High levels of circulating triiodothyronine induce plasma cell differentiation

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Abstract

The effects of hyperthyroidism on B-cell physiology are still poorly known. In this study, we evaluated the influence of high-circulating levels of 3,5,3′-triiodothyronine (T_3) on bone marrow, blood, and spleen B-cell subsets, more specifically on B-cell differentiation into plasma cells, in C57BL/6 mice receiving daily injections of T_3 for 14 days. As analyzed by flow cytometry, T_3-treated mice exhibited increased frequencies of pre-B and immature B-cells and decreased percentages of mature B-cells in the bone marrow, accompanied by an increased frequency of blood B-cells, splenic newly formed B-cells, and total $CD19^+$B-cells. T_3 administration also promoted an increase in the size and cellularity of the spleen as well as in the white pulp areas of the organ, as evidenced by histological analyses. In addition, a decreased frequency of splenic $B220^+$ cells correlating with an increased percentage of $CD138^+$ plasma cells was observed in the spleen and bone marrow of T_3-treated mice. Using enzyme-linked immunospot assay, an increased number of splenic immunoglobulin-secreting B-cells from T_3-treated mice was detected *ex vivo*. Similar results were observed in mice immunized with hen egg lysozyme and aluminum adjuvant alone or together with treatment with T_3. In conclusion, we provide evidence that high-circulating levels of T_3 stimulate plasmacytogenesis favoring an increase in plasma cells in the bone marrow, a long-lived plasma cell survival niche. These findings indicate that a stimulatory effect on plasma cell differentiation could occur in untreated patients with Graves' disease.

Key Words

- hyperthyroidism
- B lymphocyte
- plasma cell
- thyroid hormone
- spleen
- bone marrow

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Introduction

Augmentation of circulating levels of thyroid hormones (THs) can be associated with thyrotoxicosis, a clinical condition resulted from exacerbated TH action in several tissues (Ioos *et al*. 2008, Bahn *et al*. 2011). High levels of thyroxine (T_4) and/or 3,5,3′-triiodothyronine (T_3) may result from excessive exogenous TH intake, such as in overtreated hypothyroid patients, in individuals using weight loss formula, or excessive ingestion of iodine consequent to chronic use of amiodarone, an iodine-enriched drug used for treatment of cardiac arrhythmia

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(Ioos *et al.* 2008, Bahn *et al.* 2011). Hyperthyroidism is considered to be a form of thyrotoxicosis caused by increased synthesis and secretion of TH by the thyroid gland due to adenoma, multinodular goiter, or Graves' disease. The latter is an autoimmune condition consequent to excessive production of thyroid-stimulating immunoglobulins by plasma cells (Bahn *et al.* 2011, Hou *et al.* 2011).

Although it is well established that immunoglobulins secreted by plasma cells are able to stimulate TH production by the thyroid gland under certain pathological conditions (Ueki *et al.* 2011), the effects of high-circulating levels of TH on B-cell physiology and plasma cell generation are still poorly understood. The plasma cells originate from B-cells, following their activation and differentiation (Radbruch *et al.* 2006). In mice and humans, B-cells are continuously produced throughout life from the bone marrow hematopoietic stem cells, which express several membrane molecules on their surfaces. During bone marrow B lymphopoiesis, B lymphocyte precursors undergo a strictly controlled process of differentiation, passing from pre–pro B to pro-B and then to pre-B stages, phenotypically characterized as fractions A–D (Hardy *et al.* 1991). Progressively, several molecules, including cKit, are downregulated while CD43 expression is also modulated on the surface of these cells (Hardy & Hayakawa 2001, Ye & Graf 2007). Subsequently, following the successful rearrangement of the B-cell receptor light chain genes, IgM is expressed on the surface of immature B-cells (Burrows *et al.* 2002). B-cell maturation further progresses with the expression of IgD and upregulation of IgM and B220. At this stage, B-cells are capable of leaving the bone marrow to enter the blood circulation and peripheral lymphoid organs, such as the spleen, where the final steps of maturation lead to the generation of mature B-cell phenotypes (Allman *et al.* 2001, Pillai & Cariappa 2009). In the peripheral lymphoid organs, activated B-cells can further differentiate to generate plasma cells, this is accompanied by the alteration in the expression of several plasma membrane molecules, including B220 and CD138 that are downregulated and upregulated on the cell surface respectively (Justement 2001, Radbruch *et al.* 2006, Klein & Dalla-Favera 2007). Once differentiated, the plasma cells either die or migrate to survival niches, such as the bone marrow, where they may survive for long periods of time (Radbruch *et al.* 2006).

Murine and human splenocytes, including B-cells, express TH receptor (TR), which indicates that TH can act directly on lymphoid cells (Hastings *et al.* 1997, Shahrara *et al.* 1999, De Vito *et al.* 2011). Supporting the hypothesis that TH might regulate B-cell physiology, Klecha *et al.* (2006) showed that protein kinase C, a signal transduction molecule involved in lymphocyte activation, is increased or decreased in B-cells from hyperthyroid or hypothyroid mice respectively. Previously, Fabris (1973) demonstrated that thyroidectomy in neonatal or young adult rats decreased the number of peripheral blood lymphocytes and humoral immune response. Moreover, studies using TRα knockout or *hyt/hyt* mice, a model for congenital hypothyroidism, showed a decrease in the number of splenic B lymphocytes and in the bone marrow B lymphopoiesis (Foster *et al.* 1999, Arpin *et al.* 2000).

In this study, we investigated the effects of high-circulating levels of T_3 on the profile of B lymphocyte subpopulations in the spleen, bone marrow, and blood as well as on plasma cell differentiation and antibody secretion in mice. Our results indicate that high-circulating levels of T_3 alter B-cell maturation and induce increased B-cell differentiation into immunoglobulin-secreting plasma cells *in vivo*.

Materials and methods

Animals

Male and female C57BL/6 mice were maintained in a 12 h light:12 h darkness photoperiod cycle, at room temperature, with access to water and food *ad libitum*. Mice were killed in a CO_2 chamber. All experiments were done after approval by the Ethical Committee for Animal Use in research of the Federal University of Rio de Janeiro.

In vivo T_3 treatment

Mice received daily s.c. administration of T_3 (Sigma) at 5 μg/10 g of body weight (BW) for 14 days. The animals received injections of 100 μl of T_3 sterile solution (previously diluted from the stock solution at 0.5 mg/ml in sterile saline) according to their BW or of 100 μl of sterile vehicle solution. The animals were killed one day after the end of the treatment. Blood and tissues were collected for further analyses as described below.

Measurement of serum levels of T_3, T_4, TSH, and immunoglobulins

Blood was collected from the mouse trunk immediately after killing and centrifuged at 200 ***g*** at 4 °C for 5 min. Serum was stored at −70 °C until use. Determination of total serum levels of T_3 and T_4 was done using a commercial kit for RIA with specific antibodies (MP-Biochemical, Solon, OH, USA),

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according to the manufacturers protocol. Serum TSH concentration was measured by RIA, employing reagents supplied by the National Hormone and Peptide Program/ National Institutes for Health (NHPP/NIH, Torrance, CA, USA), as described previously (Oliveira *et al.* 2006).

