Chronic exposure to stress predisposes to higher autoimmune susceptibility in C57BL/6 mice: Glucocorticoids as a double-edged sword

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Stress activates the hypothalamic-pituitary-adrenocortical axis to promote the release of corticosterone (CORT), which consequently suppresses pathogenic stimulation of the immune system. Paradoxically, however, stress often promotes autoimmunity through yet unknown mechanisms. Here we investigated how chronic variable stress (CVS), and the associated alterations in CORT levels, affect the susceptibility to experimental autoimmune encephalomyelitis (EAE) in female and male C57BL/6 mice. Under baseline (nonstressed) conditions, females exhibited substantially higher CORT levels and an attenuated EAE with less mortality than males. However, CVS induced a significantly worsened EAE in females, which was prevented if CORT signaling was blocked. In addition, females under CVS conditions showed a shift toward proinflammatory Th1/Th17 versus Th2 responses and a decreased proportion of CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells. This demonstrates that whereas C57BL/6 female mice generally exhibit higher CORT levels and an attenuated form of EAE with less mortality than males, they become less responsive to the immunosuppressive effects of CORT under chronic stress and thereby prone to a higher risk of destructive autoimmunity.

Keywords: CD4 T cells · Chronic stress · Corticosterone · EAE

Introduction

It has been well established that stress may substantially affect the homeostatic regulation of the immune system [1–3]. In most animal models studied thus far, stressful triggers such as fear, maternal deprivation, social threat, or physiological challenge have been shown to induce immunosuppression associated with increased susceptibility to allergies and infectious diseases [1,4,5]. These effects are mediated by the hypothalamic-pituitary-adrenal (HPA) axis, a complex network linking the nervous, endocrine and immune systems [6, 7].

The HPA axis can be triggered by stress or by proinflammatory cytokines (e.g. IL-1, IL-6, and TNF-\textalpha) to ultimately result in
the secretion of corticosterone (CORT) from the adrenal glands to the circulation [8]. CORT, in turn, acts to suppress the activation, proliferation, and trafficking of immune cells [9, 10] and plays a role in autoimmune regulation via shifting from Th1/Th17 pro-inflammatory to Th2 anti-inflammatory responses [11–13]. Indeed, previous studies have shown that rats producing lower CORT levels (e.g. due to genetic manipulation or adrenalectomy) are more susceptible to pathogenic autoimmunity [14]. CORT is therefore often used as an immunosuppressor in the clinical treatment of inflammatory and autoimmune diseases [9, 15, 16].

Regardless of the immunosuppressive effects of CORT, chronic exposure to stress has also been linked with relapse of autoimmune diseases such as multiple sclerosis [17, 18] and psoriasis [19, 20]. Paradoxically, these diseases are characterized by a Th1/Th17 pro-inflammatory immune response [21–23], which implies that chronic stress exposure attenuates the immunosuppressive effects of CORT [24, 25]. It has also been suggested that CORT may affect regulatory T (Treg) cells which play a central role in protecting against autoimmune diseases [26–29].

The present study aims to explore the effects of chronic stress on immunoregulatory mechanisms that directly control autoimmunity. To this end, we subjected C57BL/6 mice to 24 days of chronic variable stress (CVS). This well-established paradigm consists of different stressful stimuli randomly introduced for different durations to minimize adaption, and thereby model the diversity of stressful events in daily human life [30]. As a model for autoimmune disease susceptibility we tested the mice’ susceptibility to EAE and the course of its development.

### Results

**CVS induces anxiety-like behaviors and affects HPA axis activity**

To examine the behavioral effects of CVS, we tested stressed and nonstressed C57BL/6 mice for anxiety-like behaviors. We used a CVS model that was found to affect both physiological and psychological parameters and particularly immune functions [31]. In contrast to short and predictable stress, long-lasting exposure to unpredictable stressors avoids habituation to stress and induce hallmark characteristics of overexposure to corticosteroids. The stress paradigm lasted 24 days as detailed in Table 1 and in Material and methods.

