Research article

Coordination of hypothalamic and pituitary T3 production regulates TSH expression

Tatiana L. Fonseca,[1] Mayrin Correa-Medina,[1] Maira P.O. Campos,[1] Gabor Wittmann,[2] Joao P. Werneck-de-Castro,[1,3] Rafael Arrojo e Drigo,[1] Magda Mora-Garzon,[1] Cintia Bagne Ueta,[1] Alejandro Caicedo,[1] Csaba Fekete,[2,4] Balazs Gereben,[4] Ronald M. Lechan,[2] and Antonio C. Bianco[1]

[1]Division of Endocrinology, Diabetes and Metabolism, Miller School of Medicine, University of Miami, Miami, Florida, USA. [2]Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, Tufts Medical Center, Boston, Massachusetts, USA. [3]Institute of Biophysics Carlos Chagas Filho and School of Physical Education and Sports, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. [4]Department of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary.

Type II deiodinase (D2) activates thyroid hormone by converting thyroxine (T4) to 3,5,3′-triiodothyronine (T3). This allows plasma T4 to signal a negative feedback loop that inhibits production of thyrotropin-releasing hormone (TRH) in the mediobasal hypothalamus (MBH) and thyroid-stimulating hormone (TSH) in the pituitary. To determine the relative contributions of these D2 pathways in the feedback loop, we developed 2 mouse strains with pituitary- and astrocyte-specific *D2* knockdown (pit-*D2* KO and astro-*D2* KO mice, respectively). The pit-*D2* KO mice had normal serum T3 and were systemically euthyroid, but exhibited an approximately 3-fold elevation in serum TSH levels and a 40% reduction in biological activity. This was the result of elevated serum T4 that increased D2-mediated T3 production in the MBH, thus decreasing *Trh* mRNA. That tanycytes, not astrocytes, are the cells within the MBH that mediate T4-to-T3 conversion was defined by studies using the astro-*D2* KO mice. Despite near-complete loss of brain D2, tanycyte D2 was preserved in astro-*D2* KO mice at levels that were sufficient to maintain both the T4-dependent negative feedback loop and thyroid economy. Taken together, these data demonstrated that the hypothalamic-thyroid axis is wired to maintain normal plasma T3 levels, which is achieved through coordination of T4-to-T3 conversion between thyrotrophs and tanycytes.

Introduction

The negative feedback loop between circulating thyroid hormone and the neuroendocrine system (hypothalamus and pituitary gland) is the fundamental mechanism preserving thyroid hormone homeostasis. During iodine deficiency, there is decreased synthesis of thyroid hormone that leads to a reduction in plasma thyroxine (T4), rapidly activating secretion of hypothalamic thyrotropin-releasing hormone (TRH) and pituitary thyroid-stimulating hormone (TSH), which stimulates the thyroid to grow and normalize thyroid hormone synthesis and secretion (1). As with T4, a reduction in plasma 3,5,3′-triiodothyronine (T3), the biologically active thyroid hormone, also triggers the feedback mechanism and activates TRH and TSH secretion. This has been well documented in patients acutely given large amounts of propylthyouracyl (PTU), which inhibits peripheral synthesis of T3 (2).

Given that the *TSHB* and *TRH* genes are negatively regulated by T3 (and not T4), it is crucial that T4 be converted to T3 in order to activate the negative feedback mechanism. The presence of type 2 deiodinase (D2), which converts T4 to T3 in the brain, is thought to be critical for this mechanism (3). D2 is coexpressed with TSH in pituitary cells (4) and is also expressed in astrocytes in thyroid hormone–sensitive regions of the brain, such as the cerebral cortex and hippocampus (5, 6), but its presence has also been documented in astrocytes in the mediobasal hypothalamus (MBH) (7). However, the highest expression of D2 in the MBH is in tanycytes, specialized ependymal cells lining the floor and infralateral wall of the third ventricle. In fact, tanycytes and TRH-expressing neurons in the paraventricular nucleus (PVN) are intermingled in the external zone of the median eminence (8, 9), providing the physical proximity for D2-mediated feedback signaling. Thus, hypothalamic D2 (astrocytic and tanycytic) has been proposed to have an important role in feedback regulation of thyroid hormone on hypophysiotropic TRH (10), but the relative importance of D2 expression in astrocytes versus tanycytes is not known.

Global inactivation of the *D2* gene (also known as *Dio2*) in mice leads to elevated serum TSH and T4, but serum T3 remains normal, a phenotype compatible with an impaired transduction mechanism in T4-mediated TSH suppression (11). A very similar phenotype was also observed in mice treated with amiodarone, a noncompetitive inhibitor of D2 (12). However, neither the global *D2* KO mouse nor the amiodarone-treated mouse models allow for a mechanistic understanding of the relative roles played by pituitary versus hypothalamic D2 in the thyroid hormone–mediated feedback mechanism. In addition, an unexpected finding was that both of these animal models exhibit normal PVN TRH expression, calling into question the role played by hypothalamic D2 in the T4-mediated feedback mechanism.

To address these questions, we developed the floxed *D2* (*D2*^{fl}) mouse and used it here to generate 2 mouse strains: one exhibiting near-null *D2* activity in the pituitary gland (referred to herein as pit-*D2* KO mice), and one lacking *D2* activity selectively in astrocytes (astro-*D2* KO mice). We showed that the disruption in the T4-mediated mechanism of the pit-*D2* KO mouse was bypassed by elevated serum T4 and reduced PVN TRH expression, defining a critical role for the MBH D2 pathway in the feedback mechanism. The phenotypic characterization of astro-*D2* KO mice indicated that tanycytes, rather than astrocytes, are the cell type within the MBH, where this critical D2-mediated T4-to-T3 conversion takes place.

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J Clin Invest.* 2013;123(4):1492–1500. doi:10.1172/JCI61231.

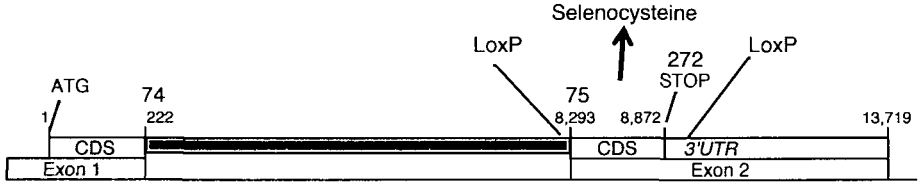

Figure 1
D2 gene. The 2 loxP sites flanking the selenocysteine insertion site, base numbers, and amino acid positions are indicated. ATG, methionine; CDS, coding sequence.

Results

Pituitary-specific D2 inactivation disrupts the hypothalamic-pituitary-thyroid axis (HPT). To determine the importance of pituitary versus hypothalamic D2 in HPT regulation, we used p-flox technology to allow for tissue-specific inactivation of the *D2* gene based on selective expression of Cre recombinase (see Methods and Figure 1). We used mice expressing Cre recombinase under the pituitary-specific glycoprotein hormone α-subunit promoter (CGA-cre mice), which led to the generation of pit-*D2* KO mice (CGA-cre/$D2^{f/f}$), a strain in which D2 activity was markedly reduced in the pituitary while remaining unaffected in the other D2-expressing tissues (i.e., cerebral cortex, hypothalamus, hippocampus, brown adipose tissue (BAT), and placenta; Figure 2, A–E, and Supplemental Figure 1M; supplemental material available online with this article; doi:10.1172/JCI61231DS1). D3 activity was unaffected throughout the brain (Supplemental Figure 1, A–C).

The pit-*D2* KO mouse exhibited about 2.8-fold higher serum TSH levels and approximately 40% higher serum T4 levels compared with CGA-cre littermate controls, whereas serum T3 concentrations were not different (Figure 2, F, G, and I). No differences in ^{125}I-T4 charcoal uptake were detected between pit-*D2* KO and littermate control animals (Figure 2H), which indicates that the higher serum T4 was associated with elevated free T4 levels. However, that the pit-*D2* KO mouse is systemically euthyroid was confirmed by studying tissue markers of thyroid hormone action. Total BW and individual organ weights in 2-month-old pit-*D2* KO animals were similar to those of CGA-cre controls (Table 1). In addition, there were no differences in total skeleton bone mineral density (BMD), lean body mass (LBM), or oxygen consumption (VO_2) (Tables 2 and 3), all sensitive markers of thyroid hormone action (13, 14). The very sensitive genetic marker of thyroid hormone signaling, liver D1 activity (15), was unaffected in pit-*D2* KO mice (Figure 2J). The only noticeable difference was the approximately 20% lower body fat mass in pit-*D2* KO animals (Table 2). Given that CGA is also expressed in gonadotrophs, we confirmed that the gonadal axis was pre-

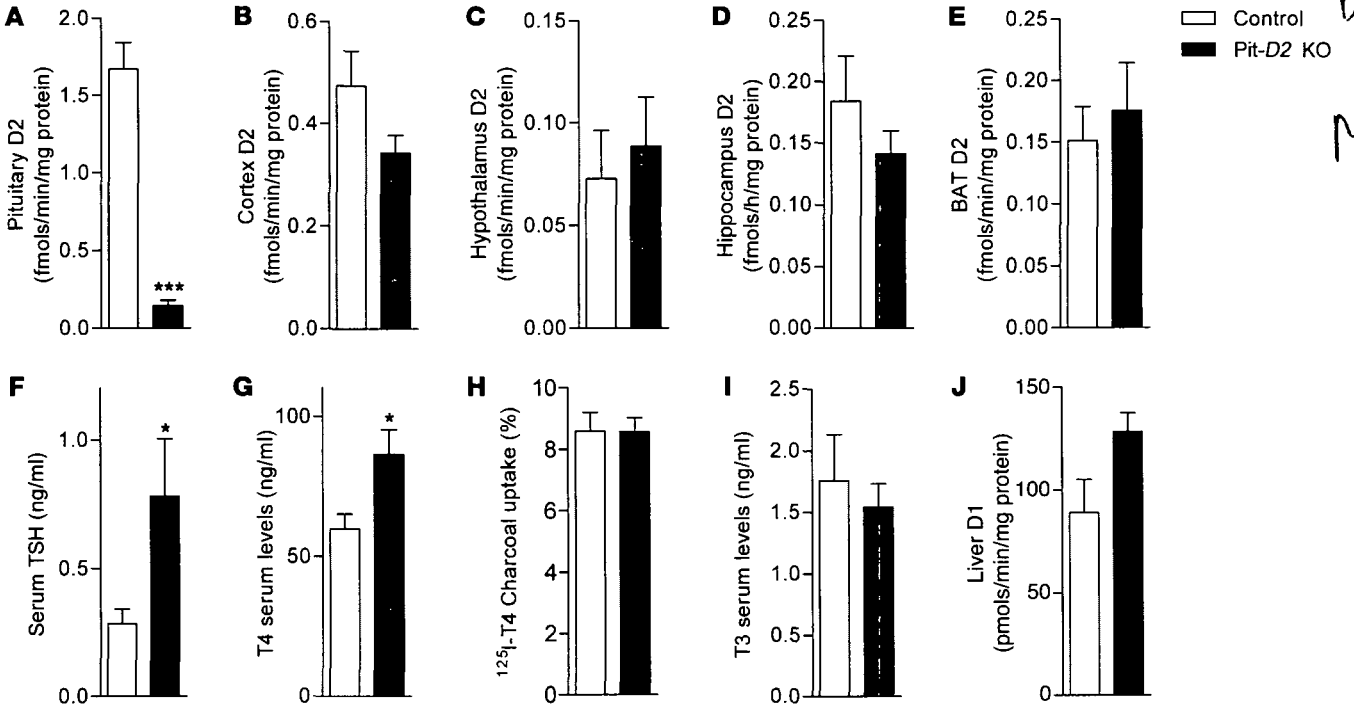

Figure 2
Deiodinase activity and thyroid function tests in pit-*D2* KO mice versus CGA-cre controls. (**A–E**) D2 activity in sonicates of pituitary, cortex, hypothalamus, hippocampus, and BAT (n = 3–5). (**F** and **G**) Serum TSH and T4 levels (n = 9–10). (**H**) ^{125}I-T4 charcoal uptake in the serum (n = 9). (**I**) Serum T3 levels (n = 9–10). (**J**) Liver D1 activity (n = 3–5). *P < 0.05, ***P < 0.001 vs. control.

Table 1
BW and organ weights

Group	BW (g)	Organ weights (mg/mg BW)			
		Brain	Epididymal fat	Liver	Muscle
CGA-cre control	27 ± 3.8	14 ± 1.5	27 ± 10.2	44 ± 6.7	5.3 ± 0.7
Pit-*D2* KO	29 ± 2.0	12 ± 0.58	24 ± 4.2	49 ± 3.1	5.6 ± 0.3
GFAP-cre control	28 ± 2.0	15 ± 1.8	13 ± 4.4	50 ± 6.3	5.4 ± 0.4
Astro-*D2* KO	29 ± 3.4	15 ± 2.2	20 ± 1.4	45 ± 3.6	5.8 ± 0.3

Values are mean ± SD of 5 animals.

served in pit-*D2* KO mice, as shown by their normal serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as well as normal gonadal weight compared with controls (Supplemental 1, G–J).

We next hypothesized that reducing D2 expression in thyrotrophs would render these cells less sensitive to T4-mediated signaling, as has been proposed for the global *D2* KO mouse (11). This was tested by assessing acute TSH responsiveness to T4 or T3. In control animals, administration of T4 elevated serum T4 approximately 7-fold and serum T3 approximately 3-fold, but reduced serum TSH by about 10-fold, to near-suppression levels (0.08 ± 0.002 ng/ml) (Figure 3, A–C). The drop in serum TSH occurred as a result of T4-to-T3 conversion in the thyrotroph and elevated serum T3. In contrast, administration of the same dose of T4 in pit-*D2* KO animals only reduced serum TSH about 4-fold (0.5 ± 0.11 ng/ml), despite similar elevations in serum T3 and serum T4 compared with control animals (Figure 3, A–C). This can only be attributed to impaired thyrotroph T4-to-T3 conversion, given the identical T3-induced TSH suppression in both groups (Figure 3C).

Notably, there were no differences between groups in 30-minute TRH-stimulated TSH secretion: in all animals, serum TSH increased to similar levels, although the calculated fold response to TRH was greater in the control animals (~7- versus ~2-fold), given the higher baseline serum TSH in pit-*D2* KO mice (Figure 3D). Furthermore, serum levels of T4 and T3 remained unaffected in these TRH-treated animals (Supplemental Figure 1, K and L).

To test D2-mediated T4 signaling in pit-*D2* KO MBH, we studied PVN *Trh* mRNA levels in both mouse groups by in situ hybridization and found an approximate 50% reduction in pit-*D2* KO mice (Figure 3, E and F). However, *Trh* mRNA levels remained unaffected in the lateral hypothalamus of pit-*D2* KO mice (Figure 3G). Given that MBH D2 remained unaffected in pit-*D2* KO animals (Figure 2C), the elevated serum T4 and consequent increase in MBH D2-generated T3 explains the decreased TRH expression. This notion was supported by the finding that the difference in PVN *Trh* mRNA was minimized in control versus pit-*D2* KO animals that were made systemically hypothyroid (105 ± 13 vs. 76 ± 20 AU; $P > 0.05$). Notably, the expression of key enzymes involved in processing the TRH peptide remained unaffected in the pit-*D2* KO hypothalamus (Figure 3, H and I), which indicates that the decreased *Trh* mRNA is not neutralized by posttranslational mechanisms, thereby decreasing TRH peptide production. This finding illustrates a marked difference between the pit-*D2* KO and global *D2* KO mouse models, defining a major role for MBH D2 in the T4-mediated TRH feedback mechanism.

Reduced PVN TRH expression is expected to decrease hypothalamic stimulation of the thyrotroph; indeed, *Tsha* mRNA levels were reduced in pit-*D2* KO mice (Figure 3J). However, pituitary *Tshb* mRNA levels were not affected in this mouse model (Figure 3K), presumably because the negative effects of T3 on TSHβ are greater than those on TSHα (16, 17). In other words, whereas *Tshb* was less stimulated by the hypothalamus, it was also less repressed by T3, due to inactivation of the local D2 pathway.

How could an elevation in serum TSH be reconciled with normal pituitary *Tshb* mRNA levels? We hypothesized that the reduction in pit-*D2* KO PVN TRH expression prolonged TSH half-life and decreased its biological activity by altering the TSH glycosylation pattern (18). To test this hypothesis, we used a previously characterized in vitro assay to estimate pit-*D2* KO TSH biological activity. Inducible cAMP production in CHO cells stably expressing TSH receptors approximately doubled with pit-*D2* KO sera and decayed linearly with sera dilution (Figure 4, A and B). However, relative to the higher gravimetric amounts of TSH used in the assay (Figure 4C), pit-*D2* KO TSH exhibited about 40% lower biological activity (Figure 4D). This is supported by our observations that thyroid structure, 2-hour ^{125}I uptake, and mRNA levels of sodium/iodide symporter (*NIS*) and thyroglobulin (*Tg*) were not different from those in control animals (Figure 4, E–I), despite the much higher serum TSH (Figure 2F). Notably, the pit-*D2* KO thyroid gland was slightly enlarged, but the elevation in serum T4 and T3 observed 2 hours after bovine TSH administration was similar in both mouse groups (Figure 4, J and K).

