^+$ Tregs and in $CD4^+$ T cells expressing Treg-related effector molecules cytotoxic T-lymphocyte antigen-4 (CTLA-4) and latency associated peptide (LAP). Finally, we observed β_1-adrenergic and glucorticoid α receptors to be overexpressed in Tregs, suggesting that these molecules might mediate stress-related effects on Tregs.

In conclusion, inhibiting components of the adaptive immune response, like Tregs, are down-regulated during a stress-induced activation of the adaptive immune response. In situations of chronic stress, this scenario might result in an exacerbation of inflammatory conditions such as autoimmune diseases.

1. Introduction

The effects of acute psychological stress on the innate immune system have widely been investigated and especially the stress-induced mobilization of natural killer cells into the periphery is a repeatedly confirmed phenomenon (Segerstrom and Miller, 2004; Ader, 2007). On the other hand, the effect of an acute stressor on T cells as key players of the acquired immune system has thus far not been examined systematically, a circumstance which seems surprising since the course of a number of T cell-mediated diseases is thought to be influenced by psychological stress. T cell-related immune dysregulation has, for example, been associated with various autoimmune disorders (Cools et al., 2007; Yamanouchi et al., 2007; Anderson and Isaacs, 2008; Costantino et al., 2008). On the other hand, autoimmune diseases, such as multiple sclerosis, asthma, and rheumatoid arthritis, have been shown to be related to psychological stress (Sandberg et al., 2000; Mohr et al., 2004; Straub et al., 2005). However, immunological mechanisms linking the effects of psychological stress to exacerbations of autoimmune diseases have not yet successfully been delineated.

T cells can be characterized by dividing them into four distinct subsets according to their expression of the lymph node homing receptor CCR7 and CD45RA (Sallusto et al., 1999; Wills et al., 1999; Sallusto et al., 2004). $CD45RA^+CCR7^+$ "naïve" as well as the $CD45RA^-CCR7^+$ "central memory" T cells circulate between the peripheral blood and lymphoid tissue in search of antigen while $CD45RA^-CCR7^-$ "memory effector" and $CD45RA^+CCR7^-$ "terminally differentiated" effector T cells migrate into peripheral tissues where they exert their effector function. We have recently shown that acute psychological stress alerts the adaptive immune response causing a redistribution of these four T cell subgroups in the peripheral blood: $CCR7^+$ naïve and central memory fractions decrease while $CCR7^-$ memory effector and terminally differentiated effector T cells increase (Atanackovic et al., 2006). Thus, under acute stress less mature T cells seem to be retained within lymphoid tissue awaiting exposure to antigen. Meanwhile, effector-type T cells are allocated into the blood, ready to rapidly migrate into tissues in case their effector function is needed.

While the immune system provides a large repertoire of T cells specific for foreign proteins such as microbial antigens, selection processes in the thymus largely prevent the escape of T cells directed against self-antigens into the periphery (Werlen et al., 2003). However, negative selection cannot fully guarantee the depletion of all autoreactive T cells. Accordingly, naturally occurring Tregs, one main group of various subgroups of immunosuppressive cells, play a vital role in maintaining peripheral self-tolerance and immune homeostasis (Sakaguchi et al., 2008).

First described in 1995 by Sakaguchi et al. (1995), $CD4^+CD25^+$ Tregs have become of central research interest in the context of autoimmune disorders (Viglietta et al., 2004; Sakaguchi et al., 2006; Yamanouchi et al., 2007; Costantino et al., 2008), tumour immunity (Shimizu et al., 1999; Woo et al., 2001; Beyer and Schultze, 2006; Zou, 2006; Curiel, 2007), and infectious diseases (Belkaid and Rouse, 2005). As CD25, which constitutes the alpha chain of the IL-2 receptor, is not exclusively expressed on Tregs but also on activated conventional $CD4^+$ T cells, the description of transcription factor forkhead box P3 (FOXP3), which is a specific intracellular marker and master regulator gene of naturally occurring Tregs, represented a major improvement for the proper identification of this T cell population (Fontenot et al., 2003; Hori et al., 2003). The major role of FOXP3-expressing Tregs in preventing autoimmune diseases is underlined by the fact that FOXP3-deficient mice develop a lethal lymphoproliferative autoimmune syndrome and defect or loss of FOXP3 in humans leads to a severe autoimmune syndrome called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennett et al., 2001). It has been shown that Tregs actively suppress the proliferative response of $CD4^+$ and $CD8^+$ T cells, dendritic cells, and B lymphocytes. Tregs seem to exert these immunsuppressive functions through cell-cell contact dependent mechanisms involving various effector molecules as the glucocorticoid-induced tumor necrosis factor family-related gene (GITR), CTLA-4, lymphocyte activation gene-3 (LAG-3), as well as by secreting immunomodulating cytokines such as transforming growth factor-β (TGF-β) (Huang et al., 2004; von Boehmer, 2005).

Very little is known about the effect of acute psychological stress on peripheral T regulatory cells. Based on our previous finding of a stress-induced mobilization of effector T cells into the peripheral blood we hypothesized that acute stress might at the same time reduce peripheral numbers of immunosuppressive Tregs. In our present study, we analyzed for the first time the effects of acute psychological stress on human $CD4^+CD8^-FOXP3^+$ T regulatory cells. We quantified numbers of Tregs and effector T cell subpopulations and we investigated T cellular expression of Treg-related effector molecules CTLA-4, GITR and LAP following a brief artificial stressor. Finally, we explored which hormone receptors might be responsible for possible stress-related changes in the numbers of peripheral Tregs.

