Preliminary evidence of immune function modulation by thyroid hormones in healthy men and women aged 55–70 years

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Abstract

A reciprocal relationship between the endocrine and immune system has been demonstrated under pathophysiological conditions. However, few studies have assessed the relationship between thyroid hormones and immune function in apparently healthy individuals. Therefore, to clarify our understanding of normal physiological endocrine–immune interactions this study aimed to examine the interrelationships between thyroid hormones and immunity in healthy individuals. Total triiodothyronine (T3), total thyroxine (T4) and markers of immune status were assessed in 93 free-living and apparently healthy individuals aged 55–70 years. T3 and T4 concentrations were determined by commercially available kits. Immune status was assessed using flow cytometry and biochemical markers. Statistical analysis was performed by partial correlation, controlling for age. Thyroid hormone concentration was positively associated with markers of inflammation (P≤0.05), natural killer-like T cells (P≤0.001), expression of interleukin–6 (IL6) by activated monocytes (P≤0.05); percentage expression of memory T-lymphocytes (P≤0.01), memory T-helper lymphocytes (P≤0.05) and memory T-cytotoxic lymphocytes (P≤0.05), and higher IL2 receptor density on CD3+ T-lymphocytes (P≤0.05). Thyroid hormone concentration was inversely associated with early lymphocyte apoptosis (P≤0.05) and the ratio of naïve–to memory T-cytotoxic lymphocytes (P≤0.05). The current study provides preliminary evidence of a role for T3 and T4, within normal physiological ranges, in the maintenance of lymphocyte subpopulations, and in mediating the inflammatory response. In conclusion, these findings highlight the potential implications of altered thyroid function in older individuals and the importance of future research examining thyroid–immune interactions.


Introduction

Bidirectional communication between the central nervous system and immune system is mediated by the endocrine system, via the hypothalamic–pituitary–adrenal (HPA) axis (Felten & Felten 1991, Bellinger et al. 2002). Evidence, predominantly from animal studies, also suggests a direct reciprocal relationship exists between the thyroid gland and the immune system (Fabris et al. 1995, Klein 2006). Thyroid hormones are involved in numerous physiological processes, such as development, growth and the metabolism of lipids and carbohydrates. While it is believed thyroid hormones are not critical to the development of a normal immune response, they are thought to be involved in the maintenance of immune function in response to environmental stimuli and stress-mediated immunosuppression (Davis 1998, Dorshkind & Horsemann 2000, Dorshkind & Horsemanelson 2001). Total triiodothyronine (T3) is able to modulate immune function via nuclear receptors (TRs) to regulate target genes, and by non-genomic interactions with membrane receptors independent of protein synthesis (Yen 2001, Csaba et al. 2005, Pallinger et al. 2005); the mechanisms that are speculated to underlie the aspects of immunity such as the homing of thymocytes to peripheral lymphoid organs (Ribeiro-Carvalho et al. 2007), and immune response to vaccination (Klecha et al. 2000). The prevalence of thyroid function disorders increases with age (Mariotti et al. 1995, Chiovato et al. 1997, Latrofa & Pinchera 2005), and hypothalamic–pituitary–thyroid (HPT) dysfunction and altered thyroid metabolism occurs even in apparently healthy older individuals (Mooradian 1995, Chakraborti et al. 1999, Magri et al. 2002). Given the relationship between thyroid and immune function, alterations in thyroid hormone concentration with age may contribute to age-related changes in immune function (Stulnig et al. 1995, Mc Nerlan et al. 1999, Santagostino et al. 1999, Bisset et al. 2004).

Previous investigation of the relationship between thyroid hormones and immune functions has been predominantly restricted to a limited range of immune markers in clinical populations with thyroid function disorders (Palmblad et al. 2000).
Material and Methods