Astrocyte-specific D2 inactivation does not affect HPT. The MBH contains high D2 activity levels, expressed in both astrocytes (7) and tanycytes (5, 6). To elucidate the contribution of astrocyte versus tanycyte D2 in the HPT, we created a second mouse strain expressing Cre recombinase driven by the glial fibrillary acidic protein (GFAP) promoter (GFAP-cre mice), a well-known marker for matured astrocytes that is rarely expressed in tanycytes (Figure 5A). Indeed, in the resulting astro-*D2* KO mice (GFAP-cre/*D2*^{fl}; see Methods), *D2* mRNA levels in tanycytes were not altered compared with GFAP-cre littermate controls (Figure 5, B and C). However, astro-*D2* KO mice exhibited a marked decrease in brain D2 activity in areas where D2 is expressed in glial cells (such as the cerebral cortex, hippocampus, and cerebellum), but not in the hypothalamus or pituitary gland, where D2 activity remained unaffected (Figure 6, A–E). In addition, D2 activity was also preserved outside the central nervous system (e.g., in BAT; Figure 6F). As with the pit-*D2* KO mouse, D3 activity was not affected throughout the astro-*D2* KO brain (Supplemental Figure 1, D–F).

Table 2
Body composition

Group	BMD (mg/cm^2)	LBM (g)	Fat mass (g)
CGA-cre control	51.8 ± 3.6	18.6 ± 2.4	6.3 ± 1.3
Pit-*D2* KO	51.5 ± 2.4	19.0 ± 1.3	5.0 ± 0.7^A
GFAP-cre control	55.7 ± 2.1	22.4 ± 1.8	7.2 ± 1.4
Astro-*D2* KO	52.8 ± 1.4^A	21.5 ± 1.8	6.2 ± 1.3

Values are mean ± SD of 5–7 animals. $^A P < 0.05$ vs. respective control.

Table 3
Indirect calorimetric analysis

	VO_2 (ml/kg/h)	
Group	**Light**	**Dark**
CGA-cre control	7.3 ± 1.1	8.8 ± 1.6
Pit-*D2* KO	8.4 ± 1.4	9.8 ± 1.6
GFAP-cre control	6.7 ± 1.0	7.6 ± 0.9
Astro-*D2* KO	6.7 ± 1.7	8.4 ± 2.1

Values are mean ± SD of 6 animals. All entries are AU from AUC for individual animals.

We found that astro-*D2* KO mice had normal HPT and thyroid economy, as evidenced by their normal serum levels of TSH, T4, and T3; thyroid morphology; and PVN *Trh* mRNA expression (Figure 6, G–L). That the animals were systemically euthyroid was confirmed by analysis of multiple tissue markers of thyroid hormone action in 2-month-old animals, including BW, organ weights, LBM, fat mass, and VO_2 (Tables 1–3). Finally, liver D1 activity was unaffected in astro-*D2* KO mice (Figure 6M). In addition, we tested the relative sensitivity of TSH secretion to T4 or T3 and found that administration of either hormone suppressed serum TSH (Figure 6N), indicative of preserved HPT sensitivity to T4 in astro-*D2* KO mice. As with pit-*D2* KO animals, in both experiments, there were no differences between the serum levels of T4 and T3 achieved after administration of T4 or T3 (data not shown). An unexpected finding was a lower total body BMD in astro-*D2* KO versus GFAP-cre control mice (Table 2), which suggests that brain D2 could be involved in bone metabolism or that D2 expression in certain GFAP-positive bone cells could play a previously unappreciated role in bone mass.

Discussion

The development and phenotypic characterization of 2 strains of tissue-specific *D2* KO mice revealed a novel aspect of HPT regulation, namely, that a decrease in D2-generated T3 in the thyrotrophs can be compensated for by changes in hypophysiotropic *Trh* gene expression, presumably via an increase in D2-generated T3 in the MBH. Decreasing TRH expression in the pit-*D2* KO mouse nullified any effect of selective pituitary D2 deficiency on *Tshb* gene expression, which remained unaffected (Figure 3K). However, the reduction in *Trh* expression (Figure 3, E and F) is likely to alter the pattern of TSH glycosylation, explaining the observed decrease in TSH biological activity (Figure 4, A–D). MBH D2 expression is present in astrocytes and in tanycytes (5–7). Thus, our observation that thyroid economy was preserved in the astro-*D2* KO mouse (Figure 6, G–I) indicates that tanycytic, and not astrocytic, D2 is required to transduce the

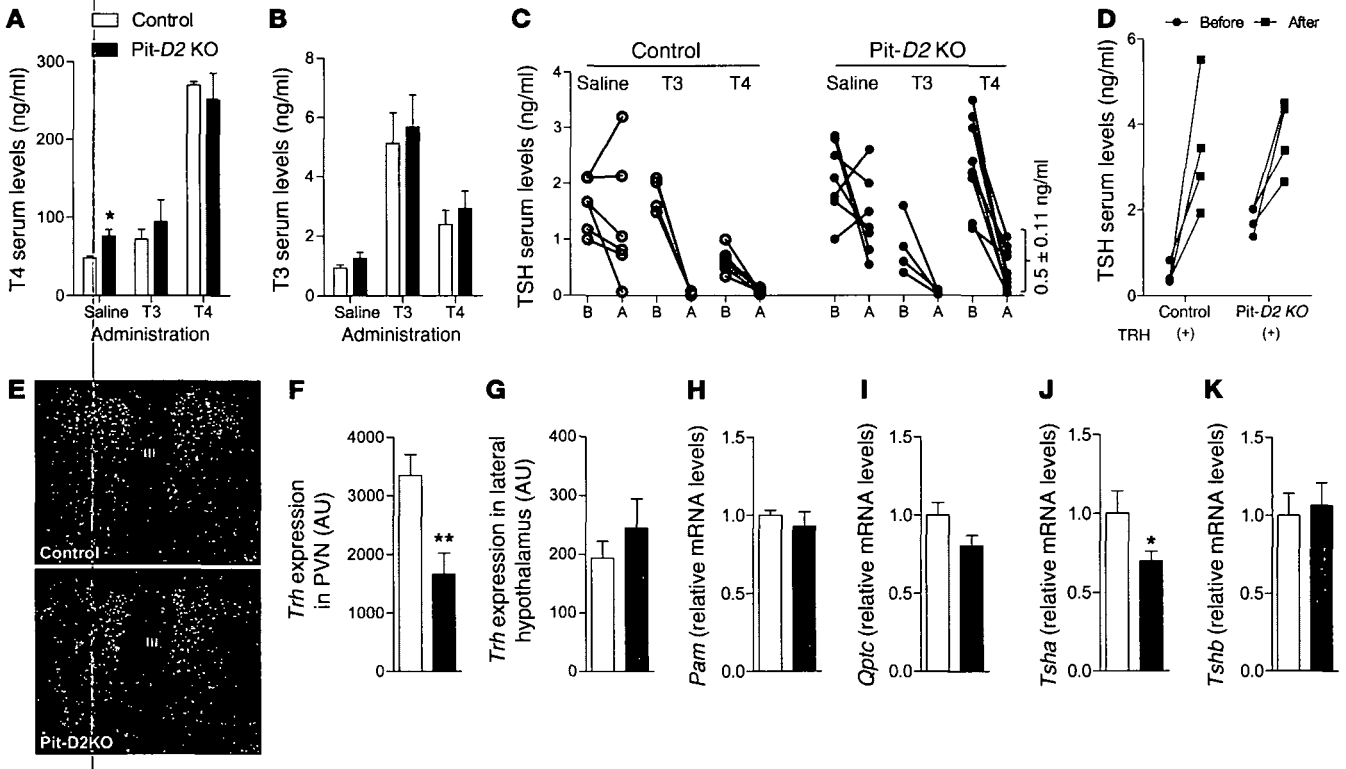

Figure 3
Suppression, stimulation, and mRNA levels of *Tsh* and *Trh* in pit-*D2* KO mice versus CGA-cre controls. (**A** and **B**) Serum T4 and T3 levels after 4-hour administration of saline, T3, or T4 (n = 3–7). (**C**) Serum TSH levels before (B) and after (A) 4-hour administration of saline, T3, or T4 (n = 4–10). Mean ± SEM serum TSH in the pit-*D2* KO group after T4 treatment is indicated at right. (**D**) TSH serum levels before and 30 minutes after TRH stimulation (n = 4). (**E**) Representative in situ hybridization of *Trh* mRNA in the MBH. III, third ventricle. (**F**) Quantification of data in **E** (n = 7–9). (**G**) Quantification of *Trh* mRNA in the lateral hypothalamus (n = 7–9). (**H** and **I**) Relative expression of the TRH biosyntheses enzymes *Pam* and *Qpct* in the hypothalamus (n = 5). (**J** and **K**) Relative mRNA expression of *Tsha* and *Tshb* in the pituitary (n = 5–7). mRNA levels were normalized to *CypA*. $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

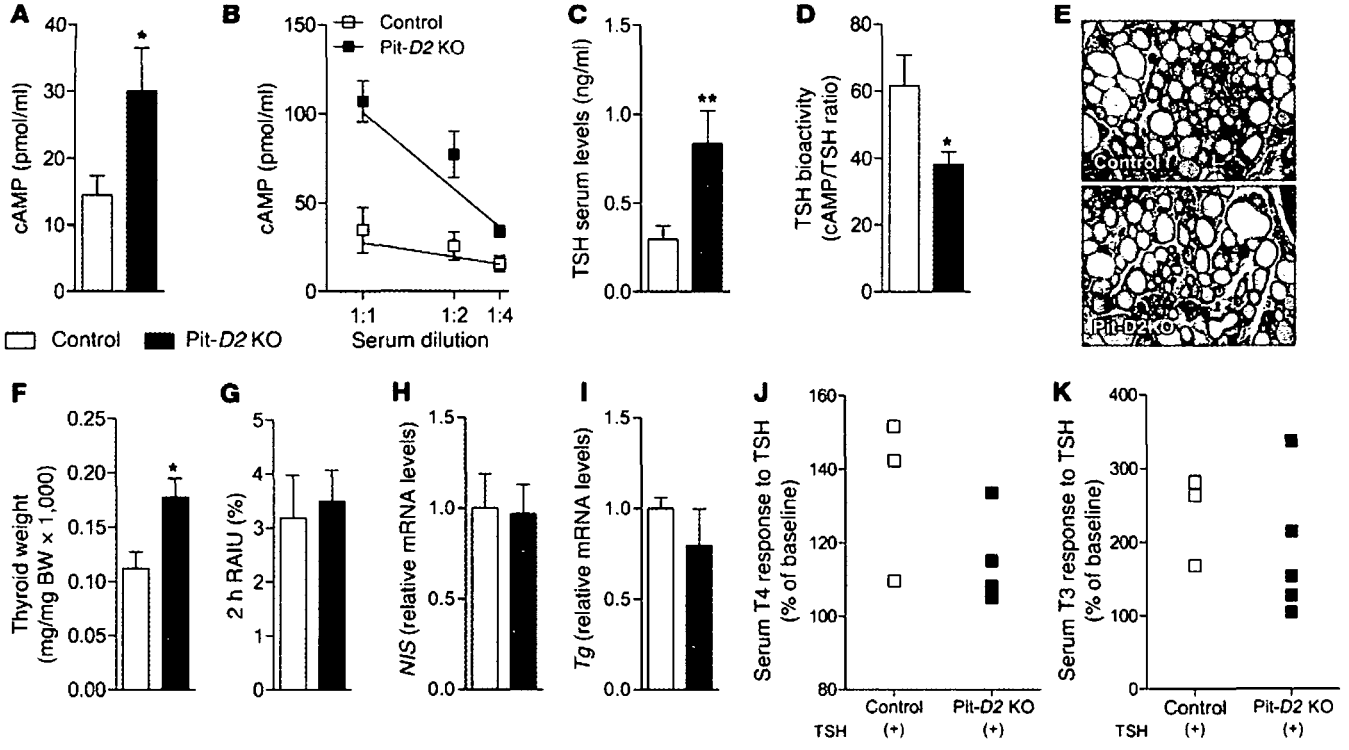

Figure 4

Thyroid parameters and response to TSH in pit-*D2* KO mice versus CGA-cre controls. (**A**) cAMP levels, (**B**) serum dilution vs. cAMP production ($n = 5$), (**C**) TSH serum levels, and (**D**) TSH bioactivity ($n = 6–8$). (**E**) Representative structural analysis of thyroid glands, as assessed by optical microscopy ($n = 3–5$). H&E staining (original magnification, ×20). (**F**) Relative thyroid weight ($n = 8–10$). (**G**) Thyroidal ^{125}I uptake (RAIU) after 2 hours ($n = 5$). Uptake was calculated as the ratio of thyroid cpm to total cpm and expressed as a percentage. (**H** and **I**) mRNA expression of *NIS* and *Tg* ($n = 7–8$), normalized to *CypA*. (**J** and **K**) T4 and T3 levels 2 hours after TSH administration. $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

T4-mediated negative feedback on PVN TRH expression. These mechanisms define a critical coordination between thyrotroph and tanycyte D2 pathways in the HPT and illustrate how preserving circulating serum T3 is indeed a centerpiece in thyroid hormone homeostasis.

The negative feedback mechanism in the thyrotroph is mediated by 2 important signals — serum T3 and serum T4 — via the D2 pathway (4). Thus, disruption of D2 in thyrotrophs would decrease their sensitivity to T4 and elevate serum TSH, which would in turn elevate serum T4 and T3 until a new steady state was reached at hyperthyroid levels. Alternatively, if serum T4 could bypass the thyrotroph and signal a negative feedback via the D2 pathway at the MBH, this would compensate for the lack of thyrotroph D2 and preserve euthyroidism. Our newly developed pit-*D2* KO mice demonstrated that the latter mechanism is the case: serum T3 remained normal in these animals, and they were systemically euthyroid (Figure 2, I and J, and Tables 1–3). That the pit-*D2* KO mouse exhibits increased T4 signaling in the MBH was confirmed by the reduction in PVN *Trh* mRNA (Figure 3, E and F). The observation that serum T4 was elevated in the pit-*D2* KO mouse (Figure 3C) indicated that this MBH-based compensatory mechanism is less sensitive to serum T4, a phenomenon that could be explained by MBH D3 activity inactivating T4 en route to the tanycytes.

However, if the MBH D2 pathway compensates for thyrotroph D2 pathway inactivation, then why were elevated serum TSH levels observed in pit-*D2* KO mice? The answer involves the posttranslational modification of the TSH molecule (i.e., TSH glycosylation), which is controlled by TRH (18). A reduction in TRH such as that seen during central hypothyroidism is expected to decrease TSH biological activity and prolong its half-life (18). In fact, pit-*D2* KO TSH demonstrated approximately 40% decreased biological activity (Figure 4, A–D). This notion is strongly supported by the finding that most thyroidal parameters tested in the pit-*D2* KO mouse were normal, including thyroid structure, 2-hour ^{125}I uptake, and *NIS* and *Tg* mRNA levels (Figure 4, E and G–I), despite much higher serum TSH (Figure 2F). Thus, the new steady state reached by the pit-*D2* KO mouse included normal pituitary-driven thyroidal activity, presumably because the elevation in serum TSH was mitigated by its reduced biological activity. Nevertheless, the pit-*D2* KO mice exhibited goiter, with an approximately 60% increase in thyroid size (Figure 4F). This raises several possibilities, including selective TSH activation of thyroidal growth versus activity resulting from the expected alteration in TSH glycosylation, or earlier thyroid growth (before the new steady state was achieved).

Despite the elevation in serum T4, pit-*D2* KO mice exhibited strong evidence for systemic euthyroidism, as documented by normal serum T3, and tissue euthyroidism, given the multiple T3-responsive parameters that were normal (Figure 2, I and J, and Tables 1–3). This indicates that in response to a disruption in pituitary T4 signaling, multiple adjustments take place in the HPT, eventually preserving systemic euthyroidism. In fact, the pit-*D2* KO mouse model represented yet another example that the HPT is wired to maintain normal serum T3 levels, in addition to most animal models of isolated or combined deiodinase deficiency (19).

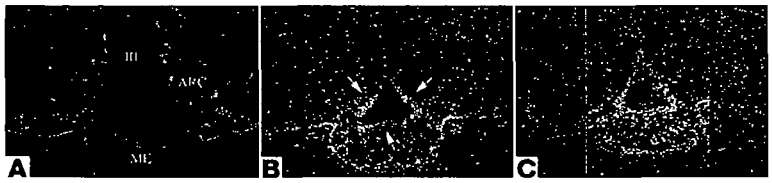

Figure 5
D2 and GFAP expression in the brain. (**A**) GFAP immunostaining demonstrated that in GFAP-cre animals, GFAP was expressed by astrocytes around the third ventricle — in the arcuate nucleus (ARC) and internal zone of the median eminence (ME) — but was not expressed by tanycytes located in the wall of the third ventricle. (**B** and **C**) Darkfield image of radioactive in situ hybridization demonstrated that *D2* mRNA was expressed at the same level in the tanycytes of GFAP-cre control (**B**) and astro-*D2* KO (**C**) mice. Arrows indicate *D2* mRNA labeling in tanycyte cell bodies.

In the MBH, D2 has access to plasma-born or cerebral spinal fluid–born T4 to produce and release T3 that may then reach the PVN or the pituitary gland via portal blood (8). Previous studies in which MBH D2 activity is stimulated, either by LPS administration (20) or as a result of fatty acid amide hydrolase gene inactivation (21), indicate that D2-generated T3 can suppress TRH expression (22). In this region, D2 is reported to be expressed both in astrocytes (7), the only D2-expressing cell population that expressed GFAP-cre in the present studies (Figure 5A), and in tanycytes (5, 6). The preservation of MBH D2 activity in the astro-*D2* KO mouse (Figure 6D) indicates that D2 is primarily expressed in tanycytes, although we cannot exclude with absolute certainty the possibility that selective ablation of D2 in hypothalamic astrocytes led to upregulation of D2 enzymatic activity in tanycytes to compensate for this loss. Nevertheless, by in situ hybridization histochemistry, no apparent increase in *D2* mRNA was observed in astro-*D2* KO versus GFAP-cre control tanycytes (Figure 5, B and C). We are confident that astrocyte D2 in the hypothalamus is markedly reduced in the astro-*D2* KO mouse, given that D2 activity was dramatically reduced in every other location in which it is expressed in glial cells (including the cerebral cortex, hippocampus, and cerebellum), but remained unaffected in the pituitary gland and outside the central nervous system (Figure 6, A–C, E, and F). At the same time, we cannot exclude the possibility that MBH D2–generated T3 also reaches the thyrotroph via portal blood and mediates, at least in part, the T4-mediated TSH negative feedback in pit-*D2* KO mice.