2. Design and methods

2.1. Test subjects

All subjects were recruited following a public announcement at the Benjamin Franklin University Hospital and the vast majority of participants were medical students at the Free and Humboldt University in Berlin. A total of 31 healthy young men (median age 26 years, range: 21—41 years, SD: 4.6) were included in the study and all subjects included completed the protocol. Before being included, participants underwent a standard physical examination to confirm that they were in good health. All test subjects were nonsmokers and were not on medication. Subjects with alcohol abuse, strenuous physical exercise, history of chronic physical or psychological diseases, recent surgery, mental stress on the test day, or needle phobia were excluded. Test subjects were asked to refrain from drinking coffee or black tea after 8 p.m. the night before the test. All participants were provided with informed consent in accordance with the revised version of the Declaration of Helsinki. The study protocol had received approval by the local ethics committee.

2.2. Test procedure and cardiovascular parameters

The stress test took place at the Benjamin Franklin hospital in Berlin, Germany. On the first day, in a crossover design, all participants were randomized either into the group that underwent the stress procedure on the first day or on the second day. This resulted in a study concept where all subjects served as their own unstressed controls. The experimental procedure started either at 9 a.m. or at 11 a.m. The starting time remained equal on both test days to avoid chronobiological variation. After written consent was obtained, a catheter was placed into a lower arm vein. The first blood sample was obtained at the end of a first ''resting period'' lasting for 25 min and served as the baseline value. Next, our computer-based mental stressor, which has previously been described in detail (Atanackovic et al., 2003) was started. The stressor consisted of a standardized computer-based information-processing task to be completed using a tracking ball. It consists of two subtests, creates a purely mental stress situation, and requires no physical movements. During the stress test, an increasing number of 3—11 clocks are presented to the test subjects who have to decide whether one or more hands of the clocks deviated more than 90° from the direction of an arrow shown on top of the screen. Wrong answers are indicated by a loud acoustic signal. At the end of the stress phase, which has a mean duration of about 12 min, a second blood sample was collected (''stress'' value). Following a second ''resting'' period, which lasts for another 25 min, the third blood sample was collected. Three tubes of heparinized blood containing a total volume of 10 ml each were collected within 1 min after the end of each study phase. Cardiovascular data were recorded for 5 min during each trial phase and averages were calculated.

2.3. Questionnaires

We used state forms of the German version of Spielberger's state—trait-anger-expression inventory (STAXI) (Schwenkmezger et al., 1992) to measure acute anger as an indicator of acute stress, and of the German translation of the state—trait anxiety inventory (STAI-G) (Laux et al., 1981). All 31 test subjects filled out the questionnaires during the first resting period, immediately following the ''stress value'' blood collection, and 30 min later at the end of the experiment. The same questionnaires were completed by all subjects ($N = 31$) on the control date.

2.4. Flow cytometry

Peripheral blood lymphocytes obtained on the ''stress'' day were available from 31 test subjects. In addition, control samples of 10 randomly selected participants were analyzed. Mononuclear cells were stained using monoclonal antibodies to CD4, CD8, CTLA-4 (BD Biosciences, San Jose, CA), CD25, CD45RA (Caltag, South San Francisco, CA), CCR7, GITR, LAP, (R&D Systems, Minneapolis, MN), and appropriate IgG isotype controls. Co-staining of intracellular FOXP3 was performed applying anti-FOXP3 mAb PCH101 (eBioscience, San Diego, CA) according to the manufacturer's instructions. Samples were analyzed using a FACSCalibur cytometer and CELLQuest software (BD Biosciences).

2.5. Real-time PCR analysis of hormone receptor expression

Tcells of 3 healthy donors were separated into $CD4^+CD25^-$ and $CD4^+CD25^+$ fractions using microbeads according to the manufacturer's instructions. Briefly, PBMCs were incubated for 15 min in MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and saturating amounts of a cocktail of biotin-conjugated antibodies (CD8, CD11b, CD16, CD19, CD36 and CD56; Miltenyi Biotec). Cells were then incubated with anti-biotin microbeads (Miltenyi Biotec) and CD4+ cells were negatively selected using appropriate magnetic separation columns. $CD25^+$ and $CD25^-$ fractions were further purified from $CD4^+$ T cells (>90% purity) using CD25 microbeads (Miltenyi Biotec) and were analyzed by quantitative real-time PCR. Extraction of total RNA and reverse transcription were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and AMV reverse transcriptase (Promega, Madison, USA). Real-time PCR was performed as described by Torrego et al. (2004) and Moniotte et al. (2001) using LightCycler (Roche Diagnostics, Branchburg, USA) technology as previously described (Atanackovic et al., 2008). Glucocorticoid receptor α and β cDNAs were amplified with specific antisense primers that shared the same sense primer. Sequences were 5′-CTTACTGCTTCTCTCTTCAGTTCCT-3′ for the sense primer, 5′-GCAATAGTTAAGGAGATTTTCAACC-3′ for the glucocorticoid receptor alpha antisense primer, and 5′-AGTGCACA-TAATCTTCTTTTTCTCA-3′ for the glucocorticoid receptor beta antisense primer. Primers for housekeeping gene glyceraldehydephosphate dehydrogenase (GAPDH) were 5′-TGATGACAT-CAAGAAGGTGG-3′(sense) and 5′-TTTCTTACTCCTTGGAGGCC-3′ (antisense). Beta-adrenergic receptors were analyzed using the primers and probes indicated below. β_1-Adrenergic receptor 5′-CAGGTGAACTCGAAGCCCAC-3′ (sense), 5′-CTCCCA-TCCCTTCCCAAACT-3′ (antisense), 5′-6-FAM-AAAGCCACG-GACCGTTGCACAAAA-TAMRA Amidid-3′ (probe). β_2-Adrenergic receptor 5′-CCGAAAGTTCCCGTACGTCA-3′ (sense), 5′-CAGCC-

