

Thyrotrophin and thyroxine support immune homeostasis in humans

Martin Jaeger,^{1,2,3} 
 Yvette J.E. Sloot,^{1,3}
 Rob Ter Horst,² Xiaojing Chu,⁴
 Hans J.P.M. Koenen,^{3,5}
 Valerie A.C.M. Koeken,^{2,3}
 Simone J.C.F.M. Moorlag,²
 L. Charlotte J.de Bree,^{2,6,7}
 Vera P. Mourits,^{2,3} 
 Heidi Lemmers,² Helga Dijkstra,²
 Marco Medici,¹ Antonius E. van
 Herwaarden,⁸ Irma Joosten,^{3,5}
 Leo A.B. Joosten,² Yang Li,^{2,4}
 Johannes W.A. Smit,¹
 Mihai G. Netea^{2,9} and
 Romana T. Netea-Maier¹

¹Department of Internal Medicine, Division of Endocrinology, Radboud University Medical Center, Nijmegen, The Netherlands, ²Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, The Netherlands, ³Radboud Institute for Molecular Life Sciences (RIMLS), Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, ⁴Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, ⁵Department of Laboratory Medicine, Laboratory of Medical Immunology (LMI), Radboud University Medical Center, Nijmegen, The Netherlands, ⁶Research Center for Vitamins and Vaccines, Bandim Health Project, Statens Serum Institut, Copenhagen, Denmark, ⁷Odense Patient Data Explorative Network, University of Southern Denmark/Odense University Hospital, Odense, Denmark, ⁸Department of Laboratory Medicine, Radboud Laboratory for Diagnostics (RLD), Radboud University Medical Center, Nijmegen, The Netherlands and ⁹Department for Immunology & Metabolism, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany

Summary

The endocrine and the immune systems interact by sharing receptors for hormones and cytokines, cross-control and feedback mechanisms. To date, no comprehensive study has assessed the impact of thyroid hormones on immune homeostasis. By studying immune phenotype (cell populations, antibody concentrations, circulating cytokines, adipokines and acute-phase proteins, monocyte–platelet interactions and cytokine production capacity) in two large independent cohorts of healthy volunteers of Western European descent from the Human Functional Genomics Project (500FG and 300BCG cohorts), we identified a crucial role of the thyroid hormone thyroxine (T4) and thyroid-stimulating hormone (TSH) on the homeostasis of lymphocyte populations. TSH concentrations were strongly associated with multiple populations of both effector and regulatory T cells, whereas B-cell populations were significantly associated with free T4 (fT4). In contrast, fT4 and TSH had little impact on myeloid cell populations and cytokine production capacity. Mendelian randomization further supported the role of fT4 for lymphocyte homeostasis. Subsequently, using a genomics approach, we identified genetic variants that influence both fT4 and TSH concentrations and immune responses, and gene set enrichment pathway analysis showed enrichment of fT4-affected gene expression in B-cell function pathways, including the CD40 pathway, further supporting the importance of fT4 in the regulation of B-cell function. In conclusion, we show that thyroid function controls the homeostasis of the lymphoid cell compartment. These findings improve our understanding of the immune responses and open the door for exploring and understanding the role of thyroid hormones in the lymphocyte function during disease.

Keywords: immune response; thyroid hormones; thyroid-stimulating hormone (TSH); adaptive immunity; innate immunity.

Abbreviations: T4, Thyroxine; TSH, Thyroid-stimulating hormone; NK, Natural killer cells; HFGP, Human Functional Genomics Project; HPT, Hypothalamus–pituitary–thyroid axis; TRH, Thyrotrophin-releasing hormone; T3, 3,5,3'-triiodo-L-thyronine; FDR, False discovery rate; CRP, C-reactive protein; MR, Mendelian randomization; IVW, Inverse-variance weighted; GWAS, Genome-wide association study; PTGER3, Prostaglandin E receptor 3; TRAF, 6TNF receptor-associated factor 6; eQTLs, Expression QTLs

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Martin Jaeger and Sloot equally contributed to this study.

Correspondence

Dr. Martin Jaeger, Department of Internal Medicine (463), Division of Endocrinology, Radboud University Medical Centre, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, The Netherlands.

Email: Martin.Jaeger@radboudumc.nl

Senior author: Dr. Romana T. Netea-Maier

Introduction

Homeostasis is defined as a dynamic state of equilibrium maintained by all living beings, which is necessary for the optimal function of the organism. The control of homeostasis of complex animals such as humans is maintained by a number of systems that collect, process and share the information needed for the normal functioning of the body: the nervous system, the endocrine system and the immune system.¹ Among them, the immune system provides the host defence responses that are crucial for defending the host against invading pathogens: innate immune cells such as monocytes, neutrophils and natural killer (NK) cells make up the first line of this response, while the adaptive immune responses are more specific and are executed by T cells and B cells. Dysregulation (either defects or hyperactivation) of different immune cell subsets or immune cell functions can result in increased susceptibility to infections, autoimmune or inflammatory diseases, and even cancer.^{2–5}

The immune system, however, does not operate isolated in the body, and important modulatory signals from the nervous and endocrine systems regulate the immune responses. There is long-standing evidence that the endocrine and the immune system interact with each other by sharing receptors for hormones and immune mediators (e.g. cytokines), cross-control and several feedback mechanisms.^{6–8} Despite this knowledge, the research on the impact of the thyroid endocrine axis on the immune system was mostly done in relatively small studies assessing specific aspects of these interactions, while a comprehensive overview is missing so far. The Human Functional Genomics Project (HFGP) aims to describe the host genetic, non-genetic and environmental factors that influence immune responses at a broad level: immune cell populations, cytokine responses from myeloid and lymphoid cells upon multiple stimuli, and humoral immune responses.⁹ We have previously described the influence of genetic,¹⁰ non-genetic¹¹ and microbiome¹² factors that influence immune responses. However, the impact of endocrine factors in regulating the immune responses in

this broad sense has not been studied so far. Furthermore, understanding the physiology of these relationships is paramount for identifying the mechanisms shaping susceptibility for immune-mediated diseases, such as infections, autoimmune diseases and cancer, among healthy individuals.

Within the endocrine system, thyroid hormones regulate numerous biological processes, including organ development and metabolism. Circulating levels of thyroid hormones are regulated by the hypothalamus–pituitary–thyroid (HPT) axis. Within this axis, thyrotrophin-releasing hormone (TRH) is secreted by the hypothalamus and stimulates the secretion of thyroid-stimulating hormone (also known as thyrotrophin) (TSH) by the pituitary. In turn, TSH stimulates synthesis of thyroid hormones, thyroxin (T4) and 3-5,32'-triiodo-L-thyronine (T3) by the thyroid gland.¹³ The thyroid gland mainly produces T4, which is converted by deiodinases to the bioactive form T3 at the tissue level.^{13,14} Increasing evidence suggests that thyroid hormones also influence specific components of the immune responses.^{15–17} In this respect, hyperthyroidism is associated with altered antibody production,¹⁸ increased lymphocyte proliferation and reduced production of pro-inflammatory cytokines such as MIP-1 α and IL-1 β ,^{17,19} while hypothyroidism is associated with immune defects and increased susceptibility to infection due to impaired cellular function.^{17,19–21} However, the effect of thyroid hormones on immune cell populations and immune cell functions under normal physiological conditions has only scarcely been investigated.