<table>
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<tr>
<th>Day</th>
<th>Stressor</th>
<th>Duration</th>
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<tr>
<td>1</td>
<td>Isolation</td>
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<td>2</td>
<td>Restrainer</td>
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<td>Predator odor</td>
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<td>4</td>
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<td>Illumination</td>
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<td>6</td>
<td>Predator odor + restrainer</td>
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<td>7</td>
<td>45° inclination</td>
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<td>8</td>
<td>No stressor applied</td>
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<td>9</td>
<td>Predator odor</td>
<td>1 h</td>
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<td>10</td>
<td>Restrainer</td>
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<td>11</td>
<td>Predator odor</td>
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<td>16</td>
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<td>17</td>
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<td>19</td>
<td>Illumination</td>
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<td>20</td>
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<td>21</td>
<td>45° inclination</td>
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<td>22</td>
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<td>23</td>
<td>Predator odor</td>
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<td>24</td>
<td>Restrainer</td>
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<td>25</td>
<td>Predator odor</td>
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Both female and male mice demonstrated clear and significant anxiety-like behaviors following the 24-day experimental period (Fig. 1A and B). Specifically, as compared with nonstressed mice, stressed male and female mice showed less entries (p < 0.001) and spent less time in the open arms of an elevated plus maze (p < 0.01) (Supporting Information Fig. 1A and B), and spent more time in the peripheral zones of an open-field arena (p < 0.001; Supporting Information Fig. 1C). Stressed mice also gained less weight during the 24-day CVS period, such that their body weight did not change significantly as compared with their initial body weight (Fig. 1C and D). This was in marked contrast to nonstressed mice, which significantly gained body weight during the 24-day experimental period (Fig. 1C and D).

To examine how CVS affects HPA axis activity we determined CORT levels in urine samples collected weekly. Overall, for the entire experimental period, cumulative urine CORT levels were significantly higher in stressed than in nonstressed mice in both females (358 ± 38 ng/mL and 138 ± 17 ng/mL, respectively; p < 0.001) and males (13.7 ± 1.4 ng/mL and 9.26 ± 0.81 ng/mL, respectively; p < 0.01; Fig. 2A). In addition, CORT levels under both basal and stressful conditions were markedly higher in females compared to males (p < 0.001 for each condition; Fig. 2A). These higher CORT levels were observed mainly during the first 3 weeks of the 24-day experimental period; in the fourth week of stress, CORT levels in stressed mice were not significantly higher than those in nonstressed mice (Fig. 2B). Of note, whereas CORT was found primarily in its free form in the urine of female and male mice (85 and 78% of total CORT, respectively), in the blood it was mostly bound to CORT-binding globulin (92 and 83% of total CORT in females and males, respectively) and was detected at significantly lower concentrations compared with urine CORT. In addition, although to a lesser extent than in the urine, blood CORT levels were significantly higher in females than in males (Fig. 2D and E).
CVS increases the susceptibility to EAE in female C57BL/6 mice

Given the overall stress-induced increase in CORT levels, and in light of previous studies [8, 32], we expected stress to induce spleen anomalies and, due to its apparent immunosuppressive activity, attenuate the susceptibility to EAE. To evaluate stress-induced spleen anomalies we measured the spleen weight and number of splenocytes in stressed and nonstressed mice following the 24-day experimental period. To determine stress-induced susceptibility to EAE, we immunized stressed and nonstressed mice with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) following the 24-day experimental period and quantified the severity of EAE-related symptoms.

As expected, stressed mice exhibited a significant decrease in splenocyte cell count compared to nonstressed controls (females: \(38 \times 10^6\) cells compared with \(52 \times 10^6\) cells; \(p < 0.01\); Supporting Information Fig. 2A. Males: \(35 \pm 2.37 \times 10^6\) cells compared with \(62 \pm 3.5 \times 10^6\) cells; \(p < 0.001\); Supporting Information Fig. 2B), as well as decreased spleen weight (females: \(75.0 \pm 3.2\) mg compared with \(97.7 \pm 5.7\) mg; \(p < 0.01\); Supporting Information Fig. 2C. Males: \(71.4 \pm 4\) mg compared with \(95.5 \pm 6.2\) mg; \(p < 0.01\); Supporting Information Fig. 2D). These differences were not due to overall differences in body weight (e.g. differences resulting from decreased weight gain in stressed mice), as the spleen weight/body weight ratio was also decreased by 15% in stressed mice compared with nonstressed mice (Supporting Information Fig. 2E and F). Although all mice showed clinical symptoms...
Figure 3. CVS exacerbates EAE in female but not in male mice, which is associated with an attenuated CORT response to MOG35-55 immunization. Mice were immunized with a high dose of MOG35-55 to induce EAE following 24 days of CVS or nonstressed conditions, as described in Materials and methods. Progression of the disease (clinical score) is shown separately for female (A) and male (B) mice, and cumulatively (C) for all groups (F: females; M: males; NS: nonstressed; S: stressed). Urine CORT levels (D) is shown before and during (days 9, 14, and 21 postimmunization) EAE. Data in (A–C) are shown as means ± SEM of 18 mice per group, pooled from three independent experiments. Data in (D) are shown as means ± SEM of 11 mice per group, pooled from two independent experiments. *p-values were calculated by Student’s t-test.