TSH has been linked to significant extrathyroidal effects, namely, lipolysis (23, 24) and control of bone mass (25–27). Interestingly, the pit-*D2* KO animals exhibited decreased fat mass and normal bone mass (Table 2), which could potentially shed some light on the roles played by TSH in these processes. However, the reduced TSH biological activity in the pit-*D2* KO mouse prevents us from reaching definitive conclusions. At least it is clear that the elevated serum TSH we detected in the pit-*D2* KO animals was not associated with increased bone mass, as would be expected by some (28, 29). Lastly, also unexpected was the observation that astro-*D2* KO mice exhibited reduced BMD (Table 2). Although it is difficult to speculate on the underlying mechanisms (the global *D2* KO has normal BMD; ref. 25), it is known that bone remodeling is under central control via the sympathetic nervous system (30, 31), a pathway that is affected by thyroid hormone and D2 expression (32, 33). Alternatively, D2 is expressed in bone and mouse osteoblasts (34) as well as in the chicken developing growth plate (35). Thus, given the GFAP expression in osteocytes and in cartilage chondrocytes (36), it is conceivable that selective D2 inactivation in these cells could have resulted in a phenotype that was not apparent in the global *D2* KO mouse.

In conclusion, our characterization of the pit-*D2* KO mouse revealed coordination between the D2-generated T3 pathways in the pituitary gland and MBH. In the absence of the pituitary D2 pathway, tanycyte D2 played a greater role within the MBH, reducing TRH expression and bringing the HPT to a new steady state in which serum T3 levels and euthyroidism were preserved. Included in this mechanism was an elevation in serum TSH and a reduction in its biological activity, which combined had a neutral net effect on thyroidal activity. Characterization of the astro-*D2* KO mouse was instrumental in allowing us to define that tanycytic D2, not astrocytic D2, is a critical component of the feedback regulation mechanism of thyroid hormone on hypophysiotropic TRH.

Methods

Animals. To establish an in vivo mouse model for cell type–specific deletion of *D2*, first we generated *D2*^{fl} mice by inserting loxP sites (37, 38) into the *D2* gene. The resulting *D2*^{fl} mice were then crossed with transgenic mice expressing Cre recombinase in a cell type–specific manner. The mouse *D2* mRNA is unusually long (~6.5 kb) and contains a 8,071-bp-long single intron between codons 74 and 75 (39), making it risky to flox the whole exon 2 in view of the possible homologous recombination inside the floxed fragment. Therefore, the 5′ loxP site was placed into the 3′ region of the intron, while the 3′ loxP site was introduced to the 5′ region of the 3′ untranslated region (UTR) (Figure 1). According to this strategy, the loxP-flanked region contained the portion of the D2 coding region located in exon 2 (from codon 75 to the end of the protein) including the active center of the D2 enzyme required for catalytic activity (40). The presence of the 3′ loxP site in the 3′ UTR did not alter D2 activity (data not shown).

D2^{fl} mice were crossed with CGA-cre (B6;SJL-Tg[Cga-Cre]3Sac/J; Jackson Laboratories; ref. 41) and GFAP-Cre (FVB-Tg[GFAP-cre]25Mes/J; Jackson Laboratories; ref. 42) transgenic mice, in order to eliminate *D2* activity in the anterior pituitary (pit-*D2* KO mice) and in GFAP-expressing astrocytes (astro-*D2* KO mice), respectively. In all experiments, Cre littermates were used as controls. Except when stated otherwise, all mice were male, between 9 and 12 weeks of age at the time of the studies. Hypothyroidism was induced in some animals, as indicated, by adding 1% sodium perchlorate and 0.1% metimazol in drinking water for 30 days.

Body composition. Animals were fasted overnight, and total skeleton area, LBM, and fat mass were measured by dual energy X-ray absorptiometry (DEXA; Lunar Pixi). For the procedure, mice were anesthetized with ketamine/xylazine (200 mg/kg and 7–20 mg/kg) before imaging, as described previously (43).

Indirect calorimetry. Mice were individually housed and acclimatized in a calorimeter for 2 days before studies. Measurements of gas exchange were performed subsequently over the next 2 days as described previously (43). These studies were performed in a comprehensive lab animal monitoring system (CLAMS; Columbus Instruments), a computer-controlled open circuit calorimetry system. Metabolic profiles were generated based on real-time data obtained in successive 14-minute cycles. Studies were performed

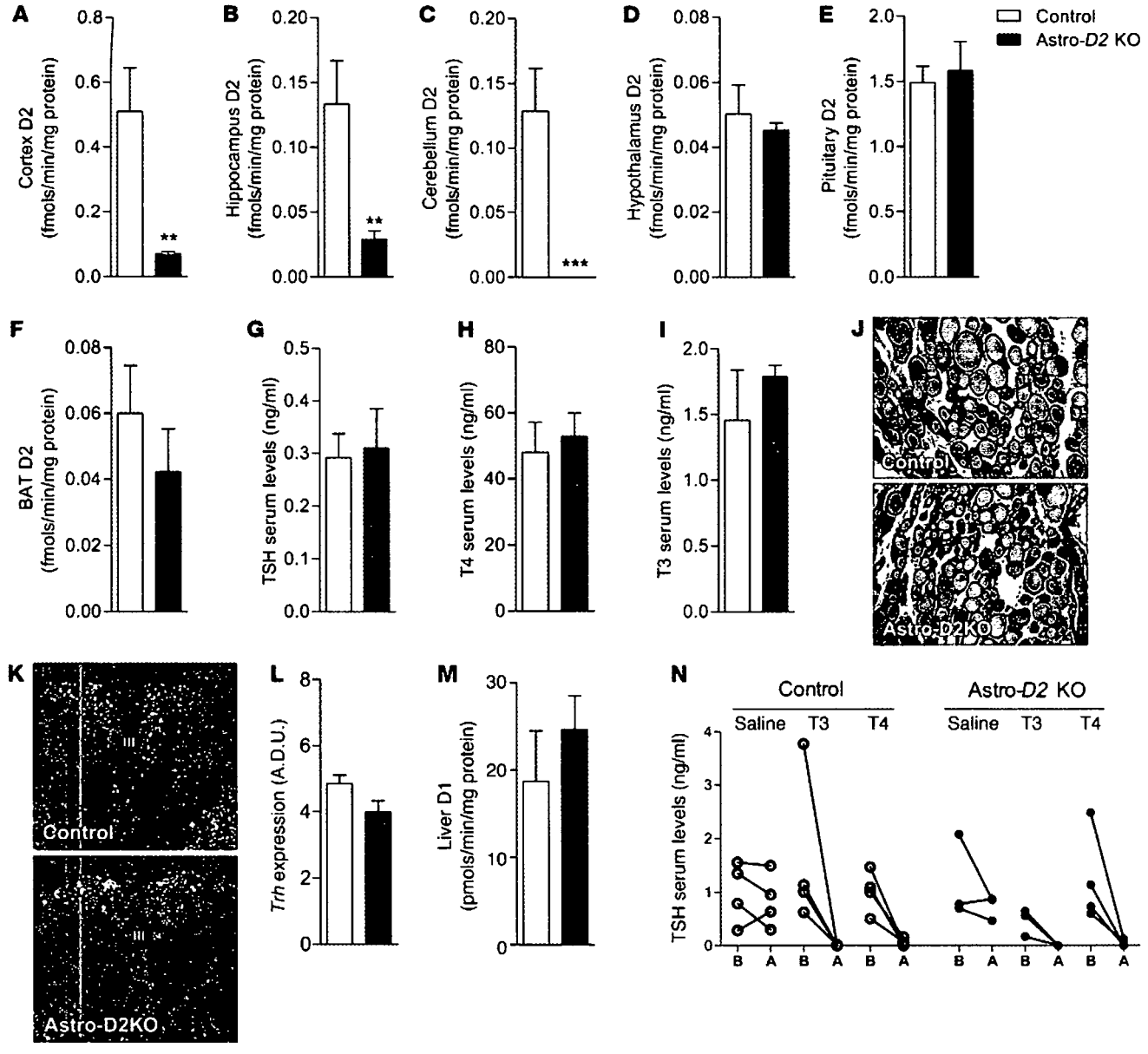

Figure 6

Thyroid phenotype in astro-*D2* KO mice versus GFAP-cre controls. (**A–F**) D2 activity in sonicates of cortex, hippocampus, cerebellum, hypothalamus, pituitary, and BAT ($n = 6$). (**G–I**) Serum TSH, T4, and T3 levels ($n = 6–7$). (**J**) Representative structural analysis of thyroid glands, as assessed by optical microscopy ($n = 3–5$). H&E staining (original magnification, ×20). (**K**) In situ hybridization of *Thr* mRNA in the PVN. Shown are coronal brain sections, including the MBH area and the third ventricle. (**L**) Computerized image analysis of the in situ hybridization autoradiograms ($n = 3–5$ per group). (**M**) Liver D1 activity ($n = 6$). (**N**) TSH serum levels before and after 4-hour administration of saline, T3, and T4 ($n = 3–4$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

at 22°C. The sensors were calibrated against a standard gas mixture containing defined quantities of O_2 and CO_2 (Airgas). VO_2 was expressed as milliliters per kilogram per hour.

TSH suppression studies. TSH suppression studies were performed as previously described (11). Pit-*D2* KO mice and CGA-cre controls, or astro-*D2* KO mice and GFAP-cre controls, were placed on PTU (1 mg/liter drinking water) for 2 weeks. After blood was obtained from the orbital plexus (~150 μl), animals were injected s.c. with PTU (1 mg/100 g BW; 0.1 ml saline containing 0.01 N NaOH). 1 hour later, animals were injected s.c. with T3 (1.2 μg/100 g BW), T4 (3 μg/100 g BW), or vehicle. 4 hours later, new blood samples were collected, and animals were killed by CO_2 inhalation.

TRH and TSH stimulation tests. TRH or TSH tests were performed as previously described (44). Control and pit-*D2* KO mice received TRH (5.0 μg/kg i.p.; Sigma-Aldrich) or bovine TSH (250 mU/100 g i.p.; Sigma-Aldrich). Serum TSH, T4, and T3 levels were measured before and after TRH (30 minutes) or TSH (120 minutes) injection.

Thyroidal ^{125}I uptake. Thyroidal ^{125}I uptake was performed as previously described (19) after injection of $Na^{125}I$ (15,000 cpm/g BW i.p.; NEN Life Science Products) into each animal. 2 hours later, mice were killed, and their thyroid glands were dissected and processed for radiometry. ^{125}I uptake was calculated as the ratio of thyroid cpm to total cpm and expressed as a percentage.

TSH biological activity. TSH biological activity was measured by bioassay using a line of CHO cells stably transfected with a human TSH receptor cDNA, as previously described (45). At the end of the incubation time, 220 µl of 10% trichloroacetic acid was added to each sample. cAMP was measured after adjusting to 6–7 pH, using cAMP ^{125}I RIA kit (PerkinElmer).

Charcoal uptake. The thyroxine binding protein was determined using a modification of previously described methods (46). 10 µl mouse serum was diluted into 0.5 ml of PBS (pH 7.4) containing approximately 7,000 cpm ^{125}I-T4, with specific activity of 135–165 µCi (5.00–6.11 MBq) per microgram (Perkin Elmer). Samples were allowed to equilibrate for 45 minutes at room temperature and then transferred to an ice water bath for 15 minutes. Prechilled 0.025% activated charcoal (0.5 ml; Sigma-Aldrich) solution in PBS was added, and samples were incubated on ice for an additional 15 minutes. Samples were spun in an Eppendorf centrifuge 5804R at 650 *g* for 15 minutes, and the charcoal-containing pellets were counted. Binding was calculated as the ratio of sample cpm to total cpm and expressed as a percentage.

Euthanasia and postmortem analyses. At the end of the experimental period, mice were euthanized by asphyxiation in a CO_2 chamber. Brain, liver, skeletal muscle, and epididymal adipose tissue was dissected, weighed, and frozen in liquid nitrogen, and organ weights were expressed relative to total BW. The thyroid gland was dissected, weighed, and fixed in 4% paraformaldehyde for 24 hours at 4°C, and then embedded in paraffin, sectioned at 5 µm, and stained with H&E. Blood was collected, and serum levels of TSH, T4, T3, FSH, and LH were measured using a MILLIPLEX rat thyroid hormone panel kit (Millipore Corp.), according to the manufacturer's instructions, and read on a BioPlex (BioRad).

In situ hybridization and analysis. In situ hybridization histochemistry was performed on every fourth section through the PVN or median eminence using a 741-base single-stranded [^{35}S]UTP labeled cRNA probe for mouse TRH (47) or mouse D2 following previously described protocols (48). The coding region of the mouse *D2* mRNA was amplified with Taq PCR (sense, ATGGGACTCCTCAGCGTAGACTT; antisense, GCTAATCTAGAATTCATCTCTTGC) and cloned into a pGEM-T vector with its 5′ end at the T7 promoter. The sequence was confirmed by sequencing. The construct was linearized with NcoI and subjected to transcription with SP6 polymerase to generate the antisense strand. In vitro transcription was performed using SP6/T7 systems (Promega Corp.) and [^{35}S]UTP (1250 Ci/mmol; PerkinElmer). The hybridization was performed under plastic coverslips in a buffer containing 50% formamide, a 2× concentration of standard sodium citrate, 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 250 µg/ml denatured salmon sperm DNA, and 5×10^5 cpm radiolabeled probe for 16 hours at 56°C. Slides were dipped into Kodak NTB autoradiography emulsion (Kodak) diluted 1:1 in distilled water, and the autoradiograms were developed after 3–7 days of exposure at 4°C. Autoradiograms were visualized under darkfield illumination using a COHU 4910 video camera (COHU Inc.). Images were captured with a color PCI frame grabber board (Scion Corp.) and analyzed with a Macintosh G4 computer using Scion Image. Background density points were removed by thresholding the image and integrated density values (density × area) of hybridized neurons on each side of the PVN measured in 3 consecutive sections of the midportion of the PVN where hypophysiotropic TRH neurons have been identified in mice (47).

Immunofluorescence for GFAP. 25-µm-thick coronal sections from the hypothalamus cut on a cryostat were collected in PBS and treated with 0.5% Triton-X-100 plus 0.5% H_2O_2 in PBS for 15 minutes to increase antibody penetration. After rinses in PBS, sections were incubated in a mouse monoclonal antibody against GFAP (catalog no. MAB360; Millipore) overnight at a dilution of 1:2,000, diluted in 2% normal horse serum, 0.2% sodium azide, and 0.2% Photo-Flo. After rinses in PBS, sections were incubated in Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch) at 1:200 dilution for 2 hours. Sections were rinsed in PBS, mounted on glass slides, and coverslipped with Vectashield mounting medium (Vector).

Deiodinase assays. D1, D2, and D3 assays were performed in tissue sonicates in the presence of 10 mM DTT and 0.25 M sucrose as described previously (49). D1 and D2 assays were performed using 500 nM ^{125}I-(5′) recombinant T3 (PerkinElmer) and 0.1 nM ^{125}I-(5′) T4 (PerkinElmer), respectively, as substrates and 1 mM PTU for D2 measurements. D3 activity was measured as previously described (50) in the presence of 0.2 nM ^{125}I-(5′) T3 (PerkinElmer) as substrate. Reactions were stopped by the addition of methanol, and the products of deiodination were quantified by UPLC (ACQUITY; Waters Corp.). Fractions were automatically processed through a Flow Scintillation Analyzer Radiomatic 610TR (PerkinElmer) for radiometry as described.

Gene expression analysis. Total RNA was extracted from pituitary and thyroid gland using RNAaqueous micro kit (Ambion; Life Technologies), according to the manufacturer's instructions. The extracted RNA was quantified with a NanoDrop spectrophotometer, and 1.0 µg total RNA was reverse transcribed into cDNA using High Capacity cDNA reverse Transcription kit (Applied Biosystems). Genes of interest were measured by RT-PCR (Bio-Rad iCycler iQ Real-Time PCR Detection System) using iQ SYBR Green Supermix (Bio-Rad) with the following conditions: 15 minutes at 94°C (hot start), 30–50 seconds at 94°C, 30–50 seconds at 55°C–60°C, and 45–60 seconds at 72°C for 40 cycles. A final extension at 72°C for 5 minutes was performed as well as the melting curve protocol to verify the specificity of the amplicon generation. Standard curves consisted of 4–5 points of serially diluted mixed experimental and control group cDNA. Cyclophilin A (*CypA*) was used as a housekeeping internal control gene. The coefficient of correlation was greater than 0.98 for all curves, and the amplication efficiency ranged between 80% and 110%. Results were expressed as the ratio of test mRNA to *CypA* mRNA. mRNA levels of the following genes were measured: *Tsha*, *Tshb*, *NIS*, *Tg*, peptidylglycine alpha-amidating monooxygenase (*Pam*), and glutaminyl-peptide cyclotransferase (*Qpct*).

Statistics. All data were analyzed using PRISM software (GraphPad). Unless otherwise indicated, data represent mean ± SEM. 1-way ANOVA followed by Student-Newman-Keuls test was used to compare differences among more than 2 groups; 2-tailed Student's *t* test was used to compare differences between 2 groups. A *P* value less than 0.05 was considered significant.

Study approval. All experimental procedures were approved by the local IACUC (Miami, Florida, USA).