In the present study, we provide a comprehensive assessment of the role of the thyroid function on immune system homeostasis. By assessing the broad immune function (cell populations, antibody concentrations, circulating cytokines, adipokines and acute-phase proteins, monocyte–platelet interactions and cytokine production capacity) in 800 healthy volunteers of Western European descent from two independent HFGP cohorts (the 500 Functional Genomics (500FG) and 300BCG cohorts), and using a multifactorial approach, we describe the impact

of TSH and free T4 (fT4) concentrations on the immune system under physiological conditions (Figure 1A).

Results

Demographic characteristics of the 500FG and 300BCG cohorts: TSH and fT4 concentrations

After quality checks considering genetic background and medication usage, immunological data of a total of 489 healthy participants from the 500FG cohort were analysed in this study. In the 300BCG cohort that served as validation cohort, a total of 321 individuals of Western European descent, consisting of 139 males and 182 females with age ranging between 18 and 71 years, were included between April 2017 and June 2018.²² Table 1 summarizes the baseline characteristics of both cohorts, and a more detailed description of both cohorts can be found in Ref. [11,23].

As age, gender and BMI are known to affect circulating levels of TSH and fT4,²⁴ we first assessed whether TSH and fT4 levels were associated with any of the demographic characteristics in our 500FG cohort (Figure 1B). As expected, fT4 levels were significantly higher in males than in females (false discovery rate (FDR) = $3.2e^{-4}$; mean men = 16.62 ± 2.11 , mean women = 16.11 ± 1.91), increased age was associated with reduced levels of fT4 (FDR = 0.011). No significant associations between TSH and age or sex were found, although the relative low number

Table 1. Baseline characteristics of both cohorts analysed in this study.

	500FG	300BCG
Gender	Male, 211 (43.1%); female, 278 (56.8%)	Male, 139 (43.3%); female, 182 (56.6%)
Age (years)	27.47 (+/-12.20)	25.94 (+/-10.74)
BMI	22.67 (+/-2.75)	22.48 (+/-2.53)
Oral contraceptive (female)	Yes, 150 (53.9%); No, 128 (46.0%)	Yes, 89 (48.9%); No, 93 (51.0%)
T4 (pmol/L)	Mean, 16.33 (+/-2.07)	Mean, 16.88 (+/-2.19)
TSH (mE/L)	Mean, 2.57 (+/-1.33)	Mean, 2.37 (+/-1.27)

of elderly individuals in our cohorts may have prevented us to identify small differences.

Oral contraceptive usage in woman correlated with higher TSH (FDR = 0.037) and higher fT4 (FDR = 0.030) concentrations in the 500FG cohort, and with higher TSH concentrations in the 300BCG validation cohort. No consistent associations of circulating concentrations of TSH and fT4 with BMI were found in the initial cohort, while a positive association of TSH with BMI was found in the 300BCG cohort (P -value = 9.7×10^{-3}) (Figure 1B). Age, gender, oral contraceptive usage, and BMI were all included as correction factors in the subsequent statistical analysis.

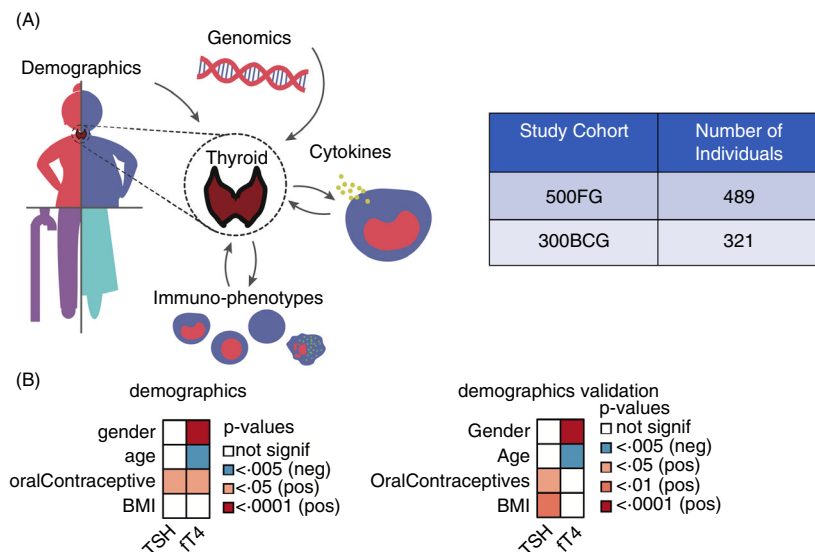


Figure 1. (A) Schematic overview of the different data sets investigated in the study. The influence of TSH and fT4 levels on immune traits, including circulating immune mediators, cytokine production and immune cell populations, was assessed in 485 individuals from the 500FG cohort and was validated in 326 individuals from the independent 300BCG cohort. Genetic analysis was performed to unravel underlying mechanisms and further strengthen observations. (B) Association between fT4 and TSH with demographic parameters. Heatmap of the Spearman correlation coefficients between TSH or fT4 and demographic characteristics for the initial cohort (500FG). Heatmap of the Spearman correlation coefficients between TSH or fT4 and demographic characteristics in the validation cohort (300BCG). Positive or negative association is depicted in brackets.

TSH, fT4 and circulating immune markers

To study the relationship between TSH and fT4 on baseline immune parameters, circulating concentrations of different immune mediators were measured: acute-phase proteins (C-reactive protein (CRP) and alpha-1-antitrypsin), adipokines (leptin, adiponectin and resistin), cytokines (IL-6, IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-18, IL-18 binding protein (IL-18BP) and VEGF) and immunoglobulin levels (IgA, IgM, IgG and four IgG + subclasses) (Figure 2A). A correlation between TSH concentrations and leptin was observed (FDR = 0.033), but no other effects of TSH and FT4 on this large array of humoral immune mediators were detected. This association was independent of BMI, since we corrected for BMI in our analysis.

TSH, fT4 and platelet function and platelet–monocyte interaction

Platelets are currently being recognized as important players in inflammation, especially through their ability to quickly release granules containing inflammatory mediators and growth factors.^{25,26} Additionally, platelets are able to interact with immune cells, including lymphocytes and monocytes.²⁷ In the present study, neither TSH nor fT4 influenced platelet–monocyte interaction (Figure 2A).

TSH, fT4 and in vitro cytokine production capacity

Cytokine production is an important parameter of immune cell function. In the 500FG cohort, ex vivo cytokine production of both monocyte-derived (IL-1 β , TNF- α and IL-6) and lymphocyte-derived (IFN- γ , IL-17 and IL-22) cytokines was measured in three different cellular systems and after different time points (Figure 2B). No significant associations were found between either TSH or fT4 and cytokine production in response to the different stimuli in any of the cellular systems.

