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Effect of acute or subchronic stress on T cell response in peripheral blood: Regulatory role of vitamin D

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The immune response, orchestrated by helper (Th1, Th2, and Th17) and regulatory (Treg) T cells, is modulated by stress and Vitamin D (Vit-D). Although the immunomodulatory functions of both are known, their specific roles on Th cells have not been fully clarified, yet. On this background, we aimed to investigate the effect of acute or subchronic stress on the distribution of peripheral T lymphocytes, as well as the immunomodulatory role of Vit-D. Young adult male, Swiss-albino mice (30–40g) were allocated to the control, acute stress (AS), subchronic stress (ChS), control+Vit-D, AS+Vit-D, and ChS+Vit-D groups (n=11/group). The combined cold (2-h at 4°C)-immobilization (2-h in a restrainer) stress protocol was employed as one day in AS groups and five consecutive days in ChS groups. Vit-D (2µg/kg ip) was applied every other day, until the end of the protocol. Serum cortisol, Vit-D and cytokine levels (IL-4, IFN-γ, and IL-17A) were measured, and lymphocytes from blood samples were subtyped by flow-cytometry. Stress exposure caused differential Th and Treg responses, acute stress shifting the response to Th1, and subchronic stress shifting the response to Th2. Th17 and Treg cells were lower in subchronic stress exposed mice. These changes became comparable to control values in Vit-D treated groups. The T cell response, crucial for immune system function, differs on the basis of stress exposure as such the Vit-D treatment. The tolerogenic profile created by Vit-D should be considered for management of stress-related diseases. Our results may help to provide a better understanding of disease pathogenesis.

Keywords: Cold-immobilization stress, Interferon (IFN-γ), Interleukins, T helper cells, T regulatory cells

Stress response is the generalized reaction of an organism to a range of challenges mediated via stress system; the hypothalamus-pituitary-adrenal (HPA) axis, and the sympathetic nervous system (SNS). In addition to regulating the activity of almost all organ systems to overcome these challenges, both HPA axis and SNS functions have been implicated in the pathophysiology of numerous diseases[1-4]. The common point of almost all of the stress-associated diseases is dys/malfunction of the immune system. The stress hormones adrenaline and cortisol, released from HPA axis or SNS, affect both the innate and adaptive immune systems and modulate the direction and magnitude of the immune response[3-6].

An effective immune response is tightly regulated, with precise timing required for its initiation, execution, and termination. This delicate control is orchestrated by helper (Th1, Th2, and Th17), and regulatory (Treg) T cells[7,8]. Naïve Th cells differentiate to Th1 and Th2 effector cells, which produce cytokines interleukin (IL)-2, -12, and interferon (IFN)-γ, and IL-4, 10, and 13, respectively. The balance between the Th1-mediated cellular and Th2-mediated humoral immune responses is via cytokinergic interactions[8]. Furthermore, the discovery of $CD4^+CD25^+$ regulatory T (Treg) cells has contributed significantly to the understanding of cellular immunity mediated immune suppression, self-tolerance, and the adaptive immune response. Treg cells play a critical role in modulating the Th1 response[9]. Another subgroup of Th cells, referred to as Th17 cells and their mediator IL-17A play key roles in a variety of autoimmune diseases and self-tolerance[10,11].

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The biologically active form of vitamin D (1,25-dihydroxyvitamin D_3; Vit-D) has a very well-known role in calcium-phosphate homeostasis[12]. Furthermore, it regulates non-calcemic pleiotropic functions, including immune regulation, inflammation, antioxidant defense, differentiation, and apoptosis[12-14]. Its immune-modulatory effects become of interest, as constitutional expression of Vit-D receptor (VDR) similar to that in bones, kidneys, and intestine have been shown on nearly all members of the immune system including monocytes, macrophages, dendritic cells, neutrophils, T and B lymphocytes[13]. The key question as to whether and how Vit-D modulates immune function has been investigated in various studies[12-14]. Furthermore, epidemiological data have clarified the relationship between Vit-D deficiency and chronic autoimmune diseases, such as type 1 diabetes mellitus, systemic lupus erythematosus, and multiple sclerosis[14]. VDR expression in T lymphocytes change with maturation and/or exposure to immunogenic stimuli indicating the major, but versatile effects of Vit-D on the immune system under different conditions[13]. Although numerous studies have shown the effect of acute or chronic stress and immunomodulating molecules on the immune response[10,11], the impact of acute or chronic stress on Treg and Th cells, and whether Vit-D plays a role in this interaction is still not clear and remains controversial. Hence, we set out to investigate Th (Th1, Th2, Th17) and Treg responses under acute and subchronic stress conditions in mice and explore the modulatory effects of Vit-D.