Figure 4. Blocking CORT signaling prevents CVS-induced EAE exacerbation. Female C57BL/6 mice (n = 7 mice per group,) were injected daily with mifepristone (mife) throughout the CVS period (2 h prior to stress exposure) and then immunized with MOG35-55 to induce EAE as described in Materials and methods. Un-injected stressed mice and nonstressed mice (n = 18 mice for each group, pooled from three independent experiments) were used as controls. (A) Disease incidence rate. (B) Daily clinical scores (asterisks refer to significant differences between stressed mice and stressed mice administered with mifepristone). Bar graphs and data points represent means ± SEM. p-values were calculated by Student’s t-test (A) and ANOVA test (B) *p < 0.05.

do EAE 12 days following MOG35-55 immunization, the clinical symptoms of the disease in stressed females were generally more severe than in nonstressed females (Fig. 3A and C). In contrast to females, male mice exhibited a more severe form of EAE than nonstressed females (Fig. 3C), which was associated with about 17% mortality rate and did not, however, exacerbate under CVS conditions (Fig. 3B and C).

The induction and progression of EAE were associated with an increase in CORT levels in both stressed and nonstressed mice (Fig. 3D). Throughout the experiment, CORT levels were persistently higher in female compared with male mice (Fig. 3D). Compared to nonstressed mice, stressed females but not stressed males, showed a lower CORT response to MOG35-55 immunization at the day of EAE onset (Fig. 3D). This suggests that an impaired CORT response may have contributed to the exacerbation of EAE in stressed female mice. We thus hereon focus on the mechanism whereby CVS increases disease severity in female mice.

To directly determine the role of CORT in stress-induced EAE exacerbation, female mice were injected daily with the glucocorticoid antagonist mifepristone 2 hours prior to stress induction (Fig. 4). Following the stress exposure period, mice were injected with MOG35-55 to induce EAE. Nonstressed and stressed mice were used as controls. As shown in Figure 4A, compared with nonstressed controls, disease incidence rate was significantly increased in stressed mice whereas no difference was observed in stressed mice administered with mifepristone. Notably, ANOVA test revealed a significant effect for treatment (F (2,38) = 3.0132, p < 0.05) and for time (F (12,456) = 30.9, p < 0.0001); Fisher post-hoc analysis confirmed that EAE severity did not exacerbate in stressed mice injected with mifepristone compared to nonstressed control mice (Fig. 4B), and was partially ameliorated compared to stressed control mice (decreased clinical score, days 11–13 post MOG35-55 immunization; p < 0.05; Fig. 4B).

CVS decreases Th1/Th17 sensitivity to CORT-induced immunosuppression

The increased EAE susceptibility and severity observed in stressed female mice could have been mediated by CORT-induced alterations in certain innate and adaptive cell subsets. To examine whether the effector functions of lymphocytes were affected following CVS in female mice, cytokine production was measured following anti-CD3 stimulation of splenocytes derived from stressed and nonstressed female mice. As shown in Table 2, no differences were found between stressed and nonstressed mice in the levels of pro- and anti-inflammatory cytokines or in the levels of the chemoattractant MCP-1, suggesting that CVS did not intrinsically affect T-cell function. Thus, and given that stress increased CORT levels for a long period of time (Fig. 2), we also tested whether stress-induced elevation in CORT levels may have desensitized the lymphocytes to the immunosuppressive effects of CORT. To this
Table 2. Adaptive- and innate-related cytokine concentrations measured in supernatants of anti-CD3 stimulated splenocytes from stressed and nonstressed mice. 