TSH, fT4 and immune cell populations

No significant associations between TSH and fT4 concentrations and absolute cell counts for three subsets of monocytes, the classical (CD14⁺ CD16⁻), intermediate (CD14⁺ CD16⁺) and non-classical monocytes (CD14⁺ CD16⁺), were found (Figure 3A–C). Likewise, we did not find a significant correlation between TSH or fT4 levels and neutrophil (CD45⁺ SSC, FSC) counts and NK-cell counts (CD56⁺ CD16⁺, CD3⁻CD56⁺, CD56⁺ CD16⁻ and NK bright CD56⁺⁺ CD16⁻). Within the innate immune cell compartment, only the NK-T-cell counts (CD3⁺ CD56⁺) were positively associated with TSH levels (FDR = 0.035). However, high concentrations of TSH and fT4 were significantly associated with CD14⁺ monocyte cell counts (Figure 3D) in the 300BCG cohort.

In contrast to the limited effects of TSH and fT4 on the myeloid cell population, a significant positive correlation was found between TSH concentrations and T-cell counts (FDR = 3.2×10^{-3}) (Figure 3A–C). T-cell counts, specifically CD45RO⁻ CD45RA⁺ terminally differentiated effector T cells, CD45RO⁺ CD45RA⁻ memory T cells, CD4⁺ CD8⁻ T cells, including CD4⁺ CD45RA⁺ CD27⁺ and CD4⁺ CD45RO⁻ CD27⁺ effector T cells and CD4⁺ CD45RO⁺ CD27⁺ and CD4⁺ CD45RA⁻ CD27⁺ central memory T cells, as well as regulatory T cells (Treg CD25⁺ CD127_{low} and CD4⁺ CD25^{high}), showed highly significant positive correlations with TSH concentrations (individual *P*-values are shown in Table S1). CD4⁻ CD8⁺ T-cell counts showed a mild positive correlation with TSH levels (FDR = 0.022). FOXP3⁺ Helios⁺ and CD45RA⁺ Treg counts were also higher in individuals with high TSH concentrations. Assessment of subset frequencies within the different T-cell compartments showed no significant associations. The obtained results from the 500FG cohort were independently confirmed in the 300BCG cohort (Figure 3D). This may indicate that TSH maintains the cell numbers in the T-cell compartments,

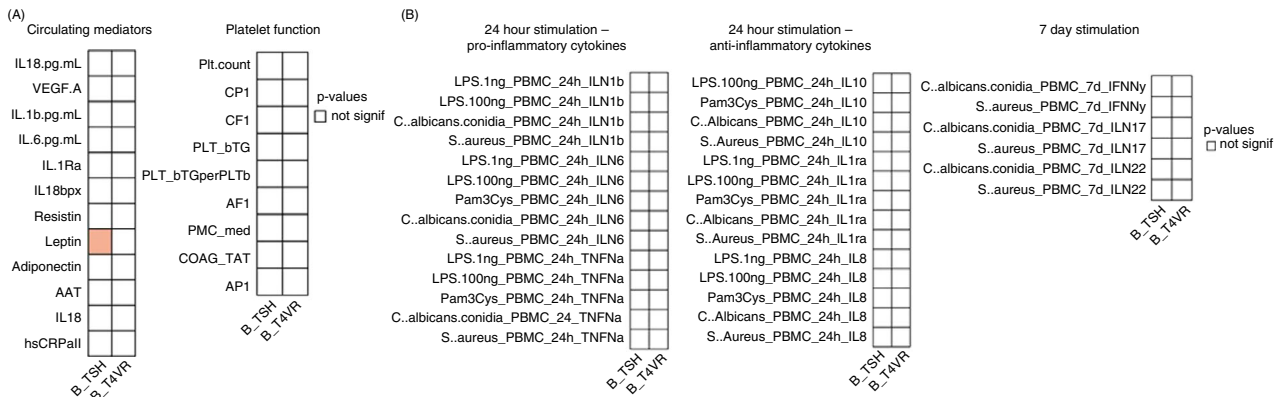


Figure 2. Effect of TSH and fT4 on: (A) circulating mediators measured in plasma and different platelet parameters. (B) Ex vivo cytokine production after stimulation with different pathogens for either 24 h or 7 days.

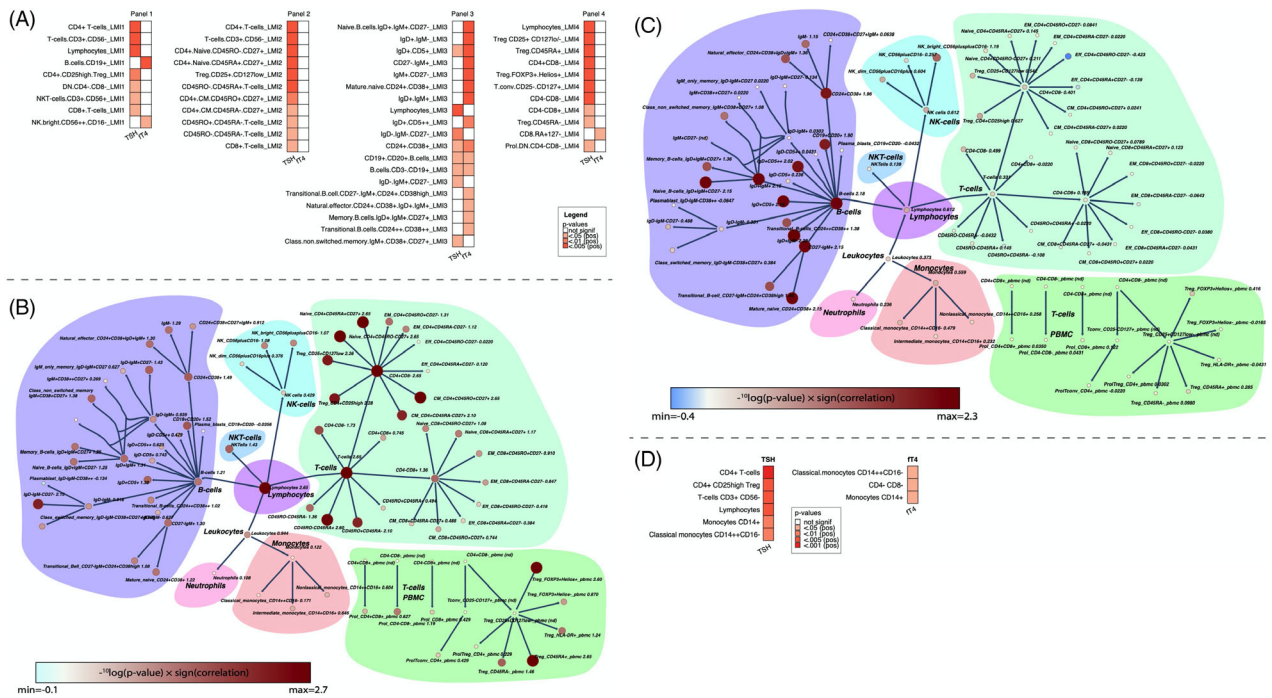


Figure 3. Effects of TSH and ft4 on cell populations in the blood of the 500FG individuals. (A) Heatmap of the Spearman correlation coefficients between TSH or ft4 and different immune cell (sub)populations. (B) Associations between cell-subtype count data and TSH, visualized in a cell-type network using Escher⁷¹; dark red indicates a more positive and significant association. Light blue indicates a negative significant association. (C) Associations between cell-subtype count data and ft4, visualized in a cell-type network using Escher.⁷¹ (D) Heatmaps of the Spearman correlation coefficients between TSH or ft4 and different immune cell (sub)populations in the 300BCG validation cohort.

but probably does not influence further differentiation of different T-cell subtypes.

