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CLINICAL STUDY

Defect of a subpopulation of natural killer immune cells in Graves' disease and Hashimoto's thyroiditis: normalizing effect of dehydroepiandrosterone sulfate

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Abstract

Background: The study of the natural killer (NK) immune compartment could provide important findings to help in the understanding of some of the pathogenetic mechanisms related to autoimmune thyroid diseases (Graves' disease (GD) and Hashimoto's thyroiditis (HT)). Within this context, it was suggested that alterations in NK cell cytotoxicity (NKCC) and NK production of cytokines might occur in subjects with GD and HT, whereas the normalization of NK functions could potentially contribute to the prevention of the onset or the progression of both diseases.

Objective: Due to the hypothesis of alterations in NK in autoimmune thyroid diseases, we were interested to evaluate NKCC in GD and HT patients and to modulate NK function and secretory activity with cytokines and dehydroepiandrosterone sulfate (DHEAS) in an attempt to normalize NK cell defect.

Design: We studied 13 patients with recent onset Graves' disease, 11 patients with Hashimoto's thyroiditis at first diagnosis and 15 age-matched healthy subjects.

Methods: NK cells were concentrated at a density of 7.75×10^6 cells/ml by negative immunomagnetic cell separation and validated by FACScan as CD16 + /CD56 + cells. NK cells were incubated with interleukin-2 (IL-2) and interferon-β (IFN-β) and co-incubated with DHEAS at different molar concentrations for measuring NKCC and the secretory pattern of tumor necrosis factor-α (TNF-α) from NK cells.

Results: Lower spontaneous, IL-2- and IFN-β-modulated NKCC was demonstrated in GD and HT patients compared with healthy subjects ($P < 0.001$). A decrease in spontaneous and IL-2-modulated TNF-α release from NK cells was also found in both groups of patients ($P < 0.001$). The co-incubation of NK cells with IL-2/IFN-β + DHEAS at different molar concentrations (from 10^{-8} to 10^{-5} M/ml/NK cells) promptly normalized NKCC and TNF-α secretion in GD and HT patients.

Conclusions: A functional defect of a subpopulation of NK immune cells, involving both NKCC and the secretory activity, was demonstrated in newly-diagnosed GD and HT patients. This defect can be reversed by a dose-dependent treatment with DHEAS. The impairment of NK cell activity in autoimmune thyroid diseases could potentially determine a critical expansion of T/B-cell immune compartments leading to the generation of autoantibodies and to the pathogenesis of thyroid autoimmunity.

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Introduction

Natural killer (NK) cells are the effectors of the innate immune response and show morphological features similar to the large granular lymphocytes which constitute about 5% of the peripheral blood lymphocytes (PBL). NK cells express CD16 (Fcq RIII) and CD56 (NKH-1 isoform of NCAM) surface antigens, but not T-cell receptor of CD3 complexes. NK cell activity mainly results in cytolysis of tumor, virus-infected and microbial cells, without a prior sensitization with target cell antigen (NK cell cytotoxicity: NKCC). Moreover, they function as killer cells that mediate antibody-dependent cellular cytotoxicity (1–3), in particular during cytokine modulation (4–7).

NK cells are a subset of mononuclear cells which have been suggested to play an immunoregulatory role in the prevention of autoimmune disease (8). Decreased NK activity, due to antilymphocyte antibodies (ALA) in patients' sera, has been found in lupus

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erythematosus (9), Sjogren's syndrome and rheumatoid arthritis (10), whereas in other diseases such as multiple sclerosis (11) and Crohn's disease (12), a role for viral antigen was suspected to cause disturbances in NK cell activity.

In autoimmune thyroid diseases such as Graves' disease (GD) and Hashimoto's thyroiditis (HT), antibody- and T cell-mediated death mechanisms and cytokine-regulated apoptotic pathways were proposed as the responsible agents for thyrocyte depletion (13–16); moreover, several abnormalities of killer cell activity have been described (17–19), while ALA, which constitute anti-asialo ganglioside membrane 1 (GMI) antibodies, a marker for NK cells, have been detected in sera of patients with GD and HT (19, 20). Moreover, the measurement of NK activity in PBL from GD patients by cytolytic assay or phenotypic analysis has produced widely different results, with reports of the activity being enhanced (21), normal (22, 23) or decreased (24–27). The reduced effector activity in PBL from hyperthyroid patients would seem to be due to a functional defect rather than to a decreased NK cell count, and the incubation of PBL of GD patients with recombinant human interleukin-2 (rhIL-2) promptly reverses the NK cell defect (27). On the other hand, increased activity was found in hyperthyroid Graves' and HT patients (28), whereas NK cell activity was found to be reduced in GD and HT patients (29). All these findings suggest that in GD patients, as in other autoimmune diseases (30), there is a functional defect involving NK maturation and/or functional activation.

The mechanism by which NK cells could influence autoimmunity is still controversial (31). However, it remains possible that autoimmune diseases could be dependent on chronic viral infections due to the decreased NKCC against virus-infected cells or to NK modulation of autoimmune responses by the regulation of B/T cells survival and/or expansion. In effect, NK cells produce some Th2-associated cytokines, such as interleukin (IL)-5 and IL-13, that may enhance B cell activity and indirectly suppress Th1 autoimmune cell-mediated responses (8).

Because of the role of NK cells in the onset and progression of autoimmunity, a great deal of attention has been focused on the objective to enhance NK cell function in order to normalize NK defects. Interferon (IFN)-α has been studied in myasthenia, and during IFN-α treatment the CD4 T lymphocytes count and the CD4/CD8 ratio increased, while NK cells underwent maturation also restoring NK cytotoxic function (32). Moreover, dehydroepiandrosterone sulfate (DHEAS) has been proposed for the treatment of systemic lupus erythematosus (33). It is well known that DHEAS exerts multiple immune functions (34, 35) also enhancing NKCC via local production of the immunoregulatory peptide insulin-like growth factor I (36, 37).

Within this context, the aim of the present study was to evaluate the functional alterations of spontaneous and IL-2-/IFN-β-mediated NKCC and of TNF-α release from circulating NK cells in subjects with GD and HT. TNF-α secretion was chosen as one of the main inflammatory markers of cytokine production by NK cells. Since a role for DHEAS could be expected in the modulation of NKCC in subjects with thyroid autoimmunity, the co-incubation of NK cells with IL-2 + DHEAS and with IFN-β + DHEAS was performed in order to normalize NKCC and NK production of cytokines.