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<th>Adaptive immune cytokines</th>
<th>Innate immune cytokines</th>
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<td></td>
<td>IL-2</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Nonstressed</td>
<td>1881 ± 52.9</td>
<td>40773 ± 3067</td>
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<tr>
<td>Stressed</td>
<td>1675 ± 226</td>
<td>39560 ± 6576</td>
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*Splenocytes were harvested from stressed and nonstressed female mice and stimulated in vitro with plate-bound anti-CD3 for 48 h. Secreted cytokines (pg/mL) were measured by multiplex ELISA (Quansys Biosciences) as described in Materials and methods. Cytokines were not detectable in control nonstimulated splenocytes. Data are shown as mean ± SEM of five mice per group, and are representative of two similar experiments performed. *p-values were calculated by Student’s t-test.

end, splenocytes harvested from stressed and nonstressed female mice were stimulated with plate-bound anti-CD3 in the presence of increasing concentrations of methylprednisolone (MP), a drug with predominantly glucocorticoid activity [33]. Overall, the levels of all secreted cytokines were significantly decreased in a dose-dependent manner in stressed as well as in nonstressed mice, demonstrating the known immunosuppressive effects of the drug (Fig. 5). Notably however, splenocytes harvested from stressed mice were less responsive to the immunosuppressive effects of MP as compared with splenocytes harvested from nonstressed mice. Specifically, reduced immunosuppressive effect of MP on splenocytes harvested from stressed mice was found for IL-2, IFN-γ, IL-17A, and IL-10, but not for IL-4 (Fig. 5). Moreover, a comparison of the IFN-γ/IL-4 ratio in the presence of increasing MP concentrations revealed that MP at 100 ng/mL tended to shift the activated lymphocytes toward a Th2 response in nonstressed but not in stressed mice (Fig. 5E). A similar comparison of the IL-17/IL-4 ratio revealed that MP did not affect this ratio in nonstressed mice but significantly shifted the activated lymphocytes toward a Th17 response in stressed mice (Fig. 5F). Such steroid resistance was also evident for the innate proinflammatory factors TNF-α and MCP-1 (Fig. 5H and I).

To further investigate the effect of CVS on immune effector functions, cytokine production was measured following stimulation of splenocytes from stressed and nonstressed mice with anti-CD3 or MOG35-55, 9 days following MOG35-55 injection. Anti-CD3 stimulation induced higher levels of secreted IFN-γ but not of IL-17A (Fig. 6A and E) and MP was significantly less suppressive of their production in splenocytes from stressed compared to splenocytes from nonstressed mice (Fig. 6A and B, E and F). Although only a trend of increased levels of IFN-γ was detected following MOG35-55-induced T-cell activation (Fig. 6C), IL-17A was significantly increased in splenocytes from stressed mice compared with splenocytes from nonstressed mice (Fig. 6G). MP completely abolished T-cell activation of splenocytes from both stressed and nonstressed mice (Fig. 6C, D, G and H), possibly due the markedly

Figure 5. CVS promotes Th1/Th17 effector functions associated with decreased T-cell sensitivity to the immunosuppressive effects of MP. Splenocytes were harvested from stressed and nonstressed female mice, stimulated in vitro with plate-bound anti-CD3 for 48 h with or without MP (10, 100, or 1000 ng/mL). Cytokine production was measured in supernatant by ELISA (A-D, G-I) and is shown as percentage of the levels measured without MP. IFN-γ/IL-4 and IL-17/IL-4 ratios were calculated (E–F). Data are shown as mean ± SEM of five mice per group, and are representative of two similar experiments performed. *p-values were calculated by Student’s t-test *p < 0.05; **p < 0.01; ***p < 0.001.
CVS decreases Treg frequency via an increase in the effector T-cell subsets

Our data demonstrate an increase in proinflammatory cytokine levels induced by MOG35-55 immunization following CVS. However, it is yet not clear whether the CD4⁺CD25⁺ Treg population, which can strongly impact the progression of EAE, is affected by CVS. We initially found that the frequency of CD4⁺ T cells was decreased by 8% in the spleen and by 33.7% in circulating PBL in stressed compared with nonstressed female mice (Supporting Information Fig. 3A and B).