Circulating concentrations of ft4 were positively correlated with total B-cell counts ($FDR = 3.5 \times 10^{-3}$). In particular, cell counts from naive B cells ($IgD^+ IgM^+ CD27^-$), memory B cells ($IgD^+ IgM^+ CD27^+$), $IgD^+ CD5^+$ B cells, $CD27^- IgM^+$ B cells, $CD19^+ CD20^+$ B cells, and natural effector B cells ($CD24^+ CD38^+ IgD^+ IgM^+$) were significantly associated with ft4 levels (individual *P*-values are shown in Table S1). In contrast, IgG-IgM-negative B cells, including plasmablasts and IgM-only B cells, were not affected by ft4 concentrations. Further analysis showed that ft4 concentrations did not influence subset frequencies of the different B-cell types. These results may indicate that ft4 mainly influences proliferation of different subsets of B cells, without affecting further differentiation into subtypes.

Causal inference from hormone to cell counts: Mendelian randomization

To test whether the circulating concentrations of TSH and ft4 have a causal effect on the changes of cell counts within the peripheral blood, Mendelian randomization (MR)²⁸ was applied to the latest GWAS summary statistics of both hormone levels (TSH and ft4)²⁹ and immune

cell counts for a total of 74 different immune cell subtypes.³⁰

Two MR approaches, that is inverse-variance weighted (IVW)³¹ and weighted median³² methods, were used, and a *p*-value of 0.05 was used as a suggestive significant threshold. Both methods identified that the increase in TSH level was a suggestive causal factor for the cell count increase in $CD8^+ EM CD45RA^- CD27^-$ T cells and $CD8^+ EM CD45RO^+ CD27^-$ T cells within the peripheral blood (Table 2).

In addition, both IVW and weighted median methods have found that the decrease in $CD4^+ CD8^+$ T-cell counts and $CD8^+ CD4^-$ T cells (summary statistics from Ref. [29]) was suggestively caused by an increase in ft4 levels (*P*-values < 0.05) (Table 2).

Genetic variants associated with TSH and ft4 levels and their influence on immune function

The individual genetic background shapes the capacity to respond to different pathogens, and numerous genetic alterations have been identified to increase disease susceptibility. We assessed the genetic variants that influence circulating ft4 and TSH concentrations, and whether they in turn impact cell population numbers in our cohort.

Table 2. Causal inference results between cell counts and TSH and fT4 levels

		Weighted median effect (b)	Weighted median (P-value)	IVW effect size (b)	IVW (P- value)
CD8 ⁺ EM CD45RA- CD27- data	TSH ²⁹	0.63	0.017	0.49	0.007
CD4 ⁺ CD8 ⁺ data	fT4 ²⁹	-0.79	0.036	-0.60	0.024
CD8 ⁺ CD4 ⁻ data	fT4 ²⁹	-0.89	0.024	-0.64	0.033

For this, we performed a Genome-wide association study (GWAS) approach in the 500FG cohort on a SNP array covering around 8.8 million SNPs. Although, likely due to the relatively small sample size, we were not able to detect in our cohort SNPs associated with TSH or fT4 levels at a $P < 1e^{-8}$, four suggestive SNPs were identified ($P < 1e^{-6}$), of which two were associated with TSH levels, and two were associated with fT4 levels (Figure 4A and B). To explore the biological role of these genetic loci, we mapped expression QTLs (eQTLs) and found that two of these SNPs have strong eQTLs influencing the expression of important genes. SNP rs6694001 is associated with TSH levels and is a (intronic) genetic variant within the *PTGER3* gene, which harbours an eQTL for the *PTGER3* gene. The *PTGER3* gene encodes the prostaglandin E receptor 3, a cell membrane-associated G protein-coupled receptor and one of the four main prostaglandin receptors. *PTGER3* is associated with analgesic and thermoregulatory responses during infection,³³ but recent studies have also shown its role in immune tolerance processes by induction of anti-inflammatory cytokines, such as IL-10.³⁴ Another strong association was found between fT4 and a (intergenic) variant between *ZC3H7A* and *TXNDC11*. Available data suggest that this variant is an eQTL for both genes. In particular, *TXNDC11* is of interest, as it is involved in the folding of DUOX proteins that participate in the H₂O₂ generating system within the thyroid (Table 3).

To further explore the biological effects of genetic alterations associated with TSH and fT4, we performed a pathway analysis. The top 10 enriched pathways associated with TSH levels mainly encompass pathways important in intracellular signalling and cellular homeostasis (Table S2). With respect to the genetic loci affecting fT4 concentration, two of the 10 top enriched pathways represent immune-related pathways (Table 4). The CD40 pathway plays an important role in several B-cell functions,³⁵ further strengthening the association we observed

between fT4 levels and B-cell counts in our cohort. Moreover, one of the genes that showed significance in both immune-related pathways is TNF receptor-associated factor 6 (TRAF6) ($P = 0.0005$) (Figure 5), which is an adaptor protein critical for development, homeostasis and activation of immune cells.³⁶ This further supports the notion that the genetics that influence fT4 level can also influence immune-related pathways.

Discussion

The influence of the endocrine system on the immune responses has received relatively little attention in comparison with the impact of genetics or the microbiome. In the present study, we aimed to partly fill this knowledge gap by investigating the effects of fT4 and TSH on immune traits in a large cohort of healthy volunteers. We used a comprehensive assessment of the human immune response, including the assessment of circulating immune mediators, cytokine production, immunophenotypes of immune cell populations and humoral factors. Here, we show that fT4 and TSH have a minimal impact on innate immune responses in healthy volunteers. In contrast, TSH and fT4 strongly influence the numbers of lymphocytes, with TSH mainly influencing the T-cell counts and fT4 the B-cell counts. Our results further suggest that the increase in TSH levels is a suggestive causal factor for the cell count increase. In addition, the impact of thyroid function on lymphoid cell homeostasis was strengthened by identification of genetic variants that on the one hand influence TSH and/or fT4 concentrations, while subsequently impacting expression of important immune-related genes. Lastly, pathway analysis showed enrichment of fT4-affected gene expression in B-cell function pathways, further supporting the importance of fT4 in the regulation of B-cell function.