CGTGCTCTGAAGAA-3′ (antisense), 5′-6-FAM-TGCACATAACGG-GCAGAACGCACT-TAMRA Amidid-3′ (probe). β_3-Adrenergic receptor 5′-CTCCCCTGGTTCCATTCCCTT-3′ (sense), 5′-TGGT-CTTTTCTACCCTGCTGC-3′ (antisense), 5′-6-FAM-TGCCACCCA-AACCCTGATGAGACCTTA-TAMRA Amidid-3′ (probe). Primers for housekeeping gene ABL were 5′-AAAACCTTCTCGCTGGACCC-3′ (sense), 5′-TTTGGGCTTCACACCATTCC-3′ (antisense) and 5′-6-FAM-GACCCGGAGCTTTTCACCTTAGTTATGCTTA-TAMRA Amidid-3′ (probe). Results were expressed as expression of the target gene relative to housekeeping gene ABL, which was used as a reference gene.

2.6. Statistical analysis

Based on the fact that most test results were unevenly distributed we used the non-parametric Friedman's test for related samples in order to find out whether any significant changes would occur throughout the course of the experiment for a given parameter. Only if this was the case, Wilcoxon's rank sum test for related samples was applied to compare baseline values to results from later timepoints in the same study phase. Spearman's rank correlation was used to analyze correlations between cardiovascular and immunological parameters. Results were considered significant if $p < 0.05$. If not stated otherwise, values given represent mean and standard error of mean (SEM).

3. Results

3.1. Increased levels of subjectively perceived stress and cardiovascular activation under acute laboratory stress

To investigate whether the laboratory stressor would cause emotional distress, we used standardized questionnaires to measure subjectively perceived anger and anxiety (state versions of STAXI and STAI-G). We found that our laboratory stressor clearly induced psychological tension indicated by increased levels of anger (Fig. 1A). We also found a trend towards an increase in levels of anxiety ($p = 0.14$). To examine whether this stress-induced psychological tension would translate into sympathetic activation we measured heart rate and blood pressure in our test subjects. We found that the increase in subjectively perceived stress was indeed paralleled by a highly significant increase in all cardiovascular parameters measured. With the exception of blood pressure, all psychological and cardiovascular indicators had returned to baseline levels only minutes after the stressful situation had ended (Fig. 1B). On the control day, we did not detect any significant changes over the course of repeated measurements except a decrease in anxiety levels and a slight decrease of blood pressure at the end of the experimental procedure.

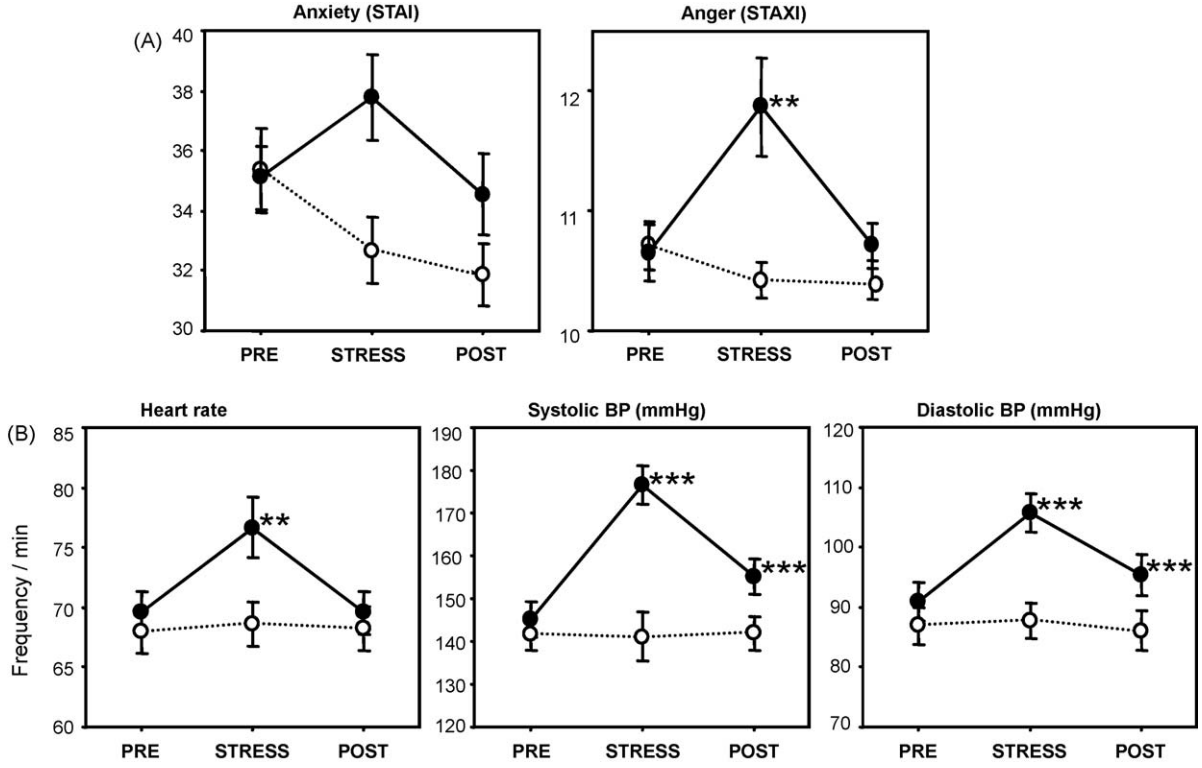

Figure 1 Stress-induced psychological and cardiovascular activation. Standardized questionnaires (STAXI and STAIG) were filled out by all subjects ($N = 31$) before (PRE), a few minutes after the stress test (STRESS) and after the second resting period (POST). Heart rate, systolic blood pressure (BP) and diastolic blood pressure were measured at the same time points. Data are given as means $\pm$ SEM. Asterisks indicate significant (Wilcoxon's rank sum test) differences compared to baseline values (**$p < 0.01$, ***$p < 0.001$). Control data of all 31 subjects are shown within the same figure. Black dots represent means on stress days, open dots represent control data.