To determine immunoglobulin serum levels, sandwich ELISA was carried out in flat-bottomed polystyrene 96-well-plates (Corning/Costar, Corning, NY, USA) coated with goat anti-mouse IgG antibodies (Southern Biotechnology, Birmingham, AL, USA). Plates coated with hen egg lysozyme (HEL; Sigma) were used to measure serum levels of HEL-specific IgM. Plates were washed three times with PBS pH 7.4 containing 0.1% Tween 20 (Vetec, São Paulo, Brazil). Unspecific binding was avoided through blocking with PBS solution containing 1% gelatin (Merck) at 25 °C for 2 h before washing with PBS. Serum diluted in PBS containing 1% gelatin and 0.1% Tween 20 was added to the plates and incubated at 4 °C overnight. The plates were washed and incubated with rabbit anti-mouse F(ab')2 or IgM conjugated to HRP (Jackson Immune Research, West Grove, PA, USA) at 25 °C for 2 h. Immunoglobulin isotype control (Southern Biotechnology) was used to generate standard curves. The plates were incubated with 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) and the absorbance was detected using an ELISA plate reader (Bio-Rad) at a wavelength of 650 nm.

Immunization

Male mice were immunized on day 0 with a single i.p. injection of 100 μg of HEL and 1.5 mg of aluminum hydroxide (Alum; EMS, São Paulo, Brazil) as adjuvant. Animals treated with HEL or saline solution were then treated or not treated with T_3, as described previously. Analyses were carried out on the 15th day after hormonal treatment in the four groups of mice: unimmunized (CTR); HEL-Alum immunized (HEL-Alum), unimmunized plus T_3-treatment (T_3); and HEL-Alum immunized plus T_3-treatment (T_3+HEL-Alum).

Cell isolation, collection of tissues, and histological staining

Bone marrow cells were obtained by flushing the femurs of the animals with culture medium (RPMI-1640, Sigma) injected through a 21 gauge needle. Spleens were removed and weighed before being smashed on a nylon membrane (Becton Dickinson, San Jose, CA, USA) in a Petri dish with culture medium supplemented with 10% fetal bovine serum (FBS; Gibco–Life Technology). The cell suspensions were centrifuged for 5 min at 200 ***g*** and the cell pellet was resuspended in 3 ml of saline or culture medium supplemented with 10% of FBS. Peripheral blood was isolated and samples were centrifuged at 200 ***g*** at 4 °C for 5 min using ammonium chloride–potassium buffer for lysis of red blood cells. Peripheral blood cells were collected from the pellet. For histological analyses, spleens were sliced into small pieces and maintained in 4% formalin overnight. The material was dehydrated in graded ethanol baths for 15 min, washed in xylene three times for 15 min before paraffin embedding. Paraffin blocks with spleens were sliced and 5 μm tissue sections were stained with Harrison's hematoxylin and eosin (Sigma).

Flow cytometry

The cell suspensions obtained from bone marrow, spleen, or blood of mice were submitted to flow cytometry analyses. A total of 10^6 viable cells was washed with PBS supplemented with 5% FBS. Bone marrow cells were incubated with Fc receptor blocker (clone 2.4G2) for 10 min for non-specific binding of primary antibodies. The cells were stained with combinations of MABs specific for anti-mouse B220 (clone RA3-6B2) conjugated to PE or PerCP, -TCR-FITC (clone H57-597), -GR1-PercP (clone RB6-8C5), -CD138-PE (clone 281-2), -CD19-PE (clone 1D3), -CD21-FITC (clone 7G6), -CD23-PE (clone B3B4), -IgM-FITC (clone II/41), -CD43-PE (clone S7), and -c-Kit-APC (clone 2B8). All antibodies were obtained from BD Pharmingen (San Jose, CA, USA). The cells were incubated in the dark at 4 °C for 30 min, washed and resuspended in PBS supplemented with FBS (5%). A total of 10 000 events were acquired in the forward scatter/side scatter (FSC/SSC) gate designed for the cell population of interest, using an Accuri C6, FACScan, or FACScalibur Cytometer (Becton Dickinson, USA). Data acquired were analyzed using C Flow (Becton Dickinson) or Win MDI 2.9 Software (The Scripps Institute, La Jolla, CA, USA). The total cellularity of the organ was quantified using a hemocytometer. The percentage of the cell population of interest in the organ was obtained following FACS analysis. The absolute number of each cell population was calculated considering the respective percentage in the organ relative to the total cellularity of the organ for each animal.

Enzyme-linked immunospot assay to enumerate antibody-secreting plasma cells *ex vivo*

Splenocytes obtained from unimmunized or HEL-Alum immunized mice treated or not treated with T_3 were used.

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Flat-bottomed polystyrene 96-well-plates (Corning/Costar) were coated with goat anti-mouse IgM antibody (Southern Biotechnology) at 4 °C overnight. The plates were washed with PBS (pH 7.4) and blocked for 1 h with PBS containing 1% gelatin (Sigma). Serially diluted cell suspension (a total of 2×10^4 cells/well) in RPMI with 5% FBS was incubated for 6 h at 37 °C in an atmosphere of 5% CO_2. The plates were washed with PBS and 0.1% Tween 20 before incubation with goat anti-mouse IgM conjugated to alkaline phosphatase (Southern Biotechnology) at 4 °C overnight. Following washing, spots generated by specific binding of secreted immunoglobulins were revealed with TMB (Sigma). The plates were washed in water and dried at room temperature. The numbers of spots were quantified by counting, using an Olympus CKX41SF inverted microscope. The numbers of counted spots were multiplied by the dilution factor indicating the number of immunoglobulin-secreting cells in the well. For each animal, the total cellularity of the spleen was considered to quantify the total number of immunoglobulin-secreting splenic plasma cells.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism, version 5 (Prism Software, La Jolla, CA, USA). The unpaired Student's *t*-test was used for the analyses of immunoglobulin and TH serum levels assays, *ex vivo* enzyme-linked immunospot (ELISpot) assay, ELISA, and flow cytometry experiments. A non-parametric non-paired *t*-test (Mann–Whitney) was used for TSH statistical analysis. A two-way ANOVA test was used to analyze differences between males and females. *P* values of 0.05 or less were considered statistically significant.

Results

Serum T_3, T_4, and TSH in T_3-treated mice

As T_3 is three to four times more potent than T_4 (Walfish 1976), the induction of thyrotoxicosis using exogenous administration of T_3 instead of T_4 has the advantage of providing the immune cells with the most biologically active TH. Indeed, the choice for T_3 administration in our study avoids the need for the conversion of intracellular T_4 to T_3 that is a required for most of T_4 actions and occurs by enzymatic deiodination, for which the activity and regulation is not clear for B-cells (Bianco 2011).