Circulating concentrations of TSH and fT4 were not associated with cell counts of innate immune cell subsets such as monocytes or neutrophils in the 500FG cohort, although a small effect was identified in the 300BCG cohort. Previous literature suggests that T4 could affect monocyte cell function, specifically phagocytosis,^{16,37-39} without affecting monocyte cell counts. Furthermore, there is only one report by Hodkinson et al., showing that neutrophil counts were significantly associated with fT4 levels in elderly,¹⁶ whereas our results show that fT4 is not associated with neutrophil counts in healthy young individuals. In addition to the lack of influence of fT4 and TSH on innate immune cell populations, no associations were found between TSH or fT4 and cytokine responses, platelet function or levels of circulating inflammatory mediators, and acute-phase proteins. However, a mild effect on leptin concentrations was found. In line with this, previous studies performed in obese individuals have shown that leptin partly regulates the HPT axis.^{40,41}

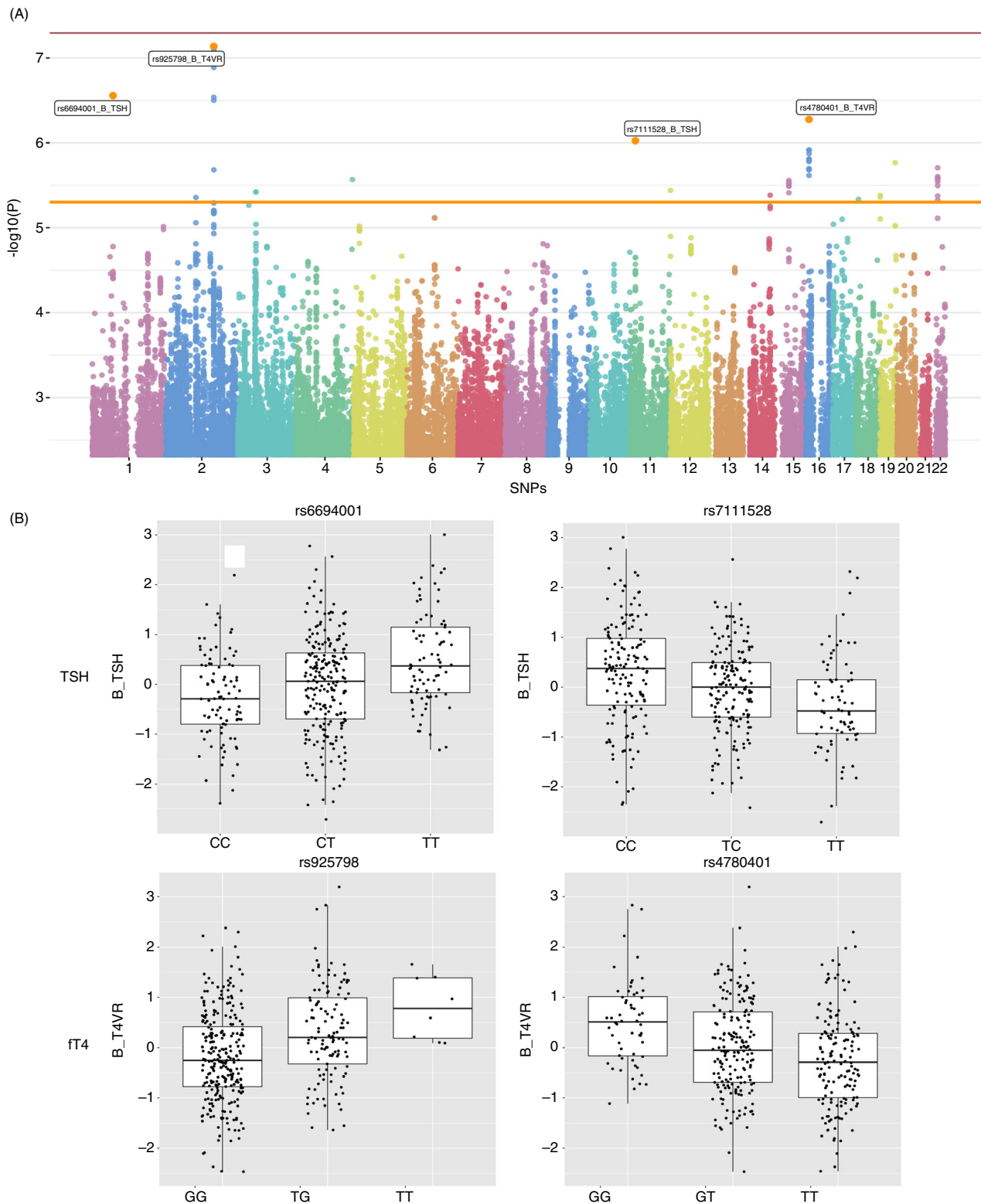


Figure 4. Genome-wide association study (GWAS) approach in the 500FG cohort on a SNP array covering around 8.8 million SNPs. (A) Manhattan plot of GWAS analysis depicting SNPs associated with TSH or fT4; the line depicts the $P < 1 \times 10^{-6}$ threshold. (B) Example plots showing the association between the genotypes of the top significant SNPs (top hits in the analysis of Figure 4A) and hormone levels in the 500FG cohort. The x-axis depicts the different genotypes, and the y-axis shows the inverse rank normalized hormone values for TSH or T4. Most significant hits for fT4: rs925798 (P -value: 7.31×10^{-8}), rs4780401 (P -value 5.3×10^{-7}), and TSH: rs6694001 (P -value 2.78×10^{-7}), rs711528 (P -value: 9.44×10^{-7}).

Table 3. Top SNPs for both TSH and fT4 and their eQTL effects of surrounding genes.

RS-number	Association	Gene	Function	eQTL
rs6694001 (intronic)	TSH	PTGER3	Prostaglandin E Receptor 3	PTGER3; RP3-333A15-2; ZRANB2-AS2
rs7111528 (within gene)	TSH	OTOG	OTOGELIN (specific to acellular membranes of the inner ear)	TRAF5; RP11-358H18-3
rs925798 (intergenic)	T4	LD with : rs144409013 rs144333826 rs10165644 rs77365095 rs4664029 rs13398362	N/A	None of the LD variants show eQTLs
rs4780401 (intergenic)	T4	N/A	Between ZC3H7A and TXNDC11. TXNDC11 is responsible for DUOX proteins folding within the thyroid H ₂ O ₂ -generating system	SNN; TXNDC11; RP11-490O6-2; ZC3H7A; ENSG00000153066.7_11785146_11785938

This, however, is the first large study in healthy euthyroid, non-obese individuals to confirm these results. The lack of association between thyroid hormones and ex vivo cytokine production by immune cells is in accordance with a previous study that showed that genetic variation explained most of the variation in stimulated cytokine production, whereas the endocrine system in general and secreted hormones in particular had only a negligible effect.⁴²

Furthermore, although clinical evidence shows that hyperthyroidism is associated with enhanced coagulation and increased risk of thrombosis⁴³ and that physiological concentrations of fT4 have been shown to activate platelets ex vivo,^{43–45} no significant association between TSH or fT4 and platelet or coagulation parameters was found in this cohort, indicating that both TSH and fT4 do not influence platelet function and platelet–monocyte interactions in euthyroid, healthy volunteers.

