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Dehydroepiandrosterone replacement in patients with Addison's disease has a bimodal effect on regulatory ($CD4^+CD25^{hi}$ and $CD4^+FoxP3^+$) T cells

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Oral replacement of the near-total deficiency of dehydroepiandrosterone (DHEA) in patients with Addison's disease (adrenal insufficiency) enhances mood and well-being and reduces fatigue. We studied the immunological effects of 12 wk of oral DHEA treatment in ten patients with Addison's disease receiving their normal mineralo- and glucocorticoid hormone replacement. We found that baseline circulating regulatory T cells were reduced in Addison's disease patients compared to controls, a hitherto unrecognised defect in this disorder. Oral DHEA treatment had a bimodal effect on naturally occurring regulatory ($CD4^+CD25^{hi}FoxP3^+$) T cells and lymphocyte FoxP3 expression. Oral DHEA replacement restored normal levels of regulatory T cells and led to increased FoxP3 expression. These effects were probably responsible for a suppression of constitutive cytokine expression following DHEA withdrawal. In contrast, oral DHEA treatment led to reduced FoxP3 expression induced by TCR engagement and so augmented the cytokine response, but without a bias towards the Th1 or Th2 phenotype. NK and NKT cell numbers fell during DHEA treatment, and homeostatic lymphocyte proliferation was increased. We conclude that DHEA replacement in Addison's disease has significant immunomodulatory properties and propose that it has a greater impact on the human immune system than would be expected from its classification as a dietary supplement.

Key words: Autoimmunity · DHEA · Human · T cells

Introduction

Dehydroepiandrosterone (DHEA) and its sulphate ester (DHEAS) are adrenally derived steroids whose physiological role is poorly understood. They may serve as precursor substrates for sex steroid (testosterone and oestrogen) biosynthesis in peripheral tissues. In addition, DHEA can act as a neurosteroid, modulating neurotransmitter signalling in the central nervous system, and it antagonises glucocorticoid-induced hippocampal neurotoxicity [1]. The foetal adrenals synthesise significant quantities of DHEAS, but production declines in early infancy before rising again with childhood adrenarche to reach a peak in young adulthood, following which there is a relentless age-related decline [2]. DHEAS is the only known steroid to show such decline in both sexes, and the fall in circulating levels or the "adrenopause" has been implicated in some of the catabolic and neurodegenerative changes of ageing, including increased cardiovascular mortality [3], malignancy [4] and risk of osteoporosis [5]. Conversely, oral DHEA replacement in 'normal' elderly individuals, which restores circulating serum levels of DHEAS and its metabolite Δ4 androstenedione, to a young adult level has been

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Abbreviations: **DHEA:** dehydroepiandrosterone · **DHEAS:** dehydroepiandrosterone sulphate ester · **IGF:** insulin-like growth factor · **NRS:** normal rabbit serum · **PHA:** phytohemagglutinin

associated with improvement in well-being [6]. Other replacement studies in this population have shown variable beneficial effects on body composition with enhanced lean body mass [7], changes in circulating insulin-like growth factor (IGF)-1 [7, 8] and improved bone mineral density and markers of bone turnover [9]. DHEA therapy may have an antidepressant effect [10], but has no effect on the cognitive impairment of Alzheimer's disease [11].

Addison's disease, or primary adrenal failure due to autoimmune gland destruction, occurs in about 1 in 25 000 individuals. It is characterised by glucocorticoid and mineralocorticoid deficiency which is life threatening, requiring oral replacement. Despite optimised therapy with these steroids, patients with Addison's disease report a reduced quality of life compared to normal individuals, often complaining of persistent fatigue and reduced well-being [12]. Hypothesising that these symptoms might, at least in part, be due to the associated near-total failure of adrenal DHEA synthesis in this disorder, which is not corrected, we undertook randomised, double-blind, placebo-controlled studies in which male and female patients with Addison's disease received 50 mg of micronised DHEA orally for 3 months [13, 14]. Following DHEA treatment, we observed significantly enhanced well-being and self-esteem together with improved mood and fatigue. In a separate study, Arlt *et al.* [15] examined the effect of DHEA replacement in females with primary or secondary adrenal insufficiency and documented significant improvement in well-being, depression and anxiety, with additional effects on sexual interest and function. Most recently, we have completed a longer-term, placebo-controlled trial of DHEA replacement for 12 months in 100 patients with Addison's disease and shown lessening of fatigue, improved well-being together with enhanced lean body mass and femoral bone mineral density.

There is increasing interest in the interaction of the endocrine and immune systems. In animal studies, the effects of DHEA on immune function are generally opposed to those of corticosteroids. In rats, DHEA restores age-related reductions in LPS-stimulated TNF-α production in alveolar macrophages and splenic mitogenic responses [16]. This activation of T cells may explain the ability of DHEA to restore the suppressed splenocyte proliferation and cytokine production seen in mice following traumatic haemorrhage [17]. Life-long treatment of mice with DHEA does not prolong survival, nor does it have any effect on T cell subsets or antibody production following erythrocyte immunisation [18]. There have been fewer studies of DHEA on human immune function, but a similar anti-glucocorticoid effect has been observed. Thus, the patterns of gene expression induced in human peripheral blood mononuclear cells (PBMC) by exposure to DHEA and testosterone *in vitro* are equivalent but the opposite of those induced by corticosteroids [19]. Most work on human immune function and DHEA has centred on its effects on NK cells. In these cells, increasing DHEAS *in vitro* promotes NK cytotoxicity in a dose-dependent way, and in parallel with increased NK cell release of IGF [20]. We have evaluated the effects of DHEA treatment in patients with primary adrenal insufficiency (Addison's disease) and reasoned that such a study would have two advantages: First, studies of DHEA administration in rodent animal models are of limited value because there are no rises or falls in DHEAS levels corresponding to adrenarche or adrenopause in non-primate species. Second, correction of the near-absolute deficiency of this steroid in younger Addison's disease patients might enable an immunological effect to be discerned more readily than with DHEA supplementation in normal, ageing subjects. The primary aim of the study was to characterise the effect of oral DHEA treatment on peripheral lymphocyte phenotypes. We showed that there was a reduction in peripheral NK and NKT cell numbers and a rise in peripheral $CD4^+$ cells bearing the markers ($CD25^{hi}$ and FoxP3) associated with one type of human regulatory T cell [21]. Although this was associated with a suppression of constitutive cytokine expression, paradoxically there was an augmented response to physiological stimuli.

Results

All patients tolerated the DHEA treatment. Serum DHEA sulphate levels in the group rose from very low baseline levels (mean 0.78 μM; SD ± 0.93 μM) to within the physiological range (1.9–8.1 μM) by wk 4 (7.9 ± 3.0 μM) and 12 (8.0 ± 3.0 μM) and fell to subnormal levels (0.74 ± 0.85 μM) by wk 16.