3.2. Acute psychological stress causes a redistribution of naive/memory T cell subsets and an increase in T cells with cytotoxic potential

Next, we examined whether our findings of a stress-induced increase in subjectively perceived stress and cardiovascular activation would translate into a specific redistribution of T cell subsets as described previously (Atanackovic et al., 2006). Since absolute cell counts were not available to us, percentages of total lymphocytes were used for statistical evaluation. There were no significant changes in the percentages of total $CD3^+CD8^+$ T cells but a slight decrease ($p < 0.05$) at the end of the experiment (baseline: $29.2 \pm 1.3\%$; stress: 29.8 ± 1.5; resting period: $27.6 \pm 1.2\%$). We observed a decrease ($p < 0.001$) in percentages of total $CD4^+$ T cells immediately after the stress procedure, followed by a return to baseline levels (baseline: $37.7 \pm 1.3\%$; stress: $31.4 \pm 1.2\%$; resting period: $35.7 \pm 1.3\%$). On control days, there were no significant changes over the course of the experiment (data not shown).

Sallusto et al. suggested that the pattern of expression of the lymph node homing receptor CCR7 and of CD45RA divides human $CD4^+$ and $CD8^+$ T cells into distinct naive/memory subsets (Sallusto et al., 1999). Confirming our previous findings (Atanackovic et al., 2006), we observed a stress-induced redistribution of human peripheral naïve/memory T cell subsets with a decrease in percentages of peripheral $CD4^+$ and $CD8^+$ naïve ($CD45RA^+CCR7^+$) and $CD8^+$ central memory ($CD45RA^-CCR7^+$) T cell fractions. $CD4^+$ central memory ($CD45RA^-CCR7^+$) T cells also decreased but had returned to baseline levels after the second resting period (Fig. 2).

At the same time, we observed a significant stress-induced increase in peripheral terminally differentiated ($CD45RA^+CCR7^-$) $CD8^+$ T cells which was accompanied by a delayed but significant increase in memory effector ($CD45RA^-CCR7^-$) $CD4^+$ T cells. Memory effector ($CD45RA^-CCR7^-$) $CD8^+$ and terminally differentiated ($CD45RA^+CCR7^-$) $CD4^+$ T cells remained on stable levels (Fig. 2). On control days, all T cell naïve/memory subpopulations remained at baseline levels (Fig. 2).

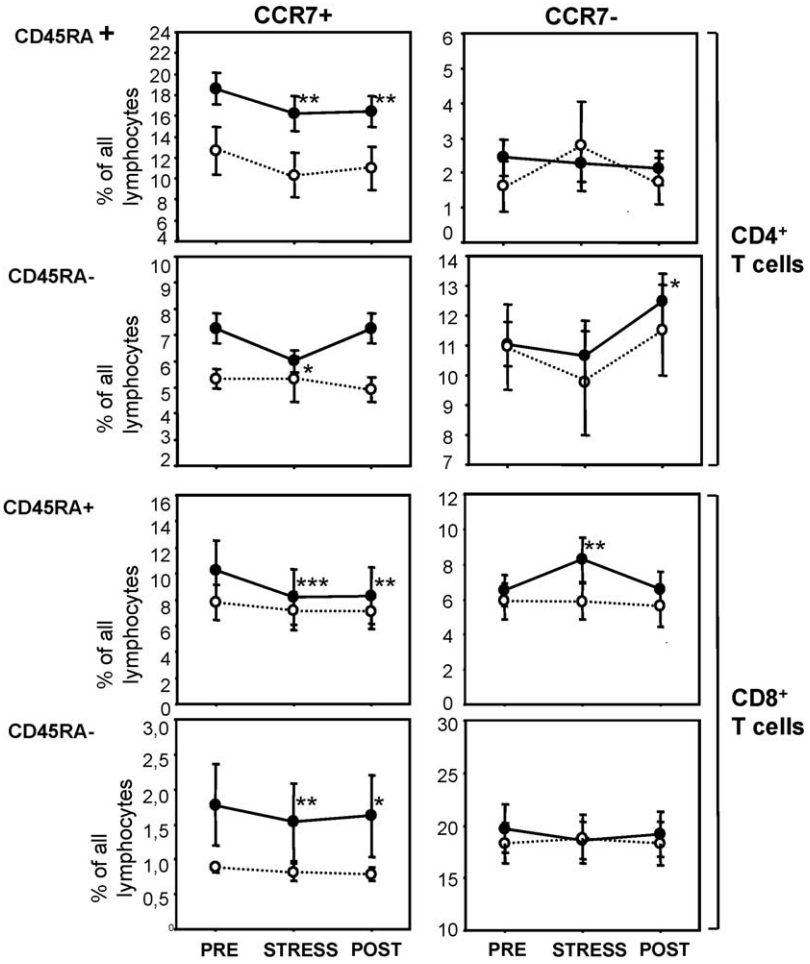

Figure 2 Psychological stress causes a redistribution of naive/memory T cells as defined by their expression of CCR7 and CD45RA. The stress-induced redistribution of different naïve/memory T cell subsets was analyzed in 31 test subjects using four-color flow cytometry. FACS analysis was performed using a combination of a morphological lymphocyte gate, a gate for $CD4^+$ T cells ($CD3^+CD4^+$) and $CD8^+$ T cells ($CD3^+CD8^+$), and gates for all four possible combinations of CCR7/CD45RA expression. Values for naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), memory effector ($CD45RA^-CCR7^-$), and terminally differenciated effector ($CD45RA^+CCR7^-$) $CD4^+$ and $CD8^+$ T cells are given as mean percentage of all lymphocytes $\pm$ SEM. Asterisks indicate significant (Wilcoxon's rank sum test) differences compared to baseline values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Control data of 10 subjects are shown within the same figure. Black dots represent means on stress days, open dots represent control data.