Regarding the effect of TSH and fT4 on acute-phase proteins, the report from Hodkinson *et al.* showed that hs-CRP levels are associated with fT4 levels in the elderly; however, there is no further evidence that TSH or fT4 is associated with levels of inflammatory markers in younger healthy individuals. Altogether, these data argue that thyroid function has a limited impact on innate immune responses and the mediators of inflammation.

In contrast, the main impact of TSH and fT4 on the immune system was at the level of the adaptive lymphoid cell populations. Our results show that TSH and fT4 levels are strongly associated with cell counts within the lymphocyte compartment: while TSH influences T-cell numbers and cell populations, fT4 strongly influences the B-cell compartment. While our data reflect the physiological situation in humans, they are supported by previous

studies in animal models and in vitro studies. In 1992, Provinciali *et al.* already showed that TSH significantly increased the mitogen-induced proliferative response of mouse lymphocytes,⁴⁶ providing a first notion of the role of TSH in T-lymphocyte homeostasis. Another study investigated the expression and distribution of the TSH receptor in murine immune cells⁴⁷: up to 10–20% of CD4⁺ 8[−] and CD4[−] 8⁺ lymph node T cells expressed the TSH receptor, which were exclusively associated with CD45RB^{high} cells, indicating that these cells are most susceptible to the effects of TSH.⁴⁷ In addition, in the hyt/hyt mouse model, a model for congenital hypothyroidism with high levels of TSH and reduced levels of T4,⁴⁸ thymus size was reduced and the percentage of CD8⁺ thymocytes was significantly lower, whereas the percentage of CD4⁺ splenocytes was significantly higher compared with euthyroid mice.⁴⁹ Furthermore, in in vitro experiments using human cells, T cells cultured with T4 underwent enhanced apoptosis, whereas this effect was not observed when TSH was added to T-cell cultures.⁵⁰ In a more recent study using human thymocytes, addition of TSH enhanced T-cell development,⁵¹ suggesting that TSH and T4 play an important role in T-cell homeostasis.

A different role seems to be played by T4 in lymphocyte homeostasis, with a strong impact on B-cell numbers. In support of these data, the group of Montecino-Rodriguez investigated the role of T4 in bone marrow B-cell development in Snell dwarf (dw/dw) mice, which are deficient in pituitary hormones.⁵² These mice had impaired B-cell development, while treatment with T4 completely restored this defect.⁵² Furthermore, in other strains of mice with defects in the HPT axis, production of pre-B cells was reduced as well, whereas myelopoiesis and thymopoiesis were normal, indicating that the B cells

Table 4. Top 10 enriched pathways associated with fT4 levels.

	GO-term	X ² P-value	P-value
1	REACTOME_NOTCH1_INTRACELLULAR_DOMAIN_REGULATES_TRANSCRIPTION	0-000399349467	0-000398
2	REACTOME_CTNNB1_PHOSPHORYLATION_CASCADE	0-000556181347	0-000443
3	BIOCARTA_CD40_PATHWAY	0-00137641097	0-00096
4	REACTOME_TRAF6_MEDIATED_IRF7_ACTIVATION_IN_TLR7_8_OR_9_SIGNALING	0-0016045688	0-0012
5	REACTOME_SIGNALING_BY_NOTCH1	0-00181636805	0-00161
6	KEGG_PATHWAYS_IN_CANCER	0-00193520053	0-00178
7	REACTOME_SIGNALING_BY_NOTCH	0-00188967961	0-0019
8	BIOCARTA_TFF_PATHWAY	0-00235013524	0-00258
9	REACTOME_P75NTR_SIGNALS_VIA_NFKB	0-00288783543	0-00284
10	REACTOME_P75_NTR_RECEPTOR_MEDIATED_SIGNALLING	0-00351062283	0-00311

specifically depended on T4 as lymphopoietic factor.⁵³ Further studies showed that the frequency of pro-B-cells in the S-G2/M phase of the cell cycle was significantly reduced in hypothyroid mice, thus resulting in a proliferation defect; treatment with T4 resulted in an increase in cycling pro-B cells.⁵⁴ In addition, mice deficient for thyroid hormone receptor T3R alpha 1 and T3R alpha 2 displayed a strong reduction in B lymphocytes in the spleen, and reduced pro/pre-B-cell numbers in the bone marrow, indicating that thyroid hormones play an important role in maintaining the B-cell pool.⁵⁵ Interestingly, in humans with autoimmune Graves' disease, thymic hypertrophy has been demonstrated,⁵⁶⁻⁵⁸ further supporting the notion that thyroid hormones play an important role in immune development. These effects on the thymus are reversible after treatment of Graves' disease.^{57,59}

The observations regarding the specific role of thyroid function on lymphoid cell numbers were further validated by integrating genomic data in the analysis. Compared with previous GWASs on thyroid function that cover more than a hundred times more subjects, this study analysed a relatively small number of subjects to draw concrete conclusions regarding fT4 and TSH levels. However, by combining genetic data with immunological parameters we pinpointed several genetic variants that, while influencing TSH and fT4 concentrations, subsequently also impact immune function. One such variant is SNP rs6694001, which was associated with TSH levels, and is an eQTL for the *PTGER3* gene that encodes the prostaglandin E receptor 3, one of the four main prostaglandin receptors. *PTGER3* is associated with analgesic and thermoregulatory responses during infection,³³ but recent studies also shown its role in immune tolerance processes by induction of anti-inflammatory cytokines such as IL-10.³⁴ Infection of *PTGER3*-deficient mice with respiratory pathogen *S. pneumoniae* resulted in enhanced survival and enhanced bacterial clearance, with lower levels of circulation leucocytes and improved phagocytic and bactericidal capacity.⁶⁰ Furthermore, 2 of the top 10

enriched pathways associated with fT4 levels represent pathways important for immune function, with TRAF6 expression being affected in both pathways. However, further research is necessary to validate these findings and further investigate the underlying processes. The concept that thyroid hormones impact immune function was further strengthened by MR analysis indicating that TSH and fT4 are causing the effects on lymphocyte numbers.

In conclusion, the present study presents solid arguments from two independent cohorts and genomic data integration to argue that TSH and T4 are important regulators of the lymphoid cell compartment in immune homeostasis. These data are relevant on several levels. Firstly, they fill an important gap in the knowledge regarding the influence of the endocrine system on immune system physiology. Secondly, they open a door for exploring and understanding the role of thyroid hormones in the lymphocyte function during disease. Thirdly, this has the potential of identifying novel avenues for diagnostic and therapies. One may even envisage the possibility to improve the efficacy of immunotherapy and/or vaccination by co-opting endocrine cues in these procedures. An experimental study suggested the possibility to recover age-dependent deterioration of immune function by levothyroxine treatment,³⁷ and our data may provide the intriguing possibility that this approach could improve the known lymphocyte defects in elderly humans as well. Future studies are warranted to explore these exciting new avenues.