Male and female mice treated for 14 days with T_3 (50 μg/10 g per day) exhibited a threefold to fourfold increase in serum T_3, as evaluated by RIA. T_3 treatment resulted in a significant decrease in serum T_4 and a trend towards decrease serum TSH (Table 1).

Table 1 Serum levels of T_3, T_4, and TSH in mice with high-circulating levels of T_3.

	Male		Female	
	Control	T_3-treated	Control	T_3-treated
T_3 (ng/dl)	98.6±6.8	384.8±47.4‡	83.4±7.9	266.3±34.4†
T_4 (μg/dl)	1.9±0.3	1.1±0.1*	3.0±0.4	Not detectable
TSH (ng/dl)	62.7±4.2	56.1±4.2	60.9±3.9	56.4±1.1

Differences in thyroid hormone (TH) circulating levels in mice treated or not with T_3 were analyzed by unpaired Student's *t*-test. Non-parametric non-paired *t*-test (Mann–Whitney) for TSH statistic analysis and unpaired *t*-test was used for TH serum levels. Values are shown as mean±S.E.M. For analyses of T_3 RIA, $n=9$ for control (CTR) and T_3-treated males, $n=7$ for CTR and $n=9$ for T_3-treated females; T_4 RIA, $n=14$ for CTR and T_3-treated males, $n=7$ for CTR, and $n=6$ for T_3-treated females; and TSH, $n=6$ for CTR and T_3-treated males, $n=4$ for CTR and T_3-treated females. *$P<0.05$, †$P<0.0005$, and ‡$P<0.0001$.

Changes in the spleen weight, cellularity, and morphology in mice with high-circulating levels of T_3

We investigated the effects of excess T_3 in the spleen, one of the peripheral lymphoid organs where B-cells reside and differentiate into plasma cells. High-circulating levels of T_3 promoted a significant increase in the spleen weight of male and female mice (Fig. 1A and B). It was also possible to identify an increase of 80.3 and 50.4% in the weight of the spleens relative to respective BWs, in male and female T_3-treated mice (Fig. 1C and D). The effect of excess T_3 on the spleen weight or on the ratio between the spleen weight and the corresponding BW was more significant in male than female mice ($P<0.05$). Moreover, T_3 promoted an enhancement in the absolute numbers of splenocytes in these animals and no significant difference was found between male and female mice (Fig. 1E and F). This effect was related to an increase in the white pulp compartment of the spleens of mice that received injections of T_3, as analyzed by hematoxylin and eosin staining (Fig. 1G and H). Interestingly, in some spleen tissue sections of T_3-treated mice, the white pulp areas, normally separated, were fused.

Influences of high-circulating levels of T_3 on the profile of splenic B lymphocyte subpopulations

To investigate the effects of high-circulating levels of T_3 on the absolute numbers and frequencies of splenic B

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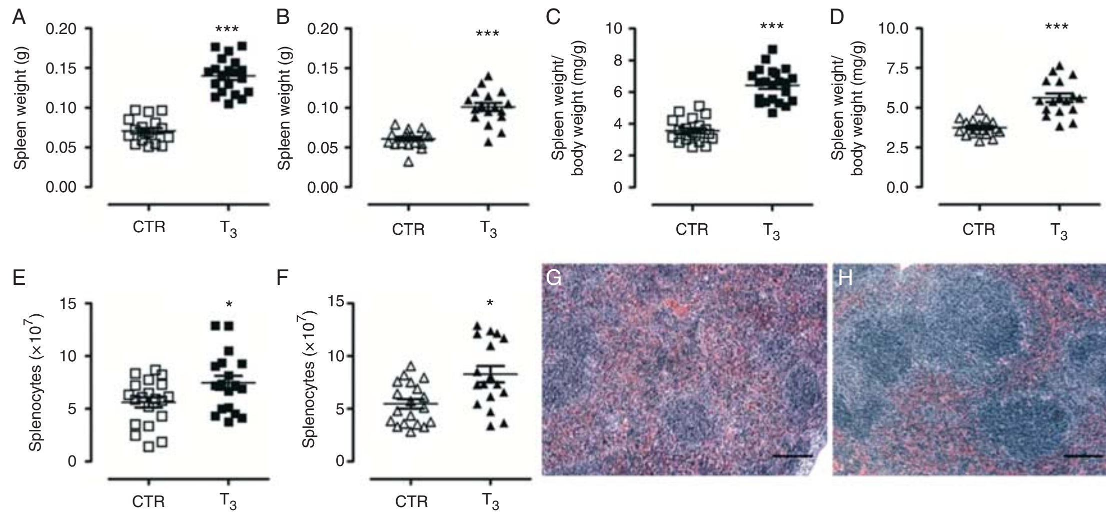

Figure 1

In vivo T_3 treatment increases the weight, cellularity, and lymphoid area of the spleen in male and female mice. Spleens were isolated from 5–6-week-old mice after treatment with T_3 or saline (CTR) for 14 days. Male groups are indicated by squares (A, C and E) and female groups by triangles (B, D and F). (A and B) Spleen weight; (C and D) spleen weight corrected per animal weight; and (E and F) total splenocyte cell numbers. Each symbol represents an animal. Values represent mean±s.e.m. $^*P<0.05$ and $^{***}P<0.001$. (G and H) Representative histological photomicrographs of the spleen of control and T_3-treated mice respectively. White pulp is stained blue and red pulp is stained dark red. Scale bar: 200 μm.

lymphocytes, we carried out flow cytometry analyses. Our results showed that excess T_3 promoted only a trend towards an increase in the absolute number of $B220^+$B cells in the spleen of male (control, $5.2\pm0.7\times10^7$ cells; T_3-treated, $5.7\pm1.2\times10^7$ cells; $P=0.16$) and female (control, $4.0\pm0.4\times10^7$ cells; T_3-treated, $5.0\pm0.6\times10^7$ cells, $P=0.7$) animals. On the other hand, the hormonal treatment reduced significantly the frequency of $B220^+$B lymphocytes in the spleen of male (19.1%) and female mice (8.0%) (Fig. 2A, B and C). In addition, we found a decrease in B220 expression per cell in splenic cells of T_3-treated male (40.9% less than control) and female mice (27.9% less than control) (Fig. 2D and E). Interestingly, we have found more pronounced effects of high-circulating levels of T_3 reducing the frequency of splenic $B220^+$ cells in male than female mice ($P<0.005$).