Participants
Individually aged 55–70 years were recruited through media coverage, and leaflets, as well as national and local organizations with members spanning this age group. Individuals were screened based on defined exclusion criteria, adapted from the SENIOR protocol (Ligthart et al. 1984) which included: body mass index (BMI; <20 and >33 kg/m²); abnormal haematology, liver and kidney function tests; dietary habits (for e.g. vegetarians and vegans); depression (score ≥11 on the geriatric depression scale); dementia (score ≤24 on the Mini Mental State Examination); pre-menopausal women; no more than three drugs per day including the use of antidepressants, laxatives, hormonal replacement therapy or other immune modulating medication; diagnosis of or treatment for pathological diseases (cancer, diabetes, insufficient renal and hepatic performance, metabolic, malabsorption and chronic inflammatory pathologies); habitual use of vitamin and/or mineral supplements within the last 6 months; alcohol consumption (>30 g/day for men and >20 g/day for women); and smoking (>10 cigarettes, cigars or pipe/day). Of the individuals screened, 15% (147) were invited to participate. Ninety-three apparently healthy late-middle aged individuals (48 females and 45 males) aged 55–70 years completed the study. The University of Ulster Research Ethical Committee granted approval for the study. All volunteers gave written informed consent in accordance with the declaration of Helsinki.

Experimental protocol and sample collection
Following an overnight (>12 h) fast, participants were asked to attend the research centre at 0830 h on the study day. Anthropometric measurements were undertaken and blood was collected. Height and weight was measured and used to calculate BMI as body weight (kg) divided by height (m) squared. Fasting, venous blood (55 ml) was collected into vacutainers containing K3EDTA and sodium-heparin and into serum separator tubes. K3EDTA and sodium-heparin whole blood was used for determination of immune status on a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK) within 4 h of collection. K3EDTA anti-coagulated whole blood was also used to assess full blood profiles, which were conducted at the Causeway Laboratory, Causeway NHSS Trust, Coleraine, UK. Serum was separated and stored at −80 °C until analysis at the end of the study.

Table 1 Anti-human conjugated monoclonal antibodies used in FACS analysis of immune markers in healthy individuals, aged 55–70 years

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker</th>
<th>Clone</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simultest γ1/γ2</td>
<td>Negative control</td>
<td>X40, X40</td>
<td>FITC/PE</td>
</tr>
<tr>
<td>Intracellular γ1</td>
<td>Negative control</td>
<td>X40</td>
<td>PE</td>
</tr>
<tr>
<td>Intracellular γ2</td>
<td>Negative control</td>
<td>X39</td>
<td>FITC</td>
</tr>
<tr>
<td>CD3</td>
<td>Pan T lymphocyte</td>
<td>SK7</td>
<td>FITC and PE</td>
</tr>
<tr>
<td>CD14</td>
<td>Monocyte</td>
<td>Mφ-P9</td>
<td>FITC</td>
</tr>
<tr>
<td>CD19</td>
<td>B lymphocyte</td>
<td>SK7, SJ25C1</td>
<td>FITC and PE</td>
</tr>
<tr>
<td>Simultest CD3/CD16/CD56</td>
<td>NK and NKT cells</td>
<td>SK7, B73-1, MY31</td>
<td>FITC/PE/PE</td>
</tr>
<tr>
<td>CD25</td>
<td>Intermediately activated cells (IL-2R)</td>
<td>2A3</td>
<td>PE</td>
</tr>
<tr>
<td>CD4</td>
<td>Late-activated T lymphocytes</td>
<td>SK7, L243</td>
<td>FITC/PE</td>
</tr>
<tr>
<td>CD8</td>
<td>T-helper lymphocytes</td>
<td>SK3</td>
<td>PerCP</td>
</tr>
<tr>
<td>CD45RO</td>
<td>Memory cells</td>
<td>SK1</td>
<td>PerCP</td>
</tr>
<tr>
<td>CD45RA</td>
<td>Naïve cells</td>
<td>UCHL-1</td>
<td>PE</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
<td>L48</td>
<td>FITC</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
<td>AS10</td>
<td>PE</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Early cellular apoptosis</td>
<td>AS12</td>
<td>PE</td>
</tr>
<tr>
<td>PI</td>
<td>Late cellular apoptosis/necrosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PHAGOTEST E. coli</td>
<td>Phagocytic capacity/activity</td>
<td>–</td>
<td>FITC</td>
</tr>
</tbody>
</table>

FACS, fluorescence activated cell sorter; CD, cluster of differentiation; FITC, fluorescein; PE, phycoerythrin; NK, natural killer; HLA, human leukocyte antigen; DR, D-related; PerCP, peridinin chlorophyll protein; IL, interleukin; PI, propidium iodide. All products were supplied by BD Biosciences, Oxford, UK, with the exception of PHAGOTEST Escherichia coli, which was supplied by ORPEGEN Pharma, Heidelberg, Germany.
Thyroid hormones

Serum T₃ and total thyroxine (T₄) were measured using a competitive immunoassay with an enhanced chemiluminescence end point (Vitros Immunodiagnostic Products, Ortho-clinical Diagnostics, Amersham, UK).