Materials and methods

Discovery cohort

534 healthy individuals with Western European descent from Nijmegen, the Netherlands (500FG cohort; see www.humanfunctionalgenomics.org), were recruited between August 2013 and December 2014 at the Radboud University Medical Center, the Netherlands. The HFGP

was approved by the ethical committee of Radboud University Nijmegen (no. 42561.091.12). Experiments were conducted according to the principles expressed in the Declaration of Helsinki. All volunteers gave written informed consent before any material was taken. All participants were asked for their medical history regarding thyroid diseases and their thyroid-interfering medication, and those known with thyroid pathology or were using medication interfering with the thyroid function were excluded. For the final analysis, 489 volunteers were used (211 males and 278 females, age ranging from 18 to 75 years, mean BMI was 22.67 ± 2.75 , 13.2% were current smokers, and of the women, 53.9% used oral contraceptives).

Validation cohort

Results were validated in a cohort of 321 (139 males and 182 females, age range 18–71 years) individuals of Western European descent (300BCG cohort, included between April 2017 and June 2018 at the Radboud University Medical Center, the Netherlands). The 300BCG study was approved by the Ethical Committee of Radboud University Medical Center (no. NL58553.091.16). Experiments were conducted according to the principles expressed in the Declaration of Helsinki, and all individuals provided written informed consent. All volunteers were asked for their medical history regarding thyroid diseases and their thyroid interfering medication, and those known with thyroid pathology or were using medication interfering with the thyroid function were excluded.

Determination of fT4 and TSH serum levels

Serum fT4 and TSH in both cohorts were measured using an Electrochemiluminescence immunoassay on a random-access analyser (Modular E170 and Cobas E801 for FG500 and 300BCG respectively, Roche Diagnostics, Rotkreuz, Switzerland).

Analysis of immune cell composition

Cells were processed immediately after blood sampling and analysed within 2–3 h, to minimize biological variability. Myeloid and lymphoid immune cell levels were measured by 10-colour flow cytometry on a Navios flow cytometer (Beckman Coulter, Indianapolis, USA) with three solid state lasers (488, 638 and 405 nm). Calibration of the machine was performed once a week, and little adjustment had to be made during the inclusion period of the study. Data were analysed using the Kaluza software version 1.3 (Beckman Coulter). Cell populations were gated manually using a hierarchical gating strategy. See Supplemental Experimental

Procedures for details on cell processing, reagents, gating and analysis.

Measurements of circulating immune mediators and immunoglobulins

The circulating mediators resistin, leptin, adiponectin, CRP and alpha-1 antitrypsin (AAT) were measured in EDTA plasma using the R&D Systems DuoSet ELISA kits following the manufacturer's protocol. The plasma cytokines IL-1Ra and IL-18 binding protein (IL-18BP) were measured using R&D Quantikine kits following the manufacturer's standard protocol. Plasma IL-1 β , IL-6, IL-18 and VEGF were measured in Simple Plex cartridges using the Ella apparatus (Protein Simple, San Jose, CA).

Serum immunoglobulin (sIg) concentrations were measured by fluorescence enzyme immunoassays (ImmunoCAP).

Assessment of platelet function

Measurements of platelet function and coagulation included platelet count, P-selectin expression (marker for platelet degranulation) and platelet–fibrinogen binding (marker for platelet aggregation), platelet–monocyte complex formation and plasma concentrations of β -thromboglobulin and thrombin–antithrombin complexes and are described in detail previously.^{61,62} Venous blood was collected in citrated Vacutainer tubes (3.2% sodium citrate; Becton Dickinson, USA).

PBMC stimulation experiments

Isolation of PBMCs was performed as described by Oosting *et al.*⁶³ After isolation by differential centrifugation in Ficoll, cells were washed twice in saline and suspended in resuspended in RPMI-1640 culture medium supplemented with 50 μ g/mL gentamicin, 2 mM glutaMAX, and 1 mM pyruvate and counted. PBMC stimulations were performed with 5×10^5 cells/well in round-bottom 96-well plates (Greiner) for either 24 h or 7 days in the presence of 10% human pool serum at 37° and 5% CO₂. Supernatants were collected and stored at –20° until used for ELISA. The stimulations used for the 24-h and 7-day experiments are shown in Table S3.

ELISA measurements of cytokine concentrations

In the supernatants of the 24-h PBMC stimulation experiments, concentrations of IL-1 β , IL-6 and TNF- α were measured following the manufacturer's protocols (PeliKine Compact or R&D Systems). Supernatants of the 7-day stimulation assays were used to measure IL-22, IL-17 or IFN- γ (PeliKine Compact or R&D Systems).