Although B220 is mainly expressed in B-cells, it can be also found in some activated T lymphocytes and myeloid granulocytic cells (Renno *et al*. 1998, Nakano *et al*. 2001). To evaluate whether the decreased levels of B220 in splenic cells could be reflecting a possible T_3 modulation of the frequency of these cell populations in the spleen, we carried out FACS analysis using B220 with TCR or GR1 cell markers in T_3-treated male mice. We found that the *in vivo* treatment with T_3 reduced frequency of B220 in splenic TCR^+T cells by 2% (control, $5.8\pm0.6\%$, $n=3$; T_3-treated, $3.8\pm0.3\%$, $n=3$; $P<0.05$) while the percentage of splenic $B220^+Gr1^+$ cells did not change significantly between control and T_3-treated mice (control, $4.8\pm0.2\%$, $n=3$; T_3-treated group, $5.2\pm0.3\%$, $n=3$). On the other hand, the percentages of $B220^+TCR^-$ cells (control, $56.0\pm1.4\%$, $n=3$; T_3-treated, $46.4\pm2.9\%$, $n=3$; $P<0.05$) significantly decreased by close to 10% while the percentage of $B220^+GR1^-$ (control, $47.5\pm2.6\%$, $n=3$; T_3-treated, $38.7\pm2.5\%$, $n=3$) showed a trend towards a decrease in the T_3-treated group in relation to controls. These results indicate that reduced expression of B220 might occur in the B lymphocyte population.

The *in vivo* effect of T_3 reducing the expression of B220 per cell raised the hypothesis that high-circulating levels of this hormone could be stimulating splenic B-cells to differentiate to plasma cells. We next analyzed the effects of excess T_3 on the expression of CD19, which is another marker of B-cells that is down-modulated in fully differentiated plasma cells and, compared with B220, maintains its expression longer (Kallies *et al*. 2004, Fairfax *et al*. 2008). We found that the frequency and absolute numbers of splenic $CD19^+$B-cells were increased in T_3-treated male mice (Fig. 3A, B and C).

To better elucidate these possible contradictory effects of *in vivo* T_3 treatment on B220 and CD19 expression on splenic B-cells, we also evaluated the B lymphocyte subpopulations in the spleen of male mice, using the cell markers CD21, CD23, and B220, which allow the identification of newly formed (NF, $B220^+CD21^-CD23^-$), marginal zone (MZ, $B220^+CD21^+CD23^-$), and follicular

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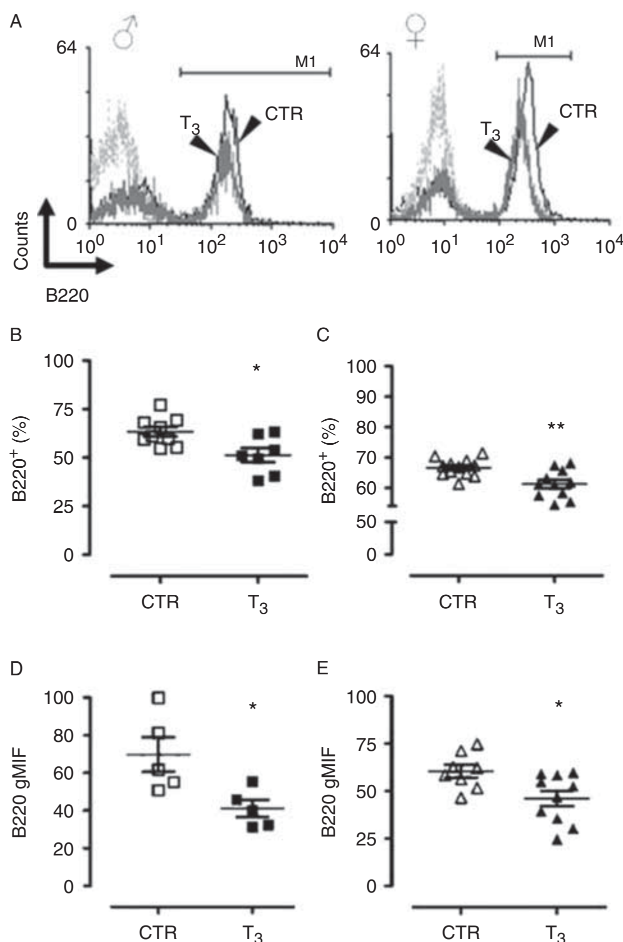

Figure 2
High-circulating levels of T_3 promote downregulation of B220 expression on splenic cells of male and female mice. Spleens were isolated from 5–6-week-old male or female mice after treatment with T_3 or saline for 14 days. Isolated splenocytes were submitted to flow cytometry using rat anti-mouse B220-PE. (A) Representative graphs for splenocytes stained for B220. Light gray lines indicate negative controls; arrowheads indicate saline (black lines); or T_3-treated animals (dark gray lines). (B and C) Percentage of $B220^+$ splenocytes. (D and E) Geometric mean of fluorescence intensity (gMIF) of B220 expression on the surface of splenic cells. Male and female mice are indicated by square and triangle symbols respectively. Each symbol represents an animal. Values represent mean±S.E.M. $*P<0.05$ and $**P<0.005$.

(FO, $B220^+CD21^+CD23^+$) B-cells (Nijnik *et al.* 2009, Su *et al.* 2011; Fig. 3). Although a significant increase in NF B-cells (22%) was detected in the spleen of T_3-treated mice, the percentages of MZ and FO B-cells were significantly decreased (21.4 and 11.8% respectively; Fig. 3D and E). In addition, the analysis of absolute numbers of these B-cell subpopulations revealed that NF B-cells were significantly increased (117%) in the spleen of mice under the influence of excess T_3 (Fig. 3C). On the other hand, the numbers of splenic MZ and FO B-cells showed only a slight trend to increase in these mice (Fig. 3F). The increase of NF B-cells in the spleen led us to question whether excess T_3 in the circulation could be regulating bone marrow B-cell differentiation and emigration to the periphery.

High-circulating levels of T_3 regulate the frequencies of bone marrow B-cell progenitors and peripheral blood B lymphocytes

Analyzing the influence of high-circulating levels of T_3 on bone marrow B-cell progenitors by flow cytometry, we did not find significant changes in the pre–pro B ($cKit^{-/lo}IgM^-B220^+CD43^+$) and pro-B ($cKit^{+/hi}IgM^-B220^+CD43^+$) cell subpopulations (Fig. 4). However, we found an increase of 13.67 and 10.1% in the mean percentage of bone marrow pre-B-cells ($cKit^-IgM^-B220^+CD43^{-/lo}$) and immature B-cells ($cKit^-IgM^+B220^{lo}CD43^-$) in the groups treated with T_3, in relation to their respective controls. Moreover, a reduction of 32.4% was observed in the mean percentage of bone marrow mature B-cells ($cKit^-IgM^+B220^{hi}CD43^-$) in the T3-treated group, as compared with control (Fig. 4A and B). No significant change occurred in the absolute numbers of bone marrow pre–pro B- and pro-B-cells (Fig. 4C). We also noticed a significant increase in the absolute numbers of bone marrow pre-B-cells and immature B-cells in the group treated with T_3, corresponding to 57.8 and 68.9% respectively. However, the absolute number of bone marrow mature B-cells did not change between control and T_3-treated mice (Fig. 4C). Because mature B-cells are capable of emigrating from the bone marrow to the blood circulation and peripheral lymphoid organs, we decided to investigate the profile of B-cells in the peripheral blood of mice treated with T_3. Interestingly, we found an increase of 8.2% in the percentage of $B220^+$B cells in the peripheral blood of T_3-treated mice (Fig. 4D and E).