DHEA reduces peripheral NK and NKT cell numbers

At 12 wk after DHEA treatment, median NK ($CD3^-CD56^+$) and NKT ($CD3^+CD56^+$) lymphocytes were significantly reduced by 37% and 43%, respectively (Fig. 1; $p < 0.05$). In the case of NK cells, this effect persisted at 16 wk.

DHEA increases human regulatory T cell numbers and FoxP3 mRNA expression

Human lymphocyte CD25 staining can be divided into negative, intermediate and "high". It is believed that the intermediate staining defines activated $CD4^+$ T cells and that high expression marks the human equivalent of the

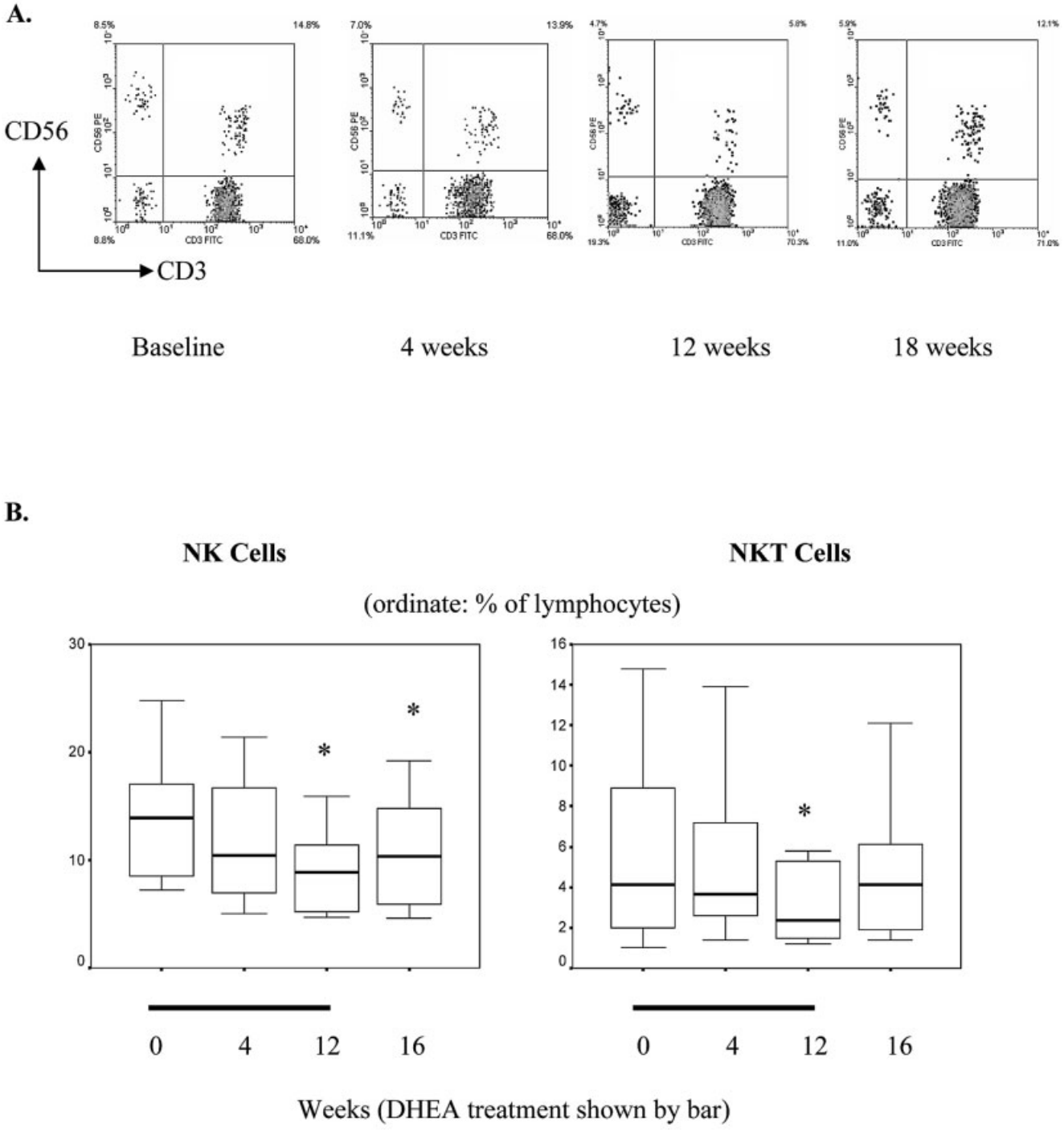

Figure 1. Peripheral NK cell numbers during oral DHEA treatment. (A) Representative FACS plots from one patient receiving oral DHEA. (B) Peripheral NK and NKT cell numbers in all patients on oral DHEA treatment, expressed as percentage of total lymphocytes (*$p < 0.05$).

murine $CD4^+CD25^+$ regulatory T cell [21]. We defined intermediate staining as any fluorescence greater than that seen with isotype controls (Fig. 2a), whereas "high" staining was any $CD25^+CD4^+$ fluorescence greater than an arbitrary level at which only 0.1% of CD4-negative lymphocytes are positive for CD25 staining. As further evidence that these were regulatory T cells, we stained for the cell surface marker CD45RO, which was positive on a mean of 94–96% of these $CD4^+CD25^{hi}$ T cells at all times during the study (data not shown). Compared to normal controls, the patients' PBMC had a greater proportion of lymphocytes with intermediate CD25 expression ($p < 0.005$) and a lesser proportion with high expression ($p = 0.37$). This suggests that Addison's disease is associated with a reduction in peripheral regulatory T cell numbers.