Inflammatory markers

Serum was analysed for C-reactive protein (CRP-high sensitivity), complement protein-3 (C3), and complement protein-4 (C4), using commercially available kits on a Hitachi 912 analyzer (Roche Diagnostics). Serum ferritin was determined at Grenoble University Hospital by sensitized immunoprecipitation using automatic nephelometry (BNII nephelometer; Dade Behring, Paris La Défence, France) as per the manufacture guidelines; laboratory quality assurance included analysis of serum from standard pools and international standards with each run.

Leukocyte immunophenotyping

Immunophenotyping of leukocyte subsets was carried out by two- and three-colour flow cytometry using panels of conjugated-monoclonal antibodies (mAb; Table 1). K₃EDTA whole blood (100 µl) was incubated with conjugated-mAbs using a lyse/wash protocol (BD Biosciences). Samples were analysed within 4 h of fixation. Two-colour analysis was performed for the following lymphocyte subsets: CD3⁺ T-lymphocytes; CD3⁺/CD19⁺ B-lymphocytes; CD3⁺/CD(16 + 56)⁺ natural killer (NK) cells; CD3⁺/CD(16 + 56)⁺ NK-like T (NKT) cells; CD3⁺/CD25⁺/CD163⁺/CD14⁻/CD38⁻/CD69⁻ memory T-lymphocytes, and CD3⁺/CD16⁻/CD56⁻/CD19⁻/CD25⁻/CD71⁻/CD62L⁻/CD11a⁻/CD45RO⁻ naive T-lymphocytes. The same gating strategy was applied for the determination of CD3⁺/CD8⁻ lymphocyte subsets.

Figure 1 Gating strategy for the three-colour analysis of lymphocyte subsets. (A) Lymphocytes (R1) were identified by FSC versus SSC scatter characteristics excluding debris, monocytes and granulocytes. (B) Gating on R1 facilitated the determination of CD3⁺/CD45RA⁺ or CD3⁺/CD45RO⁺ lymphocytes subsets. (C) CD3⁺/CD4⁺ (or CD3⁺/CD8⁺) lymphocytes were identified by gating on R1; a second gate (R2) was placed around CD3⁺/CD4⁺ lymphocytes. (D) Gates R1 + R2 enabled the determination of CD3⁺/CD4⁺/CD45RO⁺ or CD45RA⁺ lymphocytes. The same gating strategy was applied for the determination of CD3⁺/CD8⁻ lymphocyte subsets.
or CD45RA T-lymphocyte subsets, see Fig. 1a–d. The percentage of positive cells was reported as obtained from fluorescence activated cell sorter (FACS) analysis and absolute counts were calculated from the lymphocyte white blood cell differential (×10⁹/l). Ratios of lymphocyte subsets were calculated from absolute counts.

Apoptosis

The determination of early lymphocyte apoptosis by two-colour flow cytometry was conducted using the Annexin V-fluorescein (FITC) Apoptosis Detection Kit I (BD Biosciences). Briefly, peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation from sodium-heparin anti-coagulated whole blood using Accuspin System-Histopaque-1077 tubes (Sigma–Aldrich). PBMC were washed twice in fresh sterile filtered PBS and then re-suspended in buffer. PBMC were stained using Annexin V-FITC and propidium iodide (PI). Lymphocytes were gated using a FSC versus SSC dotplot. Lymphocytes staining positive for Annexin-V and negative for PI were determined using fluorescence channel 1-height (FL1-H) versus fluorescence channel 2-height (FL2-H) dotplots (see Fig. 2a–c). The percentage of lymphocytes undergoing early apoptosis was obtained from FACS analysis, and absolute counts were calculated using the lymphocyte white blood cell differential (×10⁹/l).