In conclusion, acute psychological stress seems to cause a redistribution of different naïve/memory T cell subsets in the peripheral blood leading to a decrease in less mature cells and an increase in antigen-experienced effector-type T cells. Furthermore, the expression of CCR7 once more proves to be a useful marker for the identification of T cell subsets that react towards acute psychological stress.

3.3. Psychological stress causes a decrease in naturally occurring CD4$^+$FOXP3$^+$ regulatory T cells

Next, we asked the question whether the stress-induced increase in peripheral effector T cells would be accompanied by a decrease in immunosuppressive components of adaptive immunity, namely naturally occurring CD4$^+$FOXP3$^+$ Tregs. FOXP3 represents the most specific marker of Tregs in mice and humans (Fontenot et al., 2003; Hori et al., 2003), however, the effect of acute psychological stress on peripheral numbers of Tregs expressing this marker has thus far not been examined. Analyzing the effects of our acute laboratory stressor on Tregs defined by FOXP3 expression, we indeed observed a significant stress-induced decrease in percentages of peripheral CD4$^+$FOXP3$^+$ regulatory T cells. As in the case of stress-related redistribution of other T cell subpopulations, Treg percentages returned to baseline levels after the second resting period (Fig. 3A and D).

One central role of naturally occurring regulatory T cells is the suppression of conventional effector T cells and the maintenance of peripheral tolerance (Sakaguchi et al., 2008). To investigate the relationship between these two immunological counterparts we analyzed whether the proportion of peripheral CD8$^+$CCR7$^-$ cells, representing T cell subsets with the highest cytotoxic potential (Sallusto et al., 1999; Sallusto et al., 2004), and CD4$^+$FOXP3$^+$ T regulatory cells would change under the influence of the laboratory stressor. Interestingly, we indeed observed a significant increase of the ratio between CD8$^+$CCR7$^-$ T cells and CD4$^+$FOXP3$^+$ T cells following acute psychological stress (Fig. 3B), indicating a stress-induced redistribution in the direction of the effector arm of the immune system with a dampened immunoregulatory component.

We have previously described numbers of peripheral CCR7-negative effector memory T cells as being enhanced by acute stress (Atanackovic et al., 2006), an observation which was confirmed by our current study. Importantly, almost two-thirds of CD4$^+$FOXP3$^+$ T regulatory cells, which decreased under the influence of stress, were CCR7-negative (Fig. 3C). In contrast, CCR7-negative conventional CD4$^+$ T cells, as described above, did not show a stress-induced decrease, indicating that stress-related effects are differentially regulated in conventional CD4$^+$ T cells and CD4$^+$ Tregs.

3.4. Changes in Treg-related effector molecule expression on CD4$^+$ T cells parallel the stress-induced decrease in CD4$^+$FOXP3$^+$ T regulatory cells

Several effector molecules are closely associated with the function of T regulatory cells. CTLA-4, for example, represents

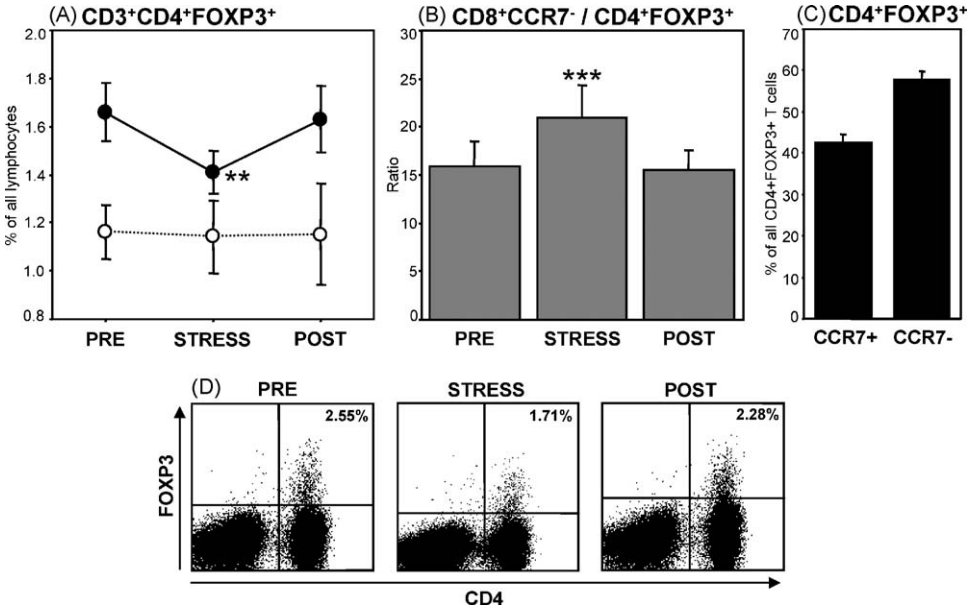

Figure 3 Acute psychological stress causes a decrease in naturally occurring CD4$^+$FOXP3$^+$ T cells. Percentages of CD3$^+$CD4$^+$FOXP3$^+$ Tregs were determined in 31 healthy male subjects after resting phase 1 (PRE), following the stress test (STRESS), and following resting phase 2 (POST). Control data of 10 subjects are shown within the same figure. Black dots represent means on stress days, open dots represent control data (A). To analyze whether the stress-induced increase in cytotoxic effector T cells and the decrease in Tregs would be correlated we analyzed the ratio of CD8$^+$CCR7$^-$ T cells and CD4$^+$FOXP3$^+$ Tregs at the same time points described above in all subjects undergoing the mental stressor ($N = 31$). Asterisks indicate significant (**$p < 0.001$, ***$p < 0.001$) differences compared to baseline values as indicated by Wilcoxon's rank sum test (B). Percentages of CCR7-negative vs. CCR7-positive Tregs were analyzed in all subjects ($N = 31$) immediately after they had received the mental stressor (C). Original data obtained by flow cytometry are shown for one representative test subject (D).