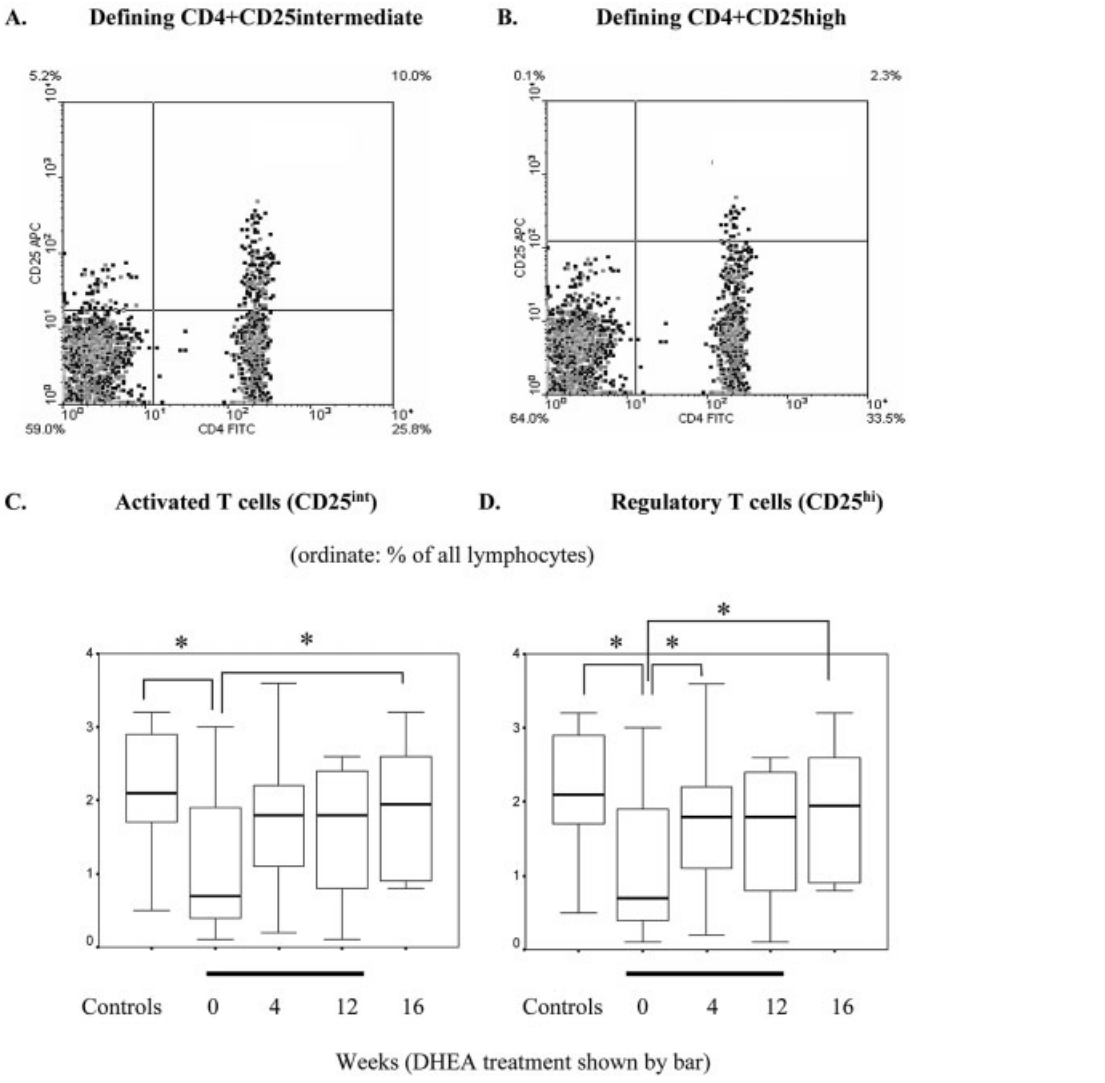

Figure 2. Activated and regulatory T cells during DHEA treatment. The thresholds for distinguishing low and intermediate CD25 staining, and intermediate and high staining, are illustrated in (A). In (B) is shown the percentage of peripheral lymphocytes expressing intermediate or high CD25 levels from patients undergoing DHEA treatment (*$p < 0.05$).

Strikingly, the median proportion of peripheral lymphocytes that were $CD4^+CD25^{hi}$ more than doubled after 4 wk of oral DHEA treatment ($p = 0.03$), with this increase persisting to 16 wk (Fig. 2b; wk 12 and wk 16 *versus* wk 0, $p = 0.241$ and $p = 0.008$, respectively). This effect restored $CD4^+CD25^{hi}$ numbers to normal control values. The numbers of $CD4^+CD25^{int}$ cells was also increased at wk 16, but only by 13% (p = 0.02), reflecting activation of some T cells.

DHEA increases homeostatic lymphocyte proliferation

Assaying cell proliferation by the sensitive technique of FACS measurement of CSFE dilution demonstrated that oral DHEA treatment significantly increased median proliferation more than tenfold in lymphocytes cultured *ex vivo* without further manipulation (Fig. 3; $p < 0.01$). DHEA has no detectable effect on proliferation induced by plate-bound anti-CD3 and soluble anti-CD28 mAb, perhaps because so many cells (>90%) are induced to proliferate by this stimulus and there is a limit to the number of generations that can be followed by CFSE. Because homeostatic lymphocyte proliferation is largely controlled by IL-7 [22], we measured serum levels of this cytokine, but there was no difference between patients and controls, nor any changes with DHEA treatment (11.4 μM for controls, 15.7 μM at baseline for patients, 13.4 μM at wk 4, 13.1 μM at wk 12 and 16.1 μM at wk 16).

DHEA increases constitutive but reduces stimulated T lymphocyte regulation

Freshly extracted unstimulated $CD4^+$ T cells from patients were lysed immediately, stored at –70°C and

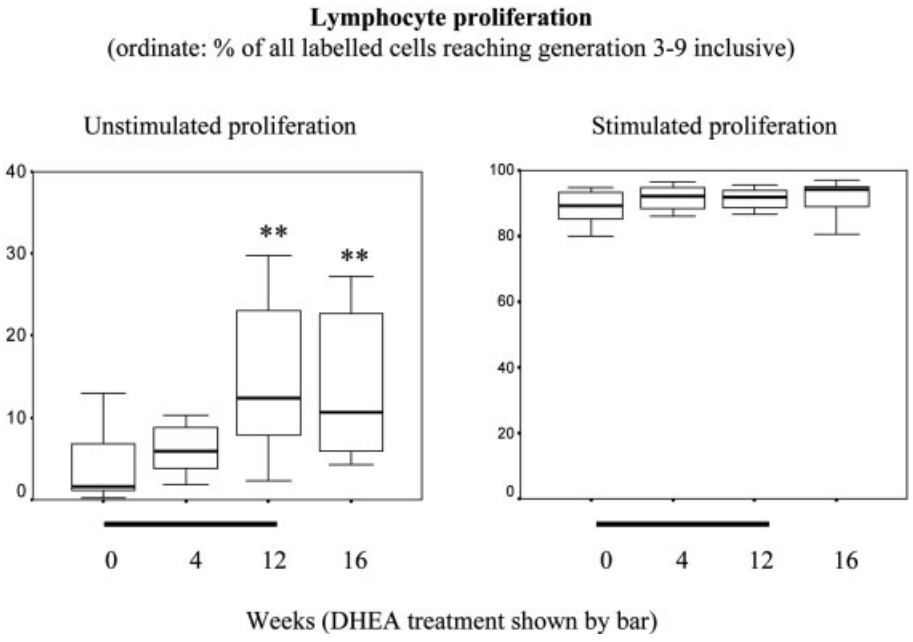

Figure 3. CFSE analysis of lymphocyte proliferation of PBMC derived from patients treated with DHEA. PBMC cultures were either stimulated through the TCR using anti-CD3/CD28 antibodies (right panel) or left unstimulated (left panel). The extent of lymphocyte proliferation is expressed as the percentage of cells reaching at least three generations of division ($^{*}p < 0.05$, $^{**}p < 0.01$).

later assayed for constitutive cytokine mRNA expression using real-time semi-quantitative RT-PCR (Fig. 4, left panels). These assays show that DHEA treatment increases constitutive $CD4^+$ lymphocyte FoxP3 expression maximally by 12 wk, after which it falls but remains significantly increased at 16 wk. This suggests that the rise in peripheral $CD4^+CD25^{hi}$ T cells on DHEA treatment (Fig. 2b) is associated with functional regulation. By the same technique, mRNA expression of the cytokines IFN-γ, IL-4 and IL-5 could be measured. (We also attempted to measure IL-10 mRNA, but consistently failed, possibly for technical reasons.) There were no significant changes in cytokine mRNA expression during DHEA treatment, but IFN-γ and IL-5 mRNA were both significantly reduced (with a similar trend in IL-4) 4 wk after DHEA withdrawal (Fig. 4). This coincides with the time when there are maximal peripheral $CD4^+CD25^{hi}$ lymphocytes and increased FoxP3 expression, compared to baseline, suggesting perhaps a period of supraphysiological immune regulation.