Phagocytosis

The quantification of phagocytic capacity and activity of granulocytes and monocytes was determined using a PHAGOTEST kit (ORPEGEN Pharma, Heidelberg, Germany). Sodium-heparin anti-coagulated whole blood (100 µl) was cooled on ice for 10 min, prior to incubation with opsonized *Escherichia coli*-FITC in capped Falcon tubes (BD Biosciences) for 10 min at 37°C with 5–7% CO₂. Monocytes and granulocytes were gated using a FSC versus SSC dotplot. SSC versus *E. coli*-FITC dotplots were used to measure phagocytic capacity and activity for both cell types (see Fig. 3a–c). Percentage of *E. coli*-FITC-positive cells was used to determine the capacity of monocytes and granulocytes to conduct phagocytosis. Mean fluorescence intensity (MFI) was used as a quantitative measure to determine the number of *E. coli* ingested per cell; this measure was used as a determinant of phagocytic activity.

Determination of intracellular cytokine production by activated monocytes

To determine intracellular cytokine production by activated monocytes, 1 ml sodium-heparin anti-coagulated whole blood was activated by incubation with lipopolysaccharide at 1 µg/ml and secretion of intracellular cytokines blocked with brefeldin A at 10 µg/ml (Sigma–Aldrich) for 4 h at 37°C with 5–7% CO₂. After activation, 100 µl activated blood was incubated with either 10 µl IgG2α FITC (isotype control) or CD14 FITC (BD Pharmingen, Oxford, UK) for 30 min in the dark at room temperature. Erythrocytes were lysed by incubation with 100 µl fixation medium A (Caltag, Invitrogen) for 30 min in the dark at room temperature. Cells were washed using cell wash solution (11 sterile PBS containing 0.5% BSA and 0.1% NaN₃), and were centrifuged at 300 g for 5 min at 4°C. Supernatant was removed, and the cell pellet re-suspended and incubated with 100 µl permeabilizing medium B (Caltag, Invitrogen) for 15 min in the dark at room temperature. Cells were then incubated with either (10 µl) of the isotype control IgG1-phycocerythrin-phycerothrin (PE), interleukin (IL1)β PE, or IL6 PE for 30 min in the dark at room temperature. Cells were washed as previously described, and fixed with 500 µl 1× Cell Fix solution (BD Biosciences). Samples were analyzed immediately.

Monocytes were gated using FSC versus SSC dotplots, and percentages of cytokine-positive CD14+ cells were obtained from FL1-H versus FL2-H dotplots (see Fig. 4a–c). Quantification of intracellular cytokine production, as determined by the antibody binding capacity, was achieved using PE QuantiBrite beads (BD Biosciences) for standardization of PE MFI.

Figure 2 Gating strategy for two-colour analysis of early lymphocyte apoptosis. (A) Lymphocytes (R1) were identified by FSC versus SSC scatter characteristics. Dotplots B and C were gated on R1 and show the expression of Annexin V and PI for the unstained negative control (B) and stained (C) test sample.
On each day of testing, three-colour calibration of the FACSCalibur was conducted using CaliBrite Beads-3 (BD Biosciences), and colour compensation levels using appropriate singly-stained cell suspensions. Flow cytometry data were acquired using CellQuest 3.3 for Power Macintosh (BD Biosciences) with 10,000 gated events analysed per sample. Cytometric data was analysed using FlowJo (version 4.5) for Macintosh (TreeStar, Ashland, OR, USA).

**Statistical analysis**

Statistical analysis was performed using a Statistical Package for Social Sciences (SPSS; Chicago, IL, USA) version 11.0. Data were transformed to approximate normal distribution where appropriate. Sex-differences in thyroid hormone concentration were assessed by independent t-test \((P \leq 0.05)\). As age was a significant determinant of immune function in this group (Hodkinson et al. 2006), the relationship between thyroid hormone concentration and immunity was explored using partial correlations, controlling for age.

**Results**

**Participants**

Participant characteristics are summarized in Table 2. At inclusion, volunteers were considered healthy and without incidence of infection in the preceding 4 weeks, as evidenced by self-reporting, a full blood profile, and normal liver and kidney function. Participants were also unaffected by clinical depression and dementia. Participants had a mean age of 62.4 (s.d. 4.48) years and a mean BMI of 26.9 (s.d. 3.22) kg/m². There was no significant difference in age, BMI, T₃ or T₄ concentration between men and women.