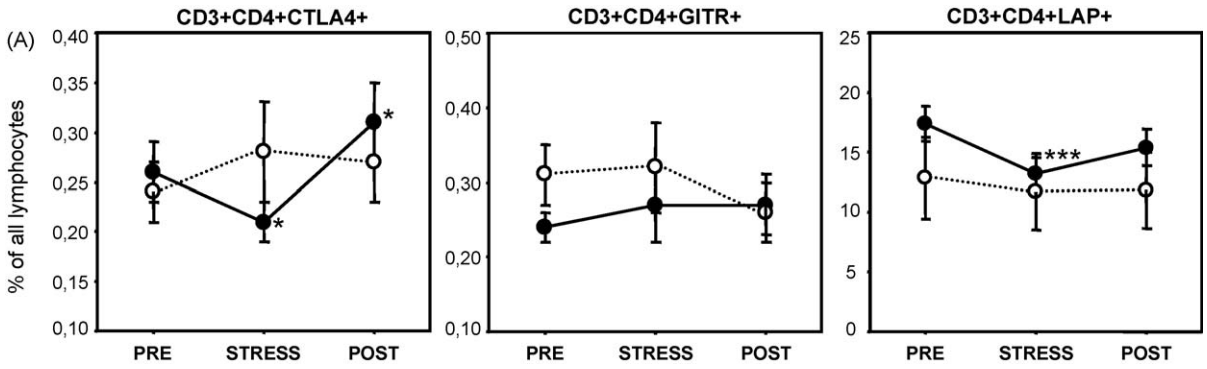

Figure 4 Acute psychological stress causes a decrease in CD4+CTLA-4+ and CD4+LAP+ T cells. To analyze whether the stress-induced decrease in Tregs would be associated with decreases in Treg-related effector molecules as CTLA-4 (A), GITR (B) and LAP (C) we investigated $CD4^+$ T cell numbers expressing these molecules after resting phase 1 (PRE), following the stress test (STRESS), and following resting phase 2 (POST) in all 31 healthy male subjects. Control data of 10 subjects are shown within the same figure. Black dots represent means on stress days, open dots represent control data. Asterisks indicate significant (Wilcoxon's rank sum test) differences compared to baseline values ($*p < 0.05$, $***p < 0.001$).

a central mediator of the immunosuppressive effects of regulatory T cells (Sansom and Walker, 2006). In agreement with the stress-induced decrease of peripheral $CD4^+FOXP3^+$ T regulatory cells we observed a similar decrease in $CD4^+CTLA\text{-}4^+$ T cells at the same time point which was followed by a slight increase during the resting period (Fig. 4A).

GITR is a surface receptor molecule expressed on regulatory T cells and conventional activated T cells (McHugh et al., 2002; Shimizu et al., 2002). Our acute laboratory stressor did not induce any significant alterations in the percentages of peripheral $CD4^+$ T cells expressing GITR (Fig. 4B).

Latency associated peptide (LAP) forms a latent complex with TGF-β1, a cytokine which is closely associated with the suppressive function of Tregs (Nakamura et al., 2004). Interestingly, we observed a significant decrease in $CD4^+LAP^+$ T cells induced by the acute laboratory stressor (Fig. 4C). As for the other parameters analyzed in this study, we did not observe any comparable changes over the course of the experiment on the control dates.

In conclusion, we show here that both $CD4^+FOXP3^+$ regulatory T cells and effector molecules that are positively related to their suppressive function are down-regulated during acute stress conditions, allowing effector T cells, which are mobilized into the periphery in stressful situations, to more rapidly exert their function.

3.5. Hormonal mechanisms mediating stress-induced decreases in peripheral Tregs

Because stress-induced increases of the heart rate in a given subject has previously been indicated to be the most reliable cardiovascular parameter for predicting changes in cellular immune parameters under stress (Herbert et al., 1994; Atanackovic et al., 2002), we next analyzed whether the extent of the test subjects' heart rate increases would correlate with the redistribution of $CD4^+FOXP3^+$ Tregs and other T cell subpopulations analyzed. We could not find any significant correlations between peripheral numbers of conventional T cells and the extent of the stress-related increase in heart rate (data not shown). Moreover there was no significant correlation between the stress-related effect on the subjects' heart rate and the decrease in $CD4^+FOXP3^+$ Tregs.

"Stress hormones" such as catecholamines and glucocorticoids are mediators of stress responses and exert their function by binding to intra- and extracellular cell receptors. Rapid changes in cardiovascular parameters and numbers of peripheral leukocyte subsets have been shown to be induced by stress hormones (Benschop et al., 1996). Since a selection of Tregs based on the intracellular expression of FOXP3 is technically impossible, we separated conventional $CD4^+$ T cells and Tregs based on their expression of CD25 from peripheral lymphocytes of three healthy blood donors (Fig. 5A). Importantly, Tregs separated based on their overexpression of CD25 indeed expressed high levels of FOXP3 RNA (data not shown).