Materials and Methods

Experimental animals

Male, Swiss albino mice (30-40 g, n=66) were housed in standard cages in a temperature-controlled environment (22±2°C) with 12-h dark/light cycles (lights on 8.00 am-8.00 pm) and *ad libitum* access to standard mice chow and tap water. All experimental protocols were approved by the Hacettepe University Ethics Board for Animal Experiments. All experimenters were certified for the care and use of laboratory animals, and Turkish bylaws for laboratory animal use were strictly adhered to. Following a week of adaptation, animals were weighed and allocated to six groups by simple randomization as; control (C), acute stress (AS), subchronic stress (ChS), control+Vit-D (D), acute stress+Vit-D (ASD), and subchronic stress+Vit-D (ChSD) (n=11/group).

Stress protocol and Vit-D application

A combined cold-immobilization stress model[15] was chosen for acute or subchronic stress exposure. To induce cold stress, mice were kept in a room at 4°C for 2-h. Immobilization stress for 2-h was performed at room temperature (22±2°C) in the restrainers which restricts movement of the animals completely. This protocol was performed once in the acute stress groups (AS, ASD), and for five consecutive days in the subchronic stress groups (ChS, ChSD). The order of the cold exposure and immobilization was randomly determined each day in the ChS groups. This ChS protocol was chosen specifically so as to not cause depression. The animals were followed daily for symptoms of depression and aggressive behaviour and no such observations were noted in the ChS or ChSD groups.

Vit-D (2µg (equivalent to 80 IU)/kg for 5 days) was dissolved in corn oil and dosed intraperitoneally to the relevant groups between 8.00 and 9.00 am, while other animals received solvent. For the Vit-D groups, the treatment was initiated 5 days before the stress protocol, and applied every other day (Fig. 1).

Leucocyte counting and lymphocyte immunophenotyping

All animals were weighed and sacrificed with exsanguination under ketamine (100 mg/kg), and xylazine (10 mg/kg) anesthesia on the last day of the stress protocol (30-min after completion of the protocol), or on the scheduled days for the control and

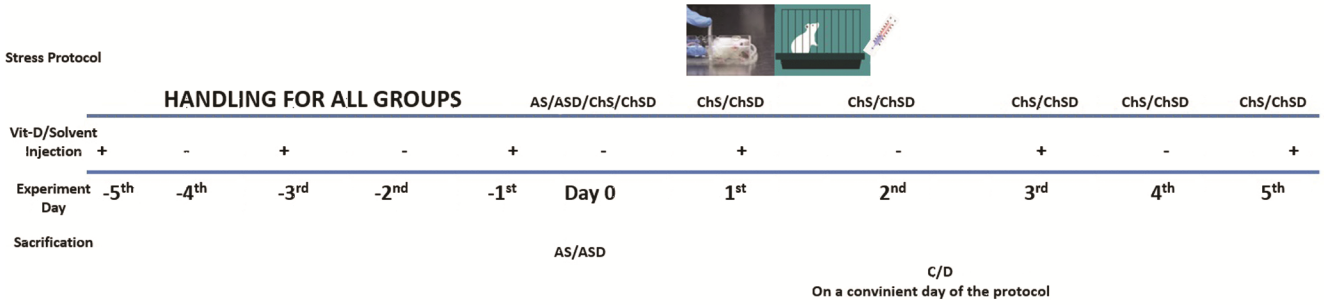

Fig. 1 — Schematic presentation of the experimental protocol

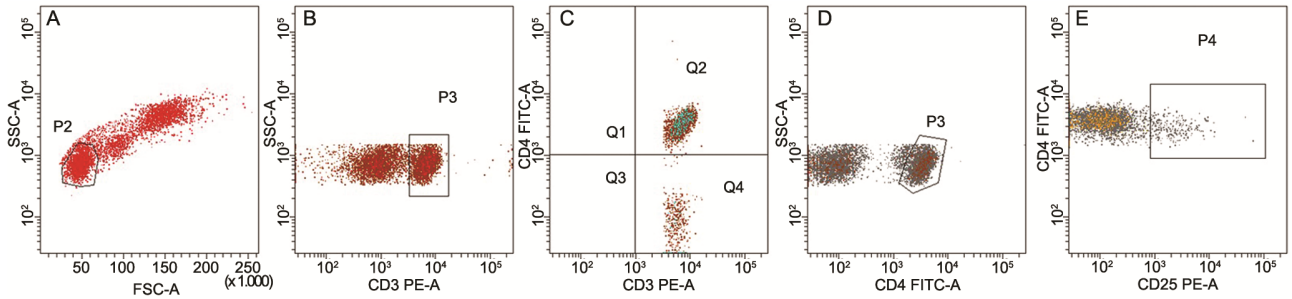

Fig. 2 — Forward scatter/side scatter (FSC/SSC) analysis of the blood samples flowcytometrically. (A) Whole white blood cells; (B) Total lymphocytes; (C) Total T lymphocytes; (D) Helper T lymphocyte subgroup; and (E) Regulatory T lymphocyte subgroup