Materials and methods

Patients with GD and HT and healthy subjects

The study concerned 13 subjects with newly diagnosed Graves' disease (GD), 11 subjects with newly diagnosed Hashimoto's thyroiditis (HT) and 15 matched healthy subjects. Diagnosis of GD was based on standard clinical (subjective and objective) criteria, high thyroid hormone levels (free-thyroxine (T4) >48 pmol/l in all subjects), suppressed basal thyrotropin (TSH) levels (<0.08 U/l in all subjects), positive anti-TSH-receptor antibodies (>22 U/l in all subjects) and a diffuse uptake on ^{99m}Tc pertechnetate scintigraphy; four GD subjects had ophthalmopathy. HT subjects were diagnosed on the basis of clinical symptoms, low thyroid hormone levels (free-T4 <6 pmol/l in all subjects), high basal TSH levels (>25 U/l in all subjects), on the presence of anti-thyroid peroxidase antibodies (>1600 U/l in all subjects) and of anti-thyroglobulin autoantibodies (>850 U/l in all subjects), and on the basis of thyroid ultrasound that revealed a diffuse reduction of echogenicity compatible with thyroiditis. Therefore, all HT subjects were hypothyroid during the recruitment period. Furthermore, as partial support for the clinical diagnosis of HT, all these subjects presented with lymphocytic thyroid infiltration established by fine needle aspiration.

All patients gave their informed consent to the study, in accordance with the Helsinki Declaration, and were investigated before any treatment. Clinical, biochemical and immunological characteristics of healthy GD and HT subjects are summarized in Table 1. Serum DHEAS was also determined in all subjects with a specific RIA (Coat-A-Count DHEA-SO_4, DPC, Los Angeles, CA, USA) (Table 1).

GD patients were studied at the time of diagnosis and after 4 and 8 weeks of treatment with the antithyroid drug methimazole (MTZ). The treatment was started with a dose of 15 mg MTZ per day for 1 week (5 mg before every meal: 0700 h, 1300 h, 2000 h) and was continued with 30 mg MTZ for 3 weeks (10 mg every meal), finally returning to 15 mg/day for the last period of 4 weeks. All the patients were euthyroid within 4 weeks of treatment. HT patients were studied at the time of diagnosis and after 4 and 8 weeks of

Table 1 Clinical, biochemical and immunological parameters of healthy subjects and GD and HT patients. Results are means±S.D.

	Healthy subjects	GD patients	HT patients	ANOVA F test
Number of subjects/patients	15	13	11	—
Women:men	9:6	10:3	7:4	—
Age (years)	38±5.3	37±9.9	41±7.4	NS
Albumin (g/l)	43.5±3	42.8±3.1	43.1±2.8	NS
Pre-albumin (g/l)	0.33±0.03	0.31±0.05	0.32±0.04	NS
Transferrin (g/l)	3.17±0.3	3.15±0.6	3.12±0.3	NS
Lymphocytes (cells/mm^3)	1997±60	1965±71	1973±77	NS
CD16 + (%)	10.1±5.2	9.8±5.6	9.6±6.1	NS
CD56 + (%)	5.3±1.6	5.7±1.8	5.8±1.7	NS
DHEAS (μg/ml)	6.3±0.8	5.3±0.5	5.1±0.6	$P < 0.01$

NS, not significant.

replacement therapy with L-thyroxine (75–100 μg/daily) and all the patients were euthyroid within 4 weeks of treatment. The study of NK cell cytotoxicity (NKCC) and of TNF-α secretion by NK cells was conducted before and after 4 and 8 weeks of treatment with MTZ and L-thyroxine.

Immunological procedure for NK separation and modulation

Complete medium containing RPMI 1640 medium (HyClone Laboratories Inc., Logan, UT, USA) enriched with 10% inactivated fetal bovine serum (FBS), 1% glutamine (HyClone Laboratories Inc.) and 50 μg/ml/gentamycin (Irvine Scientific, Santa Ana, CA, USA) was used for all cultures and cytotoxicity assays. Inactivation of FBS was performed by treatment with dithiothreitol, a procedure that eliminates all detectable growth factors (38). The human myeloid cell line, K562, was the source of sensitive targets for measurements of NK cytotoxicity (39, 40). The cell line K562 was maintained in our laboratory in suspension culture flasks at 37 °C in a 5% CO_2 incubator (Heraeus BB 6220, Hanau, Germany). All target cells used were >90% viable, as measured by Trypan Blue dye exclusion (Trypan Blue solution 0.4%, Sigma Chimica, Milano, Italy). Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood samples in all subjects fasting for 12 h before venipuncture. PBMC cells were immediately separated by Ficoll-Hypaque density centrifugation (41) (Lympholyte-H, Cedarlane Laboratories Limited, Hornby, Ontario, Canada). Plastic-adherent cells were removed by incubation at 37 °C in petri-cultured dishes for 1 h. The remaining non-adherent cell population was passed through nylon wool columns preincubated for 1 h with RPMI 1640 supplemented with 10% heat-inactivated autologous serum (RPMI/AS) at 37 °C (5% CO_2 in air). T/NK cells were obtained by rinsing the columns with tissue culture medium which leaves B cells and remaining monocytes attached to the nylon wool (42). The enriched fraction of PBMC, containing T/NK cells, was used for the separations in the magnetic field. For the immunomagnetic separation we used the magnetic cell separation (MACS) system and the NK cells isolation kit for the negative enrichment (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Washed PBMC were resuspended in 80 μl buffer (per 10^7 total cells) containing PBS and supplemented with 0.5% BSA. PBMC were incubated for 15 min at 6 °C with 20 μl reagent consisting of modified CD3, CD4, CD19, CD33 antibodies of mouse IgG1 isotype to label non NK cells. Thereafter the cells were washed once in PBS and incubated for 15 min at 6 °C with 20 μl colloidal superparamagnetic MACS microbeads recognizing non NK cells. Labeled and unlabeled cells were separated in a high gradient magnetic field, generated in a steelwool matrix inserted into the field of a permanent magnet (43, 44). The negative unlabeled cells, representing the enriched non-magnetic NK cell fraction, were eluted from the separation column outside the magnetic field in a laminar flow to ensure appropriate asepsis. The efficiency of separation was evaluated by flow cytometry, using a FACScan (Becton Dickinson, Mountain View, CA, USA). The sample obtained from the negative fraction was stained with FITC-conjugated NK cells antibodies (CD56 +, CD16 +) and counted for total NK cell number. Anti-leu 11b (anti-CD16) and anti-leu 19 (anti-CD56) were purchased from Becton Dickinson. The procedure allowed us to separate the negative NK cell population within 2 h with yields >95% and a purity of 97±1% for CD16 +, CD56 + NK cells. The viability of the NK subpopulation was determined by Trypan Blue uptake before the cytotoxicity assay against K562 cells and was >95% viable in all subjects.