Subsequently, we analyzed the RNA expression of β_1-, β_2-, and β_3-adrenergic as well as glucocorticoid α and β receptors in both lymphocyte subgroups applying real-time PCR. Confirming our previous findings (Atanackovic et al., 2006), glucocorticoid β receptors showed very low or undetectable expression levels in human T cells (data not shown) while β_3-adrenergic receptors expression evidenced the highest expression levels in both subtypes, followed by β_2-receptor and β_1-receptor RNA expression (Fig. 5B). Comparing conventional T cells and Tregs, there was a trend towards an increased β_1-receptor expression in $CD4^+CD25^+$ Tregs while β_2-receptor expression seemed to be more pronounced in conventional $CD4^+$ T cells. In contrast, expression of β_3-adrenergic receptors showed no distinct distribution between the two groups. Interestingly, the most pronounced difference between both T cell subpopulation consisted of a markedly higher expression of glucocorticoid α receptors on $CD4^+CD25^{high}$ Tregs (Fig. 5B).

4. Discussion

So far, acute psychological stress has primarily been examined regarding its effects on conventional $CD4^+$ and $CD8^+$ T cells (Segerstrom and Miller, 2004). Considering our recently observed findings of a stress-induced redistribution of human peripheral naïve/memory T cell subsets (Atanackovic et al., 2006) and findings of others indicating a stress-induced increase in antigen-experienced T cells expressing markers such as CCR5 (Bosch et al., 2003), we investigated here the

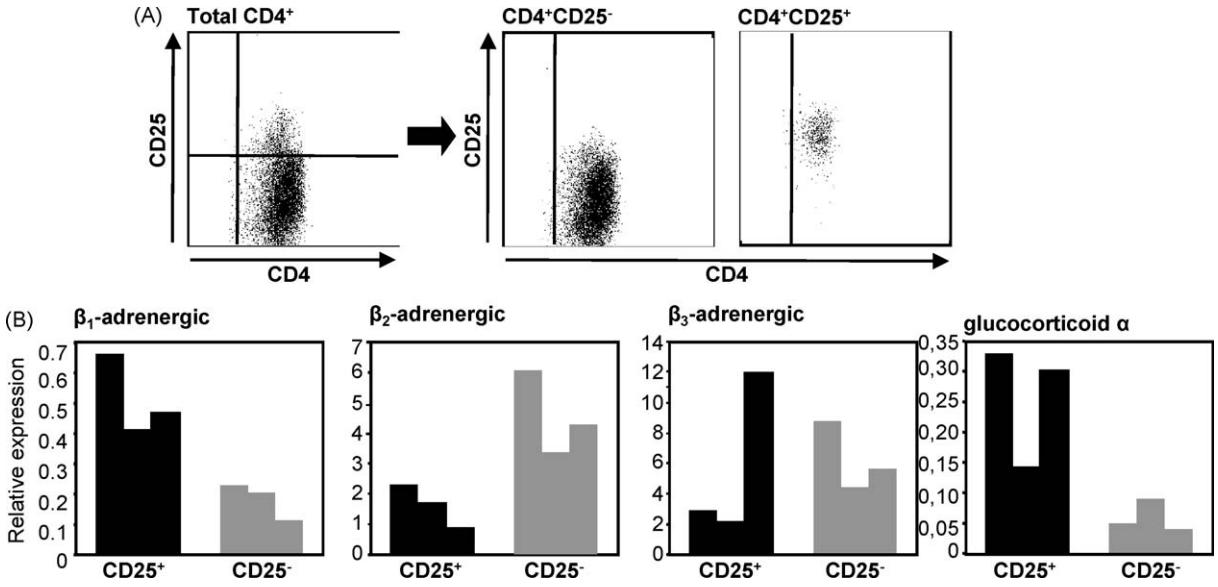

Figure 5 Hormone receptor expression in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. T cells of 3 healthy blood donors were separated into $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ conventional T cells using magnetic microbeads (A). Subsequently, expression of β_1-, β_2-, and β_3-adrenergic as well as of glucocorticoid receptors was analyzed using quantitative real-time PCR. Single black (Tregs) or grey (conventional CD4+ T cells) bars represent RNA expression in one donor (B).

role of naturally occurring $CD4^+FOXP3^+$ regulatory T cells in the context of a "fight or flight" situation.

We confirmed our previous findings of a stress-induced redistribution of peripheral naïve/memory T cell subsets (Atanackovic et al., 2006) observing a stress-induced decrease in percentages of peripheral $CD4^+$ and $CD8^+$ naïve ($CD45RA^+CCR7^+$) and a decrease in $CD4^+$ and $CD8^+$ central memory ($CD45RA^-CCR7^+$) T cells. These cells do not possess significant effector function and circulate between peripheral blood and lymphoid tissue (Jenkins et al., 2001; Wolint et al., 2004). At the same time we found a highly significant increase in the terminally differentiated ($CD45RA^+CCR7^-$) $CD8^+$ T cell fraction and a delayed increase in memory effector ($CD45RA^-CCR7^-$) $CD4^+$ T cells while memory effector ($CD45RA^-CCR7^-$) $CD8^+$ and terminally differentiated ($CD45RA^+CCR7^-$) $CD4^+$ T cells remained on stable levels. These CCR7-negative cells possess effector function i.e. against viral antigens (Chen et al., 2001; Hengel et al., 2003) and can rapidly migrate into peripheral sites of inflammation (Roman et al., 2002). Our findings, therefore, add further support to the hypothesis that in humans less mature T cells might be kept in secondary lymphoid tissues during acute stress, while antigen-experienced effector-type T cells with a strong cytolytic potential are being mobilized into the peripheral blood, probably providing a pool of immune cells which can more rapidly reach target tissues in case of injury or local inflammation. However, chronic situations of psychological stress might lead to constantly heightened levels of cytotoxic T cells in the periphery promoting inflammatory and autoimmune conditions (Viswanathan and Dhabhar, 2005). Such a scenario might be the biological explanation for the impact of psychological stress on the clinical course of these diseases (Sandberg et al., 2000; Mohr et al., 2004; Straub et al., 2005).