The effect of CVS on the frequency of CD4⁺ Treg cells was examined by either intracellular staining of Foxp3 or surface staining of CD127 as a potential bio-marker of Treg cells ([34] and Supporting Information Fig. 3 and 4). FACS analysis revealed that within the CD4⁺CD25⁺ and CD4⁺CD25⁺high subpopulations, the frequency of Foxp3⁺ T cells was significantly decreased following CVS (Fig. 7A–C). In addition, whereas stressed mice demonstrated a significant increase in the frequency of splenic CD4⁺CD25⁺ T cells as compared with nonstressed mice (17.3 and 14.7%, respectively, \( p < 0.05 \); Fig. 7D and E), the fraction of CD127⁻ cells among CD4⁺ CD25⁺ T cells was significantly lower in stressed than in nonstressed mice in the spleen (76 and 82%, respectively, \( p < 0.05 \); Fig. 7D and E) and in the blood (65.6 and 77%, respectively, \( p < 0.01 \); Supporting Information Fig. 5A and B). Comparing the frequency of cells expressing CD127⁺ with CD127⁻ within splenic (Fig. 7D and F) and blood-derived (Supporting Information Fig. 5A and C) CD4⁺ T cells revealed a significant decrease in the CD127⁺/CD127⁻ ratio in stressed mice compared with nonstressed mice. This was evident primarily within the CD4⁺CD25⁺high subpopulation and to a lesser extent within the CD25⁺/low population, but not evident in the CD25⁻ population. Notably, the frequency of CD25⁺/CD127⁺, but not CD25⁺CD127⁻, within splenic (Fig. 7G) and blood-derived (Supporting Information Fig. 5D) CD4⁺ T cells was significantly higher in stressed than in nonstressed mice. This indicates that the increased Teff/Treg ratio in stressed mice resulted from an increase in the effector T-cell population with no change in the Treg-cell population.

The frequency of Foxp3⁺ cells and the CD127⁺/CD127⁻ ratio among CD4⁺CD25⁺ T cells were then examined following EAE induction. As shown in Figure 7H, whereas the frequency of splenic Foxp3 Treg cells among CD4⁺ T cells was generally reduced in stressed mice prior to EAE induction, no difference was observed between stressed and nonstressed mice following EAE. Similarly, no difference was observed in the CD127⁺/CD127⁻ ratio among blood-derived CD4⁺CD25⁺ T cells between stressed and nonstressed mice following EAE induction or remission (Supporting Information Fig. 5E). Notably, both the frequency of Foxp3⁺ cells (Fig. 7H) and the CD127⁺/CD127⁻ ratio among CD4⁺CD25⁺ T cells (Supporting Information Fig. 5E) were reduced at EAE onset and gradually recovered toward disease remission.

Discussion

The present study aimed to test the effects of chronic variable stress on immunoregulatory processes involved in autoimmune diseases. Although stress has been traditionally considered to suppress the immune system and shift it toward an antiinflammatory response through the secretion of CORT [3, 13], our results show that prolonged stress exposure exacerbates, rather than ameliorates, EAE in female C57BL/6 mice; this phenomenon, however,
Figure 7. CVS reduces the frequency of splenic regulatory T cells. Splenocytes were harvested from stressed and nonstressed female mice, stained for CD4, CD25, and Foxp3 (A–C, H) or CD4, CD25, and CD127 (D–G), and subsequently analyzed by flow cytometry. (A–B) Splenocytes were gated for CD4+ T cells and then analyzed for the frequency of CD25+Foxp3+ and CD25 high Foxp3+ T cells. (C) The frequency of Foxp3+ cells among CD4+ T cells. (D–E) Analysis of CD4+CD25+ T cells and the frequency of CD127− cells among CD4+CD25+ T cells. (D and F) The CD127−/CD127+ ratio within CD4+ T cells. (G) The frequency of CD25+CD127− and CD25+CD127+ cells among CD4+ T cells. (H) The frequency of Foxp3+ cells among CD4+ T cells before and during EAE. (A–C) Data are shown as means ± SEM of four mice per group, representative of two similar experiments. (D–G) Data are shown as means ± SEM of 17 mice per group, pooled from three independent experiments. (H) Data represent means ± SEM of four mice per group. *p-values were calculated by Student’s t-test *p < 0.05; **p < 0.01; ***p < 0.001.

could be prevented by blocking CORT signaling throughout the stress exposure period. We also show that CORT levels under basal conditions are significantly lower in male than in female mice, which is associated with exacerbated EAE symptoms. Finally, we show that stress decreases the Treg/Teff ratio, and increases the Th1-Th17/Th2 ratio, within the Teff-cell subsets. Taken together, our findings raise the possibility that while the HPA axis provides immunosuppression under basal conditions (i.e. in nonstressed females), prolonged exposure to chronic stress results in an attenuated CORT response to stimuli, which predisposes to higher susceptibility to pathogenic autoimmunity.