Acknowledgments

This work was supported by NIDDK grants R01 65055 and R01 37021, the National Science Foundation of Hungary (OTKA K81226), the Lendulet grant of Hungarian Academy of Sciences, and the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259772. We are grateful to Judith Molina for skilled technical assistance and Susy Bianco for assistance with cAMP assay.

Received for publication October 24, 2012, and accepted in revised form January 31, 2013.

Address correspondence to: Antonio C. Bianco, Division of Endocrinology, Diabetes and Metabolism, University of Miami Miller School of Medicine, 1580 NW 10th Avenue, Suite 602, Miami, Florida 33136, USA. Phone: 305.243.5631; Fax: 305.243.7268; E-mail: abianco@deiodinase.org. Or to: Ronald M. Lechan, Division of Endocrinology, Diabetes and Metabolism, Tufts Medical Center, 800 Washington Street, Box 268, Boston, Massachusetts 02111, USA. Phone: 617.636.8517; Fax: 617.636.4719; E-mail: rlechan@tuftsmedicalcenter.org.

1. Larsen PR. Thyroid-pituitary interaction: feedback regulation of thyrotropin secretion by thyroid hormones. *N Engl J Med.* 1982;306(1):23–32.
2. Geffner DL, Azukizawa M, Hershman JM. Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin secretion in man. *J Clin Invest.* 1975;55(2):224–229.
3. Gereben B, et al. Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev.* 2008;29(7):898–938.
4. Christoffolete MA, et al. Atypical expression of type 2 iodothyronine deiodinase in thyrotrophs explains the thyroxine-mediated pituitary thyrotropin feedback mechanism. *Endocrinology.* 2006;147(4):1735–1743.
5. Guadano-Ferraz A, Obregon MJ, St Germain DL, Bernal J. The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. *Proc Natl Acad Sci U S A.* 1997; 94(19):10391–10396.
6. Tu HM, et al. Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. *Endocrinology.* 1997; 138(8):3359–3368.
7. Diano S, Leonard JL, Meli R, Esposito E, Schiavo L. Hypothalamic type II iodothyronine deiodinase: a light and electron microscopic study. *Brain Res.* 2003;976(1):130–134.
8. Fekete C, Lechan RM. Negative feedback regulation of hypophysiotropic thyrotropin-releasing hormone (TRH) synthesizing neurons: role of neuronal afferents and type 2 deiodinase. *Front Neuroendocrinol.* 2007;28(2–3):97–114.
9. Rodriguez EM, et al. Hypothalamic tanycytes: a key component of brain-endocrine interaction. *Int Rev Cytol.* 2005;247:89–164.
10. Kakucska I, Rand W, Lechan RM. Thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is dependent upon feedback regulation by both triiodothyronine and thyroxine. *Endocrinology.* 1992;130(5):2845–2850.
11. Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA. Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. *Mol Endocrinol.* 2001;15(12):2137–2148.
12. Rosene ML, Wittmann G, Arrojo e Drigo R, Singru PS, Lechan RM, Bianco AC. Inhibition of the type 2 iodothyronine deiodinase underlies the elevated plasma TSH associated with amiodarone treatment. *Endocrinology.* 2010;151(12):5961–5970.
13. Bianco AC, Maia AL, da Silva WS, Christoffolete MA. Adaptive activation of thyroid hormone and energy expenditure. *Biosci Rep.* 2005;25(3–4):191–208.
14. Silva JE. Thermogenic mechanisms and their hormonal regulation. *Physiological Review.* 2006; 86(2):435–464.
15. Zavacki AM, et al. Type 1 iodothyronine deiodinase is a sensitive marker of peripheral thyroid status in the mouse. *Endocrinology.* 2005;146(3):1568–1575.
16. Shupnik MA, Chin WW, Habener JF, Ridgway EC. Transcriptional regulation of the thyrotropin subunit genes by thyroid hormone. *J Biol Chem.* 1985;260(5):2900–2903.
17. Shibusawa N, Yamada M, Hirato J, Monden T, Satoh T, Mori M. Requirement of thyrotropin-releasing hormone for the postnatal functions of pituitary thyrotrophs: ontogeny study of congenital tertiary hypothyroidism in mice. *Mol Endocrinol.* 2000;14(1):137–146.
18. Weintraub BD, Gesundheit N, Taylor T, Gyves PW. Effect of TRH on TSH glycosylation and biological action. *Ann N Y Acad Sci.* 1989;553:205–213.
19. Christoffolete MA, et al. Mice with impaired extrathyroidal thyroxine to 3,5,3′-triiodothyronine conversion maintain normal serum 3,5,3′-triiodothyronine concentrations. *Endocrinology.* 2007;148(3):954–960.
20. Fekete C, et al. Lipopolysaccharide induces type 2 iodothyronine deiodinase in the mediobasal hypothalamus: implications for the nonthyroidal illness syndrome. *Endocrinology.* 2004;145(4):1649–1655.
21. Sipe JC, Waalen J, Gerber A, Beutler E. Overweight and obesity associated with a missense polymorphism in fatty acid amide hydrolase (FAAH) *Int J Obes (Lond).* 2005;29(7):755–759.
22. Freitas BC, et al. Paracrine signaling by glial cell-derived triiodothyronine activates neuronal gene expression in the rodent brain and human cells. *J Clin Invest.* 2010;120(6):2206–2217.
23. Iida Y, Amir SM, Ingbar SH. Stabilization, partial purification, and characterization of thyrotropin receptors in solubilized guinea pig fat cell membranes. *Endocrinology.* 1987;121(5):1627–1636.
24. Elgadi A, Zemack H, Marcus C, Norgren S. Tissue-specific knockout of TSHr in white adipose tissue increases adipocyte size and decreases TSH-induced lipolysis. *Biochem Biophys Res Commun.* 2010;393(3):526–530.
25. Bassett JH, et al. Optimal bone strength and mineralization requires the type 2 iodothyronine deiodinase in osteoblasts. *Proc Natl Acad Sci U S A.* 2010;107(16):7604–7609.
26. Bassett JH, et al. A lack of thyroid hormones rather than excess thyrotropin causes abnormal skeletal development in hypothyroidism. *Mol Endocrinol.* 2008;22(2):501–512.
27. Abe E, et al. TSH is a negative regulator of skeletal remodeling. *Cell.* 2003;115(2):151–162.
28. Sun L, et al. Intermittent recombinant TSH injections prevent ovariectomy-induced bone loss. *Proc Natl Acad Sci U S A.* 2008;105(11):4289–4294.
29. Sampath TK, et al. Thyroid-stimulating hormone restores bone volume, microarchitecture, and strength in aged ovariectomized rats. *J Bone Miner Res* 2007;22(6):849–859.
30. Fonseca TL, et al. Double disruption of alpha2A- and alpha2C-adrenoceptors results in sympathetic hyperactivity and high-bone-mass phenotype. *J Bone Miner Res.* 2011;26(3):591–603.
31. Takeda S, et al. Leptin regulates bone formation via the sympathetic nervous system. *Cell.* 2002;111(3):305–317.
32. Silva JE. Thermogenesis and the sympathoadrenal system in thyrotoxicosis. In: Braverman LE, ed. *Werner & Ingbar's The Thyroid: A Fundamental And Clinical Text.* Philadelphia, Pennsylvania, USA: Lippincott Williams and Wilkins; 2005:307–620.
33. Silva JE. Intermediary metabolism and the sympathoadrenal system in hypothyroidism. In: Braverman LE, ed. *Werner & Ingbar's The Thyroid: A Fundamental And Clinical Text.* Philadelphia, Pennsylvania, USA: Lippincott Williams and Wilkins; 2005:817–823.
34. Gouveia CH, et al. Type 2 iodothyronine selenodeiodinase is expressed throughout the mouse skeleton and in the MC3T3-E1 mouse osteoblastic cell line during differentiation. *Endocrinology.* 2005;146(1):195–200.
35. Dentice M, et al. The Hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate. *Nat Cell Biol.* 2005;7(7):698–705.
36. Kasantikul V, Shuangshoti S. Positivity to glial fibrillary acidic protein in bone, cartilage, and chordoma. *J Surg Oncol.* 1989;41(1):22–26.
37. Abremski K, Hoess R. Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J Biol Chem.* 1984;259(3):1509–1514.
38. Hoess RH, Abremski K. Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system. *J Mol Biol.* 1985;181(3):351–362.
39. Davey JC, Schneider MJ, Becker KB, Galton VA. Cloning of a 5.8 kb cDNA for a mouse type 2 deiodinase. *Endocrinology.* 1999;140(2):1022–1025.
40. Croteau W, Davey JC, Galton VA, St Germain DL. Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *J Clin Invest.* 1996;98(2):405–417.
41. Cushman LJ, Burrows HL, Seasholtz AF, Lewandoski M, Muzyczka N, Camper SA. Cre-mediated recombination in the pituitary gland. *Genesis.* 2000;28(3–4):167–174.
42. Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. *Genesis.* 2001;31(2):85–94.
43. Castillo M, et al. Disruption of thyroid hormone activation in type 2 deiodinase knockout mice causes obesity with glucose intolerance and liver steatosis only at thermoneutrality. *Diabetes.* 2011;60(4):1082–1089.
44. Yamada M, et al. Tertiary hypothyroidism and hyperglycemia in mice with targeted disruption of the thyrotropin-releasing hormone gene. *Proc Natl Acad Sci U S A.* 1997;94(20):10862–10867.
45. Moeller LC, Kimura S, Kusakabe T, Liao XH, Van Sande J, Refetoff S. Hypothyroidism in thyroid transcription factor 1 haploinsufficiency is caused by reduced expression of the thyroid-stimulating hormone receptor. *Mol Endocrinol.* 2003;17(11):2295–2302.
46. Jennings RD. A simple, rapid and precise automated method for the determination of T3-uptake. *Ann Clin Biochem.* 1977;14(5):263–268.
47. Kadar A, et al. Distribution of hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons in the hypothalamic paraventricular nucleus of the mouse. *J Comp Neurol.* 2010;518(19):3948–3961.
48. Fekete C, et al. Neuropeptide Y has a central inhibitory action on the hypothalamic-pituitary-thyroid axis. *Endocrinology.* 2001;142(6):2606–2613.
49. Curcio-Morelli C, et al. In vivo dimerization of types 1, 2, and 3 iodothyronine selenodeiodinases. *Endocrinology.* 2003;144(3):937–946.
50. Huang SA, et al. Transforming growth factor-beta promotes inactivation of extracellular thyroid hormones via transcriptional stimulation of type 3 iodothyronine deiodinase. *Mol Endocrinol.* 2005;19(12):3126–3136.

frontiers in Immunology

DL 6/2018
USA

ORIGINAL RESEARCH
published: 15 November 2017
doi: 10.3389/fimmu.2017.01573

A Role for Iodide and Thyroglobulin in Modulating the Function of Human Immune Cells

*Mahmood Y. Bilal[1,2], Svetlana Dambaeva[1,2], Joanne Kwak-Kim[2,3], Alice Gilman-Sachs[1,2] and Kenneth D. Beaman[1,2]**

[1] Clinical Immunology Laboratory, Rosalind Franklin University of Medicine and Science, North Chicago, IL, United States, [2] Department of Microbiology and Immunology, Rosalind Franklin University of Medicine and Science, North Chicago, IL, United States, [3] Department of Obstetrics and Gynecology, Rosalind Franklin University Health System, Vernon Hills, IL, United States

Iodine is an essential element required for the function of all organ systems. Although the importance of iodine in thyroid hormone synthesis and reproduction is well known, its direct effects on the immune system are elusive. Human leukocytes expressed mRNA of iodide transporters (NIS and PENDRIN) and thyroid-related proteins [thyroglobulin (TG) and thyroid peroxidase (TPO)]. The mRNA levels of PENDRIN and TPO were increased whereas TG transcripts were decreased post leukocyte activation. Flow cytometric analysis revealed that both PENDRIN and NIS were expressed on the surface of leukocyte subsets with the highest expression occurring on monocytes and granulocytes. Treatment of leukocytes with sodium iodide (NaI) resulted in significant changes in immunity-related transcriptome with an emphasis on increased chemokine expression as probed with targeted RNASeq. Similarly, treatment of leukocytes with NaI or Lugol's iodine induced increased protein production of both pro- and anti-inflammatory cytokines. These alterations were not attributed to iodide-induced *de novo* thyroid hormone synthesis. However, upon incubation with thyroid-derived TG, primary human leukocytes but not Jurkat T cells released thyroxine and triiodothyronine indicating that immune cells could potentially influence thyroid hormone balance. Overall, our studies reveal the novel network between human immune cells and thyroid-related molecules and highlight the importance of iodine in regulating the function of human immune cells.

Keywords: iodine, iodine deficiency, RNAseq, nutritional immunology, thyroid hormones, thyroglobulin, NIS, pendrin

leukocytes can convert TG to T4 and T3

INTRODUCTION

Iodine is an essential mineral required for the biosynthesis of thyroid hormones and subsequent proper function of metabolic pathways of all body organs (1). Disorders stemming from iodine deficiency or insufficiency are a worldwide issue affecting approximately two billion people including school-aged children (2, 3). The requirement for sufficient iodine levels encompasses all stages of life (2, 4). First, increased iodine levels are required during pregnancy, and reduced amounts lead to miscarriages and reproductive failures (5–8). This is in-part due to the role of iodine-derived thyroid hormones, thyroxine (T_4), and triiodothyronine (T_3), for optimal fetal brain development (9, 10). Second, congenital hypothyroidism, defined by reduced thyroid hormones leading to stunted mental and physical development during early childhood, is caused by insufficient iodine

OPEN ACCESS

Edited by:
Lorraine M. Sordillo, Michigan State University, United States

Reviewed by:
Oscar J. Cordero, Universidade de Santiago de Compostela, Spain
Yanfei Zhang, Geisinger Health System, United States

****Correspondence:***
Kenneth D. Beaman
kenneth.beaman@rosalindfranklin.edu

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *31 August 2017*
Accepted: *01 November 2017*
Published: *15 November 2017*

Citation:
Bilal MY, Dambaeva S, Kwak-Kim J, Gilman-Sachs A and Beaman KD (2017) A Role for Iodide and Thyroglobulin in Modulating the Function of Human Immune Cells. Front. Immunol. 8:1573. doi: 10.3389/fimmu.2017.01573

intakes (2–4, 11). In adults, non-optimal iodine intake causes hypothyroidism and goiter formation that could be reversed with increased iodine intakes or supplementation (2, 4, 12, 13). Therefore, iodine is required at all stages of life, and its decreased uptake will lead to potentially life threatening conditions and/or severe reduction of quality of life.

Production of thyroid hormones begins with iodide transportation into thyroid follicular cells in the thyroid gland *via* the sodium iodide symporter (NIS) (14, 15). Iodide molecules are then shuttled through another receptor, PENDRIN, into the thyroid colloid/lumen. Membrane bound thyroid peroxidase (TPO) enzymes oxidize iodide into iodine, a reaction needed for the eventual conjugation or organification of iodine into tyrosyl compounds present on the large dimeric protein thyroglobulin (TG). These biochemical pathways are the source of iodinated tyrosines that eventually form thyroid hormones T_4 and T_3. T_4 forms 80–90% of total biologically active hormones made by the thyroid and are stored in TG (15–17). TG is endocytosed by follicular cells and undergoes proteolytic degradation thereby releasing mostly T_4 and relatively low amounts of T_3 into peripheral blood. The activity of tissue bound deiodinase enzymes converts T_4 into the more biologically active hormone T_3 (15, 18). Ultimately, thyroid hormones affect the metabolic processes of cells, which include gluconeogenesis, glycogenolysis, thermogenesis, and protein metabolism (15).

Interestingly, thyroid hormones can directly affect multiple branches of the immune system by enhancing dendritic cell antitumor immunity, B cell differentiation, phagocytosis, natural killer cytotoxicity, inducing higher expression of cytokines, and increasing the frequency of T cell memory cells (19–23). The effects of thyroid hormones on immune cells are due in-part through activation of protein kinase C signaling (20). Furthermore, immune cells are able to produce TSH and utilize TSH to increase T_3 levels (24, 25). Thus, far no significant sources of *de novo* T_4 that could affect tissue or blood hormone levels have been identified other than the thyroid gland. However, intriguing findings by Nagao et al. and others demonstrated the likelihood of extrathyroidal T_4 synthesis in thyroidectomized rats (26, 27). Others have shown the presence of low intracellular levels of thyroxine in cardiomyocytes utilizing radioactive iodide ^{125}I, and the presence of "thyroxine-like" compounds in ^{131}I-pulsed leukocytes (28, 29). The prospect of extrathyroidal T_4 production is strengthened by experiments demonstrating the presence of iodinated tyrosines and thyroid biosynthesis machinery (NIS, TG, and TPO) in multiple tissues including the endometrium, placenta, mammary glands, thymus, testis, liver, and kidneys (30–34). These studies reflect the potential for iodide influx into these tissues. Overall, sources of extrathyroidal thyroxine remain elusive.

The direct effects of inorganic iodine or iodide on cellular activity of immune cells, outside of thyroid hormones, remain relatively unexplored. Evidence for possible direct role for iodine on immune cells was demonstrated by Marani et al. wherein school children deficient in iodine had reduced immune responses despite normal thyroid hormone levels (35–37). Further studies on human breast cancer cells demonstrated the effects of Lugol's solution, composed of molecular iodine (I_2) and potassium iodide (KI), on the transcriptional activity of these cells (38). Other iodine concentrating tissues have been identified including ovaries, salivary glands, and the thymus (39, 40). Similarly, a 1971 study by Stolc showed that ^{131}I-pulsed leukocytes could concentrate iodide intracellularly (28). Xiaoyi et al. studied the cytotoxic effects of molecular iodine in murine immune cells and found, increased lymphocyte survival, slightly reduced CD4/CD8 ratios, and increased IFNγ/IL4 ratio upon activation. Nonetheless, the effects and mechanisms of iodide, as well as molecular iodine, on the function immune cells remains ill explored. The immune system not only protects against foreign pathogens, tumors, and autoimmune responses but it can also modulate and provide a growth milieu during tissue repair and pregnancy through the production of growth factors and angiogenesis (41–43). During the stages of pregnancy, the balance between pro- and anti-inflammatory factors needs to be actively balanced through production of multiple cytokines and immune agents (41, 44).