Vit-D groups. Blood samples were collected into an EDTA containing tube, for lymphocyte counting and subtyping, and into a silica additive tube for cortisol, Vit-D and cytokine measurements. These tubes were centrifuged (10,000 *g* for 10 min), serum samples were separated and stored at −80°C until analysis. Leukocytes (WBCs) were diluted with Turk solution and counted with a Thoma hemocytometer (ISOLAB, Wertheim Germany). Immunophenotyping was performed via direct flow-cytometry using anti-mouse CD3 phycoerythrin (PE), anti-mouse CD4 fluorescein isothiocyanate (FITC), and anti-mouse CD25 PE. All samples were evaluated by FACS Canto II-flow cytometry (Becton-Dickinson, San Jose, CA, USA), and analyzed with FACS DIVA software (Becton-Dickinson, San Jose, CA, USA) to determine the Th cell subtypes. Gating of lymphocytes was first performed based on forward scatter/side scatter (FSC/SSC) graphics. Thereafter, the CD3 gate was used to select for T cells, with $CD3^+CD4^+$ cells considered to be Th cells, and $CD4^+CD25^+$ cells were phenotyped as Treg cells (Fig. 2)[16].

Serum cortisol measurement

Serum cortisol levels were determined with a competitive electrochemiluminescence immunoassay using two monoclonal antibodies raised against cortisol. All data were plotted using a Roche P800 Modular Analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Serum vitamin D measurement

Serum Vit-D levels were measured by liquid chromatography-mass spectrometer (LC-MS) (API 4000 QTRAP Triple Quadrupole, Linear Ion-Trap LC/MS/MS mass spectrometer, Applied Biosystems, USA) and data was quantified by Analyst 1.4.2 software (Applied Biosystems, USA)[17].

Determination of cytokines

IL-4, IL-17A, and IFN-γ (eBiosciences Inc., San Diego, CA, USA) levels were measured by ELISA method, per the manufacturer's instructions. The absorbance was read at 450 nm using a BioRad microplate reader (BioRad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics 22 for Mac software (IBM Corp, Los Angeles, CA, USA). The data were found to have a normal distribution when tested with the Kolmogorov–Smirnov test, and are expressed as mean±standard deviation (SD). Experimental groups (C-AS-ChS and D-ASD-ChSD) were compared separately using one-way ANOVA followed by post-hoc Tukey test with Bonferroni correction. The corresponding groups for the effect of Vit-D (C *vs*. D; AS *vs*. ASD and ChS *vs*. ChSD) was compared by a two-tailed Student's t-test. P <0.05 was considered statistically significant.

Materials used

Vit D, Sigma D1530, St. Louis, MO, USA; Blood Tubes, BD Vacutainer with K_2EDTA (5.4 mg) REF: 368856 and CAT REF: 367896BD Biosciences Corp., Plymouth, UK; Lymphocyte immunophenotyping antibodies, BD Biosciences, USA; ELISA kits, mouse IL-4, mouse IL-17, mouse IFN-γ, eBiosciences Inc., San Diego, CA, USA.

Results

The body weights of the mice in all groups before and after the experimental procedures were similar.

Serum cortisol levels

Serum cortisol levels were measured to confirm activation of the stress system. Acute stress significantly increased cortisol levels ($P_{C\text{-}AS}$<0.05), whereas this difference was not observed after exposure to subchronic stress ($P_{C\text{-}ChS}$>0.05, $P_{AS\text{-}ChS}$<0.05) [$F_{C\text{-}AS\text{-}ChS}$(2,21):3969; ($P$<0.05); Fig. 3A].

In the Vit-D-treated groups, same pattern was preserved so that acute stress increased serum cortisol

levels ($P_{D\text{-}ASD}$<0.05), which become compatible to the control values in subchronic exposure ($P_{D\text{-}ChSD}$>0.05, $P_{ASD\text{-}ChSD}$<0.05) [$F_{D\text{-}ASD\text{-}ChSD}$ (2,17):5968 (P<0.05)].

A pairwise comparison of the corresponding groups (C-D, AS-ASD, and ChS-ChSD) showed that Vit-D increased cortisol levels under both control and acute stress conditions, however this was not observed in the subchronic stress groups ($P_{C\text{-}D}$<0.05, $P_{AS\text{-}ASD}$<0.05, $P_{ChS\text{-}ChSD}$>0.05) (Fig. 3A).