Plasma cells increase in the spleen and bone marrow of T_3-treated mice

Our data showing a decrease in B220 expression on splenic cells after T_3 treatment (Fig. 2B and C) led us to evaluate whether the *in vivo* T_3 treatment could stimulate the expression of CD138, a surface marker for plasma cells, on splenic cells. We found an increase of ~30% in the percentage of $CD138^+$ plasma cells in the spleen of T_3-treated mice, in relation to control (Fig. 5A and B). The absolute number of $CD138^+$ splenic plasma cells numbers also increased when compared with control animals (Fig. 5C). We could identify small ($FSC^{lo}CD138^+$) and

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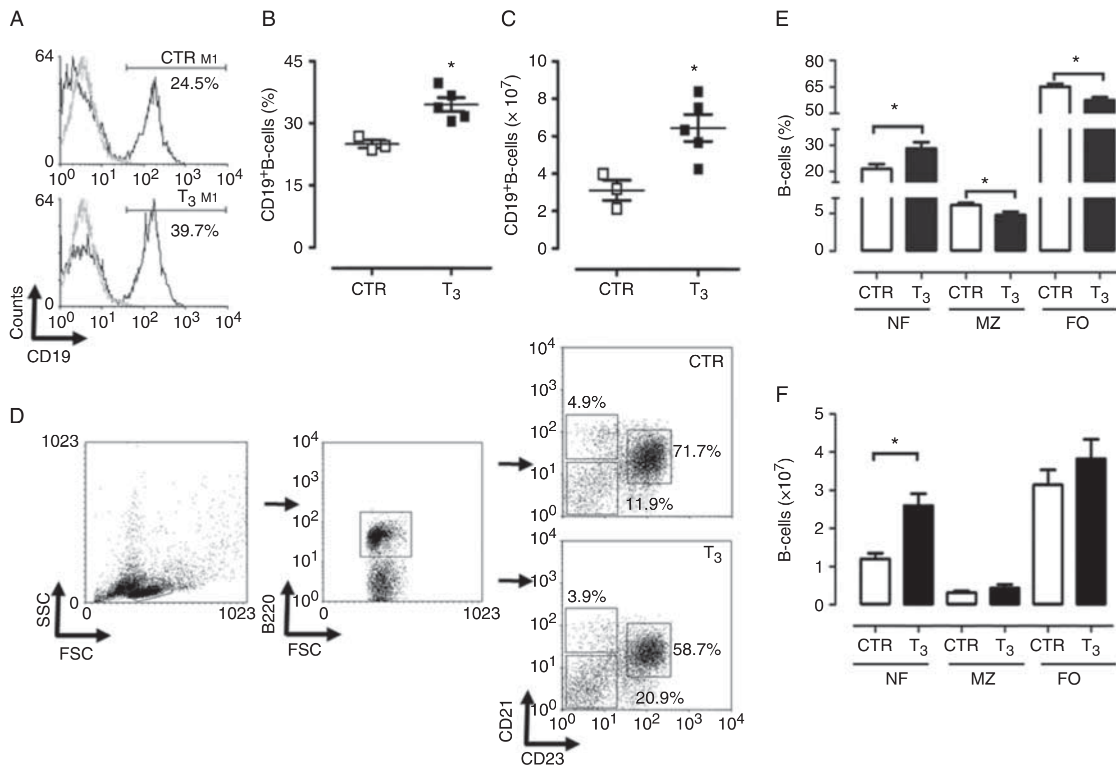

Figure 3
Excess T_3 in circulation leads to an increase in NF B-cells in the spleen. Splenocytes isolated from 5–6-week-old male mice after treatment with T_3 or saline for 14 days were submitted to flow cytometry analysis using the following rat anti-mouse antibodies: anti-CD19-PE, anti-B220-PerCP, anti-CD21-FITC, and anti-CD23-PE. (A) Graphs showing CD19 expression in splenic lymphocyte-gated populations of control (CTR) and T_3-treated mice (T_3). (B) Frequencies and (C) absolute numbers of $CD19^+$B-cell population. (D) Left panel represents gated lymphocytes in the SSC/FSC dot plot; middle panel shows the gate designed for $B220^+$B lymphocytes in the B220/FSC dot plot; and right panels show representative dot plots for CD21/CD23 expression on $B220^+$-gated cells. NF, newly formed B-cells, $B220^+CD21^-CD23^-$; MZ, marginal zone B-cells, $B220^+CD21^+CD23^-$. FO, follicular B-cells, $B220^+CD21^+CD23^+$; CTR, saline control; and T_3, T_3-treated mice. (E) Frequencies of $B220^+$B cell population and (F) absolute numbers of NF, MZ, and FO B-cells. Values represent mean ± S.E.M of at least three animals per group. *$P<0.05$.

large ($FSC^{hi}CD138^+$) plasma cells in the spleens of these mice (Fig. 5A). An increase of 42% was noticed in the small plasma cells of T_3-treated mice in relation to controls. On the other hand, only a trend towards an increase in the percentage of large plasma cells was observed in the spleen of T_3-treated mice. When we analyzed the absolute numbers of small and large splenic plasma cells in the T_3-treated and control groups, only the small plasma cells were significantly increased after treatment with T_3 (Fig. 5B and C).

Based on the knowledge that plasma cells once differentiated are able to migrate from the spleen to long-lived plasma cell niches, we investigated the possible influence of T_3 treatment on the frequency and absolute number of plasma cells in the bone marrow (Mebius & Kraal 2005, Radbruch *et al.* 2006). However, because a bone marrow B-cell progenitor also expresses CD138 (Montecino-Rodriguez *et al.* 2006), we used an anti-CD138 antibody in combination with markers for IgM and cKit in order to identify bone marrow plasma cells ($CD138^+IgM^-cKit^-$). Evaluating the *in vivo* T_3 effect on the percentage of $CD138^+IgM^-cKit^-$ plasma cells in the bone marrow (Fig. 5D, E and F), we verified an increase of 41.6% in relation to control (Fig. 5D and E). Furthermore, the absolute number of plasma cells in the bone marrow survival niche was significantly increased in mice treated with T_3 (Fig. 5F).