After the magnetic separation, NK cells were washed three times (with 0.9% saline and complete RPMI medium), and finally resuspended to a measured density of 7.75×10^6 cells/ml of complete medium. NK effector cells were incubated for 20 h (45, 46) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, with IL-2, IFN-β and IL-2 and IFN-β co-incubated with DHEAS in order to determine modulated NKCC; NK cells were also incubated without modulators

(using 100 μl of the vehicle RPMI) for measurement of spontaneous NKCC. IL-2 (recombinant human IL-2; Proleukin, Chiron Corporation, Emeryville, CA, USA) was employed at final concentrations of 50 and 100 IU/ml/(7.75×10^6) NK cells. IFN-β (recombinant human IFN-β; Betantrone, Italfarmaco S.p.A., Milano, Italy) was used at final concentrations of 325 and 650 IU/ml/(7.75×10^6) NK cells. DHEAS (Sigma Chimica, Italy) was diluted in complete fresh medium (in a 0.1 ml final volume) and used at final molar concentrations of 10^{-7}, 10^{-6} and 10^{-5} M/ml/(7.75×10^6) NK cells (36). DHEAS was also co-incubated with IL-2 at 100 IU/ml/(7.75×10^6) NK cells and with IFN-β at 650 IU/ml/(7.75×10^6) NK cells. After incubation, the NK cells were washed twice with 0.9% saline and then once with complete medium containing modified medium 199 and 5% fraction V bovine albumin (Sigma Chemical Co., St Louis, MO, USA).

Procedure of NKCC evaluation

After washing three times with 0.9% saline and complete medium (medium 199 + 5% albumin fraction V), 3×10^4 target cells in 0.1 ml complete medium were mixed in triplicate with various concentrations of NK effector cells in the wells of a round-bottomed 96-hole standard microtiter plate (TPP, Celbio, Pero-Milano, Italy), at a final total volume of 0.2 ml. These mixtures gave final effector:target ratios (E:T) of 25:1, 12.5:1, 6.25:1, 3.125:1. After a second incubation for 4 h at 37 °C in a 5% CO_2 atmosphere, the microtiter plate was centrifuged and a fixed aliquot (0.1 ml) of supernatant was extracted from each well and transferred to the corresponding wells of a flat-bottomed microtiter plate. The cytotoxicity assay of NK cells was based on the kinetic measurement, by a computer-assisted (Milenia Kinetic Analyzer DPC, Los Angeles, CA, USA) microtiter plate reader, of the amount of lactate dehydrogenase (LDH) released in the supernatant of target cells, according to the calculation of Korzeniewski and Callewaert (47). Subsequently, 0.1 ml lactic acid dehydrogenase substrate mixture (48) was added to each well with intervals of 3 s. Data on NK activity of effector cells incubated with modifiers were expressed as lytic units (LU)/10^7 cells (48) and as a percentage of increase and decrease of specific lysis. The reproducibility of the cytotoxicity assay was evaluated on triplicate measurements and was $< 2\%$.

Procedure for TNF-α evaluation

A 500 μl volume of the supernatants of cultured NK cells was centrifuged at 4 °C and 300 μl were rapidly frozen at −80 °C until assay for the cytokine TNF-α. Hence, the fluids were resuspended at 4 °C and analyzed for the TNF-α concentration by using a high sensitive colorimetric sandwich ELISA (Quantikine Human TNF-α, R&D Systems Inc., Minneapolis, MN, USA). The sensitivity of the method was 0.5 pg/ml and the intra- and interassay precisions were respectively 5% and 8%. TNF-α concentrations in the supernates of NK cells were measured in spontaneous conditions and after modulation with lipopolysaccharide (LPS) (1 μg/ml), IL-2 (50 and 100 U/ml), DHEAS (10^{-7}, 10^{-6} and 10^{-5} M/ml) and IL-2 (100 U/ml) co-incubated with DHEAS (10^{-7}, 10^{-6} and 10^{-5} M/ml).

Statistical analysis

One-way analysis of variance (ANOVA F-test) was employed to measure differences concerning clinical, metabolic, hematologic and nutritional parameters among healthy subjects and GD and HT subjects. Non-parametric Wilcoxon signed-rank and sum-rank test were employed to evaluate differences in NKCC and TNF-α release evaluated in the different experimental conditions. Correlations were performed using the parametric Pearson's regression test. A P value of less than 0.05 was considered significant. All analyses were run with the SPSS/PC + V 3.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Table 1 summarizes the clinical, biochemical and immunological characteristics and serum DHEAS concentrations of healthy subjects and GD and HT patients. No differences concerning the total number of lymphocytes and the percentage of NK cells expressing CD16 and CD56 monoclonal antibodies were demonstrated among the groups. Furthermore, nutritional parameters were found to be similar in all the subjects examined. On the other hand, a significant reduction ($P < 0.01$) in serum DHEAS levels was found in GD and HT subjects compared with the healthy group.

Figure 1 shows the mean changes (±S.D.) of spontaneous and IL-2-/IFN-β-modulated NKCC in healthy subjects and in patients with GD and HT. The results are expressed as lytic units and as percentage increase of NKCC from basal conditions. A significant reduction in both spontaneous and IL-2-/IFN-β-stimulated NKCC was found in GD and HT patients compared with healthy subjects. Figure 2 shows the mean variations (±S.D.) in spontaneous and modulated (with LPS and IL-2) TNF-α secretion by NK cells in healthy subjects and in patients with GD and HT. A significant reduction in TNF-α production was demonstrated after exposure with LPS and IL-2 in GD and HT patients. Figure 3 shows the mean changes (±S.D.) of NKCC and TNF-α secretion during modulation with DHEAS in healthy subjects and in patients with GD and HT. A dose-dependent increase in both NKCC and TNF-α release was found during modulation