The pattern of cytokine mRNA expression in $CD4^+$ cells stimulated *ex vivo* was radically different (Fig. 4, right panels). Plated anti-CD3 and anti-CD28 mAb were used as "physiological" non-antigen-specific mitogens. Oral DHEA treatment caused this stimulus to reduce lymphocyte FoxP3 expression. DHEA may sensitise regulatory T cells to the established tendency of plated anti-CD3 mAb (but not soluble anti-CD3 mAb) to reduce the regulatory effect of $CD4^+CD25^{hi}$ cells [21] with anti-CD28 mAb. Unsurprisingly then, as regulation is reduced, so DHEA treatment caused an increase in stimulated cytokine mRNA expression that was significant at 12 wk for IL-5 and IL-4.

We also measured the amount of cytokine protein secreted into the supernatant of the peripheral mononuclear cell cultures, stimulated by plated anti-CD3 and anti-CD28 mAb. Because of the possibility that the proliferation of these cultures might vary with different times after DHEA treatment (see above), we corrected the data for cell number, derived from β-actin mRNA expression, and expressed the results as a percentage of baseline expression (Fig. 5). The secretion of the cytokines IFN-γ, IL-5, IL-10 and TGF-β was significantly increased after 4 wk of DHEA treatment, with a trend towards an increase at 12 wk, although this did not reach statistical significance. Interestingly, given the significantly increased production of IL-4 mRNA at wk 12, we could not detect any IL-4 in the supernatant, despite using a sensitive ELISA, suggesting either that IL-4 production had been increased but remained below the detectable limit of our assay or that there was a post-transcriptional inhibition of IL-4 production.

DHEA has no effect on lymphocyte proliferation *in vitro*

To investigate whether the changes we observed were a direct or an indirect effect of DHEA on PBMC cultures *in vitro*, we examined lymphocyte proliferation by CFSE in supernatants containing 10^{-9} M DHEA. (This concentration was chosen as it had been used previously by other investigators [23] and because at higher concentrations of DHEA, the ethanol in which DHEA has to be suspended caused lymphocyte death). At this concentration, we did not see any increased homeostatic lymphocyte proliferation and therefore conclude that this effect is mediated indirectly by oral DHEA treatment.

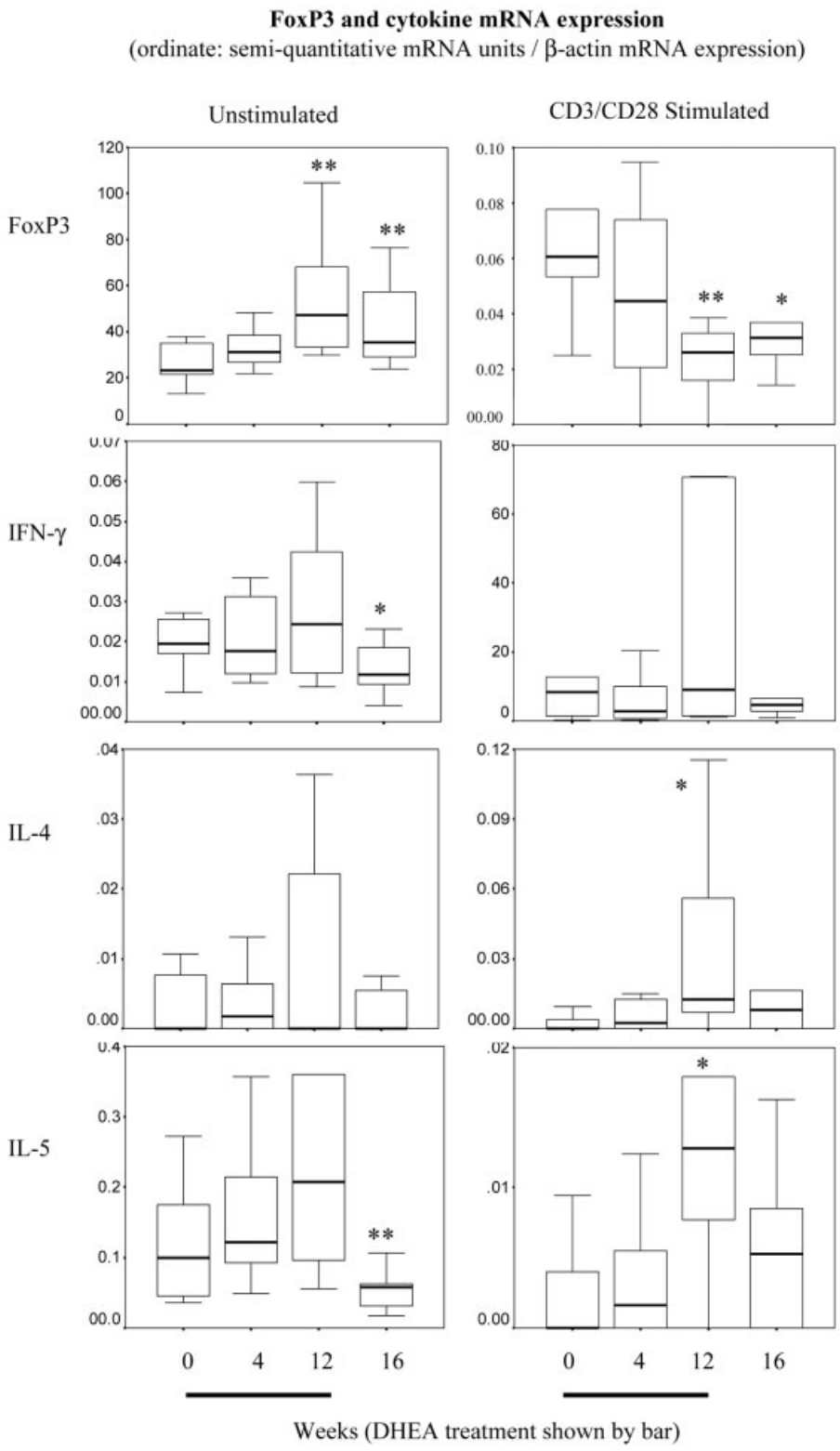

Figure 4. Cytokine and FoxP3 mRNA expression from lymphocytes derived from patients treated with DHEA. Measured by real-time PCR, corrected for cell number using β-actin mRNA expression. PBMC cultures were either stimulated through the TCR (right panels) or left unstimulated (left panels) ($^{*}p < 0.05$, $^{**}p < 0.01$).