**Thyroid hormones**

The euthyroid reference ranges for T₃ and T₄ were 1.49–2.60 nmol/l and 71.2–141 nmol/l respectively. Mean (± s.d.) values for the thyroid hormones T₃, T₄ and T₃:T₄ ratio were 1.95 (± 0.30) nmol/l, 85.3 (± 12.5) nmol/l and 0.23 (± 0.003) ratio respectively, and were within normal range.

**Markers of inflammation and immunity**

Mean (± s.d.) serum CRP, C3 and C4 for all subjects were 0.20 (± 0.36) mg/dl, 1.28 (± 0.21) g/l and 0.26 (± 0.06) g/l respectively, and were within normal ranges. Ten participants had subclinical inflammation, as indicated by elevated serum CRP concentration (>0.5 mg/dl). There was no significant difference in ferritin or immune markers between participants with normal and elevated CRP concentration, with the exception of monocyte count, which were higher in individuals with raised CRP concentration \((P=0.014)\). Medians (2.5–97.5 intervals) for all markers of immunity in this study population have previously been reported by Hodkinson et al. (2006).
Associations between $T_3$, $T_4$ and markers of immunity

Studies examining immune–endocrine interactions involving the hormones $T_3$ and $T_4$ have assessed a limited range of immune markers, predominantly in individuals with thyroid disorders which may be confounded by the presence underlying autoimmunity. Therefore, in the current study $T_3$ and $T_4$ were measured in healthy adults in addition to a comprehensive range of phenotypic and functional markers of immunity. As age was a significant determinant of immune function in this group it was necessary to include age as a co-founder in all analyses to control for any influence on immune function. Calculation of mean $T_3$, $T_4$ concentration, and $T_3$:$T_4$ ratio was used to discriminate between low- and high-thyroid hormone concentrations within normal physiological ranges.

Table 3 shows the significant associations between thyroid hormones and markers of immune function. $T_3$ concentration was closely related to immune markers involved in innate immunity. Higher $T_3$ concentrations ($>1.95$ nmol/l) were significantly associated with higher concentrations of complement proteins C3 ($P\leq0.001$) and C4 ($P\leq0.01$); increased monocyte phagocytic activity ($P\leq0.05$); increased NKT percentage expression ($P\leq0.001$) and absolute count ($P\leq0.001$); and higher percentage expression of IL6 by activated monocytes ($P\leq0.05$). In addition, $T_3$ concentration was positively associated with monocyte count, a finding that approached statistical significance ($P=0.051$), and also demonstrated a significant inverse association with the percentage expression of early lymphocyte apoptosis ($P\leq0.05$).

$T_4$ concentration was closely related to immune markers involved in both innate and adaptive immunity. Higher $T_4$ concentrations ($>85.3$ nmol/l) were significantly associated with higher concentrations of complement proteins C3 ($P\leq0.001$) and C4 ($P\leq0.05$); higher CRP concentration ($P\leq0.05$); and higher neutrophil counts ($P\leq0.001$). $T_4$ was also positively correlated with the percentage expression of total memory T-lymphocytes ($P\leq0.01$); the percentage expression of CD3+CD4+/CD45RO+memory T-helper lymphocytes ($P\leq0.05$); and the percentage expression of CD3+/CD8+/CD45RO+memory T-cytotoxic (CTL) lymphocytes ($P\leq0.05$). In addition, $T_4$ concentration was inversely correlated with the ratio of naive T-cytotoxic to memory T-cytotoxic lymphocytes ($P\leq0.05$). A higher $T_4$ concentration was associated with increased monocyte phagocytic activity ($P\leq0.05$); higher IL2 receptor density on CD3+T-lymphocytes ($P\leq0.05$); and higher absolute counts of NKT cells ($P\leq0.05$). Moreover, a higher $T_3$::$T_4$ ratio was also associated with lower neutrophil counts ($P\leq0.05$).