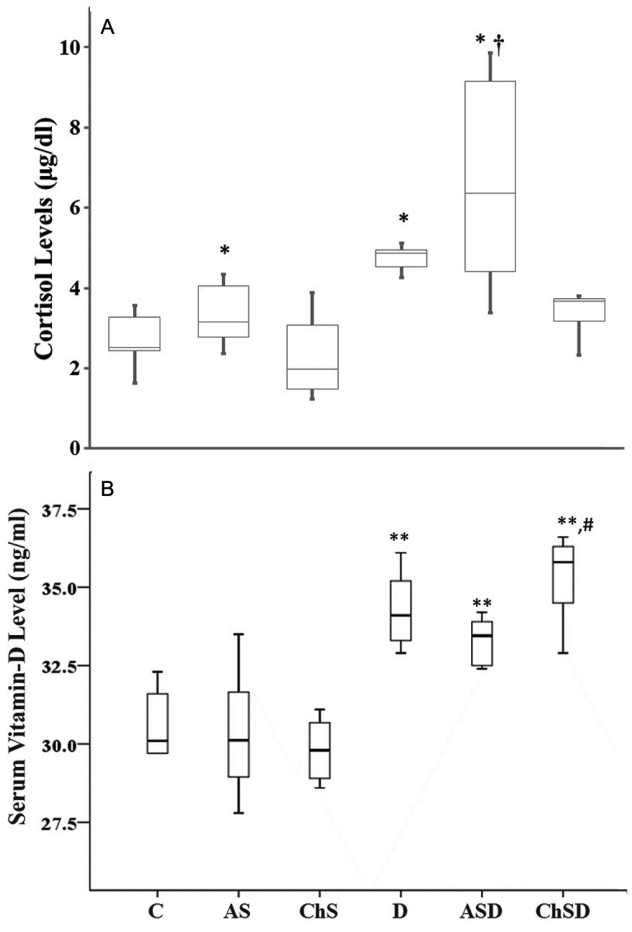

Fig. 3 — (A) Serum cortisol levels (µg/dL); and (B) Serum Vitamin D levels (ng/mL) of experimental groups measured in samples obtained after experimental procedure is completed. [Significant difference (P<0.05) compared to * C; † AS; ** Vit-D untreated groups; and #ASD]

Serum vitamin D levels

Serum Vit-D concentrations was similar in Vit-D untreated [$F_{C\text{-}AS\text{-}ChS}$ (2,17):0.485 (P>0.05) however ChSD group had higher Vit-D levels than the ASD group [$F_{D\text{-}ASD\text{-}ChSD}$ (2,19):5.784 (P<0.05); $P_{ASD\text{-}ChSD}$<0.05]. As expected Vit-D treated groups had higher serum Vit-D concentration than the untreated groups (Fig. 3B)

Response of immune cells

Total leucocyte count

Both acute and subchronic stress significantly increased WBC count (cells/mL) compared to that in the control animals [$F_{C\text{-}AS\text{-}ChS}$ (2,32):8752; (P<0.05), ($P_{C\text{-}AS}$<0.05, $P_{C\text{-}ChS}$<0.05)]. The two stress protocols were not different ($P_{AS\text{-}ChS}$>0.05). WBC count was comparable between three Vit-D-treated groups [$F_{D\text{-}ASD\text{-}ChSD}$ (2,24):0.732; (P>0.05), (Table 1)]. In regard of Vit-D effect, only subchronic stress groups revealed significant difference for leucocyte count ($P_{ChS\text{-}ChSD}$<0.05; $P_{AS\text{-}ASD}$>0.05).

Lymphocyte subtypes

The lymphocyte typing/subtyping was performed by flow-cytometer (Fig. 1), with the subtypes expressed as the percentage of the total WBCs.

The percentage of total lymphocytes was similar when compared for stress or Vit-D application [$F_{C\text{-}AS\text{-}ChS}$ (2,32):0.554; (P=0.659) and $F_{D\text{-}ASD\text{-}ChSD}$ (2,24):1243; (P=0.698); Table 1] with the exception of control and Vit D groups ($P_{C\text{-}D}$=0.021) (Table 1).

T lymphocytes' levels were also comparable under the two stress conditions within each subgroup [$F_{C\text{-}AS\text{-}ChS}$(2,32):0.713; ($P$=0.291) and $F_{D\text{-}ASD\text{-}ChSD}$(2,24):0.002; ($P$=0.817)]. In the pairwise comparison, Vit-D treatment significantly increased the percentage of T lymphocytes in the control ($P_{C\text{-}D}$ =0.043), acute ($P_{AS\text{-}ASD}$ =0.023), and subchronic ($P_{ChS\text{-}ChSD}$=0.691) stress groups (Table 1).

Table 1 — Leucocyte counts, percentages of T and Regulatory T (Treg) Lymphocytes in experimental groups (n=11/group).

Groups	WBC Count (No./mL)	% of Total Lymphocytes	% of T Lymphocytes	% of CD4 (+) T Lymphocytes	% of CD4(+) CD25(+) T Lymphocytes
Control (C)	3316.7±458.9	73.50±12.84	44.61±11.02	76±3.56	3.97±1.24
Acute stress (AS)	4475.0±807.0*	67.16±16.51	49.77±9.71	76.15±5.06	4.2±1.34
Subchronic stress (ChS)	4427.3±955.0*	66.35±23.35	50.20±16.56	74.09±12.23	3.3±0.85
Control+Vit-D (D)	3650.0±453.0	65.52±14.81*	57.46±8.87*	74.15±3.58	4.15±1.68
Acute stress+Vit-D (ASD)	3810.0±555.0	64.32±13.04	57.25±15.29†	78.18±6.67	3.89±1.18
Subchronic stress+Vit-D (ChSD)	3544.4±414.0**	56.23±12.73	57.04±15.50	77.82±2.7	3.98±1.18

[WBC , White blood cells. Significant difference (P <0.05) compared to * Control, † AS, ** ChS. Results are given as Mean ± Standard Deviation]

The percentages of $CD4^+$ T (Th) and $CD4^+CD25^+$ Treg lymphocyte were similar in all groups (Table 1).