Diminished numbers and/or a defective function of Tregs have been clearly associated with a greater susceptibility to autoimmune diseases (Kriegel et al., 2004; Viglietta et al., 2004; Lindley et al., 2005; Yamanouchi et al., 2007). In our current study, we observed a significant stress-induced decrease in naturally occurring $CD4^+FOXP3^+$ regulatory T cell. Thus, T cell immunity seems to be skewed into the direction of immunoenhancing components during acute psychological stress while inhibitory components, such as Tregs, are down-regulated.

While Tregs, as defined by the most specific marker FOXP3, have until now not been examined regarding possible effects of acute psychological stress, others have examined the role of Tregs, as defined by their expression of CD25, in the setting of chronic stress. Hoglund et al. (2006) analyzed $CD4^+CD45R0^+CD25^{bright}$ T cells in atopic and healthy individuals in response to examination stress. They found an increase in the proportion of peripheral blood T cells expressing these markers in both atopic and healthy participants. $CD4^+D45R0^+CD25^{bright}$ T cell numbers were higher in subjects with high perceived stress levels. Importantly, chronic stress has been shown to suppress both innate and adaptive immunity (Dhabhar and McEwen, 1997; Segerstrom and Miller, 2004; Ader, 2007) and, accordingly, chronic stress might indeed increase the numbers of peripheral T regulatory cells. However, it might also be that these previous observations, which are in contrast to our findings, can simply be explained by the fact that CD25 is not the most reliable marker for Tregs since it is also expressed on activated effector-type T cells.

If Tregs are central mediators of a stress-induced increases in the susceptibility to autoimmune and other diseases, which molecular mechanisms might be involved? Several effector molecules and activation markers have been associated with Treg function (von Boehmer, 2005). CTLA-4 is a CD28-family receptor expressed on Treg and other activated effector T cells. CTLA-4 represents a negative regulator of T cell function and proliferation (Sansom and Walker, 2006) and CTLA-4 deficient mice develop lethal lymphoproliferative

diseases (Tivol et al., 1995; Waterhouse et al., 1995). We observed a significant decrease in $CD4^+CTLA\text{-}4^+$ T cells corresponding to the decrease in $CD4^+FOXP3^+$ T cells, indicating that psychological stress might directly influence peripheral Tregs and other $CD4^+$ T cells expressing this marker.

Latency Associated Peptide (LAP) forms a latent complex with TGF-β1 which besides IL-10 is one of the most important cytokines involved in regulatory T cell development and function (Nakamura et al., 2004). TGF-β1 deficiency has been associated with a numeral inadequacy the loss of regulatory function of $CD4^+CD25^+FOXP3^+$ Tregs (Bommireddy et al., 2008). LAP is useful for distinguishing between Tregs and activated $FOXP3^-$ or $FOXP3^+$ non-Tregs and for the selection of functional Tregs from patient material (Tran et al., 2009). Importantly, $CD4^+LAP^+$ T cells showed a significant decrease undergoing the stress test, confirming a stress-induced effect on Tregs and other cells with immunoregulatory function.

The increased expression of glucocorticoid α receptor expression on $CD4^+CD25^+$ Tregs might strengthen the role of glucocorticoids in short-term stress responses (Atanackovic et al., 2006). A leading role of the hypothalamic—pituitary—adrenocortical axis in mediating stress-related changes in numbers and function of peripheral T cells might also be supported by the missing association between sympathetic activation, as indicated by cardiovascular activity, and the stress-induced redistribution of different T cell subsets observed in our study. Future investigations should, therefore, delineate in detail the biological role of glucocorticoid receptor expression on Tregs and its importance for stress-induced effects on these cells.

We never directly compared controls and stressed subjects regarding the composition of their lymphocyte subpopulations. Instead, we analyzed variances between baseline values and later time points within the same group. This approach helped us to minimize the relevance of baseline differences between stressed subjects and controls, which were sometimes already present at baseline. However, we specifically consider two possible reasons to be responsible for such a phenomenon. (1) 10 samples had to be randomly selected from the control samples for the final analysis and this might have biased results for lymphocyte subpopulations in the unstressed subjects. (2) Participants were informed shortly before the first study day whether they were going to receive the stressor on the particular day. Although we consider this explanation less likely, it is nonetheless still conceivable that an anticipatory effect might have confounded the study results.

Based on current findings we propose a specific model of changes in T cell-related immunity induced by acute psychological stress. According to this working hypothesis, acute stress, besides activating innate immunity, enhances adaptive immunity causing a mobilization of antigen-experienced and cytotoxic T cells into the peripheral blood. Moreover, naturally occuring $CD4^+FOXP3^+$ regulatory T cells decrease in the peripheral blood, thus allowing more pronounced effector T cell responses in "fight or flight" conditions. This organized T cell redistribution might benefit immune defence against microbial infections. On the other hand, the disturbed balance of effector T cells and Tregs might also contribute to the exacerbation of autoimmune diseases if stressful conditions persist for prolonged periods of time. Future studies will in a detailed manner evaluate the biological basis and the functional consequences of the psychoneuroendocrinological model proposed herein.

Role of the funding source

Funding sources had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflict of interest

None declared.

Acknowledgements

This work was supported by grants from the Erich un Gertrud Roggenbruck-Stiftung, Eppendorfer Krebsund Leukaemiehilfe e.V., José Carreras Leukaemie-Stiftung, and from the Cancer Research Institute (to D.A.).

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