High-circulating levels of T_3 increase the number of immunoglobulin-producing plasma cells *ex vivo*

Because we found an increase in plasma cell differentiation under the influence of T_3 *in vivo*, we decided to analyze the circulating levels of IgG in male and female mice treated with this hormone, using sera collected throughout the study. No significant changes were detected in the serum

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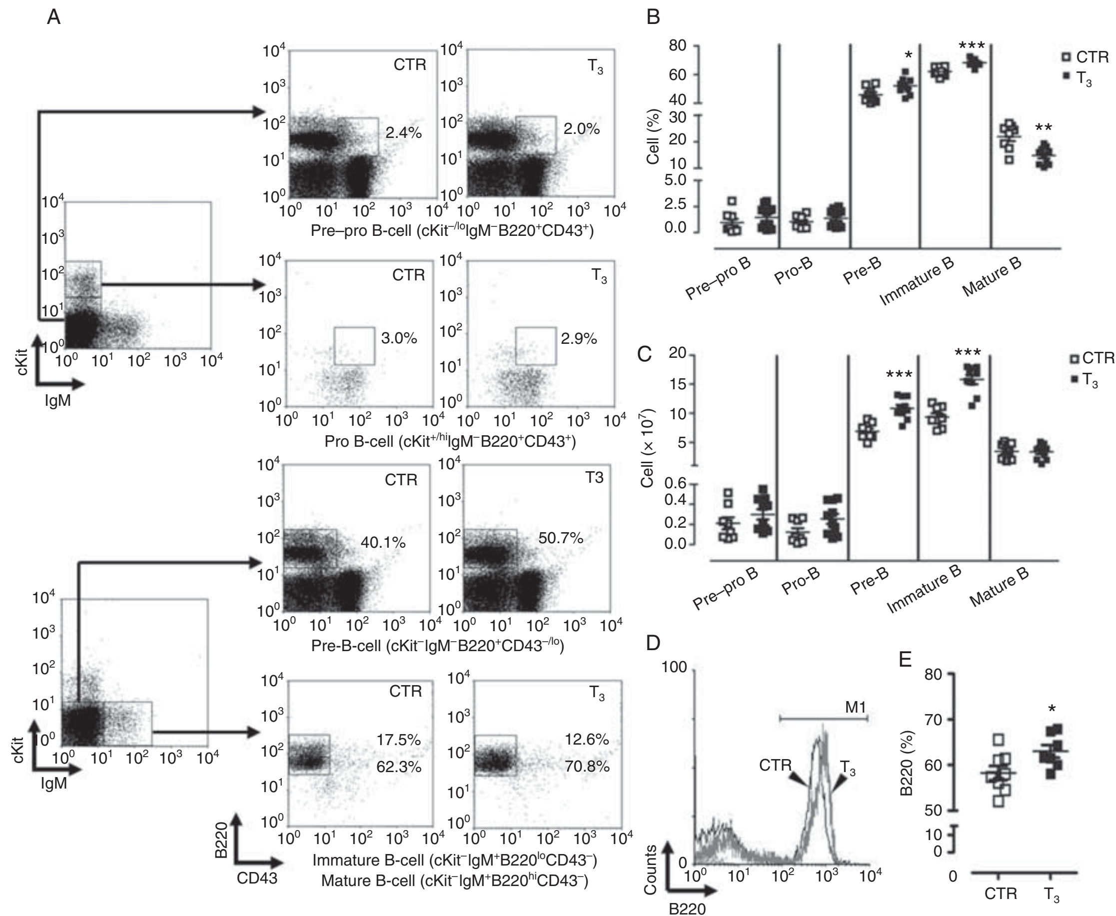

Figure 4
Effects of excess T_3 on bone marrow B lymphopoiesis and mature B-cells in the peripheral blood. (A, B and C) Bone marrow cells or (D and E) peripheral blood lymphocytes were isolated from 5–6-week-old male mice after treatment with T_3 or saline (CTR) for 14 days and submitted to flow cytometry analyses. (A) Dot plots represent bone marrow cells stained with anti-cKit-APC, anti-IgM-FITC, anti-B220-PerCP, and anti-CD43-PE antibodies. Arrows indicate $cKit^{+}IgM^{-}$, $cKit^{-/lo}IgM^{-}$, $cKit^{-}IgM^{-}$, or $cKit^{-}IgM^{+}$-gated populations for further analysis based on B220/CD43 expression to characterize distinct stages of bone marrow B-cell differentiation, for which phenotypes are indicated in the figure as pre–pro B, pro-B, pre-B, immature B, and mature B-cell subsets. (B) Frequencies and (C) absolute cell numbers of differentiating B-cells in the bone marrow. (D) Representative histogram of peripheral blood lymphocytes stained with anti-mouse B220-PE antibody. Arrowheads indicate lines corresponding to saline (CTR) or T_3-treated mice. (E) Relative numbers of peripheral blood $B220^{+}$B lymphocytes. Each symbol represents an animal. Values represent mean ± S.E.M. *$P<0.05$, **$P<0.005$, and ***$P<0.0005$.

levels of IgG in male (control, 2582±437.4 μg/ml, $n=15$; T_3-treated, 2336±550.8 μg/ml, $n=11$; $P=0.7$) and female (control, 1937±493.4 μg/ml, $n=9$; T_3-treated, 2161±486.0 μg/ml, $n=11$; $P=0.7$) animals. These data indicated that the systemic levels of IgG did not alter after 14 days of T_3 treatment.

Next, we investigated whether splenic plasma cells isolated from T_3-treated mice were able to secrete immunoglobulins (Fig. 6). We verified a significant increase in the number of total *ex vivo* IgM-secreting plasma cells in the spleens from T_3-treated mice (Fig. 6), indicating that $CD138^{+}$ splenocytes induced to differentiate under the influence of T_3 are functional plasma cells. We also evaluated the influence of the *in vivo* T_3-treatment on antigen-specific humoral response to HEL, but no influence was noted (not shown), indicating that the increase in immunoglobulin production under T_3 treatment is possibly under immune-regulatory control.

Discussion

Previous studies have revealed a pleiotropic effect of T_3 in the immune system. To our knowledge, the present work is the first demonstration that high-circulating levels of T_3 can induce B-cell differentiation into plasma cells and their accumulation in the bone marrow.