Due to the importance of iodine and thyroid hormones in pregnancy and the modulatory roles of the immune system on this process, we sought to explore the interplay between thyroid-related molecules and the immune system. In this study, we have examined the effects of inorganic iodine/iodide on cellular function and assessed if immune cells could secrete thyroid hormones. To this end, we have analyzed the expression of iodide transporters in normal donor peripheral immune cells and determined if iodide induces functional changes in the activity these cells. Our studies show a pronounced iodide-induced transcriptional and cytokine response by human peripheral blood leukocytes that were not attributed to new thyroid hormone synthesis. Interestingly, substantial amounts of thyroid hormones were released upon incubation of leukocytes with thyroid-derived TG. Altogether, these observations demonstrate a novel insight on the effects of iodide on human immune cells and highlight leukocytes as a potential source for T_4 in local tissues and peripheral blood.

MATERIALS AND METHODS

Purification and Culture of Human Leukocytes and Jurkat E6.1 Cell Line

Human leukocytes were obtained from blood samples drawn in sodium heparin where donors have consented for blood donation. The consent and documentation process associated with these donors were approved by the IRB for Rosalind Franklin University of Medicine and Science. Leukocytes were extracted with standard Ficoll-Paque method. The cells were cultured at 37°C and 5% CO_2 in complete RPMI 1640 (RPMI medium supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine) (Gibco). The E6.1 Jurkat T cell line was acquired directly from ATCC (TIB-152) and cultured at 37°C and 5% CO_2 in complete RPMI medium.

For cell counts or viability assays, 5×10^6 leukocytes were seeded into 1 mL complete RPMI media with 1 mM NaI (383112, Sigma) or PBS (control) for 3 days. Cells were counted using BIO-RAD TC20 automated cell counter. Viability was determined with standard Trypan Blue (T8154, Sigma) exclusion assay analyzed by TC20 cell counter software.

RNA Extraction from Leukocytes

5×10^6 leukocytes were suspended in 1 mL complete RPMI media, and then left unstimulated or activated with PMA (25 ng/mL) and ionomycin (1 μM) for 18 h and then total RNA was extracted from leukocytes utilizing Qiagen's mini RNeazy kits. For targeted RNASeq, leukocytes were incubated with or without 1 mM NaI or PBS (control) for 48 h.

Reverse Transcription PCR/Quantitative PCR (qPCR)

Reverse transcription was performed with 400 ng of total RNA using transcriptor first strand cDNA system (Roche). 4 μL of cDNA was amplified for 35 cycles with Amplitaq DNA polymerase kit (Life Technologies). The following primers were used for PCR amplification with all primers listed in the 5′ → 3′ direction (NIS F 287 bp: CTCTTCATGCCCGTCTTCTAC, NIS R: GA CAACCCAGAAGCCACTTA), (PENDRIN F 320 bp: TCCTG TCGGATATGGTCTCTAC, PENDRIN R: GATCTGCCAAGTA CCTCACTATG), (TPO F 274 bp: GGAAGCAGATGAAGG CTCTG, TPO R: AGTGCACAAAGTCCCCATTC), and (GAP DH F 440 bp: ACATCATCCCTGCCTCTACT, GAPDH R: CTCTCTTCCTCTTGTGCTCTTG).

For quantitative real-time PCR, 2 μL cDNA was amplified with TaqMan Fast Advanced Master Mix (Applied Biosystems) and read with StepOnePlus real-time PCR instrument (Applied Biosystems). Validated TaqMan primer mix was obtained from Invitrogen as follows: NIS: Hs00950365_m1, PENDRIN: Hs01070627_m1, TG: Hs00174974_m1, and B2M: Hs00187842_m1. Gene expression was normalized to internal B2M amplification.

Targeted RNASeq *via* Next-Generation Sequencing (NGS)

Next-generation sequencing Library preparation was performed per Qiagen's targeted RNASeq *Human Inflammation & Immunity Transcriptome* panels containing probes for 475 genes. Targeted RNASeq is more quantitative and specific than conventional qPCR due to specific transcript sequencing and the unique molecular barcoding of each transcript, detected with bioinformatics, before PCR amplification. In this way, molecular barcoding bypasses PCR bias that could affect conventional qPCR results resulting from inefficient primers and/or poor sample preparation. Briefly, cDNA was made from 400 ng of leukocyte RNA, and then unique molecular 12 nucleotide tags were incorporated into a total of 20 ng cDNA *via* gene specific primer extension. After PCR purification using magnetic beads, the barcoded cDNA was amplified utilizing gene specific primers. The purified DNA was again amplified through a second PCR reaction to insert index sequences that are unique to each sample. This step allows the combination of multiple samples in one tube for subsequent sequencing. The completed library was loaded into Illumina's reagent cartridge (150 cycle v3) with a standard flow cell and custom sequencing primer provided by Qiagen. NGS was performed on Illumina's MiSeq instrument per manufacturer's recommendations. Sequencing quality controls, including cluster density, total reads, and percent reads reaching Q30, were all within optimal ranges provided by Illumina. In addition, secondary quality controls provided by Qiagen's targeted RNASeq software that reads and quantifies the sequencing files were all within acceptable ranges. The FASTQ files obtained from the sequencing runs were uploaded to Qiagen's *GeneRead DNAseq variant calling service*. The data were then exported into a format that provides the total unique molecular barcode sequencing reads per gene. All reads/samples were normalized to 10 internal control housekeeping genes after screening negative for genomic DNA contamination. Statistical analysis was then performed on normalized data that were quantified as fold change compared with the associated controls.

Enzyme-Linked Immunosorbent Assay (ELISA)

5×10^6 leukocytes were incubated in complete RPMI media with PBS (control) or 1 mM NaI for 72 h, and then the supernatant was collected. Alternatively, the cells were treated with 500 μM of Lugol's iodine solution (32922, Sigma). Cytokines were quantified utilizing Invitrogen's ELISA kits per manufacturer's recommended protocol (Invitrogen IFNγ: 88-7316; IL6: 88-7066; IL8-CXCL8: 88-8086; IL10: 88-7106; CCL2: 88-7399). ELISA plates were read using a spectrophotometric plate reader at a wavelength of 450 nm.

Flow Cytometry

5×10^6 leukocytes were washed in PBS and then incubated in 500 μL PBS with 10% goat serum (S-1000, Vector Laboratories) for 30 min to block non-specific binding. The cells were resuspended in 100 μL PBS with 10% goat serum for 1 h at room temperature with primary or without (control) rabbit antibodies against *SLC5A5*/NIS (SAB2102220, Sigma) or *SLC26A4*/PENDRIN (MBS9215961, MyBioSource). The cells were washed and then stained for 30 min with secondary F(ab′)2 goat anti-rabbit (Invitrogen, A21246) at a 1:100 dilution and CD45 Krome orange (Beckman Coulter, A96416) at a 1:20 dilution at room temperature. Other experiments included CD14 FITC staining (BD Pharmingen, 555397) to identify monocytes. Cells were washed, resuspended in IsoFlow sheath fluid (Beckman Coulter), and then loaded onto BD FACSCanto II where 25,000 events were collected in the lymphocyte gate. The resulting data and the median fluorescence intensity (MFI) were analyzed utilizing FlowJo software.

Detection of Thyroid Hormones *via* Immunoassay

To detect T_4 or T_3 hormones, 5×10^6 leukocytes were washed and resuspended in 800 μL of complete RPMI or supplemented DMEM F-12 media (DMEM F-12 with 50 U/mL penicillin, 50 μg/mL streptomycin, non-essential amino acids, and MEM vitamin solution) (Gibco). The cells were incubated with or without native human TG (609312, Sigma) at a concentration of 20 μg/mL for 3 days. Supernatants from the cell culture were then loaded onto the Vitros ECiQ Immunodiagnostics instrument (Ortho molecular diagnostics) and assayed with Vitros reagents (total T_4: 874468; total T_3: 1322528; free T_4: 1387000; free T_3: 1315589).

Statistical Analysis

Analysis and graphs/plots of all data were performed in GraphPad prism and Microsoft Excel software using two-tailed t-test assuming equal variance. Levels of significance $p < 0.05$ and $p < 0.005$ are presented as * and **, respectively.

RESULTS

Human Leukocytes Express and Regulate Thyroid-Related Compounds

Previous work illustrated the ability of leukocytes to concentrate radio iodide (28). However, it is unclear how iodide is transported into immune cells or if leukocytes differentially express one or both of the known iodide transporters. To this end, human leukocytes were extracted from different donors, and then transcript levels of thyroid-related molecules were amplified with reverse transcription PCR. DNA gel electrophoresis analysis illustrated that leukocytes expressed expected size transcripts of NIS, and PENDRIN (**Figure 1A**). Activation of leukocytes with PMA and ionomycin (PMA-IO) induced a substantial increase of mRNA levels of PENDRIN but not NIS (**Figure 1A**). qPCR with TaqMan probes confirmed no significant change in NIS expression, but approximately eightfold increase of PENDRIN mRNA upon activation of leukocytes (**Figure 1B**). Although we could not find validated TaqMan probes that could amplify TPO, gel analysis revealed expected size TPO transcripts that were increased when leukocytes were activated (**Figure 1A**). Next, TG transcripts have been previously found in peripheral blood lymphocytes but it is not clear whether cellular activation regulates the mRNA expression of this protein (45). Since leukocytes upregulated PENDRIN and TPO, we sought to investigate the expression of the large dimeric protein TG during activation of leukocytes. Accordingly, mRNA analysis confirmed the expression of TG in leukocytes, but that activation significantly reduced its mRNA expression (**Figure 1B**).

To determine if NIS or PENDRIN are differentially expressed within peripheral blood leukocyte subsets, we utilized flow cytometric analysis of leukocytes probed with antibodies against NIS or PENDRIN. Leukocyte subpopulations were separated based on side scatter and CD45 intensity (Figure S1A in Supplementary Material), and then levels of iodide transporters were examined. NIS and PENDRIN were expressed in all leukocyte populations with the strongest expression detected in granulocyte and monocyte populations and minimal expression on lymphocytes (**Figure 1C**). Quantitative MFI analysis showed minor but non-significant MFI increase over background on lymphocytes, but strong and significant expression on granulocytes and monocytes (**Figure 1D**). Altogether, these results demonstrate that leukocytes, particularly monocytes and granulocytes, express and regulate thyroid-related compounds.

Iodide Induces Human Leukocytes to Undergo Transcriptional Modification in Immunity-Related Genes

No studies thus far have determined if iodine or iodide could directly affect the transcriptome of immune cells. Since treatment with Lugol's solution containing iodine/iodide could alter transcriptional changes in breast cancer cell lines, and leukocytes expressed iodide transporters (**Figure 1**), we asked if iodide could alter immunity-related transcriptional events in human immune cells (38). We first investigated whether iodide had any toxic effects on human leukocytes. Preceding studies in breast cancer cell lines found no toxic effects using 1 mM of Lugol's iodine solution (38). To confirm these studies in primary immune cells, we incubated leukocytes with 1 mM of NaI for 3 days. Overall, we found no significant changes in total cell counts or viability with iodide treatment (**Figure 2A**).

To determine if iodide treatment had any effect on the transcriptional activity of human leukocytes, we utilized NGS in the context of targeted RNASeq. This method allows quantification of hundreds of immunity-related genes per sample. To this end, leukocytes were incubated for 2 days with 1 mM NaI or PBS control, and then RNA was extracted. NGS library preparations were created, and then control or NaI-treated leukocytes were screened for changes in 475 inflammation and immunity genes. We found that immune cells treated with iodide had significant changes in total 29 genes with 24 being upregulated (**Figure 2B**; Table S1 in Supplementary Material). Although all transcriptional changes presented were statistically significant, IL6 and pro-platelet basic protein had a p value trending close to significance (0.085 and 0.076, respectively) due to variability despite the observed fold increase in all treatment groups (Table S1 in Supplementary Material). Genes that were upregulated included modulators that could affect survival or proliferation such as IL2, IL24, and CSF2. Interestingly, we observed increased expression of various cytokines that are considered to be pro- and/or anti inflammatory including IFNγ, IL6, IL1β, and IL13 (**Figure 2B**). However, chemokines comprised a significant fraction of the total altered genes and showed overall the highest fold increase with CCL7, CXCL5, and CXCL6 transcripts increasing by more than 10-fold in the iodide-treated groups (**Figure 2B**; Table S1 in Supplementary Material). Other transcriptional increases observed were in SRC kinase LYN, and complement C3. Next, iodide treatment significantly reduced the expression of both insulin growth factor-1 and leptin indicating the potential for iodide to affect hormonal balance. Altogether, our data illustrate the molecular immunomodulatory effects of iodide on human immune cells.

Immune Cells Increase Cytokine Secretion after Iodide Treatment

Basal level elevation of chemokine and cytokine transcripts after iodide treatment suggested that immune cells may consequently increase protein release. Accordingly, we examined the quantity of select cytokines comparing control and iodide-treated leukocytes. Basal levels of pro- and anti-inflammatory cytokines including IFNγ, IL6, and IL10 were substantially increased in the presence of iodide in cell culture 3 days after treatment (**Figure 3A**). IL6 production was the most affected with more than fivefold average increase in protein release (**Figure 3A**). Similarly, chemokines IL8 (CXCL8) and CCL2 were elevated after iodide treatment. However, changes in CCL2 were variable

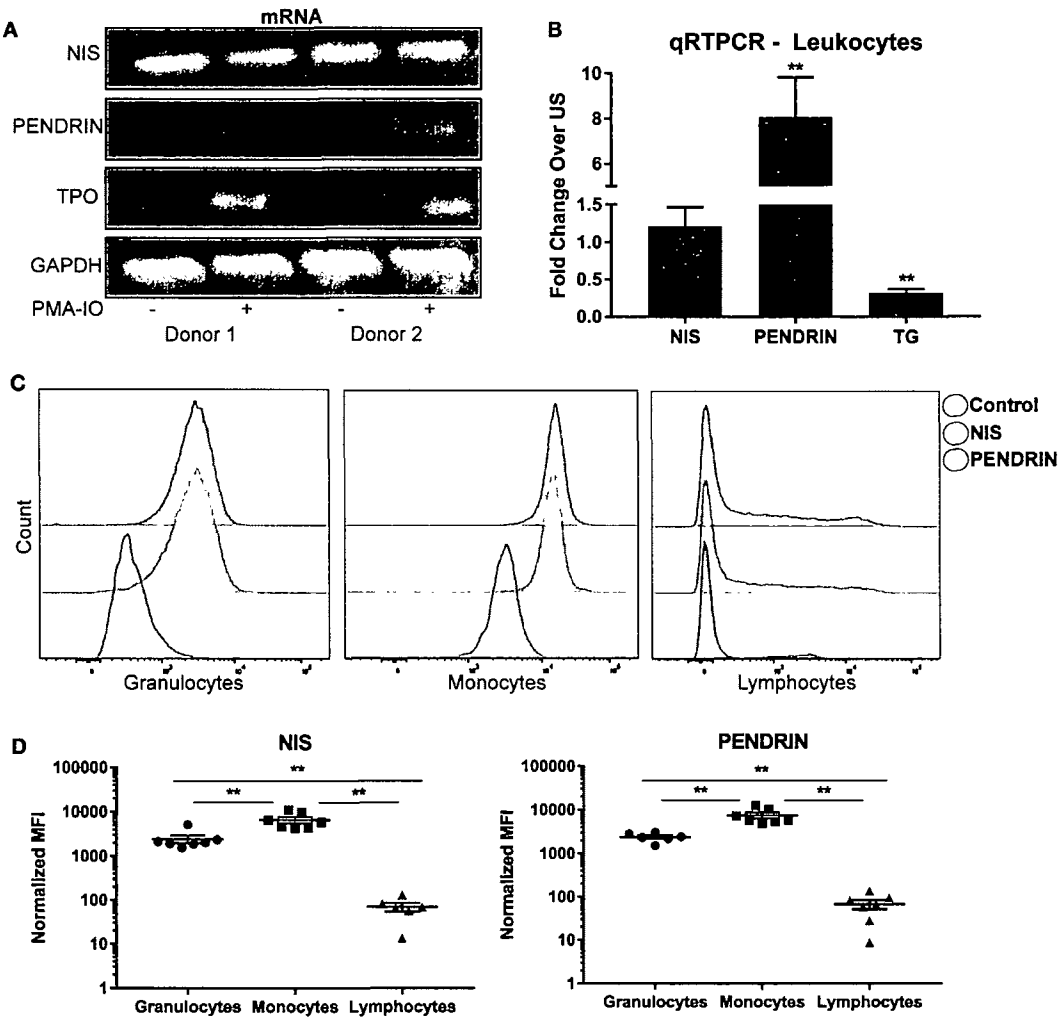

FIGURE 1 | Human immune cells express and regulate iodide transporters, thyroglobulin, and thyroid peroxidase. **(A)** Leukocytes were left unstimulated (US) (−) or activated (+) with PMA and ionomycin for 18 h, total RNA was extracted, and then the cDNA from samples was amplified with PCR. Shown is DNA gel electrophoresis image of two representative donors. **(B)** Leukocytes mRNA was amplified using real-time quantitative PCR, and then each gene was normalized to internal B2M control. Fold increase over US controls was graphed ± SEM of nine independent donors. **(C)** Leukocytes were stained with no antibody (control), NIS, or PENDRIN antibodies. The cells were then stained with secondary Alexa-647 and primary-conjugated CD45 Krome-orange antibodies. Leukocyte subsets (granulocytes, monocytes, and lymphocytes) were gated on based on CD45 and side-scatter characteristics. The gated subset histograms are shown as cell counts and antibody staining intensity representative of six donors. **(D)** Median fluorescent intensity (MFI) values from panel **(C)** were subtracted from background staining and then graphed ± SEM of six to seven independent donors.

with 3 out of 11 donors showing no change or slight reduction in cytokines levels (**Figure 3A**).

