



Hypothyroidism-related zinc deficiency leads to suppression of T lymphocyte activity

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Abstract

Purpose Hypothyroidism has been shown to induce immunosuppression and both the thyroid status and immunity are affected by zinc deficiency. However, the impact of hypothyroidism on zinc metabolism and its possible relationship with the immune status has not yet been deeply explored. Here, our aim was to study whether hypothyroidism may alter zinc metabolism and thus lead to the impairment of T lymphocyte activity.

Methods Variations in the distribution of zinc in the body were evaluated in PTU-treated hypothyroid mice. The effects of hypothyroidism and zinc deficiency were studied on T lymphocyte proliferation after stimulation both *in vitro* and *in vivo*. For *in vitro* assays, thyroid hormone-free or zinc chelator (TPEN or DTPA)-supplemented media were used. For *in vivo* assays, lymphocyte activity was evaluated in cells from hypothyroid, T3-treated, and zinc-supplemented mice.

Results Hypothyroid mice showed lower levels of zinc in femur and lymph nodes than controls. T3 and zinc supplementation reversed these effects. *In vitro*, both thyroid hormone and zinc deficiency led to a decreased response to mitogen stimulation. However, only zinc deficiency was able to induce lymphocyte apoptosis. Mitogen-stimulated T cells from hypothyroid mice showed impaired proliferation, accompanied by decreased activation of PKC and lower levels of p-ERK, effects that were reversed by T3 replacement or zinc supplementation.

Conclusions Our results show an important role of zinc deficiency in hypothyroid-mediated T-cell suppression and suggest the importance of evaluating zinc levels and restoring them when necessary to maintain an efficient immune response in hypothyroid patients.

Keywords Hypothyroidism · Zinc · Thyroid hormones · T lymphocytes

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Introduction

Immunity is modulated by a complex network of immune and neuroendocrine factors, which, in turn, require an adequate supply of micronutrients to assure the proper function of the immune system. Among these factors, the thyroid status has been demonstrated to influence the immune response, with hypothyroidism leading to immunosuppression [1–4]. Another factor closely related to immunity is zinc. Within its physiological range (12–16 µM), this trace element plays an important role in the proper functioning of the immune system [5–7]. In fact, its deficiency induces a decrease in the number and activity of lymphocytes and a greater susceptibility to infections and tumor development [8–10]. The effects of thyroid hormones and zinc deficiency on immunity have been associated with reduced intracellular signals related to lymphocyte activation [2, 7, 11]. In fact, a growing number of signaling pathways, including activation of T cells by their

T cell receptor and by the cytokine IL-2, have been discovered to involve zinc-dependent signals [12–14]. In addition, zinc has been pointed out as an important element for normal thyroid homeostasis, and several studies have shown that zinc deficiency adversely affects the synthesis, metabolism, and action of thyroid hormones [15, 16]. In fact, zinc deficiency affects both thyroid hormone synthesis and triiodothyronine (T3) binding to thyroid hormone nuclear receptors, thus leading to hypothyroidism [17–19]. Conversely, thyroid hormones are essential for the absorption of zinc, and hence hypothyroidism can result in acquired zinc deficiency [15, 20]. However, the role of this interrelationship in the immune system has not been deeply studied.

Therefore, in this context, our aim was to study whether experimental hypothyroidism in a murine model may alter zinc metabolism and whether this is related to an impairment of T lymphocyte activity associated with hypothyroid conditions. We here show that decreased levels of circulating thyroid hormones are accompanied by a significant zinc loss and that reversal of this situation by zinc replacement restores T cell function during hypothyroidism.

Materials and methods

Animal models

Animal model to evaluate hypothyroidism

A murine model of hypothyroidism was developed using 2–3 months old female BALB/c mice, which were bred and kept at the Instituto de Investigaciones Biomédicas (BIOMED, Argentina) in accordance with the ARRIVE Guidelines [21]. All experimental protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the School of Pharmacy and Biochemistry of the University of Buenos Aires and the CICUAL of BIOMED. Hypothyroid mice, fed with control diet, were obtained by daily administration of the antithyroid drug propylthiouracil (PTU, 0.5 mg/mL, Sigma-Aldrich) for 14 days in drinking water [2, 22]. It is worth to note that PTU treatment for 14 days results in high levels of thyroid stimulating hormone (TSH) (Fig. S1a) and induced the highest decrease in mitogen-mediated T cell proliferation (Fig. S1b).

Since this drug also blocks the peripheral conversion of thyroxine (T4) to T3, reversal of PTU treatment was achieved via T3 administration. To this end, animals treated with PTU also received 0.2 µg/g body weight of T3 (dissolved in 0.01 M NaOH, diluted 1:10 with PBS, pH 7.2, and Millipore filtered) per day for the last 6 days of treatment. Control animals received the vehicle alone [2]. For zinc supplementation, PTU-treated mice received an intraperitoneal injection of

3 mg/kg body weight of zinc per day [23, 24]. Mice injected with the vehicle alone were used as controls.

To confirm the thyroid status, T3 and T4 serum levels were determined using commercial RIA kits (Immunotech, Praga, Czech Republic) according to the manufacturer's instructions and TSH serum levels were determined using an ELISA kit (Uscn Life Science, Inc., Wuhan, Hubei, China).

Animal model to evaluate zinc deficiency

Two groups (10 animals per group) of 21–23-day-old female BALB/c mice bred and kept at BIOMED following the same ethical considerations as indicated above, were fed with or without zinc-containing diets for 40 days. The diets were <1 ppm of zinc (zinc-deficient diet) and without any mineral deficiency and 50 ppm of zinc as sulfate salt (control diet), respectively. Mineral contents were verified by flame atomic absorption spectrophotometry (Varian Spectra 220, Australia) after a mineralization procedure previously described [11]. The compositions of the zinc-deficient and control diets, prepared according to the American Institute of Nutrition Rodent Diets-93 [25], were identical with regard to the content of protein, energy, vitamins, and other minerals. Both groups were given free access to the diets. Because zinc deficiency can lead to anorexia-induced malnutrition, the control group was pair-fed in amounts necessary to equal the mean that the zinc-deficient mice had consumed during the preceding 24 h. Hematological parameters were determined both before and after treatment in blood samples from all animals. Hemoglobin and microhematocrit were determined by the cyanmethemoglobin method and centrifugation, respectively. Leukocyte counts were also obtained. The levels of zinc in mineralized samples of lymph nodes and femur were determined by flame atomic absorption spectrophotometry, as indicated for diets.