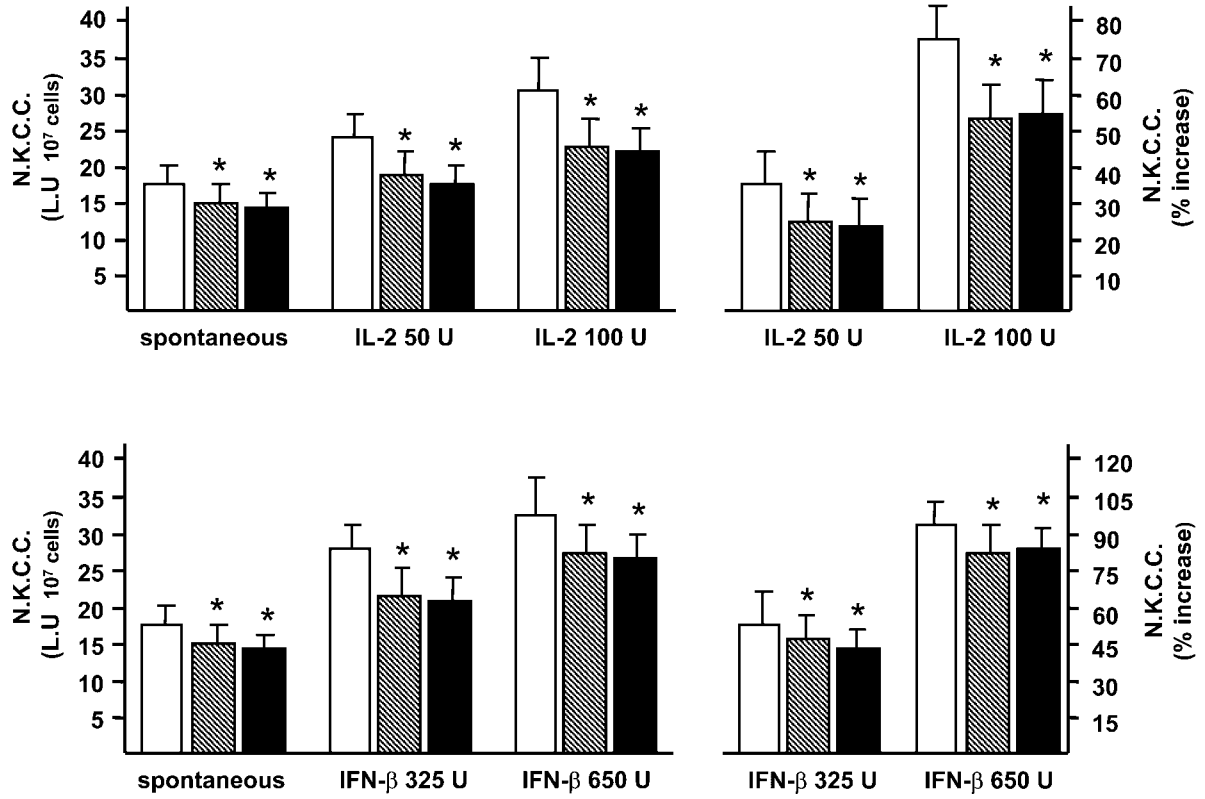

Figure 1 Mean variation in natural killer cell cytotoxicity (NKCC), expressed as lytic units (LU) and as percentage increase of NKCC from baseline, after incubation with IL-2 (50 and 100 IU/ml) and IFN-β (325 and 650 IU/ml). Data (mean±S.D.) for healthy subjects (open bars), GD patients (dashed bars) and HT patients (solid bars) are shown. $*P < 0.001$ compared with healthy subjects.

with DHEAS in all groups. Figure 4 shows the mean variations (±S.D.) in NKCC after co-incubation of NK cells with cytokines (IL-2, 100 U/ml and IFN-β, 650 U/ml) and DHEAS (from 10^{-7}M to 10^{-5}M) in healthy subjects and in patients with GD and HT. A dose-dependent increase and normalization of NKCC was found in GD and HT groups during DHEAS co-incubation with IL-2 and IFN-β. Table 2 summarizes the data concerning NKCC and TNF-α secretion by NK cells and serum DHEAS levels following IL-2 and IFN-β after 4 and 8 weeks of treatment with methimazole (GD subjects) or with replacement therapy with L-thyroxine (HT subjects). No changes in any of the immunological parameters were found after 4 weeks of treatment in either the GD and HT groups; a slight but significant improvement in NKCC and TNF-α release by NK cells was demonstrated after 8 weeks of treatment in the GD group. No changes in serum DHEAS concentration were found during either treatment.

Finally, no correlations among thyroid hormones and any of the immunological parameters (NKCC and TNF-α release by NK cells) were found in GD and HT subjects at diagnosis (data not reported).

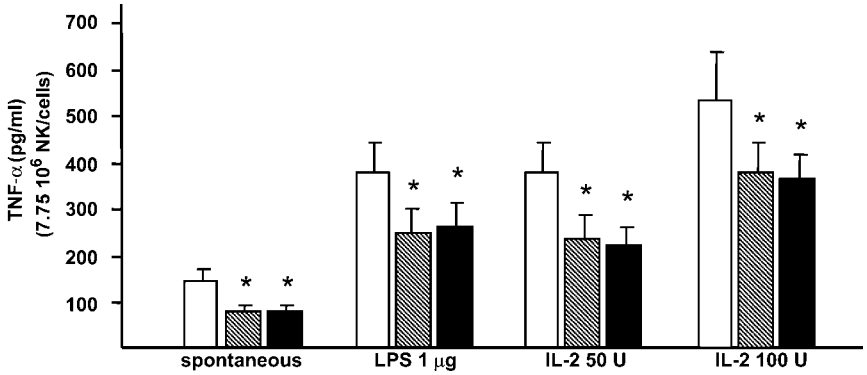

Figure 2 Mean variations in TNF-α release from NK cells, expressed as pg/ml × 7.75 × 10^6 NK cells, after incubation with LPS (1 μg/ml) and IL-2 (50 and 100 IU/ml). Data (mean±S.D.) for healthy subjects (open bars), GD patients (dashed bars) and HT patients (solid bars) are shown. $*P < 0.001$ compared with healthy subjects.