A comprehensive and widely accepted biological model linking stress, CORT and autoimmune diseases is currently lacking. Although numerous studies demonstrated that CORT suppresses autoimmune diseases in humans and in animal models [15, 35, 36], other studies indicate that low levels of CORT or certain stress paradigms may skew to proinflammatory conditions [14, 18, 19, 37–42]. In the present study we found that CVS exacerbated EAE in female mice despite the overall stress-induced increase in CORT levels, which was also reported previously [32, 43, 44]. The elevated urine CORT levels in females were, however, significantly lower on the fourth week of stress and reached those of nonstressed females. In addition, CORT levels failed to increase toward disease onset (9 days postimmunization) in stressed as compared with nonstressed mice. Following the disease onset (14 and 21 days postimmunization) CORT levels in stressed mice markedly increased to levels higher than those observed during stress, and remained similar to those observed in nonstressed mice throughout the course of the disease. These results suggest that the temporarily decreased functionality of the HPA axis in stressed female mice, which resulted in a delayed CORT response to MOG35-55 immunization, could at least partially account for the initial exacerbation of the disease over that induced in nonstressed mice.

An important finding in our study was that although stressed male mice demonstrated decreased weight gain and increased anxiety index similar to females, they showed significantly lower levels of urine CORT under basal, stress and EAE conditions.
Although to a less extent, blood CORT levels were also lower in male than in female mice. However, whereas primarily free CORT was observed in the urine, only a small fraction (less than 10%) of the blood CORT was free, with levels similar between male and female mice, while the rest was presumably bound to CORT-binding globulin [45]. Higher CORT levels were previously documented in female compared with male Sprague–Dawley rats [46]. Furthermore, CORT secretion has been previously shown to attenuate EAE severity, suggesting that the HPA axis suppresses autoimmune disease progression [47–49]. Taking together, it is reasonable to assume that although similar levels of free CORT were observed in male and female mice, the overall higher basal levels of CORT in nonstressed females attenuated their EAE severity. The role of free versus bound CORT in gender-related EAE susceptibility should be further investigated. Given the anti-inflammatory properties of CORT, we asked why CVS generally exacerbated EAE in female mice. We first observed that whereas CVS-induced spleen atrophy was associated with a reduced number of splenocytes and CD4+ cells, T-cell effector functions were not intrinsically impaired as a result of stress. Ex-vivo anti-CD3 stimulation of splenocytes revealed no differences in the levels of Teff cytokines between stressed and nonstressed mice, and there were also no significant differences in the secretion of monocyte-derived cytokines such as IL-1, TNF-α, IL-6, and MCP-1. Notably however, stimulation of splenocytes derived from stressed mice in the presence of MP revealed a significant reduction in its immunosuppressive effects compared to splenocytes derived from nonstressed mice. This was reflected by the increased levels of proinflammatory cytokines secreted from cells of both the innate and adaptive immune systems predisposing a bias toward Th1-Th17 polarization. In addition, when CORT signaling was blocked throughout the course of stress, EAE exacerbation was prevented. We therefore suggest that prolonged exposure to stress in C57BL/6 female mice exhibiting a highly active HPA axis consequently induces desensitization to CORT stimuli, which otherwise shifts toward Th2 polarization as observed either following CORT administration or under various stress paradigms [11,29,50,51].

Having observed the impact of CORT-resistance on the effector function of Th1 and Th17 cells, we sought to determine the effect of CVS on the Treg population, which plays a key role in the regulation of EAE. In general, our findings show that stress increases the frequency of CD4+CD25+ T cells. This has also been shown previously in humans [52] and in animal models [53]. Accordingly, some studies demonstrated that direct administration of steroid analogues (such as dexamethasone) enhances the proportion of CD4+CD25+ T cells in lymphoid organs [54]. However, our results demonstrate that within the CD4+CD25+ T cells, stress decreases the fraction of Foxp3 Treg cells. In addition, the ratio between CD4+CD25-CD127+ and CD4+CD25+CD127- T cells was significantly lower in stressed as compared with nonstressed mice. Comparing the frequencies of CD25-CD127- and CD25+CD127- T cells (within the CD4+ T cells) between stressed and nonstressed mice revealed that CD127+ effector T cells were those which increased in stressed mice, while the CD127- T-cell population did not change. Thus, our results point to a decreased Treg/Teff ratio (rather than modulation of Treg-cell frequency per se) in response to CVS, resulting from an increase in the Teff subset. Whether this transient decrease in the Treg-cell fraction promotes EAE exacerbation should be further investigated by means of their regulatory function following CVS.