Cell suspension and culture conditions

Lymphoid cell suspensions were purified from lymph nodes of mice of all groups: euthyroid mice, PTU-treated mice (hypothyroid), PTU + T3-treated mice, PTU + Zinc-treated mice, control-diet mice, and zinc-deficient mice. Also, T-cells isolated by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA, USA) were prepared aseptically as described elsewhere. Cells were cultured at a concentration of 1×10^6 cells/mL, in RPMI 1640[®] medium (Gibco Co.), supplemented with 10% fetal bovine serum (Gibco[™]), 2 mM glutamine (Gibco[™]), 100 U/mL penicillin (Gibco[™]), and 100 mg/mL streptomycin (Gibco[™]) alone or in the presence of optimal mitogenic concentrations of the selective T-cell mitogen concanavalin A (Con A, 2 µg/mL Sigma Chemical Co.). Cells were cultured for the

times indicated in results, at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air, final volume of 0.2 mL in 96-well flat-bottom microtiter plates (Corning, NY, USA). Alternatively, a medium free of thyroid hormones, AIM-V (Gibco™), containing L-glutamine, 50 µg/mL streptomycin and 10 µg/mL gentamicin was used.

Where indicated, the cells were cultured for 24 h, and then the medium was replaced with fresh medium, in the absence (Basal) or the presence of the cell membrane permeable zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) (20 µM) or the membrane impermeable zinc chelator diethylenetriaminepentacetic acid (DTPA) (60 µM), added to cultures either alone or co-incubated with equimolar amounts of exogenous zinc, or zinc alone, at different times depending on each test.

T cell proliferation assays: [³H]- thymidine incorporation

T cell proliferative responses were tested by [³H]-thymidine (Perkin Elmer, 25 Ci/mmol) incorporation during the last 16 h before the end of the culture incubation. The cells were harvested on filter paper with an automatic cell harvester (Micromate 196, Packard) and the radioactivity was counted by liquid scintillation. Results are expressed as stimulation index (SI), calculated as the rate between dpm values in experimental cultures and dpm values in controls obtained with unstimulated cells.

Apoptosis assessment

Cells (1 × 10⁶ cells) were resuspended in staining buffer (10 mM HEPES/NaOH, pH 7.5; 0.14 M NaCl; 2.5 mM CaCl₂) and incubated for 15 min in the dark with 50 ng/mL Annexin V-FITC (Sigma Chemical Co.) and 100 ng/mL of propidium iodide (PI, Sigma-Aldrich). Labeled cells were analyzed by flow cytometry (BD Accuri™ C6, BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using BD Accuri C6 software and expressed as the percentage of cells in each condition (Annexin V and PI–, Annexin V+ and PI–, Annexin V and PI+) relative to the total number of cells (20,000 events) analyzed.

Alternatively, the cells were fixed and stained with Hoechst 33258 (Molecular Probes, Eugene, OR, USA) and visualized under fluorescence microscopy to evaluate the morphological changes in the nuclei as a measure of apoptosis.

Western blot analysis

Cells were lysed for 30 min at 4 °C in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1% NP40, 1 mM NaF, 1 mM Na₃VO₄, 0.25% sodium deoxycholate, 1 µM

phenylmethylsulfonyl fluoride (PMSF), 10 µM aprotinin, 10 µM pepstatin, and 10 µM leupeptin). Lysates were resuspended in SDS-sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris–HCl, pH 6.8, 0.2% bromophenol blue, and 1% 2-mercaptoethanol) and 30 µg of protein was separated by SDS–PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Nonspecific binding sites on the PVDF membranes were blocked using blocking buffer (5% nonfat dried milk containing 0.1% Tween 20 in 100 mM Tris–HCl, pH 7.5 and 0.9% NaCl) for 1 h at room temperature. Then, the PVDF membranes were incubated for 18 h with rabbit anti-caspase-3 antibody (1:1000, Abcam), rabbit anti-protein kinase C alpha (PKCα), PKCβ or PKCθ (1:1000, Santa Cruz Biotechnology Inc.), total or phosphorylated ERK (p42/44) (1:1000, Abcam Co.), and rabbit anti-β-actin antibody (1:2000, Santa Cruz Biotechnology Inc.), the latter of which was used as a protein loading control. Goat anti-rabbit horseradish peroxidase-conjugated (1:2500, Abcam) antibody was then added for 1 h, and detected through the ImageQuant LAS 4000 digital imaging system (GE Healthcare, UK), using an enhanced chemiluminescence system (ECL Plus, GE Healthcare). The bands were densitometrically analyzed using the ImageJ software (version 5.1, Silk Scientific Corporation, NIH, Bethesda, MA, USA) and the resulting values were normalized to the corresponding β-actin bands.

Determination of PKC activity

T lymphocytes from euthyroid, hypothyroid (PTU-treated), PTU + T3-treated and PTU + Zinc-treated mice were incubated alone or in the presence of Con A (2 µg/mL) for 10 min and immediately frozen at a concentration of 1 × 10⁷ cells/sample in liquid N₂.

To purify PKC from subcellular fractions, the cells were washed once in ice-cold PBS and suspended in 1.0 mL of an ice-cold hypotonic lysis medium (1 mM NaHCO₃, 5 mM MgCl₂, pH 7.5) with the addition of protease inhibitors, PMSF (1 mM) and leupeptin (10 µg/mL). This suspension was vortexed for 2 min and sonicated for another 2 min. The lysate was centrifuged at 20,000 × g for 10 min at 4 °C (Eppendorf 5810R centrifuge). This allowed obtaining the cytosol fraction (supernatant) and a pellet, which was washed with PBS and resuspended in 0.5% (w/v) Nonidet P-40 in PBS containing 0.5 mM EGTA, 1 mM PMSF, and 10 µg/mL leupeptin. The particulate pellet was stirred for 30 min at 4 °C, and then centrifuged at 200 × g for 10 min and at 20,000 × g for 30 min. The supernatant was used as the membrane fraction.