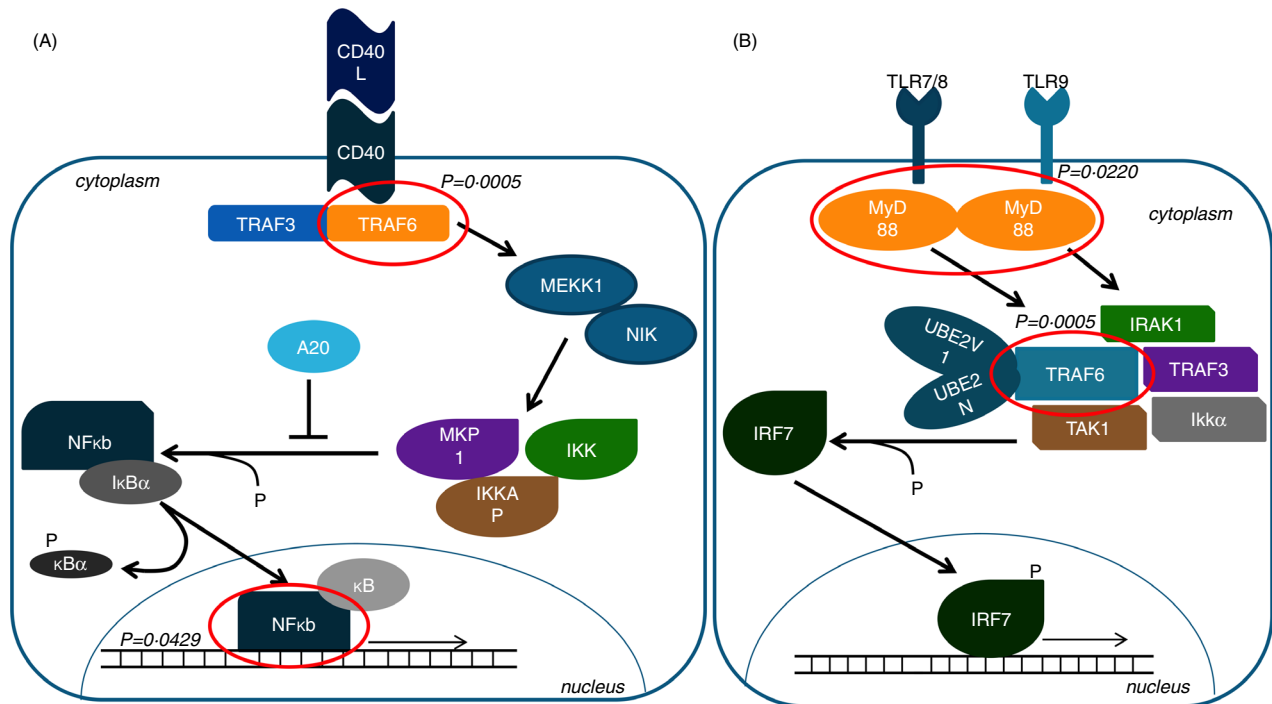


Figure 5. Gene set enrichment pathway analysis revealing significantly affected gene expression by fT4 in two different pathways. (A) The main differentially expressed genes affected by fT4 in the CD40 pathway, TRAF6 and RELA (NFκB subunit) (circled red). (B) The main differentially expressed genes affected by fT4, MyD88 and TRAF6 (circled red) in the TRAF6-mediated IRF7 activation in TLR-7, TLR-8 or TLR-9 signalling pathway.

Genotyping, quality control and imputation

Isolated DNA samples were genotyped as described before.¹⁰ Briefly, DNA samples were genotyped with default settings using the Illumina HumanOmniExpress-Exome-8 v1.0 SNP chip. After quality control checks and filtering (samples with a call rate % 0.99, variants with a Hardy–Weinberg equilibrium (HWE) % 0.0001, call rate % 0.99 and minor allele frequency (MAF) % 0.001 were excluded), 17 outliers had to be excluded. In total, 441 samples with genotype information of 518 900 variants were used for further imputation.¹⁰ Data were imputed with Impute 2 using the reference Genome of the Netherlands (GoNL, Genome of the Netherlands Consortium, 2014) data set GoNL reference panel and genome build b37. Only SNP with MAF > 0.1 HWE < 5e-7 and r2(imputation quality) > 0.3 was retained.

Data preprocessing

TSH and fT4 levels were transformed using a rank-based inverse normal transformation (IRT).⁶⁴ The same method was applied to scale the cell count data, cytokine production capacity data, platelet data and immunoglobulin data. The following R code was used:

```
transformed = rank(original).
transformed = qnorm(transformed/(length(transformed)+0.5)),
```

where 'transformed' is the transformed data and 'original' the original data.

Associations

Associations between TSH/T4 levels and other measurements were evaluated using linear regression models. Analyses were performed using the R programming language (*R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/*). The 'rfit' function and 'lm' function from the stats package were used to create regression models. Age, gender, oral contraceptive usage, BMI, smoking (binary, yes/no) and seasonal variation were included as correction factors. In cases where a subgroup was chosen based on gender or oral contraceptive usage, that factor was removed from the list of correction factors. Analyses on the discovery cohort (500FG) were corrected for multiple testing using the Benjamini–Hochberg false discovery rate procedure.⁶⁵ Corrections were performed for each set of comparisons separately. Validations in the validation cohort (300BCG) were not corrected for multiple testing.

Genetics

Quantitative trait loci (QTLs) were calculated using the MatrixEQTL package.⁶⁶ SNP allele dosages were used in a

linear model correcting for age, gender, BMI and oral contraceptive usage. Briefly, dosages are a linear transformation of the posterior genotype probabilities, if A is the reference and B the effect allele:

$$\text{dosage} = A/A * 0 + A/B * 1 + B/B * 2,$$

where A/A, A/B and B/B are the posterior probabilities.

Pathway enrichment analyses were performed using the Pascal tool in 'sum' mode.⁶⁷ The gene region was defined 50 kilobases up- and downstream of the gene. Other settings were left to default, as defined in Pascal (version current as of February 2019).

Cell Count QTL Mapping

We calculated parental and grandparental percentages from the measured cell counts as described in Ref. [30]. Absolute cell counts and percentages were transformed by IRT.⁶⁴ We then corrected the IRT cell counts using a linear model correcting for age, gender, and month of sample collection. In the following, QTL mapping was performed using a linear model as implemented in the MatrixEQTL R package,⁶⁶ where we associated immune traits with genotype information. A p-value lower than 1e-6 was considered to be suggestive genome-wide.

Mendelian randomization

To test whether there is causal relationship between measured hormone concentrations and cell counts, Mendelian randomization (MR) was performed. Summary statistics of TSH and fT4 QTLs were obtained from literature,²⁹ and cell count QTLs were obtained from 500FG cohort.³⁰ 46 and 23 independent variants achieving genome-wide significance in GWAS of TSH level and FT4 level (threshold $p = 5 \times 10^{-8}$) were used as instrument variables, respectively.

Two analytical methods including inverse-variance weighted (IVW)³¹ and weighted median regression³² methods were applied to infer causal relationship in MR analysis (R package TwoSampleMR).⁶⁸

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

M.J., Y.J.E.S., R.t.H., L.A.B.J., J.W.A.S., M.G.N. and R.T.N.M. conceptualized the idea. L.A.B.J., M.G.N., Y.L., H.K and X.C. were responsible for methodology. R.t.H. performed formal analysis. M.J., Y.J.E.S., R.T.N.M., X.C., S.J.C.F.M.M., V.A.C.M.K., L.C.J.d.B., V.P.M., H.L. and H.D. underwent investigation. Y.J.E.S., M.J., R.t.H., M.G.N. and R.T.N.M. wrote the original draft. Y.J.E.S., M.J., R.t.H., X.C., H.J.P.M.K, S.J.C.F.M.M., V.A.C.M.K., L.C.J.B.d.B., V.P.M., H.L., H.D., M.M., A.E.v.H., I.J., L.A.B.J., Y.L., J.W.A.S., M.G.N. and R.T.N.M. wrote, reviewed and edited the manuscript. R.t.H. performed visualization. R.T.N.M, M.G.N. and L.A.B.J. underwent supervision. M.G.N. was responsible for project administration. M.G.N. and L.A.B.J. were responsible for funding acquisition.

Data Availability Statement

All data used in this project have been meticulously catalogued and archived in the BBMRI-NL data infrastructure (<https://hfgp.bbmri.nl/>) using the MOLGENIS open source platform for scientific data.⁶⁹ This allows flexible data querying and download, including sufficiently rich metadata and interfaces for machine processing (R statistics, REST API) and using the FAIR principles to optimize Findability, Accessibility, Interoperability and Reusability.⁷⁰

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. FDR corrected p values of the correlation between lymphocyte subsets and circulating levels of TSH or fT4.

Table S2. Top 10 enriched pathways associated with TSH levels.

Table S3. Stimulations used for the in vitro experiments in PBMCs and Macrophages.