Th subgroups

To investigate the effect of stress and/or Vit-D application on the functional profile of the Th (Th1, Th2, and Th17), the concentrations of specific cytokines (IFN-γ, IL-4, and IL-17A, respectively) were determined. While both stress protocols significantly increased IFN-γ levels, it was significantly lower in the ChS than in the AS group [$F_{C\text{-}AS\text{-}ChS}$ (2,14):243001; P <0.005) ($P_{C\text{-}AS}$< 0.001 $P_{C\text{-}ChS}$<0.005; $P_{AS\text{-}ChS}$<0.005)]. On the other hand, Vit-D attenuated the effect of acute stress and completely abolished the effect of subchronic stress [$F_{D\text{-}ASD\text{-}ChSD}$ (2,14):136790; P<0.005; $P_{D\text{-}ASD}$<0.005; $P_{D\text{-}ChSD}$<0.005; $P_{ASD\text{-}ChSD}$<0.005), (Figure 4A)], so that IFN-γ levels were measured considerably low in ASD and ChSD groups, although it significantly increased IFN-γ levels by itself.

Serum IL-4 levels were higher in both the acute and subchronic stress groups; the IL-4 levels were significantly higher in the ChS group [$F_{C\text{-}AS\text{-}ChS}$(2,9):49423; P <0.005)]. Similarly, IL-4 levels were increased in stress groups with Vit-D applications [$F_{D\text{-}ASD\text{-}ChSD}$(2,15):166700; P <0.005), (Fig.3B)]. However, effects of Vit D on IL-4 levels in acute and subchronic stress exposed animals were different. IL-4 concentration in ASD group was higher and ChSD group was lower compared to Vit-D untreated counter parts ($P_{C\text{-}CD}$>0.05; $P_{AS\text{-}ASD}$<0.05; $P_{ChS\text{-}ChSD}$<0.05). (Fig. 4B)].

IFN-γ/IL-4 ratio as an indicator of Th1/Th2 activity ratio

The IFN-γ/IL-4 ratio reflects the balance between the Th1 and Th2 subtypes. In AS group, prominently increased ratio indicates facilitated Th1 response, whereas subchronic stress shifted the immune response towards the Th2 response. Vit-D treatment also favoured the Th2 response under stress conditions. On the other hand, the effect of Vit-D treatment was in the opposite direction in stress free control groups (Table 2).

IL-17A levels were highly variable in almost all of the groups. Nevertheless, the changes caused by stress protocols were significant [$F_{C\text{-}AS\text{-}ChS}$(2,21):7911; ($P$=0.03)]. Although serum IL-17A level was higher in the AS group, it was comparable to C group, on the other hand it was significantly lower in the ChS group ($P_{AS\text{-}ChS}$<0.05, $P_{C\text{-}ChS}$<0.05). The lowest value was measured in the ChS group. Vit-D treatment equalized the groups for the effects of acute or subchronic stress on IL-17A levels [$F_{D,\ ASD,\ ChSD}$ (2,19):0.236; (P=0.792)]. A pairwise comparison of the groups for Vit-D treatment revealed a significant difference between the subchronic stress groups ($P_{ChS\text{-}ChSD}$<0.05, $P_{C\text{-}D}$>0.05, $P_{AS\text{-}ASD}$>0.05) (Fig. 4C).

Table 2 — IFN-γ/IL-4 ratio of the experimental groups as an indicator of Th1/Th2 activity

Groups	IFN-γ/IL-4 ratio
Control (C)	9.9± 1.5
Acute stress (AS)	37.8± 4.7*
Subchronic stress (ChS)	1.8± 0.7*†
Control+Vit-D (D)	20.6± 5.9*
Acute stress+Vit-D (ASD)	3.4± 0.9†,#
Subchronic stress+Vit-D (ChSD)	0.4± 0.1**,#,¥

[Significant difference (P<0.05) compared to * Control; † AS; ** ChS; # D; and ¥ASD. Results are given as Mean ± SD]

Discussion

Stress affects all organ systems of the body and initiates a stress response that can be either beneficial or harmful[4,18-20]. Immune system dys/malfunction associates stress-triggered diseases, from a simple