Discussion

We have described the immunological consequences of oral DHEA treatment of ten people with DHEA insufficiency due to Addison's disease. DHEA treatment restored circulating levels of DHEA to within normal limits, which returned to baseline levels 4 wk after stopping the treatment.

The immunological effects of exogenous administration of DHEA in humans have not been studied in detail. In one trial, oral DHEA administration to nine elderly normal men caused a marginal increase in B cell and

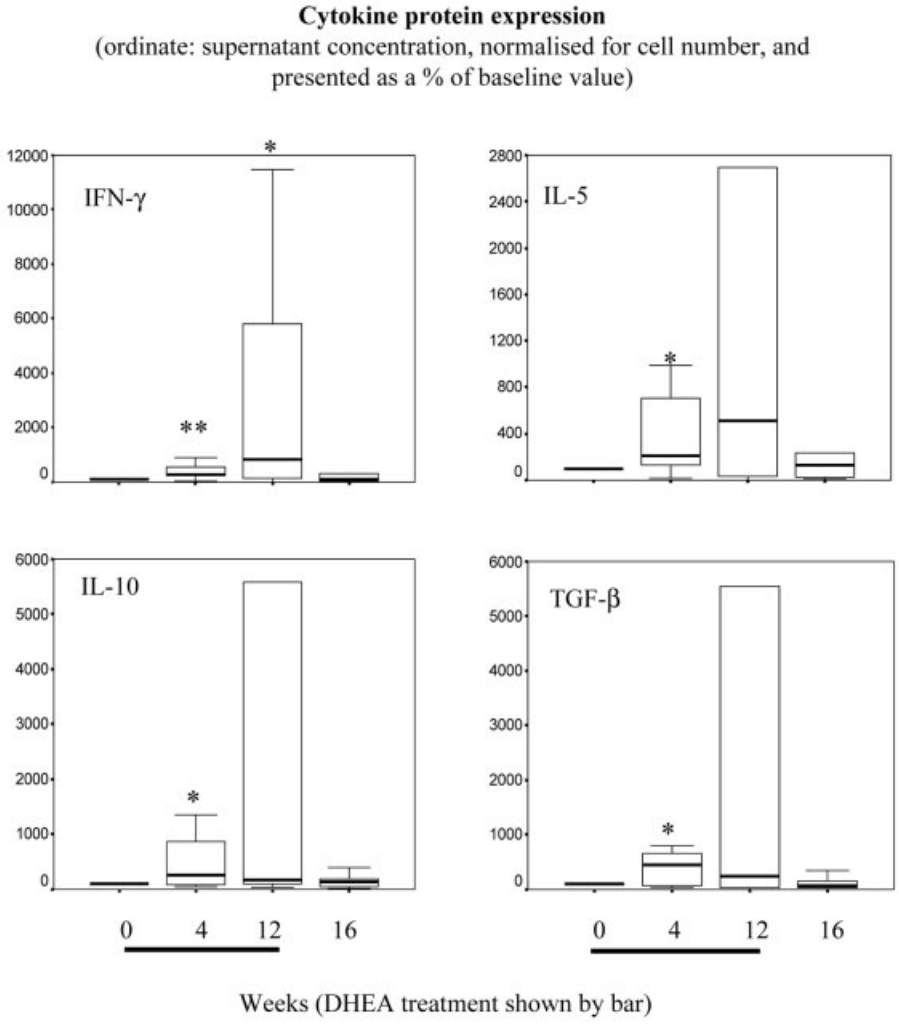

Figure 5. Stimulated PBMC cytokine secretion during DHEA treatment. Supernatant cytokine protein induced by anti-CD3/CD28 mAb stimulation of PBMC, corrected for cell number and expressed as percentage of the baseline value (see methods) (*p <0.05, **p <0.01).

monocyte numbers in the peripheral blood, increased the proliferative response of PBMC cultures to phytohemagglutinin (PHA) and pokeweed mitogen, raised the induced IL-6 secretion from PHA-stimulated cultures, increased the percentage of lymphocytes bearing the CD25 marker, and increased NK cell cytotoxicity [24]. No attempt was made in this study to define any change in lymphocyte phenotype.

Our most striking finding was that patients with Addisons's had a significantly lower number of $CD4^+CD25^{hi}$ cells and that oral DHEA treatment doubled the number of circulating lymphocytes with the $CD4^+CD25^{hi}$ phenotype, which is increasingly being used as a marker of naturally occurring human regulatory T cells [21]. This was associated with a significant rise in constitutive lymphocyte Foxp3 expression, a transcription factor found to be both sufficient and necessary for $CD4^+CD25^{hi}$ cell function [25, 26], which is deleted in a rare human disease associated with autoimmunity [27, 28]. Not many agents have been demonstrated to have this effect. Murine $CD4^+CD25^+$ T cells are increased following 1α,23-dihydroxyvitamin D (vitamin D3) alone [29] or in combination with mycophenolate mofetil [30]. The combination of vitamin D3 and dexamethasone also induces the "Tr1" form of regulatory cells, which are IL-10-producing $CD4^+$ T cells [31].

We observed a reciprocal relationship between the regulatory T cell number and FoxP3 expression and cytokine secretion. For instance, 4 wk after DHEA withdrawal, when peripheral $CD4^+CD25^{hi}$ cells remained high and constitutive FoxP3 expression was increased, constitutive lymphocyte cytokine mRNA expression was reduced below baseline; this showed that peripheral lymphocyte cytokine mRNA expression had been relatively unregulated before DHEA treatment.

We investigated whether the increased regulatory T cells would influence the response of PBMC to a physiological stimulus mediated through the TCR *ex vivo*. Somewhat paradoxically, given the effects of DHEA on constitutive lymphocyte cytokine production, oral DHEA treatment induced a fall in stimulated FoxP3 expression and an increase in both cytokine mRNA expression and protein secretion. The explanation for

this may lie in the loss of the regulatory function of $CD4^+CD25^{hi}$ cells with plate-bound anti-CD3 and anti-CD28 mAb, the stimulus we used in these experiments to provoke lymphokine release [21]. It seems that the increased cytokine protein production *ex vivo* in response to anti-CD3/CD28 mAb at wk 4 is due to post-transcriptional mechanisms, perhaps an action of DHEA on stored cytokine protein, because associated mRNA is not elevated. However, 12 wk of DHEA treatment does increase cytokine mRNA and thereby the cytokine product (Fig. 4, 5).