To determine if other forms of iodine could affect basal cytokine release, we incubated leukocytes with 500 μM of Lugol's iodine. That is, instead of NaI, the cells would be incubated with a mixture of KI and I_2. Similar to our observations with NaI, leukocytes exposed to Lugol's iodine had even greater increase of protein release in IL6, IL10, and CXCL8 (**Figure 3B**). However, we were surprised to find that Lugol's iodine significantly reduced release of IFNγ (0.47-fold) compared with controls. Next, though not statistically significant, CCL2 secretion displayed a similar pattern to NaI-treated cells wherein five out of nine donors had reduced levels (**Figure 4B**). The subtle differences between NaI and Lugol's can be explained by the distinct iodine-derivatives between the two treatments. These data suggest that iodine could affect the functional activity of human immune cells.

Iodide-Induced Cytokine and Transcriptional Alteration Is Not due to *De Novo* Thyroid Hormone Synthesis or Deiodination

Thyroid hormones are important for the metabolic function of all cells, but also carry the ability to affect the cytokine and chemokine profiles, phenotypes, and function of immune cells (20, 22, 23, 46). One possible reason for the iodide-induced

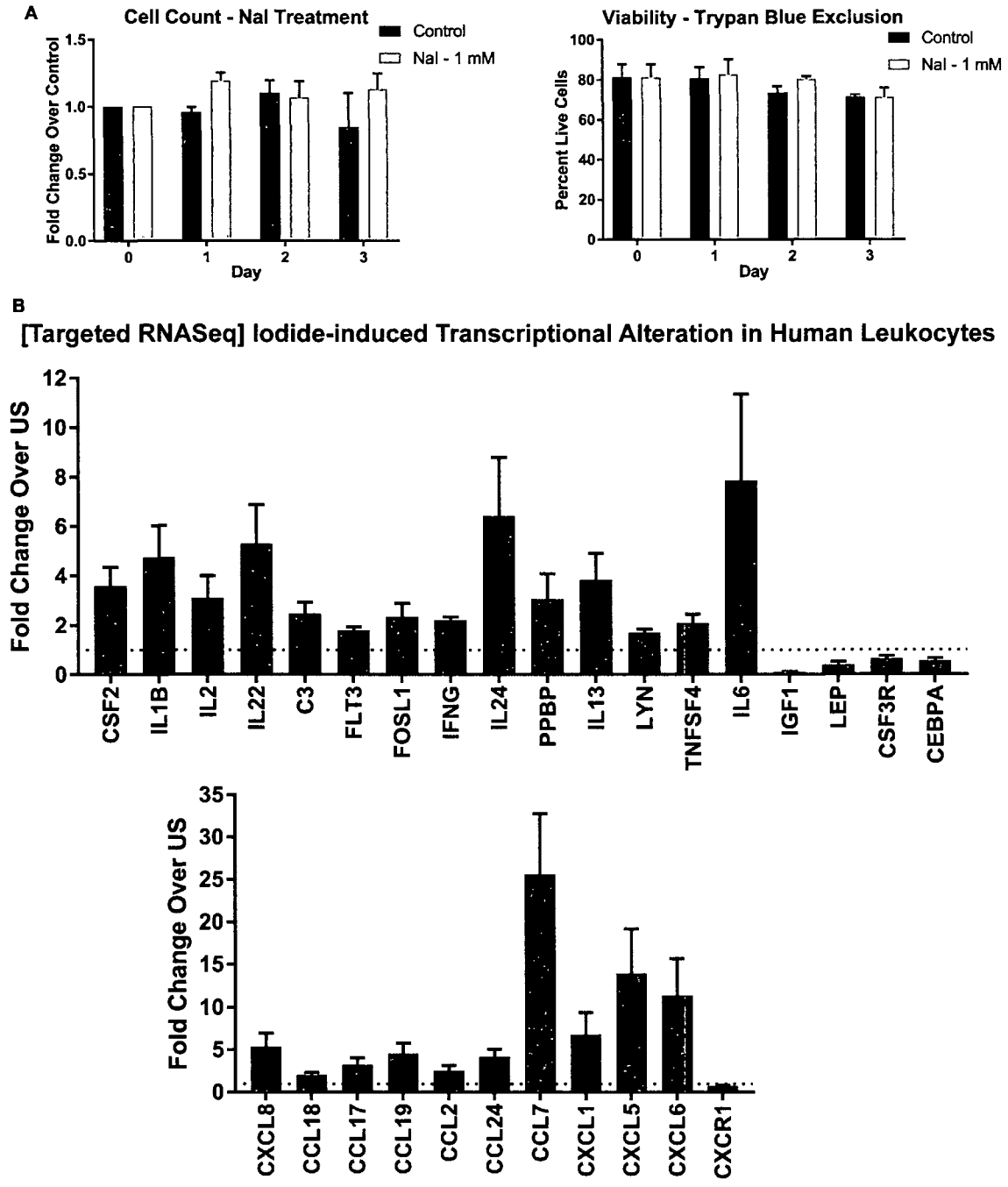

FIGURE 2 | Targeted RNASeq analysis of iodide-treated leukocytes *via* next-generation sequencing. **(A)** Viability analysis of iodide-treated leukocytes—5 x 10^6 leukocytes were left untreated (control—PBS) or incubated with 1 mM NaI for up to 3 days. Cell counts and viability utilizing trypan blue exclusion were determined with TC20 automated cell counter. **(B)** Targeted RNASeq—leukocytes were left unstimulated with PBS or incubated with 1 mM NaI for 48 h, total RNA was extracted, and then targeted RNASeq libraries were created for a total of 475 genes. The libraries were indexed (multiplexed) and then loaded onto Illumina MiSeq sequencer. The data were de-multiplexed, and unique molecular tags were identified utilizing Qiagen's RNASeq bioinformatics software. Total molecular tag counts were normalized to counts of 10 housekeeping genes and then quantified based on fold expression relative to each untreated control. Average fold increases were obtained from 5 to 10 independent donors. Genes that were significantly increased or decreased ($p < 0.05$) were selected and displayed in bar graph ± SEM of at least five independent donors. See Table S1 in Supplementary Material for quantifications and p values.

elevation of mRNA and cytokine release in leukocytes would be an increase of *de novo* T_4 synthesis and/or by the known ability of immune cells to deiodinate T_4 present in media into T_3 (18, 47–49). Therefore, we analyzed supernatants of NaI-pulsed leukocytes for possible fluctuations of thyroid hormones. Utilizing immunodiagnostics immunoassay ECiQ instrument, we could

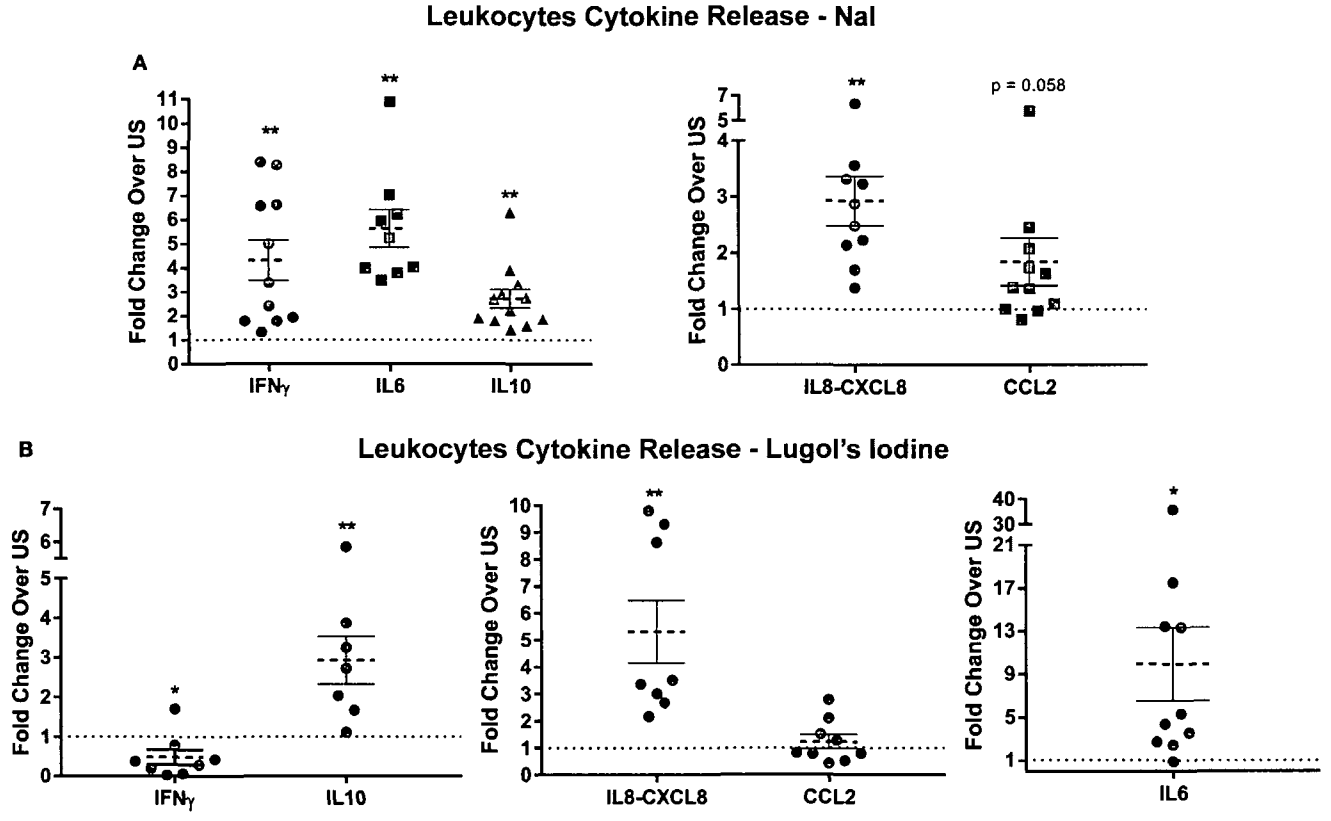

FIGURE 3 | Increased release of cytokines and chemokines by iodide-treated leukocytes. **(A)** 5 × 10^6 leukocytes were left unstimulated (US) with PBS or treated with 1 mM NaI for 72 h. The supernatants were collected, and protein levels of cytokines/chemokines were analyzed utilizing enzyme-linked immunosorbent assay. **(B)** Same as in panel **(A)**, but instead leukocytes were incubated with 500 μM Lugol's iodine for 72 h, and then the supernatant was analyzed for cytokine levels. Detected ranges for cytokine secretion are (IFNγ: 5–1,005 pg/mL, IL6: 10–2,500 pg/mL, IL10: 26–482 pg/mL, IL8-CXCL8: 0.3–230 ng/mL, and CCL2: 15–65 ng/mL). Cytokine concentrations were normalized based on fold changes over each US pair and was then averaged and graphed as fold increase ± SEM of at least seven donors.

not detect any significant differences in free and total forms of thyroid hormones between controls and NaI-treated leukocytes (**Figure 4A**). Next, we have attempted to expose leukocytes to various experimental settings in an effort to expose potential *de novo* T_4 synthesis and release. To this end, leukocytes were incubated in serum free media (to remove serum hormones) supplemented with amino acids with added vitamins and minerals along with 1 mM NaI and/or 500 μM Lugol's solution. These experiments could not produce any detectable T_4 or T_3 in the supernatant (data not shown). In addition, stimulating leukocytes with PMA-IO or anti-TCR antibodies in the presence of NaI or Lugol's iodine could not produce detectable levels of T_4 or T_3 in the supernatant (data not shown). These observations indicate that the increased specific transcription and cytokine release by Lugol's iodine and NaI-treated leukocytes is not driven by new synthesis of T_4 or T_3.

Immune Cells Utilize TG to Increase the Levels of Thyroid Hormones T_4 and T_3

During an iodine deficiency TG is substantially increased in the blood causing it to potentially interact with cells and tissues (50–52). Thus, far no reports have demonstrated if TG could also be utilized by immune or other cells types. Since thyroid hormones affect the immune system, we asked if human leukocytes could utilize thyroid-derived TG to increase surrounding levels of T_4 and T_3 (20, 22, 23, 53, 54). To this end, we incubated leukocytes with TG for 3 days in serum free media, to avoid interference with hormones present in serum, and then levels of T_4 and T_3 were detected. Interestingly, leukocytes incubated with TG released substantial amounts of T_4 and T_3 wherein no hormones were detected in untreated cells (**Figure 4B**). We were surprised to observe that this effect was not carried on to the Jurkat T cell line suggesting a non T cell-mediated mechanism (**Figure 4B**). These results suggest that a leukocyte subset, likely phagocytes, could endocytose TG, release T_4 and then produce T_3 by deiodination. Next, the thyroid is estimated to contain hormones consisting of 80–90% T_4 and approximately 10–20% T_3 stored mostly within TG (15–17). Therefore, we assume that the increased levels of T_3 after TG incubation is due to T_4 deiodination by immune cells. To confirm that human leukocytes could deiodinate T_4, the cells were instead given T_4 and then levels of T_3 were analyzed. Confirming previously reported observations, leukocytes could produce significant amounts of T_3 *via* deiodination (**Figure 4C**) (47–49, 55). Our data reflect the interaction between human

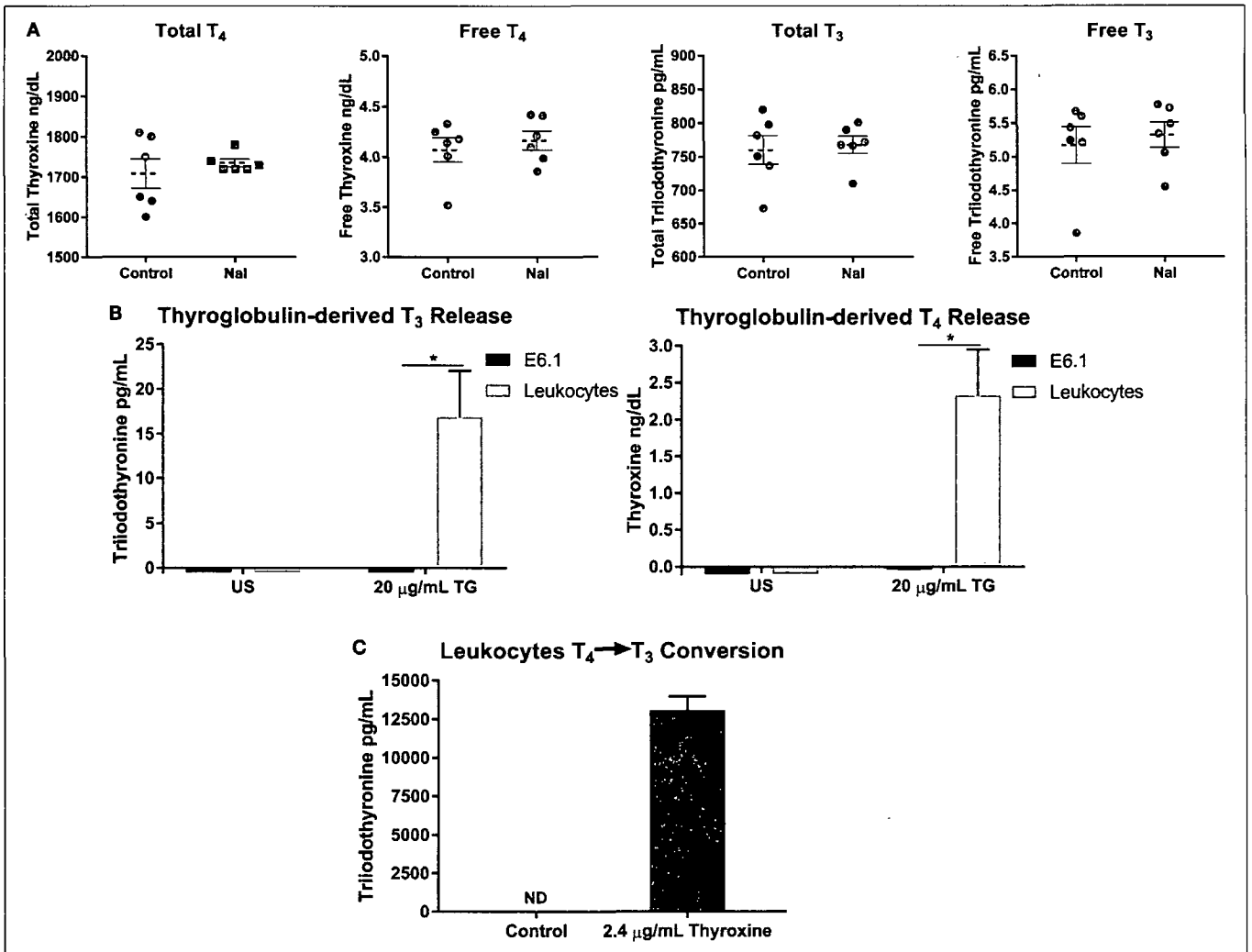

FIGURE 4 | Thyroglobulin (TG) is utilized by leukocytes to increase levels of thyroid hormones. **(A)** 5×10^6 leukocytes were left unstimulated with PBS or treated with 1 mM NaI for 72 h, and then levels of total and free thyroid hormones in media were detected utilizing ECiQ diagnostic immunoassay instrument. Shown are averaged concentrations ± SEM of six donors. **(B)** 5×10^6 leukocytes were incubated with TG derived from human thyroid tissue at a concentration of 20 µg/mL for 3 days in serum free media, and then levels thyroid hormones (T_4 and T_3) in media were detected utilizing ECiQ diagnostic immunoassay instrument. The data were averaged after background subtraction of TG signal. Shown are averaged concentrations ± SEM of six donors. **(C)** Same as in panel **(B)**, but instead leukocytes were incubated with thyroxine (T_4) at 2.4 µg/mL for 2 days in serum free media. Levels of T_3 were analyzed utilizing diagnostic immunoassay instrument ECiQ. The data were averaged after background subtraction of T_4 signals. Data shown represent averaged quantifications ± SEM of six donors.

immune cells and TG, and that immune cells could potentially raise levels of T_4 and T_3 in tissues and peripheral blood.