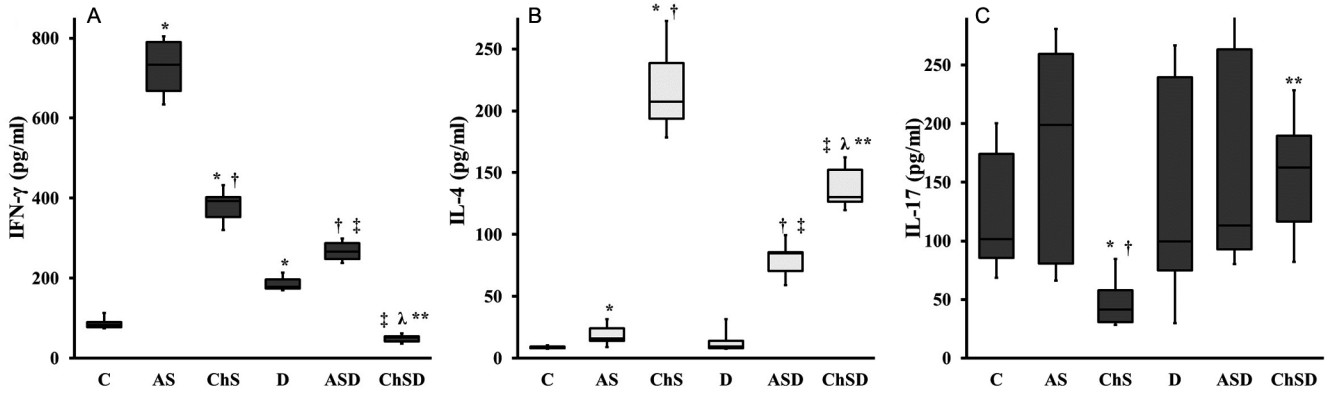

Fig. 4 — Concentrations (pg/mL) of the cytokines representing helper T cell subgroups. (A) IFN-γ; (B) IL-4; and (C) IL-17A. [Significant difference (P <0.05) compared to *C; †AS; ‡D; λASD; and **ChS]

common cold to autoimmune diseases and even cancer, and is usually accepted as being causative. This study addressed the different responses of Treg and Th (Th1, Th2, Th17) cells, the main regulators of the immune response, under both acute and subchronic stress conditions, as well as their modulation by Vit-D. Both acute and subchronic stress increased leucocyte cell numbers, and resulted in a pro-inflammatory condition, whereas Vit-D treatment reversed this effect. A closer look into T lymphocytes indicated that the immune response primarily shifted towards Th1 cells in acute stress exposed mice and towards Th2 cells under subchronic stress and with Vit-D treatment, regardless of the duration of stress. In addition, the diverse changes in Th17 and Treg cells in response to acute and subchronic stress became similar to control animals by Vit-D treatment.

Stress model

Owing to its combination of physical and psychological stressors, the cold-immobilization stress model was selected for use in this study[15,21]. This protocol has been used previously, including several studies examining the interaction between stress and the immune response[18, 21,22]. The model was preferred since the results of preliminary studies indicated no sign of depression in subchronic exposure.

The serum corticosteroid (cortisol or corticosterone) concentration is one of the most reliable parameters used to assess stress levels, and so it can be used to follow activation of the stress system. Corticosterone is considered as the main corticosteroid, which take part in stress responses in rodents, however cortisol measurement is frequently preferred since it has been shown that they correlate well under various physiological and stress-related conditions[23,24]. As, maximum cortisol levels have been reported to be reached in 20-40 min following a stress protocol[25], we set the blood sampling at 30th min. in the current protocol. In the present study, the stress protocol was uniform for modality and intensity and varied only for the repetition of the protocol, i.e. it was the only variable which may modulate the response. The highest cortisol levels, reflecting maximum stress system activation, measured in the acute stress groups. In support of our data, Bowers *et al*.[20] also showed that acute (1 day) cold-immobilization induced stress, increased cortisol levels to a greater extent than chronic stress (15 days). Reber *et al*.[25] also reported increased cortisol levels on the second day of stress, followed by a progressive decrease in levels after repeated exposure to stress. As observed in the subchronic stress group of this study, the decreased cortisol levels in repeated stress exposure presumably reflect an adaptive response of the stress system.

Increased serum cortisol levels with Vit-D treatment under all conditions is in conflict with the reports of an inverse correlation between Vit-D and glucocorticoids (GC)[26,27]. However, this and many other studies address pharmacological levels of cortisol, and not the levels of endogenously released GC and mainly effect of GC on Vit-D levels. Regarding the interaction of interest, the results are diverse there are reports implicating no effect of Vit-D supplementation on serum cortisol[28], Vit-D induced disturbance in steroidogenesis and decreased GC synthesis[26] or increased serum concentration of GC mainly due to relative and/or temporary glucocorticoid resistance[27]. Expression of VDR on adrenocortical cells[26] and alternate effects of Vit-D[12,28,29] are probably the main explanations for the different reports. As Vit-D stimulates activation of neuroendocrine pathways[30,31], the stress induced activation of HPA axis may help to explain the increased cortisol levels measured in this study.

Stress and the immune system

As has been very well documented previously, stress is a potent immunomodulator[4,6,19]. In this study, we initially measured WBC counts, and then determined the total number of total lymphocytes, T lymphocytes, Th lymphocytes, and Treg lymphocytes.

Although different results are reported, in general, acute stress is associated with a mild inflammatory process and increased WBC count, but this is not seen for longer exposures[32,33]. For example, total lymphocyte counts decreased following exposure to acute stress, which was reversed following exposure to long term chronic stress in rats[34]; acute (once for 30-min) and chronic (12 days, 30-min/day) immobilization stress caused an increase in total lymphocyte counts[35]. Another study, which investigated the effects of different stressors under both acute and chronic conditions in mice, reported a lower number of lymphocytes following exposure to acute restraint-induced stress, however, this effect attenuated as the animals chronically exposed to the same stress protocol[20]. Our findings indicating increased WBC count and lowered total lymphocyte

numbers under stress are in support of Bowers *et al*.[20], the inconsistency of WBC and total lymphocyte counts can be explained by the changes in other cell types such as natural killer (NK) cells, as has been reported previously[36,37].