Discussion

To our knowledge this is the first study to examine associations between thyroid hormone concentration and markers of immunity in healthy men and women, using a comprehensive panel of immune markers. Thyroid hormone

Table 2 Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>Males ($n=45$)</th>
<th>Females ($n=48$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.8 ± 4.68</td>
<td>62.2 ± 3.22</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.2 ± 2.8</td>
<td>26.7 ± 3.55</td>
</tr>
<tr>
<td>WBC ($\times 10^9$/l)</td>
<td>5.48 ± 1.28</td>
<td>5.10 ± 1.09</td>
</tr>
<tr>
<td>$T_3$ (nmol/l)</td>
<td>1.89 ± 0.22</td>
<td>1.99 ± 0.36</td>
</tr>
<tr>
<td>$T_4$ (nmol/l)</td>
<td>85.6 ± 11.9</td>
<td>85.0 ± 13.1</td>
</tr>
<tr>
<td>$T_3$:$T_4$ ratio</td>
<td>0.022 ± 0.003</td>
<td>0.024 ± 0.003</td>
</tr>
<tr>
<td>Afternoon (1430 h)</td>
<td>5.12 ± 6.35</td>
<td>5.83 ± 3.13</td>
</tr>
<tr>
<td>Evening (2200 h)</td>
<td>4.63 ± 10.4</td>
<td>3.35 ± 5.61</td>
</tr>
<tr>
<td>salivary cortisol (nmol/l)a</td>
<td>6.35 ± 5.83</td>
<td>13.1 ± 3.10</td>
</tr>
</tbody>
</table>

All results mean ± S.D.; n, number of participants; BMI, body mass index, WBC, white blood cell count.

aThe number of participants included in the analyses were 21 males and 15 females. No significant differences were observed between male and female participants.

Figure 4 Gating strategy for two-colour analysis of lymphocyte intracellular cytokine expression and production by activated monocytes. Intracellular cytokine expression was determined from the percentage of IL-1β PE or IL6 PE positive monocytes. Quantification of intracellular cytokine production, as determined by the antibody binding capacity (ABC), was achieved using PE QuantiBrite beads for standardization of PE MFI. (A) Monocytes (R1) were identified by FSC versus SSC scatter characteristics. Dotplots B and C were gated on R1 and show the expression of CD14 and IL-1β monocytes for the isotype negative control (B) and stained (C) test sample. The same gating strategy was applied for intracellular IL6 expression and production by activated monocytes.
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Table 3  Associations between serum thyroid hormones concentration and immune function in healthy individuals, aged 55–70 years

<table>
<thead>
<tr>
<th>Immune parameter</th>
<th>n</th>
<th>Mean ± s.d.</th>
<th>T₃ (nmol/l)</th>
<th>T₄ (nmol/l)</th>
<th>T₃:T₄ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3 (g/l)</td>
<td>89</td>
<td>1.28 ± 0.21</td>
<td>0.375</td>
<td>0.329</td>
<td>0.004</td>
</tr>
<tr>
<td>C4 (g/l)</td>
<td>90</td>
<td>0.26 ± 0.06</td>
<td>0.297</td>
<td>0.254</td>
<td>0.004</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>90</td>
<td>0.20 ± 0.36</td>
<td>0.173</td>
<td>0.208</td>
<td>-0.083</td>
</tr>
<tr>
<td>Neutrophils (× 10⁹/l)</td>
<td>90</td>
<td>2.87 ± 0.87</td>
<td>0.151</td>
<td>0.398</td>
<td>-0.249</td>
</tr>
</tbody>
</table>

Gated lymphocytes

| Annexin-V+/PI-Lymphocytes (%)             | 33 | 33.1 ± 10.7 | -0.382      | -0.170      | -0.186      |
| CD3+ T-lymphocyte IL2 receptor density (MFI) | 92 | 69.0 ± 13.1 | 0.060       | -0.167      | 0.213       |
| CD3+ /CD16+/CD56+ (%)                     | 90 | 6.49 ± 9.17 | 0.364       | 0.136       | 0.202       |
| CD3+ /CD16+/CD56+ (× 10⁹/l)              | 90 | 0.12 ± 0.22 | 0.333       | 0.095       | -0.212      |
| CD3+ /CD45RO+ (%)                        | 88 | 51.1 ± 13.2 | 0.181       | 0.286       | -0.097      |

Gated CD3+/CD4+ lymphocytes

| CD45RO+ (%)                              | 88 | 55.9 ± 15.0 | 0.170       | 0.242       | -0.057      |

Gated CD3+/CD8+ lymphocytes

| CD45RO+ (%)                              | 87 | 45.9 ± 16.7 | 0.105       | 0.272       | -0.164      |

Gated monocytes

| Monocyte phagocytic activity (MFI)       | 89 | 234 ± 112   | 0.227       | -0.035      | 0.231       |
| CD14+/IL-6+ monocytes (%)               | 86 | 52.1 ± 22.5 | 0.271       | 0.052       | 0.180       |

n, number of participants; CD, cluster of differentiation. *P ≤ 0.05, †P ≤ 0.01, ‡P ≤ 0.001.