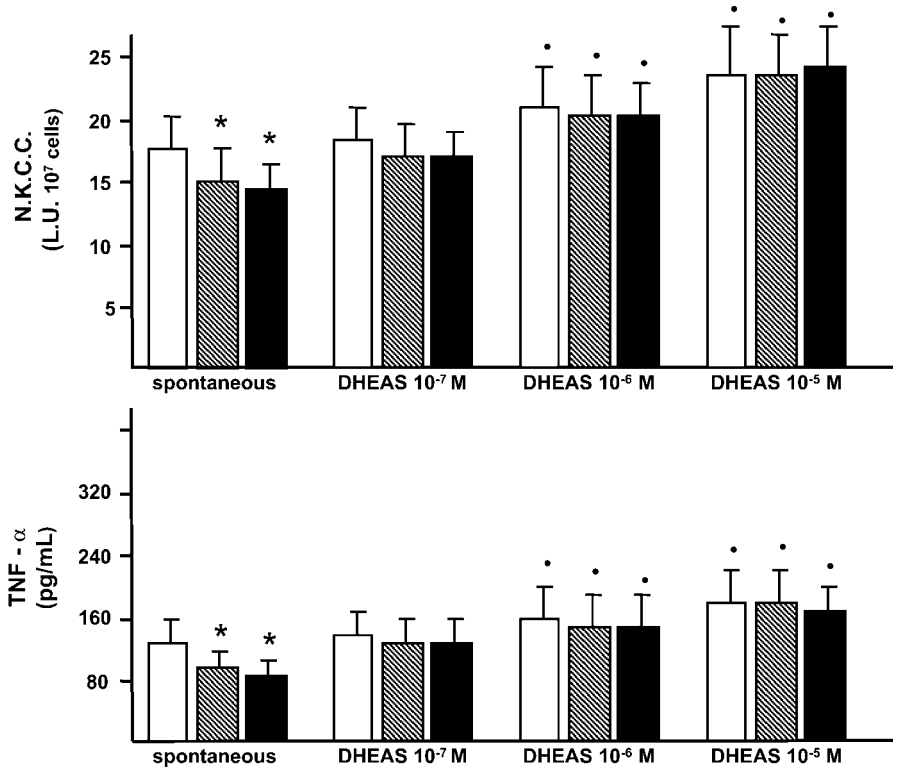

Figure 3 Mean variations in NKCC and TNF-α release from NK cells, after incubation with different molar concentrations of DHEAS. Data (mean±S.D.) for healthy subjects (open bars), GD patients (dashed bars) and HT patients (solid bars) are shown. $*P < 0.001$ compared with healthy subjects; $\bullet P < 0.001$ compared with spontaneous conditions and to 10^{-7} M DHEAS.

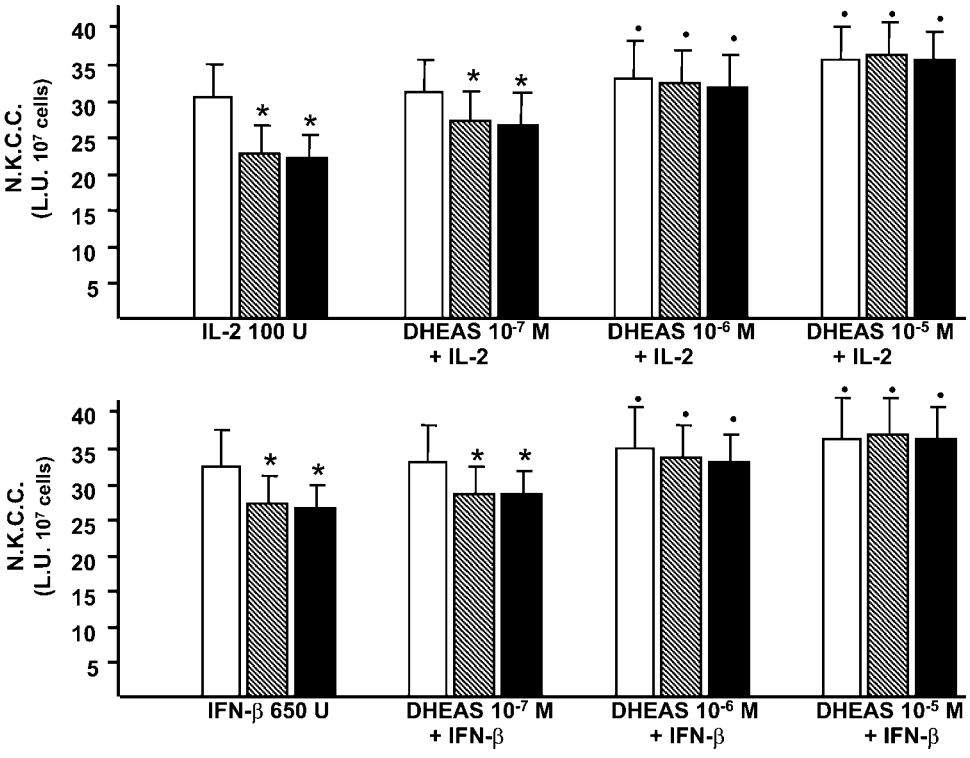

Figure 4 Mean variations in NKCC, after co-incubation of IL-2 or IFN-β with different molar concentrations of DHEAS. Data (mean±S.D.) for healthy subjects (open bars), GD patients (dashed bars) and HT patients (solid bars) are shown. $*P < 0.001$ compared with healthy subjects; $\bullet P < 0.001$ compared with spontaneous conditions and to 10^{-7} M DHEAS.

Table 2 Immunological parameters evaluated during treatment with methimazole (GD subjects) and L-thyroxine (HT subjects). Data are presented as means±S.D.

	GD patients			HT patients		
	Baseline	4 weeks	8 weeks	Baseline	4 weeks	8 weeks
Number of patients	15	15	15	13	13	13
NKCC (LU) after IL-2 50 U	18.0±3.3	18.1±3.5	19.3±4.1	17.1±3.3	17.3±3.2	18.2±3.6
NKCC (LU) after IL-2 100 U	23.6±4.1	24.9±4.6	26.7±5.9*	22.9±4.5	23.7±4.8	24.2±5.2
NKCC (LU) after IFN-β 325 U	22.8±6.1	23.3±6.1	23.8±5.7	22.8±5.7	23.1±5.5	23.6±5.8
NKCC (LU) after IFN-β 650 U	27.1±5.2	27.4±5.6	28.6±6.2*	27.0±4.7	27.1±5.1	27.9±5.8
TNF-α (pg/ml) after LPS 1 μg	278±31	290±33	308±51	282±44	303±40	311±49
TNF-α (pg/ml) after IL-2 100 U	384±45	396±52	417±62*	378±72	381±66	399±71
Lymphocytes (cells/mm^3)	1965±71	1960±73	1982±82	1973±77	1978±71	1974±74
CD16 + (%)	9.8±5.6	9.9±5.9	9.5±6.7	9.6±6.1	9.7±5.7	9.8±7.7
CD56 + (%)	5.7±1.8	5.5±1.7	5.7±1.9	5.8±1.7	5.9±1.4	5.6±1.5
DHEAS (μg/ml)	5.3±0.5	5.2±0.4	5.2±0.6	5.1±0.6	5.2±0.5	5.2±0.7

LU, lytic unit.
$P < 0.05$ versus baseline (before treatment).