The augmented cytokine response to anti-CD3/CD28 mAb induced by oral DHEA is not biased towards cytokines of either Th1 or Th2 type. *In vitro* DHEA was interpreted to cause a shift in murine cells towards the Th2 phenotype by antagonising the production of IL-12 by antigen-presenting cells [32]. Somewhat conflicting with that effect, DHEA ameliorates experimental autoimmune myasthenia gravis in rats [33]. If anything, *endogenous* human DHEA levels correlate with Th1 cytokines. In a study of 31 patients with HIV infection, there was a positive association between serum DHEAS levels and the amount of IFN-γ (but not IL-4) secreted by PBMC stimulated *ex vivo* with PHA [34]. A similar result was obtained by following the changes in Th phenotype throughout the menstrual cycle: IL-4-secreting PBMC numbers correlated with oestrogen concentration, but the number of IFN-γ-secreting cells correlated with DHEAS levels [35]. It was concluded that DHEA induces a Th2 to Th1 shift. However, analysis of a broader range of cytokines in the current study of *exogenous* DHEA administration does not allow that conclusion. We conclude that DHEA induces a broad up-regulation of cytokine secretion in response to TCR signalling, without favouring the Th1 or Th2 phenotype.

Our assays of unstimulated lymphocyte proliferation by CFSE measurement demonstrated that oral DHEA treatment enhances homeostatic lymphocyte proliferation through unknown mechanisms. It has no measurable effect on stimulated lymphocyte proliferation. We established that this effect is mediated indirectly by DHEA as it is not mimicked by DHEA-containing PBMC cultures. Nor is it due to a change in the serum level of IL-7, the principal regulator of homeostatic lymphocyte proliferation [22].

We found that DHEA induced a significant fall in both the NK and NKT lymphocyte subgroups. It is established that DHEAS *in vitro* promotes NK cytotoxicity in a dose-dependent way, and in parallel with increased NK cell release of IGF [20, 36, 37], but we are not aware of a previous demonstration of a reduction in NKT cell numbers.

We conclude that oral DHEA in Addison's disease has complex immunological effects. It doubles the number of constitutive peripheral regulatory ($CD4^+CD25^{hi}$-FoxP3) T cells and increases constitutive lymphocyte FoxP3 expression, correcting a regulatory T cell fault not previously described in Addison's disease. This leads, on DHEA withdrawal, to a suppression of lymphocyte constitutive cytokine mRNA expression. Paradoxically, DHEA treatment also augments the response of lymphocytes to a physiological stimulus through the TCR *ex vivo* by reducing the induced expression of FoxP3. This response does not favour either the Th1 or the Th2 lymphocyte phenotype. In addition, NK and NKT cell numbers are reduced and there is increased homeostatic proliferation of lymphocytes. We propose that DHEA has a greater impact on the human immune system than would be expected from its classification as a dietary supplement. Such immunomodulatory properties may mediate some of its beneficial effects, but the immunological consequences of long-term DHEA supplementation need further evaluation.

Methods

Patients and treatment

Ten patients (five male, five female, mean age 41 years) with Addison's disease of mean duration of 16 years (5–34) were given 50 mg of ionised DHEA for 12 wk. All patients continued their treatment with corticosteroids (20–30 mg hydrocortisone), fludrocortisone (0.1–0.15 mg) and, in seven cases, thyroxin. Heparinised blood (40 mL) was taken from each patient at baseline (wk 0), 4 and 12 wk after taking DHEA and then at 16 wk, 4 wk after discontinuing DHEA treatment. DHEA was started at least 1 month after a vaccination or any infective illness, in order to prevent confounding effects on the immune assays. For the CD25 FACS and CFSE proliferation data, there were 11 healthy controls (six female, mean age 38 years). The study was approved by the Local Research Ethics Committee (LREC 02/260).

Measurement of serum IL-7 level

The concentration of serum IL-7 was measured using the Quantikine HS human Interleukin 7 ELISA kit (R&D Systems). The plate was read using a microplate reader (model 680; Bio-Rad).

PBMC cultures

PBMC were extracted from blood across a Ficoll gradient (Beckman-Coulter). Per well, 10^6 cells in RPMI medium supplemented with 5% serum and anti-CD28 mAb at a final concentration of 1 μg/mL were dispensed into plates that had been pre-coated with anti-CD3 mAb at a final concentration of 1 μg/mL for 2 h in coating buffer [1.59 g/L Na_2CO_3 (anhydrous) and 2.93 g/L $NaHCO_3$ in distilled water pH 9.6] and then washed with RPMI including 10% autologous patient's serum. The cells and supernatants were harvested 48 h later. At each time point, there were data from all ten patients.

Lymphocyte cell surface phenotyping

Cells (10^6) in 100 μL PBS/1% BSA/0.01% azide/1% normal rabbit serum (NRS, R-9133; Sigma)) were incubated for 1 h in the dark at room temperature with the following mouse anti-human antibodies in separate combinations of CD3/CD56 and CD4/CD45RO/CD25, at 5 μL (CD3 and CD56), 10 μL (CD4) or 20 μL (CD25, CD45RO) per 10^6 cells: anti-CD3-FITC (555332; PharMingen), anti-CD56-PE (555516; PharMingen), anti-CD4-FITC (MCA1267F; Serotec), anti-CD25-allophycocyanin (APC) (555434; PharMingen), anti-CD45RO-PE (555493; PharMingen), mouse IgG2a-PE monoclonal isotype control (555574; PharMingen) (for anti-CD45RO-PE), mouse monoclonal IgG1κ-APC isotype control (555751; PharMingen) (for anti-CD25-APC), mouse IgG1-FITC negative control (MCA928; Serotec) (for anti-CD4- and anti-CD3-FITC), mouse IgG1-RPE negative control (MCA928PE; Serotec) (for anti-CD56-PE).

Cells were then washed two times in 100 μL PBS/1% BSA/0.01% azide/1% NRS, fixed in 2% formaldehyde in PBS and studied using a Becton Dickinson FACSCalibur. FACS analysis was performed with WinMDI (freeware; http://facs.scripps.edu/software.html). Lymphocytes were gated on the basis of forward and side scatter and fluorescence measured within that gate. Results were compared to those gained from ten healthy volunteers.

Peripheral mononuclear cell cytokine protein secretion

Cytokine concentration in the supernatants of PBMC cultures was measured by ELISA using IL-10 Human Quantikine kit (D1000; R&D Systems), human IL-5 DuoSet (DY205; R&D Systems), IFN-γ DuoSet (DY285; R&D Systems), human IL-4 Duoset (DY204; R&D Systems) and human TGF-β1 DuoSet (DY240; R&D Systems). We corrected the whole supernatant concentration for varying cell numbers in the cultures by dividing the cytokine concentration by the β-actin content of the same culture measured by real-time RT-PCR. This results in arbitrary units that reflect the mean amount of cytokine secretion per PBMC. Results are expressed as a percentage of the baseline value.