PKC activity was determined by measuring the incorporation of ³²P from [³²P]-ATP into histone H1. Incubation was performed at 30 °C for 30 min at a final volume of 85 µL, containing 25 mM ATP (0.4 mCi), 10 mM

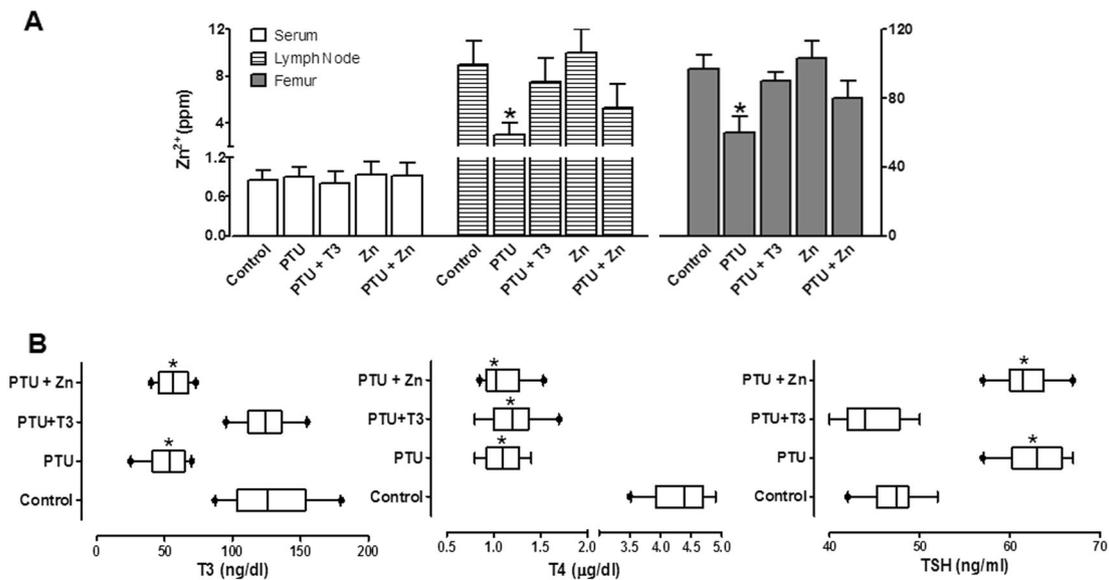


Fig. 1 Variations in the distribution of zinc (Zn) in the body, according to the circulating levels of thyroid hormones. **a** Serum (empty bars), lymph nodes (horizontal stripes), and femur (gray solid bars) samples were collected from BALB/c mice grouped as follows: euthyroid (control), hypothyroid (PTU-treated), PTU + T3-treated, Zn-supplemented controls (Zn), and PTU + Zn-treated mice. Zn content in mineralized samples was measured by using atomic absorption

spectroscopy. Zinc levels are depicted as mean \pm standard deviation (SD). *Differs from control value with $P < 0.01$. **b** Circulating levels of T3, T4, and thyroid-stimulating hormone (TSH) measured with radiometric techniques in serum samples from control, PTU-treated, PTU + T3-treated, and PTU + Zn-treated mice. The values shown are the mean of five independent experiments. *Differs from the control value with $P < 0.01$

magnesium acetate, 5 mM β -mercaptoethanol, 50 mg histone H1, 20 mM HEPES, pH 7.5, and, unless otherwise stated, 0.2 mM CaCl_2 and 10 mg/mL of phosphatidylserine vesicles. The incorporation of ^{32}P from [^{32}P]-ATP into histone was linear for at least 30 min. The reaction was stopped by the addition of 2 mL ice-cold 5% trichloroacetic acid with 10 mM H_3PO_4 . The radioactivity retained on GF/C glassfiber filters after filtration was determined by counting the filters in 2 mL of scintillation fluid. ^{32}P incorporation in the absence of Ca^{2+} and phospholipids was subtracted to determine PKC activity. Data are expressed as picomoles of phosphate incorporated into the substrate per minute and per 10^7 cells (pmol/min per 10^7 cells).

Mixed lymphocyte reactions

Mitomycin C-treated lymph node cells from C3H mice were used as allogeneic stimulators of one-way mixed lymphocyte reactions and BALB/c lymphocytes from euthyroid, PTU-treated, PTU + T3-treated, and PTU + Zinc-treated mice were used as the responding cell populations. All cell suspensions were cultured at a concentration of 1×10^6 cells/mL in a final volume of 0.2 mL of RPMI 1640 medium (Gibco Co.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and antibiotics in 96-well flat-bottomed microtiter plates (Corning, NY, USA) following standard procedures [3]. Cell suspensions were

then kept at 37 °C in a 5% CO_2 atmosphere for 6 days. Proliferation was evaluated by [^3H]-thymidine incorporation during the last 16 h before the end of the culture incubation, as described before.

Statistical analysis

Means of the different experimental groups were analyzed for statistical significance with the software GraphPad PRISM 4.0 (GraphPad Software), using unpaired two-tailed Student *t*-test or one-way analysis of variance followed by Tukey's post-hoc test for multiple comparisons. Differences between means were considered significant if $P \leq 0.05$.

Results

Consequences of the modulation of the thyroid axis on zinc metabolism

Hypothyroidism is associated with immunosuppression, and zinc deficiency has a profound effect on both thyroid status and immunity. However, the impact of hypothyroidism on zinc metabolism and its possible relationship with the immune status has not yet been explored. Thus, we evaluated the zinc levels in hypothyroid mice and compared