Discussion

NK immune alterations can be associated with the pathogenesis of thyroid autoimmune diseases (9–12), such as GD and HT (24–27). In particular, the impairment of NK functions could induce the expansion of B/T cell subsets and activity by means of enhancing Th1 autoimmune cell-mediated responses and by increasing some Th2-associated cytokines, such as IL-5 and IL-13, that may indirectly suppress the Th1 autoimmune mechanism (8). Within this context, the demonstration of NK cell dysregulation, affecting both NKCC and NK secretory activity, could have important implications in the onset and progression of thyroid autoimmunity.

In the present study, we clearly found that in subjects with Graves' and Hashimoto's disease NKCC is depressed and that the secretion by NK of the inflammatory cytokine TNF-α is reduced under stimulation with LPS and IL-2. The defect of NK cells can affect both cytotoxic function and the ability of NK to produce cytokines. The depression of NK cells is, therefore, related to all of the functional aspects linked to the immune activity expressed by these cells (i.e. cytolytic and secretory functions).

As suggested in a previous study (27), the NK defect would seem to be due to a functional alteration rather than to a decreased number of NK cells. In effect, the percentage of CD16 + /CD56 + cells observed in GD and HT patients was similar to that found in healthy subjects, so suggesting that the count of peripheral blood NK cells was within normal limits in all the experimental conditions related to our study.

The functional alteration of NK cells, demonstrated in GD and HT subjects, has been correlated not only to an impaired ability of NK cells to respond to IL-2 but also to increased NKCC during modulation with IFN-β. Therefore, NKCC impairment could depend on a multiple post-transcriptional mechanism affecting either interleukin or interferon stimulatory pathways inside NK cells. In effect, it is very interesting to observe that the NKCC abnormality found in GD and HT patients can involve multiple excitatory signals related to different concentrations of IL-2 and IFN-β and the ability of NK cells to release the inflammatory cytokine TNF-α. Moreover, the reduced release of TNF-α, demonstrated in NK cells of GD and HT subjects, could be responsible for a further progressive failure in the NK maturation and cytotoxic response against K562 tumoral target cells.

Our results originally suggest, in agreement with prevoius studies (27, 30, 49), that in subjects with clinical thyroid autoimmunity there is a functional defect involving a subpopulation of mature cytotoxic NK lymphocytes either in the stage of basal pre-activated function (spontaneous NKCC) or during the specific dose-dependent activation with cytokines and LPS. Nevertheless, further studies should be performed in order to confirm these results in other subpopulations of NK cells (e.g. CD16 or CD56 bright cells) of patients with thyroid autoimmune disorders.

Since our study demonstrated the absence of correlations between serum levels of thyroid hormones and immunological parameters, NK alterations would seem to be directly associated with the autoimmune condition linked to GD and HT pathogenesis. This evidence can also be supported by the demonstration of the persistance of the NK defect even during the normalization of thyroid metabolic patterns with methimazole in GD subjects and with L-thyroxine in HT patients. Data concerning methimazole are in agreement with other studies that found no effect on NK cell function during *in vitro* pharmacologic exposure with relevant concentrations of methimazole (50).

Altogether, all the data presented in our study are in accordance with some preliminary experimental evidence that indicates a systemic immune alteration and a peripheral NK immune deficiency in thyroid

autoimmune diseases (51). We suggest that the depression of NK activity could imply the potential expansion of T/B cell functions with a consequent up-regulation of auto reactive T lymphocytes, the production of thyroid-specific auto antibodies and lymphocytic migration and infiltration into the thyroid gland. Therefore, the complexity of NK functional depression could potentially be related to the pathophysiology of thyroid autoimmunity, also suggesting NK dysregulation as a trigger factor for GD and HT immunopathogenesis. Further studies concerning the correlations between NK and cells of the acquired immune system should be performed in order to confirm this preliminary hypothesis.

The normalization of NK cell activity in these clinical conditions could be very important in the prevention, or the delay, of some pathogenetic aspects related to the onset, progression and relapse of thyroid autoimmune disorders. In other words, a modulatory effect able to improve NKCC and NK secretory mechanisms, or boost NK cell number and functions, could represent a novel immunotherapeutic approach to GD and HT.

On these grounds, important studies indicate the role of dehydroepiandrosterone (DHEA) and its conjugate ester DHEA-sulfate (DHEAS) in the positive regulation of T/B immune cells and of NK cell activity (52, 53), the latter by means of IL-2 modulation (6, 53). Furthermore, our previous investigation demonstrated an excitatory dose-related mechanism of DHEAS on NKCC of healthy subjects in young and old age (36). Therefore, we hypothesized that DHEAS could be successfully employed in order to normalize NKCC and NK secretory function in newly diagnosed GD and HT subjects, so improving the potential disequilibrium between NK and T/B immune cells in these pathological conditions.

In effect, our investigation originally indicated that DHEAS restored, in a dose-dependent fashion, the physiological pattern of NKCC and NK secretory function in GD and HT subjects. Moreover, resulted of a certain importance the evidence that the positive effect of DHEAS towards NK was obtained in GD and HT patients with reduced serum DHEAS levels and that serum DHEAS remained unchanged during treatment with methimazole and L-thyroxine. The immune activity of DHEAS was prompt in the spontaneous conditions (i.e. without the use of immune modulators) and during the co-incubation of DHEAS with IL-2 and IFN-β. The effects of DHEAS on NKCC and TNF-α release from NK cells was therefore not entirely dependent on the increased availability and activity of IL-2 or IFN-β within NK cells (6), but was also dependent on a direct mechanism involving basal NK cytolytic function and hence spontaneous NKCC. Anyway, DHEAS demonstrated a wide spectrum of physiological effects able to restore and normalize the derangement of NK function in subjects with associated thyroid autoimmune diseases and low serum DHEAS levels. Moreover, these results could suggest the possibility of a pharmacological intervention on NK function in GD and HT only in those subjects with evident NK cell dysregulation.