To conclude, the findings hereby reported indicate that exposure to chronic variable stress impairs the communication between the neuroendocrine and immune systems, which in turn induces CORT resistance in proinflammatory T-cell lineages and thereby increased susceptibility to autoimmune diseases. Translated clinically, this suggests that patients suffering from autoimmune diseases may develop steroid resistance due to persistent CORT exposure; in the absence of careful control over steroid resistance measures, patients may thereby enter a vicious cycle where they become dependent on increasing doses of steroids.

### Materials and methods

#### Animals

Eight-week-old C57BL/6 mice were purchased from Harlan (Jerusalem, Israel) and were allowed to acclimatize to our animal facility for 7 days prior to the experimental period. All mice were housed under standard environmental conditions (12:12 light:dark cycle with light onset at 7:00 a.m.) and were allowed free access to food and water throughout the experimental period. Surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Ben-Gurion University of the Negev, Israel. To detect intracellular FoxP3 we used C57BL/6 transgenic mice expressing enhanced green florescent protein under the control of the mouse FoxP3 promoter. The mice were kindly provided by Dr. Eli Lewis.

#### Experimental design

Mice were randomly assigned into two groups: (i) a group of isolated mice exposed to CVS for 24 days as described below, (ii) and a group of nonstressed mice, kept in groups of 4–8 mice per cage and manipulated only once a week for urine collection and body weight measurement. Following the 24-day experimental period, mice in the stressed and nonstressed groups were further divided into three groups: (i) mice subjected to behavioral tests, after which they were killed for immunological analysis; (ii) mice injected with MOG35-55 emulsified in CFA to induce EAE as described below; and (iii) mice injected with the CORT antagonist mifepristone (Sigma, Israel) daily, 2 hours before exposure to the stressful conditions, throughout the stress period. Mifepristone was dissolved in 100% ethanol and diluted to 5% ethanol in corn oil to a final concentration of 3 mg/mL. A daily dose of 30 mg/kg was injected subcutaneously.
Clinical immunology

CVS paradigm

Chronic unpredictable stress paradigms typically follow a schedule of repeated exposure to several randomly assigned stressors a day. The CVS procedure was developed based on several paradigms previously validated as stress inducers in rodents. These included isolation [55]; exposure to cat urine [56]; restraint (placing the mouse in a well-ventilated 50 mL polypropylene tube, 2.8 cm in diameter and 11.5 cm in length) [57]; swimming in cold (4 °C) water [58]; illumination during the dark phase, and tilting the home cage at a 45° inclination for 24 hours [30]. Stressor types and stress durations throughout the experiment are provided in Table 1.

Behavioral paradigms

Stressed and nonstressed mice were tested to evaluate anxiety-like behaviors 24 hours after termination of the experimental protocol (i.e. on day 25) using the following behavioral tests.

Open field test

Mice were individually placed in the corner of a square arena (40 cm × 40 cm × 35 cm) with white opaque Plexiglas walls, and were allowed to freely explore for 10 min. The percent time each mouse spent in the central and peripheral zones of the arena was quantified by an EthoVision automated tracking system (Noldus Information Technology, Wageningen, The Netherlands) and an anxiety index was calculated by dividing the time spent in peripheral zones by the time spent in the central zone. The arena was cleaned with 70% ethanol and thoroughly dried between sessions.

Elevated plus maze test

Mice were individually placed in a Plus Maze apparatus elevated 40 cm above the ground. This apparatus consisted of four arms (each 35 cm long and 5 cm wide), two of which enclosed by 15 cm high walls (“closed arms”) and two without walls (“open arms”). A mouse was allowed to freely explore for 5 min, during which the total number of entries into the open and closed arms, as well as the time spent in each arm, was recorded by the experimenter. An anxiety index ranging from 0 (low anxiety) to 100 (high anxiety) was calculated based on the following formula:

Body and spleen weight measurements

Individual body weight was measured weekly throughout the experimental period. Individual spleen weight was measured following the 24-day experimental period and immediately after killing the mouse.