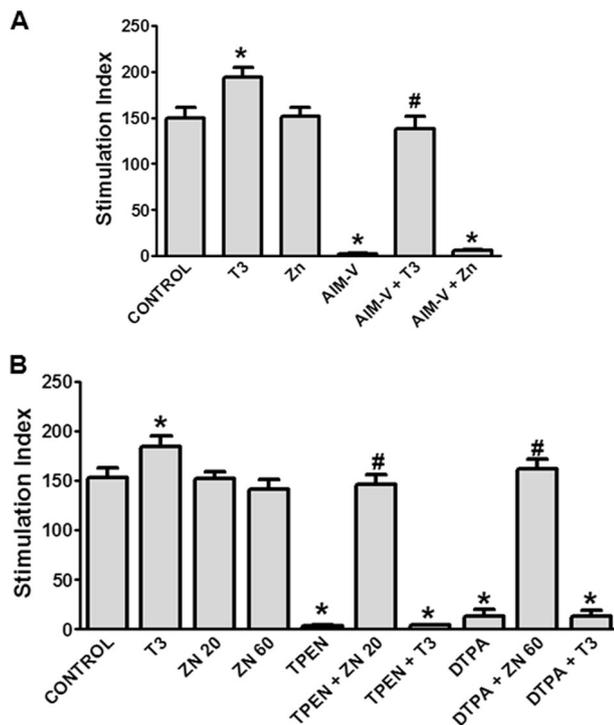


Fig. 2 Effects of thyroid hormone and Zn deficiency on T lymphocyte proliferation in vitro. Lymph node cells (1×10^6 cells/mL) were cultured in RPMI 1640 medium with 10% FBS and antibiotics in the presence or absence of concanavalin A (Con A) ($2 \mu\text{g/mL}$). Where specified, AIM-V, a thyroid hormone-free medium was used instead of RPMI 1640. As indicated in “Materials and methods”, proliferation was evaluated by [^3H]-thymidine incorporation, and results of six experiments performed in triplicate are expressed as stimulation index \pm standard error of the mean (SEM). **a** Control cells, cells plus T3 (10^{-9} M) and cells plus Zn ($20 \mu\text{M}$) in RPMI medium compared with cells grown in AIM-V medium alone, AIM-V plus T3 (10^{-9} M), or AIM-V plus Zn ($20 \mu\text{M}$). *Differs from the control value with $p < 0.01$ and # differs from the AIM-V value with $p < 0.01$. **b** Control cells, cells plus T3 (10^{-9} M), cells plus Zn (20 or $60 \mu\text{M}$), cells plus TPEN ($20 \mu\text{M}$), cells plus TPEN and Zn ($20 \mu\text{M}$), cells plus TPEN and T3, cells plus DTPA ($60 \mu\text{M}$), cells plus DTPA and Zn ($60 \mu\text{M}$) and cells plus DTPA and T3. Cells in the presence of TPEN or DTPA were cultured for 24 and 48 h respectively. *Differs from control values with $p < 0.01$ and #differs from the corresponding chelator value with $p < 0.01$

them with those of euthyroid controls. Results in serum, lymph nodes, and femur samples from the mice evaluated showed that hypothyroidism leads to an alteration in the distribution of zinc in the body. PTU-treated mice had lower levels of zinc in femur and lymph nodes than euthyroid controls, but showed no differences in serum zinc concentrations (Fig. 1a). These effects were reversed both by T3 administration and by zinc supplementation (Fig. 1a). Conversely, zinc administration in hypothyroid mice was unable to modify the low levels of T4 and T3, and the high levels of circulating TSH observed in hypothyroid conditions, thus suggesting that mineral addition is not enough to restore the function of the thyroid axis (Fig. 1b). These

results indicate that hypothyroidism leads to a mild mineral deficiency, characterized by a reduction of zinc in bone, and that this deficiency impacts on immune-relevant compartments such as lymph nodes.

To further characterize the possible relationship of zinc deficiency and hypothyroidism with immunity, we next evaluated how low levels of zinc and T3 affect the activity of T lymphocytes, the central players in immunity.

Effects of zinc and hormone deficiency on Con A-induced T cell proliferation

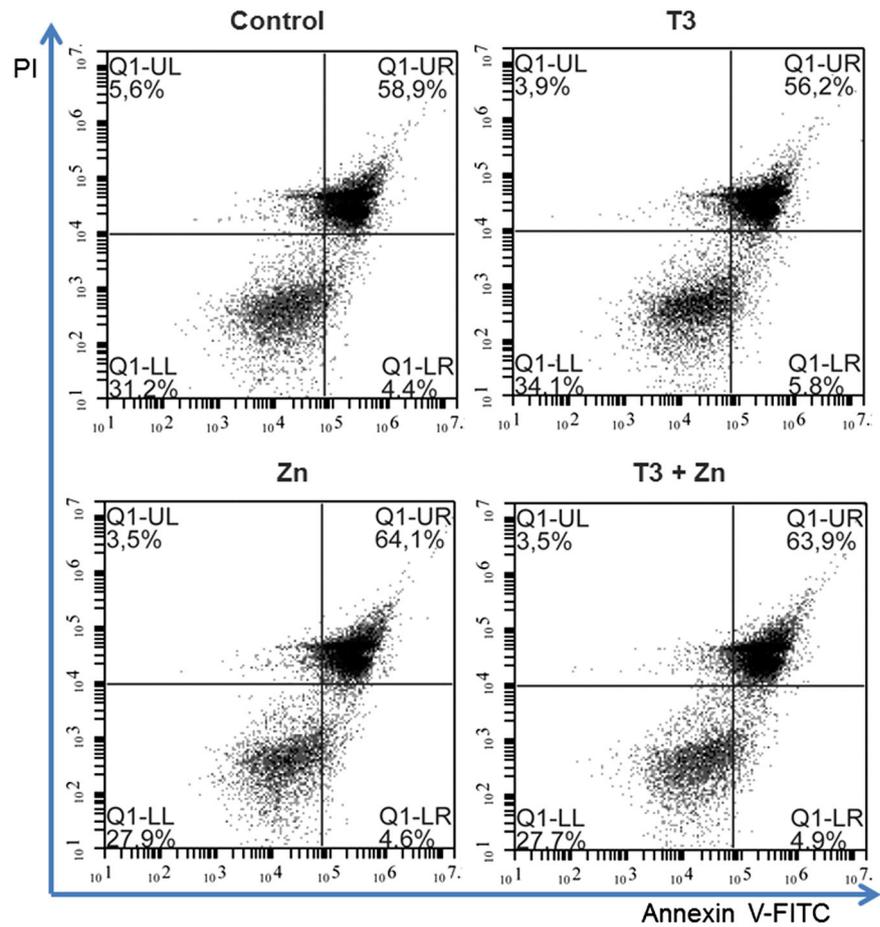
Since T-cell functions are critically regulated by changes in zinc concentrations [26], we next analyzed the action of zinc and hormone deficiency on T cell proliferation and viability. Thus, we evaluated T cell proliferation after Con A stimulation. To evaluate zinc deficiency, we used TPEN and DTPA, whereas to evaluate hormone deficiency, cells were cultured in a hormone-free medium. T3 replacement was used to evaluate specific thyroid hormone dependence of the effects observed.

Con A selective stimulation of T lymph node cells was unable to induce cell proliferation in the hormone-free medium (Fig. 2a), an effect that was reversed by the addition of physiological concentrations of T3, but not by zinc supplementation. In addition, in agreement with that described before [27], T3 per se was able to increase the Con A-dependent proliferation of T cells.

In addition, TPEN ($20 \mu\text{M}$) strongly inhibited the proliferation of T cells. The extracellular chelator DTPA was also able to suppress the proliferative response to Con A, but, unlike TPEN, concentrations equal to or larger than $60 \mu\text{M}$ were necessary to observe significant differences (Fig. 2b). The inhibitory effects of both chelators were reversed by addition of zinc in equimolar quantities, but not by T3 supplementation, and zinc alone at those concentrations showed no effect on proliferation.