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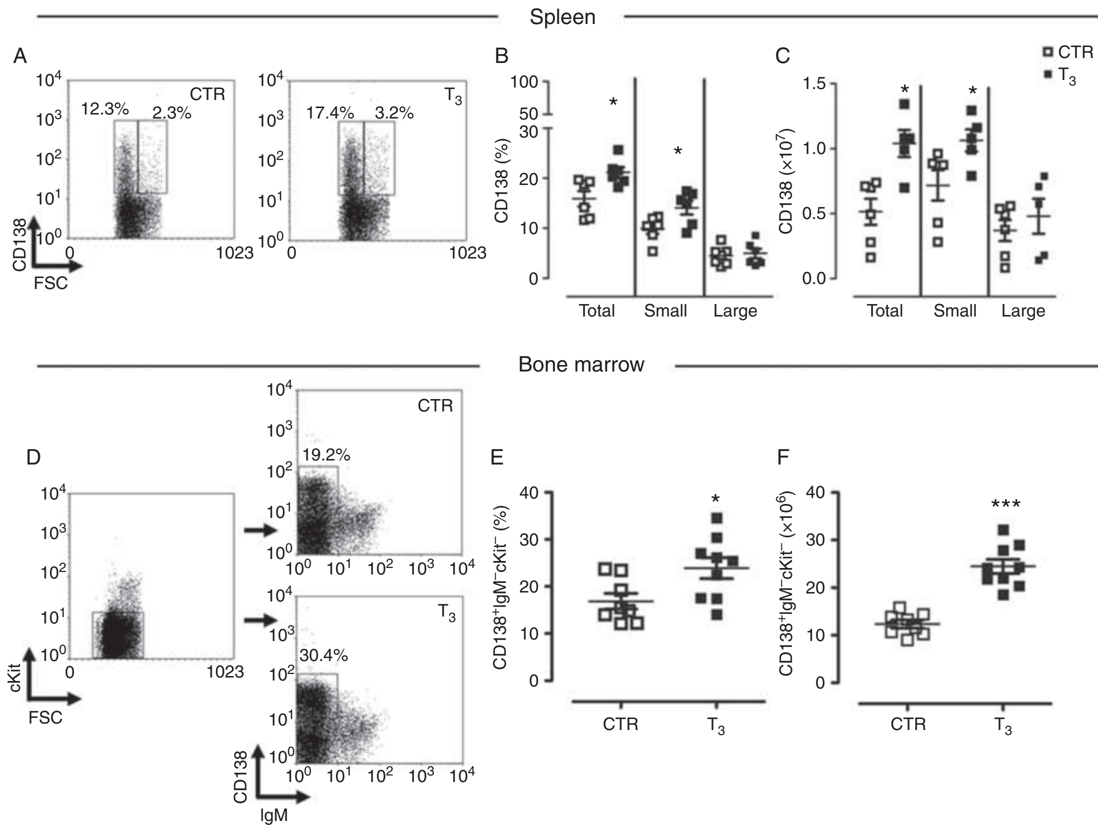

Figure 5

Increased percentage and number of plasma cells in the spleen and bone marrow of mice with high-circulating levels of T_3. Male mice were treated with saline (CTR) or T_3 for 14 days. For FACS analyses, cells were isolated from (A, B and C) spleen or (D, E and F) bone marrow and stained with anti-CD138-PE or anti-cKit-APC, anti-CD138-PE, and anti-IgM-FITC respectively. (A) Representative CD138-PE vs FSC dot plots gated for the splenic lymphocyte population. (B) Frequencies and (C) absolute numbers of total ($CD138^+$), small ($CD138^+FSC^{lo}$), or large ($CD138^+FSC^{hi}$) splenic plasma cells. (D) Dot plots for cKit-APC vs FSC and further analyses based on CD138-PE vs IgM FITC expression in the gate for the bone marrow lymphocyte population indicates bone marrow plasma cells ($CD138^+IgM^-cKit^-$). (E) Frequencies and (F) absolute cell number of bone marrow plasma cells. Each symbol represents an animal. Values represent mean±S.E.M. *$P<0.05$ and ***$P<0.0005$.

In this study, we found that mice with high-circulating levels of T_3 showed an increase in the weight of their spleens, in agreement with the results observed by Baldridge & Peterson (1927) in rats. We also noted that the increase in the spleen weight and cellularity observed in T_3-treated mice was, at least in part, due to an increase in the number of splenic B lymphocytes in these animals; however, other cell types may also be affected. In this regard, we previously showed that intrathymic injection of T_3 stimulates the output of T-cells to peripheral lymphoid organs in mice (Ribeiro-Carvalho *et al.* 2007). In addition, Klecha *et al.* (2005) demonstrated an increase in the mitogen-induced T-cell proliferation in mice previously treated with T_4.

Alterations of B-cell numbers in our T_3-treated mice were not limited to the spleen, as shown by the enhanced percentage of bone marrow pre-B-cells and immature B-cells. These results corroborated previous studies showing opposite effects on B lymphopoiesis in animal models for hypothyroidism or *TRα* knockout mice (Foster *et al.* 1999, Arpin *et al.* 2000), reinforcing the hypothesis that high-circulating levels of T_3 can stimulate bone marrow B lymphopoiesis.

We observed a decreased percentage of mature B-cells in the bone marrow accompanied by an increased percentage of B-cells in the peripheral blood of mice with high-circulating levels of T_3. These results may be due to a stimulatory effect of T_3 on the homing of B-cells from the bone marrow to the peripheral lymphoid organs. According to this hypothesis, we found an increase in the percentage and absolute numbers of splenic NF B-cells in mice under the influence of T_3. Interestingly, NF B-cells include the transitional 1 B-cell subset (T1), which corresponds to recent B-cell immigrants from the bone

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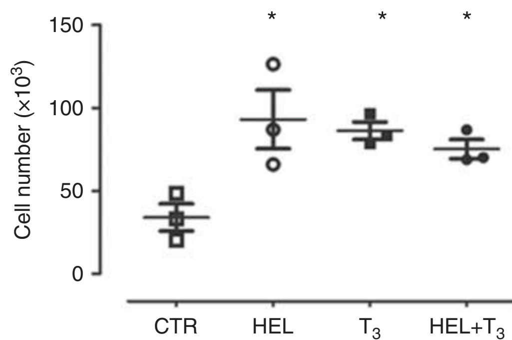

Figure 6
High-circulating levels of T_3 promote an increase in the number of immunoglobulin-secreting splenic plasma cells *ex vivo*. IgM-secreting plasma cell numbers in the spleens isolated from male mice immunized or not with HEL-Alum and subsequently treated or not with T_3 for 14 days. Control mice received injections of vehicle solution only. Each symbol represents the number of immunoglobulin-secreting cells in an animal, based on the corresponding number of spots counted, following ELISpot assay, in relation to the total cellularity of the spleen. Values represent mean ± s.e.m. *$P<0.05$ compared with the control group.

marrow to the spleen (Loder *et al.* 1999, Hu *et al.* 2004). Once in the spleen, these cells differentiate into two subsets of mature B-cells, FO, or MZ B-cells, depending on the anatomical region to which they migrate and are positioned in the spleen (Loder *et al.* 1999, Pillai & Cariappa 2009). These processes are regulated, at least in part, by extracellular matrix and chemokine molecules (Mebius & Kraal 2005, Lokmic *et al.* 2008). In this regard, we have previously shown that mice treated with T_3 exhibited modulation of extracellular matrix components in the spleen, increasing the expression of laminin and fibronectin glycoproteins (Ribeiro-Carvalho *et al.* 2007). However, additional studies are still necessary to verify whether TH excess regulates B-cell homing by modulating the levels of extracellular matrix molecules and chemokine expression and responsiveness.

We found an increase in the splenic $CD19^+$B lymphocyte population associated with an enhanced frequency and number of bone marrow pre-B-cell progenitors and mature B-cells as well as with the increase in blood $B220^+$ cells and splenic NF B-cells in T_3-treated mice. These results support the hypothesis that high-circulating levels of T_3 stimulate bone marrow B lymphopoiesis. Opposite effects showing a reduction in the percentage of B-cell progenitors were observed in TRα-/- mice (Arpin *et al.* 2000). The authors also showed a decrease in the number of $B220^+$ cells in TRα-/- mice. In our work, we did not observe a significant change in the absolute number of $B220^+$ cells after treatment with T_3. However, we observed a decreased frequency and expression per cell of B220 cells in the spleen, as demonstrated by FACS and geometric mean of fluorescence intensity (gMIF) analyses.