Determination of urine corticosterone levels

To avoid stressing mice in the nonstressed group, CORT levels were determined in urine (rather than by drawing blood) by gently massaging the urinary bladder to induce urination. Urine was collected daily at 9:00 a.m. and prior to applying the stressor. For mice in which EAE was induced, urine was also collected during the development of the disease. To determine the fraction of free CORT in urine and blood of male and female C57BL/6 mice, samples were centrifuged in centrifuge micropartition tubes (Ultrasilot Y-T cellulose membrane with a 30,000 MW cut-off) purchased from Millipore (Co. Cork, IRL). CORT levels were determined by CORT ELISA kit (Endocrine Technologies Inc, CA) according to manufacturer’s instructions.

Flow cytometry analysis of peripheral blood lymphocytes and splenocytes

For peripheral blood analysis, 50 µL of fresh blood were drawn into heparinized tubes and incubated with 100 µL of ACK lysis buffer at 37 °C for 10 min to eliminate red blood cells. For splenocyte analysis, spleens were removed, weighed and dissociated in DMEM medium containing 10% fetal calf serum, 10 µM HEPES, 1 mM sodium pyruvate, 10 mM nonessential amino acids, 1% Pen/Strep, and 50 µM β-mercaptoethanol. ACK lysis buffer was added for 1 min to eliminate red blood cells. Viable mononuclear cells were counted in a haemocytometer using trypan blue and adjusted to 5 × 10^5 cells/mL in medium containing PBS supplemented with 2% fetal bovine serum. Cell surface staining was performed was performed using anti-CD4 (FITC or PERCP), anti-CD25 (PE), and anti-CD127 (allophycocyanin) antibodies, all purchased from BioLegend (San Diego, CA). To detect intracellular FoxP3 we used anti-FoxP3 (FITC or allophycocyanin) antibodies according to manufacturer’s instructions (BioLegend) or used transgenic mice expressing enhanced green florescent protein under the control of the mouse FoxP3 promoter. Data were collected on a FACs Calibur machine and analyzed with CellQuest Pro software (BD bioscience, San Jose, CA).

Splenocyte stimulation assay

Splenocytes were cultured in anti-CD3 coated flat-bottom 96-well plates (0.5 × 10^6 cells/well) in the presence of increasing concentrations (0–1000 ng/mL) of the immunosuppressive drug MP [15]. For MOG35-55 stimulation, splenocytes were harvested from EAE mice, cultured at 0.5 × 10^6 cells/well in a U-shape 96-well plates and stimulated with 10 μg/mL MOG35-55. Culture plates were incubated at 37 °C in a 5% CO₂ atmosphere. After 48 h incubation, supernatants were harvested and stored at –80 °C until cytokine analysis.
Measurements of cytokine production

Levels of IL-2, IFN-γ, IL-4, IL-6, IL-10, IL-1, TNF-α, MCP1, and IL-17A were measured either with a multiplex ELISA kit (Quansys Biosciences, Logan, Utah) or with individual cytokine sandwich ELISA kits (Biolegend, San Diego, CA) as indicated in figure legends and according to manufacturer’s instructions. The immuno-suppressive effect of MP is presented as percent of cytokine production without MP.

EAE induction

Mice were immunized by subcutaneous injection into flanks of 100 µg MOG35-55 emulsified in CFA (Difco, Detroit, MI). Pertussis toxin (List Biological Laboratories, Campbell, CA) was injected intraperitoneally (500 ng/mouse) immediately following MOG35-55 injection and again 48 hours later. From day 9 post-immunization, mice were examined daily for clinical signs of the disease and the manifestation of the disease was graded on a 0–5 scale according to the following parameters: 0 = no clinical signs; 0.5 = loss of tail tonus; 1 = tail paralysis; 2 = partial hind-limb paralysis; 3 = hind-limb paralysis; 4 = complete paralysis; 5 = death.

Statistical analyses

All statistical analyses were performed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). All variables are expressed as mean ± SEM. p-values were calculated with Student’s t-test or ANOVA test as indicated in figure legends.

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Abbreviations: CORT: corticosterone · CVS: chronic variable stress · HPA: hypothalamic-pituitary-adrenal · MP: methylprednisolone · Teff: effector T cell

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