To further characterize the inhibitory action of these treatments, we next analyzed the possible induction of cell apoptosis. The absence of hormones in the culture medium had no effects on cell apoptosis (Fig. 3). However, the presence of zinc chelators led to an increase in T cell apoptosis, as demonstrated by the evaluation of nuclear morphology by Hoechst 33258 staining, flow cytometry analysis of Annexin V-PI fixed cells, and active caspase 3 levels. Both TPEN and DTPA caused typical apoptotic changes in the nuclear morphology, with pronounced condensation of chromatin and DNA fragmentation (Fig. 4a). Similarly, an increase in the number of double positive Annexin-V-stained and PI-stained cells (Fig. 4b) and an increase in the levels of active caspase 3 (Fig. 4c) were observed under these conditions. All these effects were reversed by zinc addition.

Fig. 3 Effect of thyroid hormone absence on T lymphocyte apoptosis in vitro. Lymph node cells (1×10^6 cells/mL) were cultured in AIM-V, thyroid hormone-free medium (Control), medium plus T3 (T3), medium plus zinc (Zn), or both (T3 + Zn), then labeled with Annexin V-FITC and propidium iodide (PI) as indicated in “Materials and methods”, and analyzed by flow cytometry. Representative dot blots of five independent experiments depicting PI staining (y-axis) vs. Annexin V-FITC (x-axis) are shown. LL indicates double negative viable cells; LR indicates the percentage of early apoptotic cells (Annexin V+ cells); UR indicates the percentage of double positive late apoptotic cells; and UL indicates the percentage of necrotic cells (PI+ cells). The attached table presents data of the five independent experiments as mean \pm standard deviation (SD)



	% Total \pm 2 SD			
	Hormone-free Culture Medium			
	Control	T3	Zn	T3 + Zn
Annexin V- PI-	29.0 \pm 5.40	30.6 \pm 7.20	32.3 \pm 6.80	29.5 \pm 5.60
Annexin V+ PI-	4.80 \pm 1.65	5.00 \pm 1.70	4.20 \pm 1.60	4.70 \pm 1.75
Annexin V+ PI+	60.5 \pm 8.50	59.0 \pm 9.10	62.4 \pm 8.70	61.1 \pm 8.25
Annexin V- PI+	5.10 \pm 1.60	4.70 \pm 1.70	3.90 \pm 1.80	4.00 \pm 1.70

T lymphocyte activity in zinc-deficient and hypothyroid mice

To evaluate whether similar effects on the thyroid status and on the immune response take place with zinc deficiency in vivo, we next developed an experimental model of zinc deficiency. To corroborate that the onset of zinc deficiency was successful, we evaluated the zinc concentration in typical body compartments, such as serum, bone, and lymph nodes. Zinc deficiency led to a dramatic decrease in zinc concentration in lymph nodes and bone (zinc store), but not in serum (Fig. 5). This decrease in zinc concentration was enough to cause a significant reduction in

leukocyte count but preserved other hematological parameters, preventing more serious health impairment.

The impact of zinc deficiency on T lymphocyte activity was evaluated on Con A-induced proliferation and apoptosis and compared to that observed in our experimental model of hypothyroidism. In vivo zinc deficiency led to Con A-dependent T cell proliferation impairment, similar to that observed with low circulating levels of thyroid hormones (Fig. 6a). This was related to the induction of T cell apoptosis in zinc-deficient mice, but not in hypothyroid ones (Fig. 6b), thus indicating that zinc deficiency during a hypothyroid status would be related to defective T-lymphocyte signaling as indicated before [2].

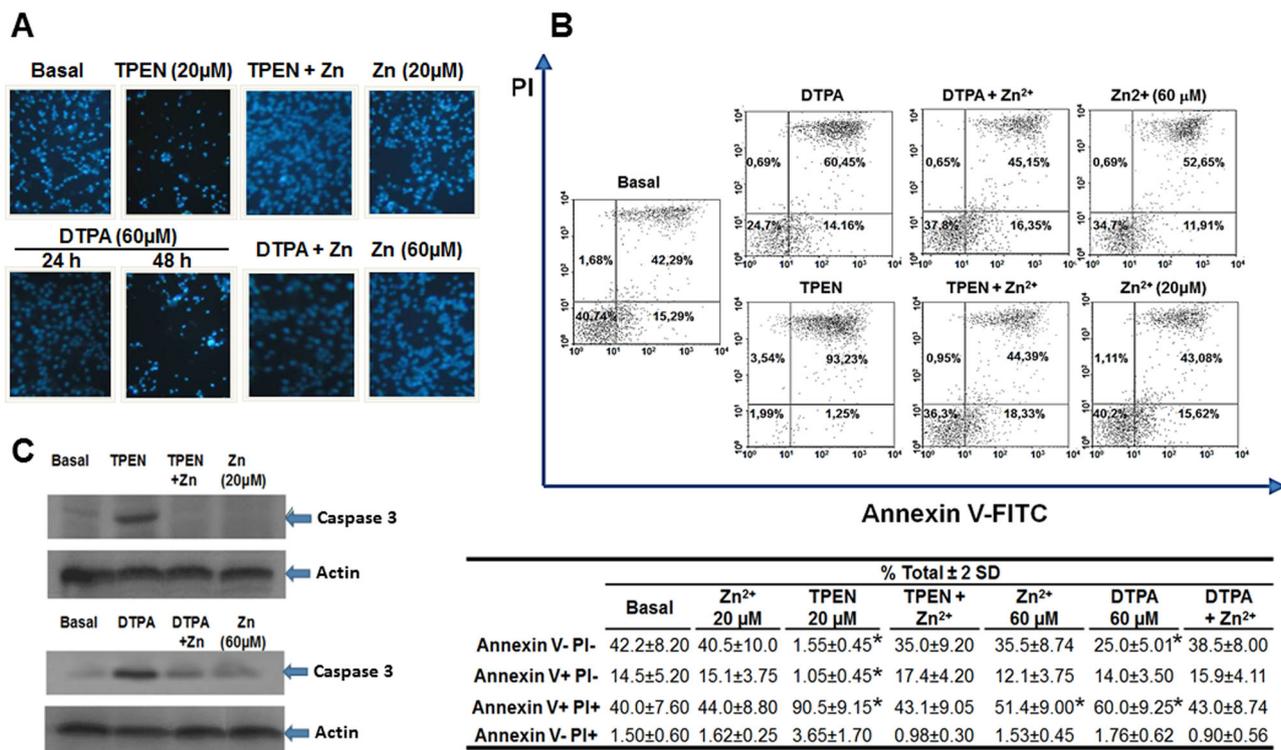


Fig. 4 Effect of zinc deficiency on T lymphocyte apoptosis in vitro. Lymph node cells (1×10^6 cells/mL) were either untreated (basal) or treated with TPEN (20 μ M) or DTPA (60 μ M), with the correspondent chelator plus equimolar concentration of Zn or with Zn alone. **a** Images under fluorescence microscope from fixed cells stained with Hoechst 33258, representative of five independent experiments. Condensed pyknotic nuclei are distinguished in the apoptotic T lymphocytes. **b** Flow cytometry analysis of cells with dual staining shown by representative