Although we found $B220^+TCR^+$T cells decreased by 2% in the spleen under the influence of T_3, a decrease of almost 10% in B220 expression was observed in TCR^-B220^+ cells. In addition, a significant effect of T_3 was observed on $B220^+GR1^+$ cells, which include the plasmocytoid cell population (Nakano *et al.* 2001). These results gave support to the hypothesis that the decrease in B220 expression might occur on the B-cell surface, which correlates with the stimulatory effect of excess T_3 on plasma cell differentiation.

A significant increase in the percentage and number of $CD138^+$ plasma cells was observed in the spleens of mice treated with T_3. It was possible to define two subpopulations of plasma cells according to their size. We propose that small $CD138^+$ plasma cells, which showed a significant increase under the effect of T_3, might correspond to plasmablasts, plasma cells at early stage of differentiation, that have down-modulated B220 but still express CD19 in the cell surface (Radbruch *et al.* 2006, Mei *et al.* 2012). In contrast, large $CD138^+$ plasma cells, which might correspond to cells at a later stage of differentiation, showed only a trend to increase in number after T_3 treatment. Our data also indicated a significant increase in the percentage of plasma cells in the bone marrow of T_3-treated mice. Taken together, these effects suggest that excess T_3 may stimulate generation of splenic plasma cells followed by their migration from the spleen to the bone marrow, where they accumulate, possibly because of the existence of long-lived plasma cell survival niches (Radbruch *et al.* 2006, Tokoyoda *et al.* 2010).

Other studies have shown a stimulatory effect of T_3 on the synthesis of IL6, which is a potent plasma cell differentiation inducer (Korholz *et al.* 1992, Jones *et al.* 1997, Pontikides & Krassas 2007). Moreover, serum levels of IL6 are increased in hyperthyroid patients (Celik *et al.* 1995, Pedro *et al.* 2011). It is thus possible that excess T_3 could act systemically through circulating cytokines that can induce plasma cell differentiation. It is interesting to speculate that TH may act upon B-cells, both directly and indirectly, in combination with IL6. This question deserves further investigation.

In our experiments, mice treated with T_3 have not shown increased levels of IgG in the serum, indicating that plasma cells in T_3-treated animals do not undergo immunoglobulin-class switching. In addition, an ELISpot assay using splenocytes isolated from mice treated with T_3 alone showed an increase in the number of IgM-secreting plasma cells, in comparison with control

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mice, and the magnitude of the increase in secreting plasma cells indicates a polyclonal activation. Interestingly, we found that antigen-specific humoral response was not affected in mice immunized with HEL at day 0 of the 14-day treatment with T_3. It would also be interesting to evaluate a possible enhanced effect on humoral response in mice immunized with HEL during or after treatment with T_3. Although our observation needs to be extended to other experimental conditions and model antigens, these results indicate that the augmentation of immunoglobulin production under T_3 treatment cannot be assumed to hold in general to foreign-antigen-specific stimuli, indicating a more complex process. The data are consistent with the notion that B-cell clones involved in the polyclonal activation induced by T_3 could be under immune-regulatory control. For instance, previously established plasma cells and those newly originated by antigen immunization may compete for niches and factors. It is interesting to consider whether the intrinsic state of the plasma cells, long-lived vs short-lived, ontogeny, differentiation and T-cell help may alter their response to T_3, possibly favoring plasma cells normally present under physiological conditions, such as those involved in the production of natural antibodies (Gronwall *et al*. 2012, Ouchida *et al*. 2012, Montaudouin *et al*. 2013). It would be also relevant to investigate the effects of T_3 in mouse models of lupus-like autoimmune diseases, characterized by elevated levels of polyclonal autoantibodies (Shlomchik *et al*. 1987, Chan *et al*. 1999).

Previously, Paavonen (1982) used human peripheral blood mononuclear cells from euthyroid individuals to evaluate the combinatory effects of T_3 plus mitogens on the levels of immunoglobulin secreted *in vitro*. Although he did not analyze the effects of T_3 alone, he found an increase in the levels of IgG, IgA, and IgE in the culture supernatant of human cells treated with T_3 plus T or B lymphocyte mitogens, as compared with control cultures treated only with mitogens. The combination of T-cell-secreted interleukins with T_3-committed plasma cells may explain the presence of immunoglobulin switching.

The animal model used in this study does not aim to mimic Graves' disease but to investigate the specific effects of high-circulating levels of T_3 on B-cells. In animal models of Graves' disease, it is difficult to analyze the effects of high-circulating levels of TH on B-cell physiology due to the interference of autoimmune B lymphocyte activity (Ludgate 2000). In our model of T_3-injected mice, serum T_3 reached values threefold to fourfold higher than euthyroid levels. Only a trend towards decreased levels of TSH was observed in T_3-injected mice. The reduced levels of T_4 also observed in these mice could be due to the presence of less-bioactive TSH produced in response to the high levels of T_3 in circulation (Dahlberg *et al*. 1987, Persani 1998).

Even though the findings of our studies may not be directly extrapolated to human diseases, it is possible that our results have clinical implications for the understanding of thyrotoxicosis and Graves' disease. An increase of more than threefold to fourfold in the plasma-circulating levels of T_3 can be found in several hyperthyroid patients with Graves' disease, one of the most common forms of thyrotoxicosis, or in T_3-predominant Graves' disease (Takamatsu *et al*. 1988), while TH ingestion in humans can raise the levels of circulating T_3 more than twofold (Ohye *et al*. 2005). Therefore, excess T_3 resulting from exogenous administration of T_3 may be a valid model to study thyrotoxicosis in the absence of autoimmunity.

In conclusion, excess T_3 induces B-cell differentiation into plasma cells *in vivo* and increases the percentage of these cells in the bone marrow, a long-lived plasma cell survival niche. These data bring insights of relevance to the understanding of clinical observations regarding the remission of autoimmunity in patients with Graves' disease associated with the recovery of the euthyroid state, regardless of the type of treatment (Laurberg *et al*. 2008). Based on our findings, we propose the existence of a possible mechanism of positive feedback in untreated Graves' disease, involving the activities of thyroid follicular cells and autoimmune B-cells, through the T_3-enhanced generation of plasma cells secreting autoreactive antibodies. In this context, it is known that antibodies produced against TSH receptor by activated autoimmune B-cells stimulate the thyroid follicular cells and the consequent increase in the circulating levels of TH. In turn, it is possible that high-circulating levels of TH could act to stimulate the activity of autoimmune plasma cells, thus aggravating the state of Graves' disease in untreated patients. Further studies in autoimmune mouse models and/or human B-cells of autoimmune patients might help to elucidate the answer to this question.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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