Real-time semi-quantitative RT-PCR of $CD4^+$ lymphocyte cytokine mRNA

$CD4^+$ cells were purified from freshly extracted and cultured PBMC using magnetic beads (MS columns 130-042-202, CD4 MicroBeads 451-01; Milteny Biotec), giving a post-separation purity of 92%, and the cells were stored in lysis buffer at –70°C. RNA was extracted using the SV Total RNA Isolation kit (Z3100; Promega) and cDNA prepared using the ProSTAR First-Strand RT-PCR Kit with random hexamers (200420; Stratagene). Primer/probe triplets were identified using Primer Express Software (Applied Biosystems): IFN-γ (For CCAACGCAAAGCAATACATGA; Rev TTCGCTTCCCTGTTTTAGCTG; probe CATCCAAGTGATGGCTGAACTGTCGC); IL-5 (For GACGCAGTCTTGTACTATGCACTTTC; Rev AGAAGCATCCTCATGGCTCTGA; probe TTGCCAAAGGCAAACGCAGAAC); IL-4 (For CGACTGCACAGCAGTTCCA; Rev AGGTTCCTGTCGAGCCGTT; probe AGGCACAAGCAGCTGATCCGATTCC); β-actin (For CCTGGCACCCAGCACAAT; Rev GCCGATCCACACGGAGTACT; probe: TCAAGATCATTGCTCCTCCTGAGCGC); FoxP3 (For CCCACAAGCCAGGCTGAT; Rev GGCATCGGGTCCTTGTCC; probe TTTCTGTCAGTCCACTTCACCAAGCCTG).

Real-time quantification of IFN-γ, IL-5, IL-4 and β-actin mRNA expression was performed using PerkinElmer ABI Prism 7700 Sequence Detection System. Cytokine probes were labelled with FAM probes at 100 nM, and the β-actin probe was labelled with JOE (Oswel DNA Service). Primers were used at 300 nM (MWG Biotech; except IL-5 primers supplied by Oswel DNA Service) with qPCR Mastermix containing ROX (Eurogentec RT-QP2X-03). Each mRNA quantity was corrected for cell numbers by dividing by β-actin expression.

CFSE measurement of PBMC proliferation

PBMC cultures were set up for each patient. Cells (2×10^6) were placed in 500 μL RPMI without serum and 5 μL CFSE (5 μM stock). These were incubated at 37°C for 15 min. The reaction was then halted by adding ice-cold RPMI with 10% serum, centrifuging at 250 × g for 10 min. Cells (10^6) were stimulated with anti-CD3/CD28 mAb as above and another 10^6 cells were left unstimulated, in RPMI with 5% serum. After 4 days, the cells from each well were washed twice in PBS/1% BSA/0.01% azide/1% NRS (R-9133; Sigma) before fixing in 2% formaldehyde. CFSE fluorescence was read on a Becton Dickinson FACSCalibur flow cytometer. The extent of lymphocyte proliferation was determined from flow cytometric data using MODFIT LT 3.0 PC software (Verity Software House, Topsham, MA). A gate was drawn to exclude cells other than lymphocytes using a forward and side scatter plot; the CFSE content of those cells was visualised using a histogram plot. The parental population was identified using the sample that had not been exposed to antibodies against CD3 and CD28. Generations were calculated to be spaced 19.19 channels apart; the module describes a Gaussian distribution at these intervals and calculates the percentage of cells that fall within each Gaussian curve. The start of true proliferation was deemed to be at the third generation.

The effect of DHEA *in vitro*

DHEA was kindly donated by Prof. Joe Herbert (University of Cambridge, UK). DHEA (20 mg) was dissolved in ethanol and added to 10^6 PBMC in culture, prepared with CFSE as above, to achieve final concentrations of 10^{-9} M and 10^{-10} M as has been used previously [23], with ethanol-only media in similar concentrations acting as controls.

Statistics

Group data was not assumed to be normally distributed, so non-parametric techniques were used. Data are expressed graphically in box plots, with median and quartiles shown. Intergroup differences were analysed by Wilcoxon signed ranks test using SPSS software.

References

1 **Kimonides, V. G., Spillantini, M. G., Sofroniew, M. V., Fawcett, J. W. and Herbert, J.,** Dehydroepiandrosterone antagonizes the neurotoxic effects of corticosterone and translocation of stress-activated protein kinase 3 in hippocampal primary cultures. *Neuroscience* 1999. **89:** 429–436.

2 **Orentreich, N., Brind, J. L., Rizer, R. L. and Vogelman, J. H.,** Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. *J. Clin. Endocrinol. Metab.* 1984. **59:** 551–555.

3 **Barrett-Connor, E., Khaw, K. T. and Yen, S. S.,** A prospective study of dehydroepiandrosterone sulfate, mortality, and cardiovascular disease. *N. Engl. J. Med.* 1986. **315:** 1519–1524.

4 **Ebeling, P. and Koivisto, V. A.,** Physiological importance of dehydroepiandrosterone. *Lancet* 1994. **343:** 1479–1481.

5 **Sambrook, P., Birmingham, J., Champion, D., Kelly, P., Kempler, S., Freund, J. and Eisman, J.,** Postmenopausal bone loss in rheumatoid arthritis: Effect of estrogens and androgens. *J. Rheumatol.* 1992. **19:** 357–361.

6 **Morales, A. J., Nolan, J. J., Nelson, J. C. and Yen, S. S.,** Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J. Clin. Endocrinol. Metab.* 1994. **78:** 1360–1367.

7 **Morales, A. J., Haubrich, R. H., Hwang, J. Y., Asakura, H. and Yen, S. S.,** The effect of six months treatment with a 100 mg daily dose of dehydroepiandrosterone (DHEA) on circulating sex steroids, body composition and muscle strength in age-advanced men and women. *Clin. Endocrinol. (Oxf.)* 1998. **49:** 421–432.

8 **Casson, P. R., Santoro, N., Elkind-Hirsch, K., Carson, S. A., Hornsby, P. J., Abraham, G. and Buster, J. E.,** Postmenopausal dehydroepiandrosterone administration increases free insulin-like growth factor-I and decreases high-density lipoprotein: A six-month trial. *Fertil. Steril.* 1998. **70:** 107–110.

9 **Labrie, F., Diamond, P., Cusan, L., Gomez, J. L., Belanger, A. and Candas, B.,** Effect of 12-month dehydroepiandrosterone replacement therapy on bone, vagina, and endometrium in postmenopausal women. *J. Clin. Endocrinol. Metab.* 1997. **82:** 3498–3505.

10 **Wolkowitz, O. M., Reus, V. I., Keebler, A., Nelson, N., Friedland, M., Brizendine, L. and Roberts, E.,** Double-blind treatment of major depression with dehydroepiandrosterone. *Am. J. Psychiatry* 1999. **156:** 646–649.

11 **Wolkowitz, O. M., Kramer, J. H., Reus, V. I., Costa, M. M., Yaffe, K., Walton, P., Raskind, M. *et al.*,** DHEA treatment of Alzheimer's disease: A randomized, double-blind, placebo-controlled study. *Neurology* 2003. **60:** 1071–1076.