dot blots depicting PI (y-axis) vs. Annexin V-FITC (x-axis) with the percentages of cells indicated on each quadrant. Data of five independent experiments are shown on the attached table as the mean of total percentages \pm standard deviation (SD) of viable cells (Annexin V- PI-), early apoptotic cells (Annexin V+ PI-), late apoptotic cells (Annexin V+ PI+) and necrotic cells (Annexin V- PI+), all listed according to the kind of diet or thyroid status. *Differs from control with $p < 0.01$

So, the possible involvement of intracellular signals, activated by Con A stimulation, was evaluated in T lymphocytes from hypothyroid mice both before and after T3 or zinc supplementation. PTU-treated mice showed decreased levels of PKC θ and PKC α (Fig. 7a) and decreased translocation of PKC to cell membranes respect to euthyroid control mice (Fig. 7b). Also, lower levels of p-ERK were found after Con A stimulation (Fig. 7c). Both T3 and zinc supplementation were able to restore the PKC levels and activity and ERK phosphorylation to control values after Con A stimulation.

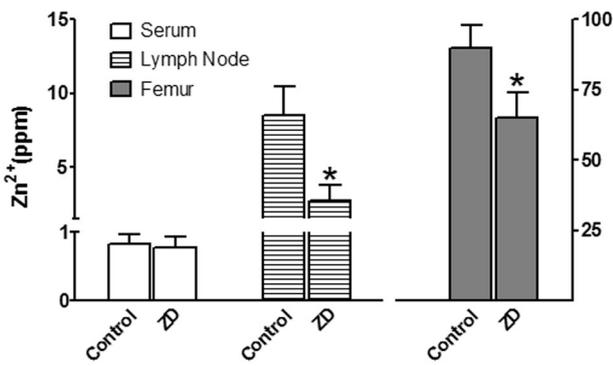
Functional consequences of zinc supplementation in T lymphocytes from hypothyroid mice

To analyze the participation of zinc deficiency in hypothyroid-mediated suppression of T cell activity more deeply, we also evaluated whether zinc was able to restore the function of hypothyroid T cells. Thus, T cell proliferation was evaluated in PTU-treated mice both before and after zinc supplementation and compared with T cells from PTU + T3-treated mice. Zinc supplementation was able to

re-establish proliferative responses to Con A in a manner similar to T3 supplementation (Fig. 8a). Similar results were obtained when analyzing an antigen-specific response in an allogeneic mixed lymphocyte reaction, where T cells from PTU-treated mice had lower reactivity than those of controls and both T3 and zinc supplementation were able to restore the proliferative allogeneic response (Fig. 8b), pointing out the importance of zinc levels in hypothyroid mice.

Discussion

Hypothyroidism is well recognized to cause immunosuppression and is suggested to be able to result in acquired zinc deficiency. In turn, the absence of this trace element most severely affects the immune response. The main finding of our study shows that low circulating levels of thyroid hormones lead to decreased levels of zinc, which, in turn are related to low T lymphocyte activity, pointing out the important role of this element in hypothyroidism-associated immunosuppression. Thus, experimental hypothyroidism induced by PTU



Hematological Parameters			
	Hemoglobin (g/dl)	Hematocrit (%)	Leukocyte count (cells/ml)
Control Diet	14.90±1.43	47±5	6992±1068
ZD Diet (Zinc Deficient)	14.60±1.50	45±5	2630±670**

Fig. 5 Indicators of mineral status in mice fed with a control or a zinc-deficient diet. The zinc content values in serum (empty bars), lymph nodes (horizontal stripes), and femur (gray solid bars) of mice fed with a control diet (Control) and of mice fed with a zinc-deficient diet (ZD) are depicted in the bar graph and expressed in parts per million (ppm). The attached table shows three hematological parameters, hemoglobin, hematocrit, and leukocyte count for both Control and ZD mice. *Differs from the control with $p < 0.01$. **Differs from the control with $p < 0.001$

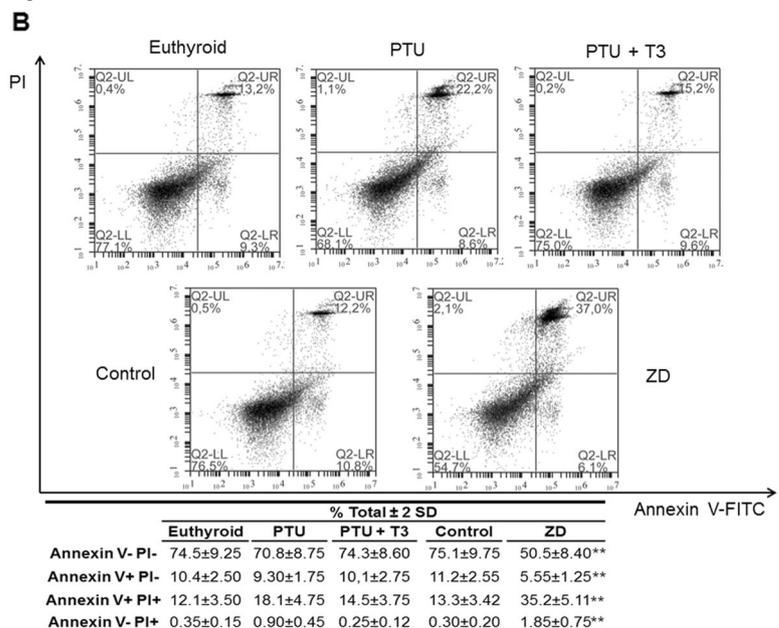
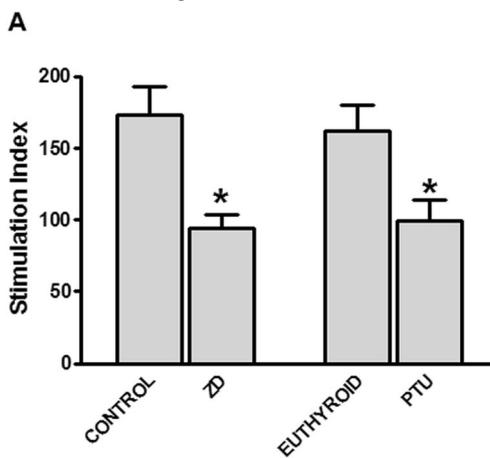