Keeping in mind, that one of the main purposes of this study is to evaluate T cell and specifically Th and Treg response to acute and subchronic stress, the data analysis revelaed comparable Th lymphocyte percentages among groups. Although these results are in line with the results of Atanackovic *et al*.[36] the number of Th cells have been reported to decrease significantly with exposure to acute stress, and increase following chronic stress, with a significant negative correlation with corticosterone levels and percent of Th cells. The conflict between different studies may be due to the kinetics of changes in T lymphocyte numbers that occur in response to stress; T lymphocyte numbers decreases significantly during stress, starts increasing again upon termination of the stress, and return to normal at around the fourth hour after the stress procedure[31]. Since the blood samples in our study were obtained 30 min after stress exposure, we might have sampled too early to see possible changes in T lymphocyte numbers. Taking these data together, it is clear that modulation of the immune system by stress can vary according to the duration and modality of the stressors, but no clear picture exists from the published data.

The differentiation of naive Th cells, and the development of Th1 and Th2 effector cells are mainly controlled via cytokines, and a dysregulation in this system results in altered Th1/Th2 response[8]. The activities of Th1 and Th2 cells were assessed by measuring the proinflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-4, respectively and the IFN-γ/IL-4 ratio was used as an index of Th1/Th2 cell activation, as has previously been reported[8,37].

Th1 response following stress was much higher in the AS groups than in the ChS groups, reflecting a shift in the immune response towards cellular immunity. On the other hand, the IL-4 levels were much higher in the ChS groups compared to the control and AS groups, indicating increased Th2 cell activity and the dominance of humoral immunity.

These results are supported by studies suggesting that short-term exposure to stress hormones, or to acute stress, facilitates the Th1 response, protecting the individual from infections[38]. On the other hand, individuals under chronic stress, or with chronic diseases, are more prone to infection[8,10,38], all of which can be explained by the differential Th response[3].

However, contrary to our data, both *in vivo* and *in vitro* exposure to glucocorticoids and catecholamines suppress the production of various pro-inflammatory cytokines such as TNFα, IFN-γ, and IL-2[4], which suppresses Th1 mediated cellular immunity and shifts the immune response to Th2 mediated humoral immunity[4]. However, in these studies, there was either direct use of exogenous glucocorticoids, and the effects were examined *in vitro*, or the duration of the stress exposure was neglected.

The third effector Th cell subtype, IL-17A expressing Th17 cells, plays both pro- and anti-inflammatory roles in the immune system[7,39] and in autoimmune inflammation[40]. The Th17 cells carry out this anti-inflammatory effect by suppressing the T cell activity and differentiation of the Th1 cells[41]. Few studies have focused on the effects of stress on Th17 activity, but it has been shown that IL-17A expression is increased following either 24-h exposure to cold stress in chickens, or following acute (12 h) restraint stress in mice[42,43].

Studies in animals[43] and stressed children[44] have shown there is an increased activity of both Th1 and Th17 cells, which are the key players in autoimmune diseases such as DM, rheumatoid arthritis, and Crohn's disease, shifting the immune balance toward pro-inflammatory Th1-Th17 dominance versus an anti-inflammatory Th2 response and a decreasing the proportion of Treg cells.

Our data support these limited data, showing increased IL-17A levels following exposure to acute stress and a decrease in IL-17A levels following exposure to subchronic stress. These data may help to explain why autoimmune diseases are triggered and/or exacerbated, especially under acute stress conditions.

Acute cold-immobilization stress increased the function of Th1/Th17 cells mediating the pro-inflammatory response, and decreased the number of Th2 cells, in mice. There was also an increase in the percentage of Treg cells following exposure to acute stress. In response to subchronic stress the immune response was completely shifted towards Th2 cell dominancy, along with a decrease in the proportion of Treg cells. Treg cells are responsible for self-tolerance, together with the negative selection of

autoreactive lymphocytes in thymus[45] explaining why either a decreased number, or a deterioration in function, of Treg cells are associated with increased susceptibility to autoimmune diseases[46]. In this study, the percentage of Treg cells increased in animals exposed to acute stress, whereas the levels dropped back to control levels following subchronic stress. The number of Treg cells has been shown to increase both in atopic and healthy participants experiencing acute examination (academic) stress, which was more prominent in participants with higher perceptions of stress[47]; possibly through the activation of the glucocorticoid-induced tumor necrosis factor-related receptor (GITR)[48].

Generally, there is an opposing relationship between Treg and Th17 cells[7]. However, our data showed an alteration in the same direction for Th17 and Treg cells. These controversial findings may be explained by the difference in the focused cells. The previous studies have examined peripheral/inducible Treg cells, whereas the whole Treg population examined in our study. In addition, identification methods for Treg cells vary, such that either the levels of CD25, or intracellular marker, FOXP3 can be chosen[49]. In this study, we used CD25, since we aimed to evaluate all peripheral Treg cells. The differences in glucocorticoid sensitivity in T cells should also be kept in mind when interpreting the results of studies on stress-immune system interactions[50].