DISCUSSION

In this study, we have identified immunomodulatory effects of iodide on human immune cells. We have demonstrated that, in the presence of iodide, human immune cells undergo specific molecular changes in certain cytokines and chemokines, and that this effect is translated into higher protein release (**Figure 5A**). These events are not caused by increased thyroid hormone synthesis, but instead by an unclear iodide specific mechanism. However, our investigation into the possibility of thyroid hormone synthesis by leukocytes revealed the capability of immune cells to process TG and release the thyroid hormones T_4 and T_3 (**Figures 4B** and **5B**).

Analysis of iodide receptor expression on leukocyte subsets demonstrated that phagocytes, monocytes, and granulocytes harbor the highest expression of iodide transporters (**Figures 1C,D**). Earlier studies reported the interplay between leukocyte myeloperoxidase and H_2O_2, with the halides chloride and iodide (56–58). The authors illustrate the antibacterial effect of myeloperoxidase mediated iodination, which was more effective in killing bacteria than chloride. The observed increase in PENDRIN post activation of immune cells, a transporter specific for both chloride and iodide, suggests that both ions are important for the killing mechanisms of monocytes and granulocytes (**Figures 1A,B**) (57). Therefore, it is likely that the mechanism of iodide intracellular

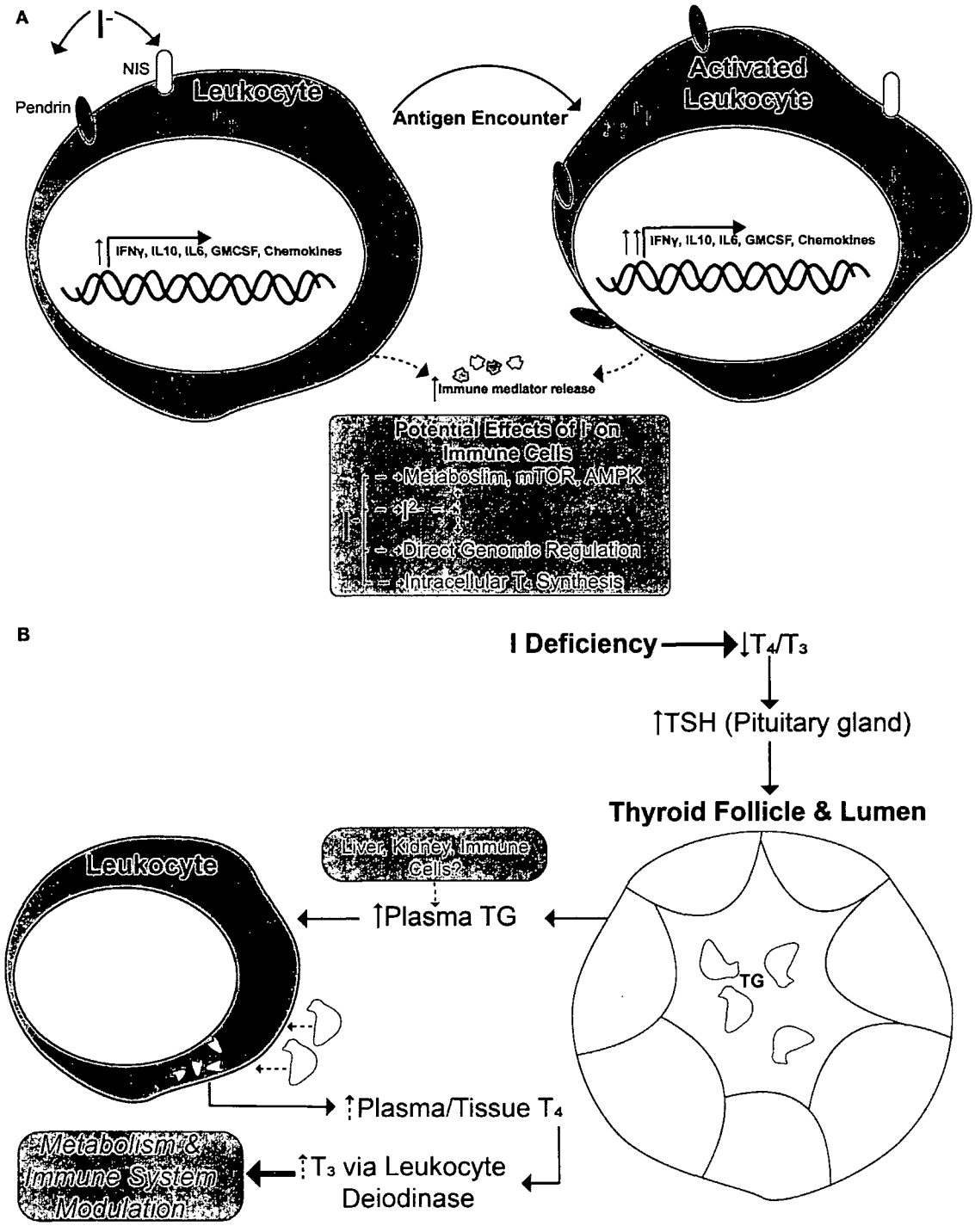

FIGURE 5 | Current model—immune cells regulate their function *via* iodide and increase levels of thyroid hormones by processing thyroid-derived thyroglobulin (TG). **(A)** Immune cells express surface iodide transporters (NIS and PENDRIN) that are upregulated during cellular activation. The cells are able to accumulate iodide that could alter the transcription of multiple immune mediators and possibly other genes. The changes are functional since the higher mRNA levels correlate with increased cytokine release at the basal state. The effect is systemic and is not polarized to either pro- or anti-inflammatory genes. During an immune response, the presence of sufficient amounts of iodide allows for a "primed" state of cells that are ready to proliferate upon activation. The effects of iodide on immune cells could have an impact during early conception wherein immune cells could release more factors to support blood vessel and optimal pregnancy. **(B)** In an iodine deficiency state, TSH is secreted to command an increase of mostly T_4 and some T_3 by the thyroid. The thyroid responds by elevating NIS and PENDRIN surface expression and the production of more TG, some of which is released in the blood stream. Increased severity of iodine deficiency raises TG levels in the blood accordingly. Based on our findings, we propose that leukocytes could uptake TG from the blood or tissues and release T_4, which will eventually raise T_3 levels by deiodinase activity. This has a systemic effect since it increases levels of hormones to local tissues and/or in the blood for increased metabolism. Released thyroid hormones are also known to affect the immune system by enhancing cytokine expression and altering phenotypes of immune cells.

transport would be used by phagocytes to clear infections. The idea of iodide receptor regulation is not new as the effect of hormones and cytokines on iodide transporters have been previously illustrated (59–61). We cannot confirm if the increase in PENDRIN and TPO in our experimental setting was due to intracellular activation by PMA-IO or secondary to cytokine release by leukocytes post activation. The increase of PENDRIN and TPO expression post PMA-IO stimulation implies that leukocytes may require increased iodide uptake when activated during an immune response. This hypothesis is strengthened by our results where increased iodide substantially raises the secretion levels of multiple cytokines. Surprisingly, lymphocytes exhibited relatively minimal expression of NIS and PENDRIN (**Figures 1C,D**). However, this does not imply that lymphocytes are unresponsive to iodide as it has been clearly demonstrated that exposure to iodide produces increases in immunoglobulin synthesis by lymphocytes with even lower doses of iodide used in this study (62).

Assessment of immunity-related genes *via* targeted RNASeq in iodide-treated leukocytes revealed significant transcriptional changes in 29 out 475 analyzed genes. The transcriptional changes observed in leukocytes were not polarized but instead constituted a mix of cytokines that were pro- and anti-inflammatory including IFNγ, IL6, and IL13. Differentiation and survival factors such as IL2, IL24, and CSF2 were also increased *via* iodide stimulation. The effects of iodide, however, were striking with regards to chemokines and their receptors which constituted 11 out of the 29 altered genes (**Figure 2B**). The most substantial transcriptional fold changes were associated with chemokines CCL7 (22.5), CXCL5 (13.9), and CXCL6 (11.3) (**Figure 2B**; Table S1 in Supplementary Material). The concept that iodine/iodide causes transcriptional changes is not new as iodine stimulation was shown to induce multiple transcriptional changes in human breast cancer and trophoblastic cell lines (38, 63). It is therefore very likely that non-immunity transcriptional changes would be occurring in leukocytes in the presence of iodine/iodide. Similar to breast cancer and trophoblastic cell lines, these changes may include genes associated with estrogen metabolism, cyclins, and transcription factors (38, 63). Next, although we have analyzed a short list of cytokines *via* ELISA, the cytokine release profile was not skewed to either pro- or anti-inflammatory, but instead seems to be systemic thereby mimicking the observed transcriptional changes. It is unclear how iodide induces these changes, but it is likely a combination of mechanisms such as an enhancement of metabolic pathways or intracellular conversion of iodide into iodine (**Figure 5A**) (64, 65). We also suggest the possibility of iodine influencing transcription by directly or indirectly activating transcription factors, or by altering enhancer/promoter sequences in the genome. In this case, iodine would mirror a similar role to zinc which could activate transcription factors to increase cytokine expression as well as interact directly with DNA (66–68).

One limitation of our study is that we have utilized a mixed leukocyte population and therefore it is not clear which cell population is contributing to the transcriptional changes. Moreover, some cell types (i.e., granulocytes) are short lived and may not represent the transcriptional changes presented in this study. Similarly, monocytes adhere to cell culture plates and could be lost during the extraction phase from culture plates. To this end, we compared leukocyte populations on the day of extraction and 2 days after cell culture *via* flow cytometry. There were no significant differences on the percentages of monocyte or lymphocyte populations 2 days after cell culture (Figures S1B,C and Table S2 in Supplementary Material). However, the monocyte population had increased side scatter but maintained exclusive expression of CD14 as in day 0. Treatment of cells for 2 days with 1 mM NaI did not cause significant changes to the percentages of leukocyte subsets. Analysis of the granulocyte fraction, however, illustrated significant reduction in cell percentage 2 days post cell culture. Therefore, our analyses in this study represent lymphocytes, monocytes, and a relatively small number of granulocytes. Importantly, in our experimental setting, the leukocyte populations utilized in targeted RNASeq had similar viabilities and were overall comparable in percentage to initial day of extraction (**Figure 2A**; Figures S1B,C in Supplementary Material).

An objective of this study was to determine if leukocytes could produce *de novo* thyroid hormones that could potentially affect systemic and/or local hormone levels. Previous experiments with radioiodine provided an insight on synthesis of low intracellular T_4 by cardiomyocytes (29). The authors argue that the low level of intracellular T_4 would only affect the synthesizing cardiomyocyte and not surrounding cells (29). Likewise, the presence of "thyroxine-like" compounds in ^{131}I-pulsed leukocytes suggested the possibility of thyroid hormone synthesis by extrathyroidal tissues (28). Under our experimental settings, we could not detect any increase of T_4 or T_3 when the cells were incubated with 1 mM of NaI for 72 h in complete media (**Figure 4A**). Likewise, addition of Lugol's iodine or NaI to leukocytes in amino acid and vitamin supplemented serum free media with or without cellular activation did not yield any detectable levels of T_4 or T_3 in the supernatant (data not shown). We cannot, however, exclude the possibility of intracellular *de novo* T_4 synthesis by leukocytes or very low levels of T_4 below the sensitivity range of our immunoassay instrument. On the other hand, we observed a marked increase of T_4 and T_3 in culture media after leukocytes were incubated with TG (**Figure 4B**). These hormones were already present on TG since it was derived from human thyroid that under normal conditions should contain 80–90% T_4. These observations indicate that immune cells could implement the last two out of the three following steps performed by the thyroid glands for hormone synthesis: (1) organification of iodine into TG, (2) endocytosis of TG containing thyroid hormones, and (3) release of T_4 and some T_3 into peripheral blood. Next, during the process of T_4 release, leukocytes were able to deiodinate TG-derived T_4 into T_3 thereby increasing levels of the active thyroid hormone T_3 (**Figure 4C**). The interaction of immune cells with TG is physiologically relevant since TG is present in the blood and is significantly increased during an iodine deficiency (51, 52). Altogether, these results shed light into the long-sought question of whether tissues other than the thyroid could influence blood or local levels of T_4 and demonstrate that at least immune cells could potentially affect blood thyroid hormone levels.

We have utilized in this study a concentration of 1 mM NaI (~125 μg/mL of iodide) that was non-toxic to primary human

immune cells. In fact, though not significant, NaI-treated cells had slightly higher cell number counts (**Figure 2A**). Moreover, this dose was non-toxic when used on human breast cancer cell lines (38). For these reasons, we opted to use this concentration for targeted RNA sequencing and functional studies in primary human immune cells. Observed population plasma levels of inorganic iodine (i.e., non hormonal iodine) are relatively low with total iodine in the range of 50–130 μg/L and an inorganic iodide range of 5–15 μg/L (69). In comparison with plasma iodide levels, thyroidal cells are exposed to 50- to 400-fold more of inorganic iodide (69). It is highly feasible that tissue resident immune cells, including intestinal or thymic cells, could be exposed to substantially higher levels of iodine relative to levels observed in plasma (37). Blood iodine levels reflect recent ingested iodine intakes and are not typically utilized for determining long-term iodine sufficiency status due partially to the kinetics of inorganic iodide metabolism where it is either absorbed and stored by tissues or excreted rapidly by the kidneys (69, 70). This is observed in persons with sufficient iodine levels where typically an iodine urinary loading test shows excretion of 90% or more of the ingested iodine within 24 h and much less in iodine deficient populations (71, 72). In fact, studies focusing on assessment of long-term levels of iodine status suggest biomarkers such as TG or urinary iodine as the more sensitive markers for iodine levels (50–52, 73, 74). Ingested iodine is therefore either rapidly absorbed by the thyroid and tissues expressing iodine transporters that are widely distributed or is excreted by the urine in quantities inversely correlated with whole-body sufficiency (28, 69, 72). Above all, optimal whole-body sufficiency levels of iodine are unknown, and the current RDA recommendations are provided primarily as preventative for goiter formation (2, 69). Future clinical investigations are needed to determine the safe daily quantity of iodine necessary for extrathyroidal tissue sufficiency.

Next, iodine is required for a successful healthy pregnancy, and its loss leads to miscarriages, reproductive failures, abnormal brain development, and congenital hypothyroidism (3, 9, 10). Epidemiological studies and surveys by the World Health Organization demonstrate that iodine deficiencies are occurring worldwide including in women of reproductive age (2, 5, 7, 75, 76). Recent findings by the National Health and Nutrition Examination Survey found that up to 35% of women in reproductive age have an iodine insufficiency (75, 76). The issue of iodine sufficiency during pregnancy is compounded as a result for higher iodine intake requirements during pregnancy and lactation (2, 7). Based on our findings and the role of the immune system in regulating the process of pregnancy, we suggest further investigation between immune cell dysfunction in female reproductive organs and the possibility of iodine deficiency in women with reproductive failures of unknown etiology.

In conclusion, we have presented evidence for the immunomodulatory effects of iodide on human peripheral blood immune cells. Iodide alters the transcriptional immune signature of these cells and induces stronger cytokine and chemokine responses. Accordingly, iodine/iodide levels that optimally saturate the cells should therefore enhance the immune system and improve trafficking, clearance of infections, and support the process of reproduction. Finally, we identify immune cells as a potential source of extrathyroidal thyroid hormones capable of performing functions typically known to be specific to the thyroid glands.

ETHICS STATEMENT

The research presented here was performed according to the principles indicated in the declaration of Helsinki. The consent and documentation process associated with donors utilized in this study were approved by the IRB for Rosalind Franklin University of Medicine and Science.

AUTHOR CONTRIBUTIONS

MB conceived, designed, and performed the experiments; interpreted the data; and wrote the manuscript with input from all other authors. SD, JK-K, AG-S, and KB contributed to study design and data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01573/full#supplementary-material.

FIGURE S1 | Leukocyte populations and viabilities post iodide treatment. **(A)** Leukocytes were stained with primary-conjugated CD45 Krome-orange antibodies. Leukocyte subsets (granulocytes, monocytes, and lymphocytes) were gated on based on CD45 and side-scatter characteristics. **(B)** Leukocytes were stained with CD45 Krome orange and CD14 FITC on the day of isolation or after 2 days of culture on 12-well culture dishes with or without 1 mM NaI. Shown is a representative donor with color coded scatter plots illustrating granulocytes, monocytes, and lymphocytes. **(C)** Leukocytes population percentages from panel **(B)** were averaged and graphed. Data shown represent averaged quantifications ± SD of six donors. See Table S2 in Supplementary Material for quantifications and *p* values.