Fig. 6 Effect of the zinc-deficient diet and thyroid status on T lymphocyte proliferation and apoptosis. **a** Lymph node cells (1×10^6 cells/mL) from mice fed with a control or a zinc-deficient diet (ZD) and from euthyroid or hypothyroid mice (PTU) were cultured in RPMI 1640 medium with 10% FCS and antibiotics in the presence or absence of Con A ($2 \mu\text{g/mL}$). As indicated in “Materials and methods”, proliferation was evaluated by [^3H]-thymidine incorporation and results of six experiments performed in triplicate are expressed as stimulation index ± SEM. *Differs from control value with $p < 0.01$. **b**

leads to a loss in the zinc deposits in femur and lymph nodes, without affecting the circulating levels of this trace element. These unmodified levels of serum zinc levels in hypothyroid mice would be related to the length of the treatment with PTU (14 days), as it has been shown that treatment with this anti-thyroid drug for 4 weeks leads to a decrease in the serum levels of zinc in rats as well [28, 29]. We chose 14 days of PTU treatment as the time point of our studies for several reasons. When PTU was administered to mice, serum TSH levels became significantly higher than control at day 14, and this was coincident with the maximum inhibitory effect on T cell reactivity upon mitogen stimulation. Also, the levels of zinc have been found to be directly related to serum-TSH in thyroid tissues [30], and at 21 days of PTU treatment higher levels of TSH were found than in 14-days PTU-treated hypothyroid mice. Levels of zinc in hypothyroid mice were reversed by both T3 and zinc supplementation, but only hormone administration but not zinc was able to restore the thyroid status. Similarly, a previous report showed no modification of TSH, T3, or T4 levels after zinc supplementation in competitive athletes [31]. However, another report showed that the zinc status and serum thyroid hormone levels in goitrous patients improved after 6 months of treatment with zinc supplementation [32], thus strengthening the relationship between the thyroid status and zinc levels.

Flow cytometry analysis of cells with dual staining shown by representative dot blots depicting PI (y-axis) vs. Annexin V-FITC (x-axis) with the percentages of cells indicated on each quadrant. Data of five independent experiments are shown on the attached table as the mean of total percentages ± standard deviation (SD) of viable cells (Annexin V- PI-), early apoptotic cells (Annexin V+ PI-), late apoptotic cells (Annexin V+ PI+) and necrotic cells (Annexin V+ PI+), all listed according to the kind of diet or thyroid status. **Differs from control with $p < 0.01$

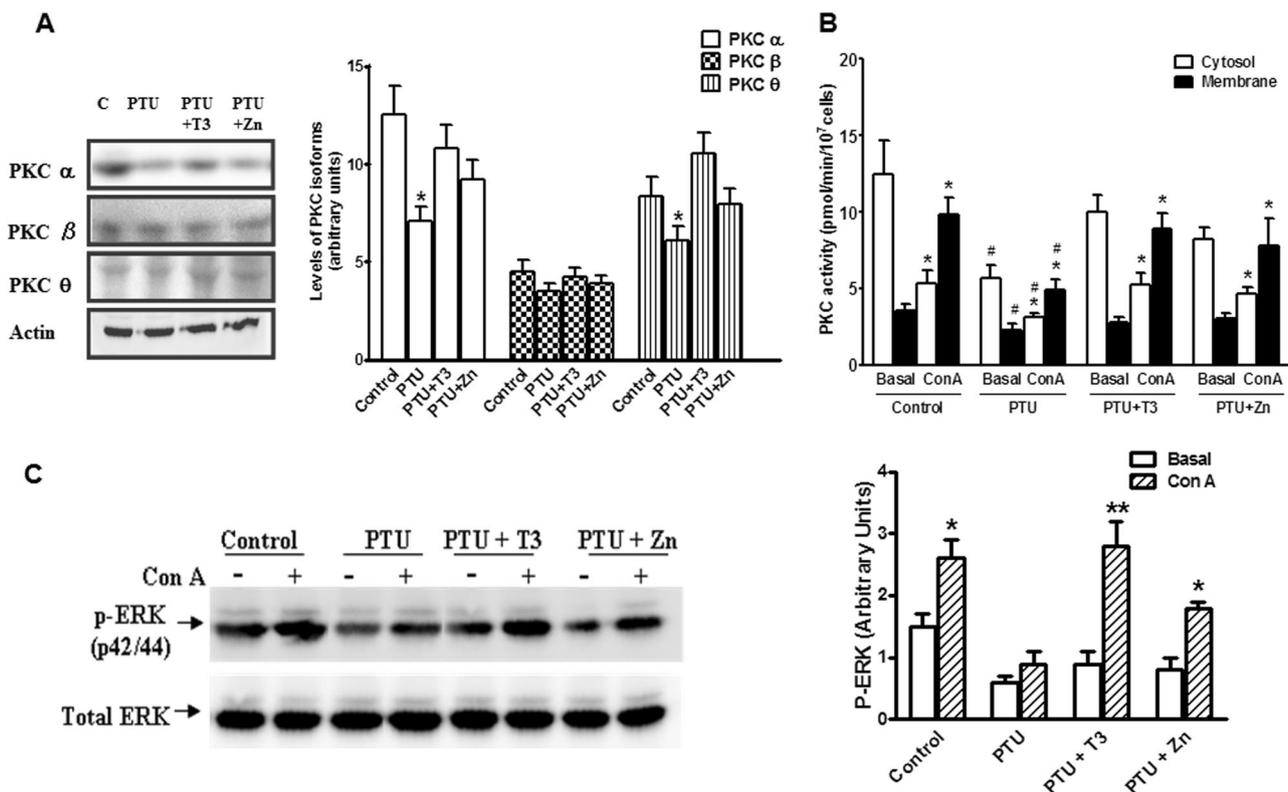


Fig. 7 Intracellular signals activated by Con A stimulation in T lymphocytes from hypothyroid mice before and after T3 or zinc supplementation. The involvement of intracellular signals (PKC and ERK) was evaluated in T lymphocytes from hypothyroid (PTU-treated), PTU + T3-treated, and PTU + Zn-treated mice. **a** Western blot of PKC isoenzymes α (empty bars), β (square pattern), and θ (vertical stripes) in T cells representative of five independent experiments, including the density analysis performed with the ImageQuant software, using actin as loading control. *Differs from control with $p < 0.01$. **b** PKC activity in the cytosol (empty bars) and membrane (solid black bars) was