The effect of subchronic stress on Treg cells in the present study support the findings of Harpaz *et al*.[43], who showed that the proportion of Treg cells decreases in mice exposed to chronic stress.

Effects of vitamin D on stress and immunity

Vit-D is recently qualified an important immunomodulator, enhancing the innate immune system and inhibiting the adaptative immune response with a shift to Th2 and Treg response[12,13,50,51]. Epidemiological studies have also reported a relationship between Vit-D insufficiency and chronic infections and autoimmune diseases[14]. The VDR is expressed on different immune cells including monocytes, macrophages, dendritic cells, as well as T and B-lymphocytes, and significantly higher in immature innate immune cells[13,50,51], and in T cells following immunogenic stimulation and through differentiation[52,53]. This suggests major effects of Vit-D on the immune system, especially with respect to modulating the phenotype of Th cells[13,14,51]. However, the effect of Vit-D on how stress or the HPA axis affects the immune response is not known.

The interaction between innate and adaptive immunity, antigen presenting cells (APC); macrophages and DC and cytokines, promote Th differentiation in cooperation with other factors. T cells progress towards Th1 or Th2 cells, which regulates immune responses[12]. In addition to the shift from Th1 (cellular immunity)[52] to Th2 (humoral immunity) [53], Vit-D inhibits the development and function of Th17 cells[54] and attenuates IL-17A expression[55].

Moreover, low serum Vit-D levels have been shown to be correlated with impaired Treg activity[54] where it appears to be related to increased Th1/Th17 activity, in contrast to adequate Vit-D concentrations which facilitates Th2/Treg activity[13]. The effect of acute stress was to shift the Th response towards Th1/Th17 dominance, whereas treatment with Vit-D reversed this shift. Subchronic stress acted in the opposite direction, so that it induced a Th-2 response, and treatment with Vit-D augmented the shift toward a humoral immune response. The results of Chang *et al*.[54] correlated that Vit-D decreases IL-2 and interferon (IFN)-γ (inflammatory cytokines) shows immune-suppressive effect also supports these differences in the Vit-D effect suggesting that stress may alter the mechanism of action of Vit-D[56]. so that the decreased ratio of IFN-γ/IL-4 seen following Vit-D treatment induces an overall shift of the immune response to a humoral (Th2) response following both acute and subchronic stress exposure, whereas under control conditions it has the opposite effect. Our results are in support of previous data where the effect of Vit D reported to change in individuals with immune suppression and animal models compared to the normally functioning immune system, as the Vit D behaved differently in stress groups where immune function is modulated than the control animals[57,58] These findings may be attributed to the mutual interaction between Vit-D and glucocorticoids[57].

The major limitations of this study are not to be able to measure more cytokines from all cell groups of interest, and try longer periods of stress exposure which we plan for our next studies. On the other hand, major strengths are, to examine the effect of acute and subchronic stress specifically on T helper cell population and its modulation by Vit-D, which to our knowledge is the first time.

Conclusion

The mechanisms underlying stress and its associated health hazards, unquestionably involve a dys/malfunction of the immune system. In this study, we tried to contribute to clarification of these mechanisms, and suggest an approach to reduce the harmful effects of stress. In this regard, the importance of Vit-D on stress-induced interactions between cytokines associated with the immune response was investigated. Although we have as yet an incomplete understanding of this, several clues were uncovered and our data indicating the Th1 dominance in AS and Th2 in subchronic stress modulated by Vit-D and a facilitated anti-inflammatory and tolerogenic profile is achieved. Similarly, the stress associated changes in Th17 and Treg cells normalized by Vit D, in support of our hypothesis. Since Vit-D deficiency is a major worldwide public-health problem, our results also contribute to the stress literature by showing the differences in acute and subchronic stress exposure and their modulation by Vit-D treatment. Further more detailed studies in this area is required to address the physiopathology of stress related diseases, and the role of Vit-D as a preventive and/or therapeutic agent.

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Animal rights

All experimental protocols were approved by the Hacettepe University Ethics Board for Animal Experiments (Issued by 11/03-1). All experimenters were certified for the care and use of laboratory animals, and all the animals received humane care in strict compliance with the “Guide for the Care and Use of Laboratory animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23 revised 1985) and Turkish bylaws for laboratory animal use.

Author Contributions

Murat Dogan: Conceptualization, investigation, methodology, performing experiments, formal analysis, writing (original draft and review and editing); Ayse Meltem Sevgili: Investigation, methodology, performing experiments, formal analysis, writing (review and editing); Ilknur Kozanoglu: investigation, methodology, resources, writing (review and editing); and Bilge Pehlivanoglu: Conceptualization, investigation, methodology, resources, supervision, funding acquisition, writing (review and editing).

Conflict of Interest

Authors declare no competing interests.

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