In conclusion, our data demonstrated that important alterations in NK cell function are present in thyroid autoimmune disorders such as Graves' and Hashimoto's diseases, and that these changes are probably related to the set and progression of the autoimmune mechanism. These functional disorders are present before treatment and persisted during the normalization of thyroid function by methimazole and replacement therapy with L-thyroxine. NK immune cells are altered either during spontaneous conditions and IL-2/IFN-β modulation or during the intracellular pathway leading to synthesis and release of the inflammatory cytokine TNF-α (54). In both GD and HT, the reversibility of these alterations was reached after *in vitro* incubation of NK cells with different molar concentrations of DHEAS thus suggesting a potential novel therapeutical approach in the correction of the immunopathogenetic disorders found in thyroid autoimmunity.

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References

1 Herberman RB & Ortaldo JR. Natural killer cells: their role in defences against disease. *Science* 1981 **214** 24–30.
2 Robertson MJ & Ritz J. Biology and clinical relevance of human natural killer cells. *Blood* 1990 **76** 2421–2438.
3 Bonnema JD, Rivlin KA, Ting AT, Schoon RA, Abraham RT & Leibson PJ. Cytokine enhanced NK cell mediated cytotoxicity. Positive modulatory effects of IL-2 and IL-12 on stimulus-dependent granule exocytosis. *Journal of Immunology* 1994 **152** 2098–2104.
4 Gidlund M, Orn A, Wigzell H, Senik A & Gresser I. Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature* 1978 **273** 759–761.
5 Trinchieri G & Santoli D. Enhancement of human natural killer cell activity by interferon. *Journal of Immunology* 1978 **120** 1845–1850.
6 Henney CS, Kuribayashi K, Kern DE & Gillis S. Interleukin-2 augments natural killer cell activity. *Nature* 1981 **291** 335–337.
7 Trinchieri G, Kobayashi M, Seehra J, London L & Perussia B. Response of resting human peripheral blood natural killer cells to interleukin 2. *Journal of Experimental Medicine* 1984 **160** 1147–1169.
8 Baxter AG & Smyth MJ. The role of NK cells in autoimmune disease. *Autoimmunity* 2002 **35** 1–14.
9 Yabuhara A, Yang FC, Nakazawa T, Iwasaki Y, Mori T, Koike K, Kawai H & Komiyama A. A killing defect of natural killer cells as an underlying immunological abnormality in childhood systemic lupus erythematosus. *Journal of Rheumatology* 1996 **23** 171–177.
10 Goto M, Tanimoto K, Chihara T & Horiuchi Y. Natural cell-mediated cytotoxicity in Sjogren's syndrome and rheumatoid arthritis. *Arthritis and Rheumatism* 1981 **24** 1377–1382.

11 Hauser SL, Ault US, Levin MJ, Garovoy MR & Weiner HL. Natural killer cell activity in multiple sclerosis. *Journal of Immunology* 1981 **127** 1114–1117.

12 Auer IO, Ziemer E & Sommer H. Immune status in Crohn's disease. *Clinical and Experimental Immunology* 1980 **42** 41–47.

13 Stassi G & De Maria R. Autoimmune thyroid disease: new models of cell death in autoimmunity. *Nature Reviews. Immunology* 2002 **2** 195–204.

14 Itoh M, Uchimura K, Makino M, Kobayashi T, Hayashi R, Nagata M, Kakizawa H, Fujiwara K & Nagasaka A. Production of IL-10 and IL-12 in CD40 and interleukin-4 activated mononuclear cells from patients with Graves' disease. *Cytokine* 2000 **12** 688–693.

15 Massart C, Caroff G, Maugendre D, Genetet N & Gibassier J. Peripheral blood and intrathyroidal T cell clones from patients with thyroid autoimmune disease. *Autoimmunity* 1999 **31** 163–174.

16 Heuer M, Aust G, Ode-Hakim S & Scherbaum WA. Different cytokine mRNA profiles in Graves' disease, Hashimoto's thyroiditis, and nonautoimmune thyroid disorders determined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). *Thyroid* 1996 **6** 97–106.

17 Mori H, Amino N, Iwatani Y, Izumiguchi Y, Kumahara Y & Miyai K. Decrease of immunoglobulin G-Fc receptor-bearing T lymphocytes in Graves' disease. *Journal of Clinical Endocrinology and Metabolism* 1982 **55** 399–402.

18 Amino N, Mori H, Iwatani Y, Asari SI, Zumiguchi YI & Myiai K. Peripheral K lymphocytes in autoimmune thyroid disease: decrease in Graves' disease and increase in Hashimoto's disease. *Journal of Clinical Endocrinology and Metabolism* 1982 **54** 587–591.

19 Sawada K, Saturami T, Imura H, Iwamori M & Nabai Y. Anti-asialo-GMI antibody in sera from patients with Graves' disease and Hashimoto's thyroiditis [letter]. *Lancet* 1980 **2** 198.

20 Pruzanski W, Capes H, Baur R, Wenzel BE, Row VV & Volpe R. Biological activity of lymphocytotoxic antibodies in Graves' disease and Hashimoto's thyroiditis. *Journal of Endocrinological Investigations* 1984 **7** 7–13.

21 Calder EA, Irvine WJ, Davidson NM & Wu FT. B and K cells in autoimmune thyroid disease. *Clinical Experimental Immunology* 1976 **25** 17–23.

22 Tezuka H, Eguchi K, Fukuda T, Otsubo T, Kawabe Y, Ueki Y, Matsunaga M, Shimamura C, Nalao H & Ishikawa N. Natural killer and natural killer-like activity of peripheral blood and intrathyroidal mononuclear cells from patients with Graves' disease. *Journal of Clinical Endocrinology and Metabolism* 1988 **66** 702–707.

23 Pedersen BK, Feldt-Rasmussen U, Bech K, Perrild H, Klarlund K & Hoier-Madsen M. Characterization of the natural killer activity in Hashimoto's and Graves' diseases. *Allergy* 1989 **44** 477–481.

24 Wenzel B, Chow A, Schleusener H & Wall JR. NK cell activity in autoimmune thyroid disorders. *Proceedings of the 57th Annual Meeting of the American Thyroid Association*, Minneapolis 1981. Abstract T26.

25 Papic M, Stein-Streilein J, Zakarija M, McKenzie JM, Guffee J & Fletcher MA. Suppression of peripheral blood natural killer cell activity by excess thyroid hormone. *Journal of Clinical Investigation* 1987 **79** 404–408.

26 Wang PW, Luo SF, Huang BY, Lin JD & Huang MJ. Depressed natural killer cell activity in Graves' disease and during antithyroid medication. *Clinical Endocrinology* 1988 **28** 205–214.

27 Marazuela M, Vargas JA, Alvarez-Mon M, Albarran F, Lucas T & Durantez A. Impaired natural killer cell cytotoxicity in peripheral blood mononuclear cells in Graves' disease. *European Journal of Endocrinology* 1995 **132** 175–180.