REFERENCES

1. Rousset B, Dupuy C, Miot F, Dumont J. Thyroid hormone synthesis and secretion. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, Koch C, Korbonits M, McLachlan R, New M, Purnell J, Rebar R, Singer F, Vinik A, editors. *Endotext*. (Chap. 2), South Dartmouth, MA (2000). p. 2–3. Available from: http://www.thyroidmanager.org/wp-content/uploads/chapters/chapter-2-thyroid-hormone-synthesis-and-secretion.pdf
2. Benoist BD, Andersson M, Egli I, Takkouche B, Allen H. *Iodine Status Worldwide*. Geneva: World Health Organization (2004).
3. Verheesen RH, Schweitzer CM. Iodine deficiency, more than cretinism and goiter. *Med Hypotheses* (2008) 71(5):645–8. doi:10.1016/j.mehy.2008.06.020
4. Ahad F, Ganie SA. Iodine, Iodine metabolism and Iodine deficiency disorders revisited. *Indian J Endocrinol Metab* (2010) 14(1):13–7.
5. Dillon JC, Milliez J. Reproductive failure in women living in iodine deficient areas of West Africa. *BJOG* (2000) 107(5):631–6. doi:10.1111/j.1471-0528.2000.tb13305.x
6. Perez-Lopez FR. Iodine and thyroid hormones during pregnancy and postpartum. *Gynecol Endocrinol* (2007) 23(7):414–28. doi:10.1080/09513590701464092

7. Zimmermann MB. The effects of iodine deficiency in pregnancy and infancy. *Paediatr Perinat Epidemiol* (2012) 26(Suppl 1):108–17. doi:10.1111/j.1365-3016.2012.01275.x
8. Sukkhojaiwaratkul D, Mahachoklertwattana P, Poomthavorn P, Panburana P, Chailurkit LO, Khlairit P, et al. Effects of maternal iodine supplementation during pregnancy and lactation on iodine status and neonatal thyroid-stimulating hormone. *J Perinatol* (2014) 34(8):594–8. doi:10.1038/jp.2014.62
9. Kapil U. Health consequences of iodine deficiency. *Sultan Qaboos Univ Med J* (2007) 7(3):267–72.
10. Pharoah P, Buttfield IH, Hetzel BS. Neurological damage to the fetus resulting from severe iodine deficiency during pregnancy. *Int J Epidemiol* (2012) 41(3):589–92. doi:10.1093/ije/dys070
11. Laurberg P, Andersen SL. Keep an eye on iodine and the thyroid and save the brain. *Horm Res Paediatr* (2014) 81(6):361–2. doi:10.1159/000360700
12. Wilders-Truschnig MM, Warnkross H, Leb G, Langsteger W, Eber O, Tiran A, et al. The effect of treatment with levothyroxine or iodine on thyroid size and thyroid growth stimulating immunoglobulins in endemic goitre patients. *Clin Endocrinol (Oxf)* (1993) 39(3):281–6. doi:10.1111/j.1365-2265.1993.tb02367.x
13. Dumont JE, Ermans AM, Maenhaut C, Coppee F, Stanbury JB. Large goitre as a maladaptation to iodine deficiency. *Clin Endocrinol (Oxf)* (1995) 43(1):1–10. doi:10.1111/j.1365-2265.1995.tb01886.x
14. Spitzweg C, Heufelder AE, Morris JC. Thyroid iodine transport. *Thyroid* (2000) 10(4):321–30. doi:10.1089/thy.2000.10.321
15. Barrett EJ. Synthesis of thyroid hormones. In: Boron WF, Boulpaep EL, editors. *Medical Physiology - A Cellular and Molecular Approach*. 2nd ed. Philadelphia, PA: Saunders Elsevier (2012).
16. Laurberg P. Mechanisms governing the relative proportions of thyroxine and 3,5,3'-triiodothyronine in thyroid secretion. *Metabolism* (1984) 33(4):379–92. doi:10.1016/0026-0495(84)90203-8
17. Mortoglou A, Candiloros H. The serum triiodothyronine to thyroxine (T3/T4) ratio in various thyroid disorders and after levothyroxine replacement therapy. *Hormones (Athens)* (2004) 3(2):120–6. doi:10.14310/horm.2002.11120
18. Bianco AC, Kim BW. Deiodinases: implications of the local control of thyroid hormone action. *J Clin Invest* (2006) 116(10):2571–9. doi:10.1172/JCI29812
19. Balazs C, Leovey A, Szabo M, Bako G. Stimulating effect of triiodothyronine on cell-mediated immunity. *Eur J Clin Pharmacol* (1980) 17(1):19–23. doi:10.1007/BF00561672
20. Klecha AJ, Genaro AM, Gorelik G, Barreiro Arcos ML, Silberman DM, Schuman M, et al. Integrative study of hypothalamus-pituitary-thyroid-immune system interaction: thyroid hormone-mediated modulation of lymphocyte activity through the protein kinase C signaling pathway. *J Endocrinol* (2006) 189(1):45–55. doi:10.1677/joe.1.06137
21. Hodkinson CF, Simpson EE, Beattie JH, O'Connor JM, Campbell DJ, Strain JJ, et al. Preliminary evidence of immune function modulation by thyroid hormones in healthy men and women aged 55–70 years. *J Endocrinol* (2009) 202(1):55–63. doi:10.1677/JOE-08-0488
22. De Vito P, Incerpi S, Pedersen JZ, Luly P, Davis FB, Davis PJ. Thyroid hormones as modulators of immune activities at the cellular level. *Thyroid* (2011) 21(8):879–90. doi:10.1089/thy.2010.0429
23. Alamino VA, Montesinos MM, Rabinovich GA, Pellizas CG. The thyroid hormone triiodothyronine reinvigorates dendritic cells and potentiates anti-tumor immunity. *Oncoimmunology* (2016) 5(1):e1064579. doi:10.1080/2162402X.2015.1064579
24. Bagriacik EU, Zhou Q, Wang HC, Klein JR. Rapid and transient reduction in circulating thyroid hormones following systemic antigen priming: implications for functional collaboration between dendritic cells and thyroid. *Cell Immunol* (2001) 212(2):92–100. doi:10.1006/cimm.2001.1846
25. Csaba G, Pallinger E. Thyrotropic hormone (TSH) regulation of triiodothyronine (T(3)) concentration in immune cells. *Inflamm Res* (2009) 58(3):151–4. doi:10.1007/s00011-008-8076-8
26. Obregon MJ, Mallol J, Escobar del Rey F, Morreale de Escobar G. Presence of L-thyroxine and 3,5,3'-triiodo-L-thyronine in tissues from thyroidectomized rats. *Endocrinology* (1981) 109(3):908–13. doi:10.1210/endo-109-3-908
27. Nagao H, Imazu T, Hayashi H, Takahashi K, Minato K. Influence of thyroidectomy on thyroxine metabolism and turnover rate in rats. *J Endocrinol* (2011) 210(1):117–23. doi:10.1530/JOE-10-0484
28. Stolc V. Stimulation of iodoproteins and thyroxine formation in human leukocytes by phagocytosis. *Biochem Biophys Res Commun* (1971) 45(1):159–66. doi:10.1016/0006-291X(71)90064-7
29. Meischl C, Buermans HP, Hazes T, Zuidwijk MJ, Musters RJ, Boer C, et al. H9c2 cardiomyoblasts produce thyroid hormone. *Am J Physiol Cell Physiol* (2008) 294(5):C1227–33. doi:10.1152/ajpcell.00328.2007
30. Ullberg S, Ewaldsson B. Distribution of radio-iodine studied by whole-body autoradiography. *Acta Radiol Ther Phys Biol* (1964) 2:24–32. doi:10.3109/02841866409134127
31. Spitzweg C, Joba W, Eisenmenger W, Heufelder AE. Analysis of human sodium iodide symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland, and gastric mucosa. *J Clin Endocrinol Metab* (1998) 83(5):1746–51. doi:10.1210/jcem.83.5.4839
32. Wapnir IL, van de Rijn M, Nowels K, Amenta PS, Walton K, Montgomery K, et al. Immunohistochemical profile of the sodium/iodide symporter in thyroid, breast, and other carcinomas using high density tissue microarrays and conventional sections. *J Clin Endocrinol Metab* (2003) 88(4):1880–8. doi:10.1210/jc.2002-021544
33. Di Cosmo C, Fanelli G, Tonacchera M, Ferrarini E, Dimida A, Agretti P, et al. The sodium-iodide symporter expression in placental tissue at different gestational age: an immunohistochemical study. *Clin Endocrinol (Oxf)* (2006) 65(4):544–8. doi:10.1111/j.1365-2265.2006.02577.x
34. Catalano RD, Critchley HO, Heikinheimo O, Baird DT, Hapangama D, Sherwin JR, et al. Mifepristone induced progesterone withdrawal reveals novel regulatory pathways in human endometrium. *Mol Hum Reprod* (2007) 13(9):641–54. doi:10.1093/molehr/gam021
35. Marani L, Venturi S, Masala R. Role of iodine in delayed immune response. *Isr J Med Sci* (1985) 21(10):864.
36. Marani L, Venturi S. [Iodine and delayed immunity]. *Minerva Med* (1986) 77(19):805–9.
37. Venturi S, Venturi M. Iodine, thymus, and immunity. *Nutrition* (2009) 25(9):977–9. doi:10.1016/j.nut.2009.06.002
38. Stoddard FR II, Brooks AD, Eskin BA, Johannes GJ. Iodine alters gene expression in the MCF7 breast cancer cell line: evidence for an anti-estrogen effect of iodine. *Int J Med Sci* (2008) 5(4):189–96. doi:10.7150/ijms.5.189
39. Venturi S, Venturi M. Iodine in evolution of salivary glands and in oral health. *Nutr Health* (2009) 20(2):119–34. doi:10.1177/026010600902000204
40. Riesco-Eizaguirre G, Leoni SG, Mendiola M, Estevez-Cebrero MA, Gallego MI, Redondo A, et al. NIS mediates iodide uptake in the female reproductive tract and is a poor prognostic factor in ovarian cancer. *J Clin Endocrinol Metab* (2014) 99(7):E1199–208. doi:10.1210/jc.2013-4249
41. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci* (2011) 1221:80–7. doi:10.1111/j.1749-6632.2010.05938.x
42. Beaman KD, Ntrivalas E, Mallers TM, Jaiswal MK, Kwak-Kim J, Gilman-Sachs A. Immune etiology of recurrent pregnancy loss and its diagnosis. *Am J Reprod Immunol* (2012) 67(4):319–25. doi:10.1111/j.1600-0897.2012.01118.x
43. Beaman KD, Dambaeva S, Katara GK, Kulshrestha A, Gilman-Sachs A. The immune response in pregnancy and in cancer is active and supportive of placental and tumor cell growth not their destruction. *Gynecol Oncol* (2017) 145(3):476–80. doi:10.1016/j.ygyno.2017.04.019
44. Bowen JM, Chamley L, Keelan JA, Mitchell MD. Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. *Placenta* (2002) 23(4):257–73. doi:10.1053/plac.2001.0782
45. Amakawa M, Kato R, Kameko F, Maruyama M, Tajiri J. Thyroglobulin mRNA expression in peripheral blood lymphocytes of healthy subjects and patients with thyroid disease. *Clin Chim Acta* (2008) 390(1–2):97–103. doi:10.1016/j.cca.2008.01.007
46. Davis PJ, Glinsky GV, Lin HY, Mousa SA. Actions of thyroid hormone analogues on chemokines. *J Immunol Res* (2016) 2016:3147671. doi:10.1155/2016/3147671
47. Holm AC, Lemarchand-Beraud T, Scazziga BR, Cuttelod S. Human lymphocyte binding and deiodination of thyroid hormones in relation to thyroid function. *Acta Endocrinol (Copenh)* (1975) 80(4):642–56.
48. Smekens L, Golstein J, Vanhaelst L. Measurement of thyroxine conversion to triiodothyronine using human lymphocytes. A useful and simple laboratory technique. *J Endocrinol Invest* (1983) 6(2):113–7. doi:10.1007/BF03350582

49. Bianco AC, Nunes MT, Marone MS, Correa PH. Conversion of thyroxine (T4) to T3 and rT3 in human leucocyte suspension: its application to clinical investigation. *Endocrinol Exp* (1985) 19(1):53–61.
50. Pezzino V, Vigneri R, Squatrito S, Filetti S, Camus M, Polosa P. Increased serum thyroglobulin levels in patients with nontoxic goiter. *J Clin Endocrinol Metab* (1978) 46(4):653–7. doi:10.1210/jcem-46-4-653
51. Vejbjerg P, Knudsen N, Perrild H, Laurberg P, Carle A, Pedersen IB, et al. Thyroglobulin as a marker of iodine nutrition status in the general population. *Eur J Endocrinol* (2009) 161(3):475–81. doi:10.1530/EJE-09-0262
52. Bath SC, Pop VJ, Furmidge-Owen VL, Broeren MA, Rayman MP. Thyroglobulin as a functional biomarker of iodine status in a cohort study of pregnant women in the United Kingdom. *Thyroid* (2017) 27(3):426–33. doi:10.1089/thy.2016.0322
53. Chen Y. Effect of thyroxine on the immune response of mice in vivo and in vitro. *Immunol Commun* (1980) 9(3):260–76. doi:10.3109/08820138009065999
54. Paavonen T. Enhancement of human B lymphocyte differentiation in vitro by thyroid hormone. *Scand J Immunol* (1982) 15(2):211–5. doi:10.1111/j.1365-3083.1982.tb00640.x
55. Woeber KA. L-triiodothyronine and L-reverse-triiodothyronine generation in the human polymorphonuclear leukocyte. *J Clin Invest* (1978) 62(3):577–84. doi:10.1172/JCI109163
56. Klebanoff SJ. Iodination of bacteria: a bactericidal mechanism. *J Exp Med* (1967) 126(6):1063–78. doi:10.1084/jem.126.6.1063
57. Klebanoff SJ. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J Bacteriol* (1968) 95(6):2131–8.
58. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J Leukoc Biol* (2013) 93(2):185–98. doi:10.1189/jlb.0712349
59. Uyttersprot N, Pelgrims N, Carrasco N, Gervy C, Maenhaut C, Dumont JE, et al. Moderate doses of iodide in vivo inhibit cell proliferation and the expression of thyroperoxidase and Na+/I- symporter mRNAs in dog thyroid. *Mol Cell Endocrinol* (1997) 131(2):195–203. doi:10.1016/S0303-7207(97)00108-1
60. Spitzweg C, Joba W, Morris JC, Heufelder AE. Regulation of sodium iodide symporter gene expression in FRTL-5 rat thyroid cells. *Thyroid* (1999) 9(8):821–30. doi:10.1089/thy.1999.9.821
61. Dohan O, De la Vieja A, Paroder V, Riedel C, Artani M, Reed M, et al. The sodium/iodide symporter (NIS): characterization, regulation, and medical significance. *Endocr Rev* (2003) 24(1):48–77. doi:10.1210/er.2001-0029
62. Weetman AP, McGregor AM, Campbell H, Lazarus JH, Ibbertson HK, Hall R. Iodide enhances IgG synthesis by human peripheral blood lymphocytes in vitro. *Acta Endocrinol (Copenh)* (1983) 103(2):210–5.
63. Olivo-Vidal ZE, Rodriguez RC, Arroyo-Helguera O. Iodine affects differentiation and migration process in trophoblastic cells. *Biol Trace Elem Res* (2016) 169(2):180–8. doi:10.1007/s12011-015-0433-1
64. Carling D, Thornton C, Woods A, Sanders MJ. AMP-activated protein kinase: new regulation, new roles? *Biochem J* (2012) 445(1):11–27. doi:10.1042/BJ20120546
65. Chapman NM, Chi H. mTOR signaling, Tregs and immune modulation. *Immunotherapy* (2014) 6(12):1295–311. doi:10.2217/imt.14.84
66. Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ. Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. *J Leukoc Biol* (2009) 86(2):337–48. doi:10.1189/jlb.1208759
67. Klug A. The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annu Rev Biochem* (2010) 79:213–31. doi:10.1146/annurev-biochem-010909-095056
68. Foster M, Samman S. Zinc and regulation of inflammatory cytokines: implications for cardiometabolic disease. *Nutrients* (2012) 4(7):676–94. doi:10.3390/nu4070676
69. Risher JF, Keith S. *Iodine and Inorganic Iodides: Human Health Aspects*. Geneva: World Health Organization (2009).
70. Vought RL, London WT, Lutwak L, Dublin TD. Reliability of estimates of serum inorganic iodine and daily fecal and urinary iodine excretion from single casual specimens. *J Clin Endocrinol Metab* (1963) 23:1218–28. doi:10.1210/jcem-23-12-1218
71. Pearce EN, Andersson M, Zimmermann MB. Global iodine nutrition: where do we stand in 2013? *Thyroid* (2013) 23(5):523–8. doi:10.1089/thy.2013.0128
72. Haap M, Roth HJ, Huber T, Dittmann H, Wahl R. Urinary iodine: comparison of a simple method for its determination in microplates with measurement by inductively-coupled plasma mass spectrometry. *Sci Rep* (2017) 7:39835. doi:10.1038/srep39835
73. Ma ZF, Skeaff SA. Thyroglobulin as a biomarker of iodine deficiency: a review. *Thyroid* (2014) 24(8):1195–209. doi:10.1089/thy.2014.0052
74. Ershow AG, Goodman G, Coates PM, Swanson CA. Research needs for assessing iodine intake, iodine status, and the effects of maternal iodine supplementation. *Am J Clin Nutr* (2016) 104(Suppl 3):941S–9S. doi:10.3945/ajcn.116.134858
75. Caldwell KL, Makhmudov A, Ely E, Jones RL, Wang RY. Iodine status of the U.S. population, National Health and Nutrition Examination Survey, 2005–2006 and 2007–2008. *Thyroid* (2011) 21(4):419–27. doi:10.1089/thy.2010.0077
76. Leung AM, Pearce EN, Braverman LE. Iodine nutrition in pregnancy and lactation. *Endocrinol Metab Clin North Am* (2011) 40(4):765–77. doi:10.1016/j.ecl.2011.08.001

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.