measured by ³²P incorporation to H₁ histone from [γ-³²P]-ATP, as indicated in “Materials and methods”. *Differs from own basal value with $p < 0.01$; #Differs from control mice with $p < 0.05$. **c** Western blot for ERK phosphorylation in T cells in the presence (bars filled with diagonal stripes) or absence (empty bars) of Con A, representative of three independent experiments, including density analysis with the ImageQuant software corrected with Total ERK expression. *Differs from the correspondent basal value with $p < 0.01$. #Differs from PTU value with $p < 0.01$

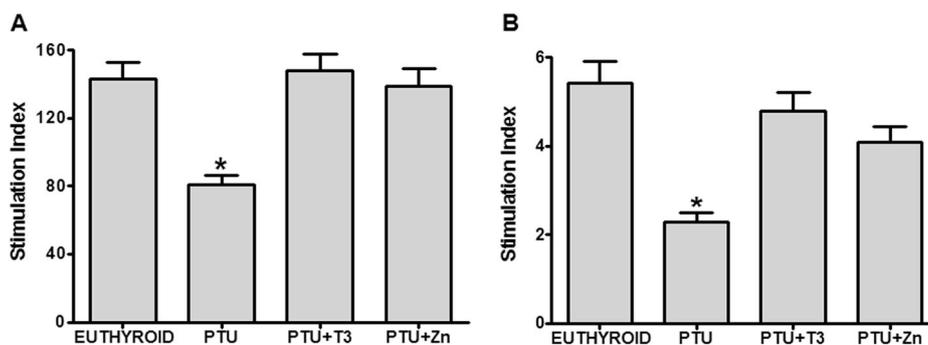


Fig. 8 Effect of Zn restoration on impaired T cell activity in hypothyroid mice. Lymph node cells (1×10^6 cells/mL) from euthyroid control mice, PTU-treated mice, or PTU-treated mice either under hormone replacement treatment or supplemented with zinc, were cultured in RPMI 1640 medium with 10% FCS and antibiotics in the presence or absence of the mitogen Con A (2 μg/mL) **a** or with or

without lymph node cells from C3H mice as allogeneic stimulus **b**. As indicated in “Materials and methods”, proliferation was evaluated by [³H]-thymidine incorporation and results of six experiments performed in triplicate are expressed as stimulation index ± standard error of the mean (SEM)

Both thyroid hormone and zinc deficiency *in vitro* and *in vivo* showed to negatively affect T cell reactivity, but only zinc deficiency led to T cell apoptosis, as shown by nuclear morphology, Annexin-PI staining, and increased levels of active caspase. Several reports have indicated that zinc chelators induce T cell apoptosis [33, 34] and that zinc deficiency *in vivo* leads to impairment of immunity [7, 35]. However, low levels of thyroid hormones have no effect on cell apoptosis and this is in agreement with the fact that both hyperthyroidism [36, 37] and *in vitro* long-lasting treatment of T cells with thyroid hormones [37, 38] lead to cell apoptosis. So, these results point out that hypothyroidism-mediated zinc deficiency would be related to defective T lymphocyte signaling, as previously demonstrated for zinc deficiency *in vivo* [11]. The evaluation of the activation of intracellular signals triggered by polyclonal activation in T cells from hypothyroid mice before and after T3 or zinc supplementation demonstrated that, similarly to T3 supplementation, zinc was able to restore the decreased activation of PKC and ERK induced by hypothyroidism. Our results also showed that the levels of PKC isoenzymes involved in T cell activation [39] in T cells from hypothyroid mice were decreased, and that these decreased levels were reversed to euthyroid values by both T3 treatment and zinc supplementation. Zinc has been reported to affect components of the T cell receptor signaling pathway [11, 40], similarly to that observed in hypothyroid T lymphocytes [2]. In fact, zinc treatment stimulates the kinase activity of PKC, its affinity to phorbol esters, and its binding to the plasma membrane, while zinc chelators inhibit the induction of these events (as reviewed in ref. [9]). Also, low zinc concentrations (nanomolar) promote the dephosphorylation of ERK1/2 in human T lymphocytes upon CD3 stimulation [41]. So, it is possible that zinc deficiency in hypothyroid conditions account for altered signaling events in these cells. Moreover, the participation of zinc deficiency in the hypothyroidism-mediated alteration of T cell function was demonstrated by the reestablishment of T cell proliferative responses not only to polyclonal activation by Con A, but also to an antigenic-stimulus in a mixed-lymphocyte reaction. It is worth noting that high concentrations of zinc (about eight times higher than the physiological level) lead to cytotoxic effects, with impairment of all T cell functions [5], whereas pharmacological doses of zinc (three to four times higher than the physiological level) have been shown to suppress the allogeneic reaction, without affecting the antigenic response [42–44]. We here restored the physiological levels of zinc, as shown by the values of zinc obtained in femur, serum, and lymph nodes, which are probably necessary to restore the alloantigenic response.

Our results show, for the first time, the relationship between hypothyroidism-mediated immunosuppression and

the decreased levels of zinc in organs of the immune system and strengthen the importance of zinc levels in hypothyroid mice. Our results are in agreement with a previous study in a model of wound healing in rats, which demonstrated that zinc supplementation gives better results than hormone replacement alone [45]. Moreover, zinc supplementation has also been demonstrated to improve thyroid function in children with Down's syndrome with plasma zinc levels below the normal range [46] and with increased susceptibility to infections [47]. The results of these previous studies suggest that zinc deficiency affects the immune responses of these children. In agreement with these previous findings, our results strongly suggest that measuring zinc levels and restoring them, when necessary, to maintain an efficient immune response in hypothyroid patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All studies using animals were performed in accordance with the ARRIVE Guidelines [21] and all the experimental protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the School of Pharmacy and Biochemistry of the University of Buenos Aires and the CICUAL of BIOMED. This article does not contain any studies with human participants performed by any of the authors.

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