28 Hidaka Y, Amino N, Iwatani Y, Kaneda T, Nasu M, Mitsuda N, Tanizawa O & Miyai K. Increase in peripheral natural killer cell activity in patients with autoimmune thyroid disease. *Autoimmunity* 1992 **11** 239–246.

29 Wenzel BE, Chow A, Baur R, Schleusener H & Wall JR. Natural killer cell activity in patients with Graves' disease and Hashimoto's thyroiditis. *Thyroid* 1998 **8** 1019–1022.

30 Manzano L, Alvarez-Mon M, Abreu L, Vargas JA, Morena E, Corugedo F & Durantez A. Functional impairment of natural killer cells in active ulcerative colitis: reversion of the detective natural killer activity by interleukin 2. *Gut* 1992 **33** 246–251.

31 Flodstrom M, Shi FD, Sarvetnick N & Ljunggren HG. The natural killer cell - friend or foe in autoimmune disease? *Scandinavian Journal of Immunology* 2002 **55** 432–441.

32 Bolay H, Karabudak R, Aybay C, Candemir H, Varli K, Imir T & Kansu E. Alpha interferon treatment in myasthenia gravis: effects on natural killer cell activity. *Journal of Neuroimmunology* 1998 **82** 109–115.

33 van Vollenhoven RF. Dehydroepiandrosterone for the treatment of systemic lupus erythematosus. *Expert Opinion on Pharmacotherapy* 2002 **3** 23–31.

34 Khorram O, Vu L & Yen SS. Activation of immune function by dehydroepiandrosterone (DHEA) in age-advanced men. *Journal of Gerontology* 1997 **52** M1–M7.

35 Nawata H, Yanase T, Goto K, Okabe T & Ashida K. Mechanism of action of anti-aging DHEA-S and the replacement of DHEA-S. *Mechanisms of Ageing and Development* 2002 **123** 1101–1106.

36 Solerte SB, Fioravanti M, Vignati G, Giustina A, Cravello L & Ferrari E. Dehydroepiandrosterone sulfate enhances natural killer cell cytotoxicity in humans via locally generated immunoreactive insulin-like growth factor I. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 3260–3267.

37 Solerte SB, Fioravanti M, Schifino N, Cuzzoni G, Fontana I, Vignati G, Govoni S & Ferrari E. Dehydroepiandrosterone sulfate decreases the interleukin-2-mediated overactivity of the natural killer cell compartment in senile dementia of the Alzheimer type. *Dementia and Geriatric Cognitive Disorders* 1999 **10** 21–27.

38 Van Zoelen EJ, Van Oostwaard TM, Van der Saag PT & De Laat SW. Phenotypic transformation of normal rat kidney cells in a growth-factor-defined medium: induction by a neuroblastoma-derived transforming growth factor independently of the EGF receptor. *Journal of Cell Physiology* 1985 **123** 151–160.

39 Timonen T, Ranki A, Saksele E & Hayry P. Fractionation, morphological and functional characterization of effector cells responsible for human natural killer activity against cell-line targets. *Cellular Immunology* 1979 **48** 133–139.

40 Robertson MJ & Ritz J. Biology and clinical relevance of human natural killer cells. *Blood* 1990 **76** 2421–2438.

41 Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation* 1968 **21** (Suppl) 31–50.

42 Julius MH, Simpson E & Herzenberg LA. A rapid method for isolation of functional thymus-derived murine lymphocytes. *European Journal of Immunology* 1973 **3** 645–649.

43 Miltenyi S, Muller W, Weichel W & Radbruch A. High gradient magnetic cell separation with MACS. *Cytometry* 1990 **11** 231–238.

44 Pflueger E, Mueller EA & Anderer FA. Preservation of cytotoxic function during multi-cycle immunomagnetic cell separations of human NK cells using a new type of magnetic bead. *Journal of Immunological Methods* 1990 **129** 165–173.

45 Gatti G, Cavallo R, Sartori ML, Del Ponte D, Masera R, Salvadori A, Carignola R & Angeli A. Inhibition by cortisol of human natural killer (NK) cell activity. *Journal of Steroid Biochemistry* 1987 **26** 49–58.

46 Holbrook NJ, Cox WI & Horner HC. Direct suppression of natural killer activity in human peripheral blood leukocyte cultures by glucocorticoids and its modulation by interferon. *Cancer Research* 1983 **43** 4019–4025.

47 Korzeniewski C & Callewaert DM. An enzyme-release assay for natural cytotoxicity. *Journal of Immunological Methods* 1983 **64** 313–320.

48 Pross HF, Baynes MG, Rubin P, Shragge P & Patterson MS. Spontaneous human lymphocyte-mediated cytotoxicity against tumor

target cells. IX. The quantification of natural killer cell activity. *Journal of Clinical Immunology* 1981 **1** 51–63.
49 Solovera J, Alverez-Mon M, Casas J, Carballido J & Durantez A. Inhibition of human natural killer (NK) activity by calcium channel modulators and a calmodulin antagonist. *Journal of Immunology* 1987 **139** 876–880.
50 Weetman AP, Gunn C, Hall R & McGregor AM. The absence of any effect of methimazole on *in vitro* cell-mediated cytotoxicity. *Clinical Endocrinology* 1985 **22** 57–64.
51 Ciampolillo A, Guastamacchia E, Amati L, Magrone T, Munno I, Jirillo E, Triggiani V, Fallacara R & Tafaro E. Modifications of the immune responsiveness in patients with autoimmune thyroiditis: evidence for a systemic immune alterations. *Current Pharmaceutical Design* 2003 **9** 1946–1950.
52 Daynes RA, Dudley DJ & Araneo BA. Regulation of murine lymphokine production *in vivo*. II. Dehydroepiandrosterone is a natural enhancer of interleukin-2 synthesis by helper cells. *European Journal of Immunology* 1990 **20** 793–802.
53 Suzuki T, Suzuki N, Daynes RA & Engleman EG. Dehydroepiandrosterone enhances IL-2 production and cytotoxic effector function of human T-cells. *Clinical Immunology and Immunopathology* 1991 **61** 202–211.
54 Solerte SB, Cravello L, Ferrari E & Fioravanti M. Overproduction of IFN-γ and TNF-α from natural killer (NK) cells is associated with abnormal NK reactivity and cognitive derangement in Alzheimer's disease. *Annals of the New York Academy of Sciences* 2000 **917** 331–340.

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