12 **Baker, J. R., Jr.,** Autoimmune endocrine disease. *JAMA* 1997. **278:** 1931–1937.

13 **Gurnell, E. M. and Chatterjee, V. K.,** Dehydroepiandrosterone replacement therapy. *Eur. J. Endocrinol.* 2001. **145:** 103–106.

14 **Hunt, P. J., Gurnell, E. M., Huppert, F. A., Richards, C., Prevost, A. T., Wass, J. A., Herbert, J. and Chatterjee, V. K.,** Improvement in mood and fatigue after dehydroepiandrosterone replacement in Addison's disease in a randomized, double blind trial. *J. Clin. Endocrinol. Metab.* 2000. **85:** 4650–4656.

15 **Arlt, W., Callies, F., van Vlijmen, J. C., Koehler, I., Reincke, M., Bidlingmaier, M., Huebler, D. *et al.*,** Dehydroepiandrosterone replacement in women with adrenal insufficiency. *N. Engl. J. Med.* 1999. **341:** 1013–1020.

16 **Corsini, E., Lucchi, L., Meroni, M., Racchi, M., Solerte, B., Fioravanti, M., Viviani, B. *et al.*,** *In vivo* dehydroepiandrosterone restores age-associated defects in the protein kinase C signal transduction pathway and related functional responses. *J. Immunol.* 2002. **168:** 1753–1758.

17 **Catania, R. A., Angele, M. K., Ayala, A., Cioffi, W. G., Bland, K. I. and Chaudry, I. H.,** Dehydroepiandrosterone restores immune function following trauma-haemorrhage by a direct effect on T lymphocytes. *Cytokine* 1999. **11:** 443–450.

18 **Miller, R. A. and Chrisp, C.,** Lifelong treatment with oral DHEA sulfate does not preserve immune function, prevent disease, or improve survival in genetically heterogeneous mice. *J. Am. Geriatr. Soc.* 1999. **47:** 960–966.

19 **Maurer, M., Trajanoski, Z., Frey, G., Hiroi, N., Galon, J., Willenberg, H. S., Gold, P. W. *et al.*,** Differential gene expression profile of glucocorticoids, testosterone, and dehydroepiandrosterone in human cells. *Horm. Metab. Res.* 2001. **33:** 691–695.

20 **Solerte, S. B., Gornati, R., Cravello, L., Albertelli, N., Oberti, S., Perotta, D., Rossi, G. *et al.*,** Dehydroepiandrosterone-sulfate (DHEA-S) restores the release of IGF-I from natural killer (NK) immune cells in old patients with dementia of Alzheimer's type (DAT). *J. Endocrinol. Invest.* 1999. **22:** 32–34.

21 **Baecher-Allan, C., Brown, J. A., Freeman, G. J. and Hafler, D. A.,** $CD4^+CD25^{high}$ regulatory cells in human peripheral blood. *J. Immunol.* 2001. **167:** 1245–1253.

22 **Tan, J. T., Dudl, E., LeRoy, E., Murray, R., Sprent, J., Weinberg, K. I. and Surh, C. D.,** IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. USA* 2001. **98:** 8732–8737.

23 **Suzuki, T., Suzuki, N., Engleman, E. G., Mizushima, Y. and Sakane, T.,** Low serum levels of dehydroepiandrosterone may cause deficient IL-2 production by lymphocytes in patients with systemic lupus erythematosus (SLE). *Clin. Exp. Immunol.* 1995. **99:** 251–255.

24 **Khorram, O., Vu, L. and Yen, S. S.,** Activation of immune function by dehydroepiandrosterone (DHEA) in age-advanced men. *J. Gerontol. A Biol. Sci. Med. Sci.* 1997. **52:** M1–M7.

25 **Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y.,** Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* 2003. **4:** 330–336.

26 **Hori, S., Nomura, T. and Sakaguchi, S.,** Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003. **299:** 1057–1061.

27 **Bennett, C. L., Brunkow, M. E., Ramsdell, F., O'Briant, K. C., Zhu, Q., Fuleihan, R. L., Shigeoka, A. O., Ochs, H. D. and Chance, P. F.,** A rare polyadenylation signal mutation of the FOXP3 gene (AAUAAA–>AAUGAA) leads to the IPEX syndrome. *Immunogenetics* 2001. **53:** 435–439.

28 **Gambineri, E., Torgerson, T. R. and Ochs, H. D.,** Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr. Opin. Rheumatol.* 2003. **15:** 430–435.

29 **Gregori, S., Giarratana, N., Smiroldo, S., Uskokovic, M. and Adorini, L.,** A 1alpha,25-dihydroxyvitamin D(3) analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice. *Diabetes* 2002. **51:** 1367–1374.

30 **Gregori, S., Casorati, M., Amuchastegui, S., Smiroldo, S., Davalli, A. M. and Adorini, L.,** Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J. Immunol.* 2001. **167:** 1945–1953.

31 **Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., Waal-Malefyt, R. *et al.*,** *In vitro* generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 2002. **195:** 603–616.

32 **Du, C., Guan, Q., Khalil, M. W. and Sriram, S.,** Stimulation of Th2 response by high doses of dehydroepiandrosterone in KLH-primed splenocytes. *Exp. Biol. Med. (Maywood.)* 2001. **226:** 1051–1060.

33 **Duan, R. S., Link, H. and Xiao, B. G.,** Dehydroepiandrosterone therapy ameliorates experimental autoimmune myasthenia gravis in Lewis rats. *J. Clin. Immunol.* 2003. **23:** 100–106.

34 **Schifitto, G., McDermott, M. P., Evans, T., Fitzgerald, T., Schwimmer, J., Demeter, L. and Kieburtz, K.,** Autonomic performance and dehydroepiandrosterone sulfate levels in HIV-1-infected individuals: Relationship to Th1 and Th2 cytokine profile. *Arch. Neurol.* 2000. **57:** 1027–1032.

35 **Verthelyi, D. and Klinman, D. M.,** Sex hormone levels correlate with the activity of cytokine-secreting cells *in vivo*. *Immunology* 2000. **100:** 384–390.

36 **Solerte, S. B., Fioravanti, M., Vignati, G., Giustina, A., Cravello, L. and Ferrari, E.,** Dehydroepiandrosterone sulfate enhances natural killer cell cytotoxicity in humans *via* locally generated immunoreactive insulin-like growth factor I. *J. Clin. Endocrinol. Metab.* 1999. **84:** 3260–3267.

37 **Solerte, S. B., Fioravanti, M., Schifino, N., Cuzzoni, G., Fontana, I., Vignati, G., Govoni, S. and Ferrari, E.,** Dehydroepiandrosterone sulfate decreases the interleukin-2-mediated overactivity of the natural killer cell compartment in senile dementia of the Alzheimer type. *Dement. Geriatr. Cogn. Disord.* 1999. **10:** 21–27.