As age was a significant determinant of immune function, age was used as a co-factor in all correlations between thyroid hormone concentration (total triiodothyronine, T₃; total thyroxine, T₄) and immune markers.

Previous studies have reported elevated levels of soluble IL-2R in hyperthyroid patients (Nakanishi et al. 1991), and after oral T₃ administration (Mariotti et al. 1992). Our observation of increased surface IL-2R density on lymphocytes in healthy individuals with a higher T₃:T₄ ratio suggests that higher concentrations of the metabolically active thyroid hormone T₃, within normal physiological concentrations, may contribute to the expression of IL-2R on lymphocytes. T₃ was also associated with lower incidence of early lymphocyte apoptosis indicating that this thyroid hormone may also facilitate maintenance of the lymphocyte population. Although evidence for thyroid hormone regulation of lymphocyte responses has been demonstrated previously in animal models (Klecha et al. 2000, 2006), the underlying cellular mechanisms are not clearly understood.

The current study included an extensive panel of phenotypic markers of immunity that provided comprehensive information on immune status and, importantly, functional assays were included which are associated with clinical endpoints and also present a mechanistic understanding of the immune response (Albers et al. 2005). While, clearly as an observational study, the current study cannot discriminate cause-and-effect relationships between immune and endocrine function, it does highlight the requirement for further studies of these complex interactions in healthy individuals. Future studies should include a comprehensive assessment of immune status as described in the current study, but would benefit from the addition of further functional markers such as NK cell activity, phagocyte oxidative burst and the measurement of other pro- and anti-inflammatory markers within normal physiological ranges. Overall, the current study provides preliminary evidence to suggest that higher concentrations of T₃ and T₄, within normal physiological ranges, enhance innate and adaptive immunity through maintenance of specific cell populations and greater responsiveness to immune stimuli.

Our observation of a positive relationship between thyroid hormone concentration and markers of inflammation is supported by evidence for the binding of C4 to thyroperoxidase (TPO), a key enzyme required for thyroid hormone synthesis, that activates the complement cascade (Blanchin et al. 2003). Furthermore, in vivo clinical studies have indicated that altered thyroid hormone concentration is associated with serum IL6 concentration (Bartalena et al. 1994a,b). Furthermore, ex vivo studies have shown that thyroid hormones stimulate free-radical production in polymorphonuclear leukocytes (Mezosi et al. 2005), and that phagocytosis is impaired in hypothyroid patients (Palmblad et al. 1981). Our observations show that NKT cell numbers are associated with higher thyroid hormone concentrations within the normal physiological range. Altered NKT cell numbers and function are associated with increased incidence of autoimmune disorders (Miyake & Yamamura 2007), and increased NKT cell numbers have been observed in successfully aged individuals with normal thyroid function (Paolisso et al. 2000, Mocchegiani & Malavolta 2004, Abedin et al. 2005). However, studies in non-autoimmune thyroid disorders are necessary to clarify the relationship between altered thyroid hormone concentration and NKT biology.

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cytokines. To place the effects of T₃ and T₄ concentration on immune function in context free T₃ and T₄, TSH, antiTPO, antiTG, ACTH and lymphocyte TR assessment should also be incorporated into study designs to assess thyroid and adrenal gland function in relation to HPA- and HPT-axis activity.

In summary, the current study suggests a role for thyroid hormones, within the normal physiological range, in the maintenance of lymphocyte subpopulations, and in mediating the inflammatory response. This study suggests that some of the same immune alterations observed in thyroid disorders are also observed under normal physiological conditions, albeit within normal reference ranges. These preliminary findings indicate the potential benefit of maintaining optimal thyroid hormone concentrations for the maintenance of immune function with advancing age. Overall, the study reveals a complex relationship between the endocrine and immune systems that warrants further study in this vulnerable population of individuals.

Declaration of interest
The author's declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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