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Association of increased eomesodermin, BCL6, and granzyme B expression with major clinical manifestations of Hashimoto's thyroiditis – an observational study

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ABSTRACT

Purpose: Studies of cytotoxic T cells and their respective lineage master regulators have been limited in Hashimoto's thyroiditis (HT). It is unclear whether their transcriptomes are changed in HT patients and how these changes are associated with the thyroid damage, major clinical manifestations, and disease progression.

Methods: We explored the gene expression patterns of selected transcription factors [eomesodermin (EOMES), BACH2, BCL6, TCF1] and cytolytic molecules [granzyme B (GZMB)] in peripheral blood (PB) T cells of 10 healthy controls and 30 HT patients of various subtypes (hypothyroid, untreated HT; L-thyroxine (T4)-treated HT, and spontaneously euthyroid HT) using real-time quantitative PCR.

Results: *EOMES* (Mann–Whitney P = 0.044), *GZMB* (P = 0.028), and *BCL6* mRNA (P = 0.001) were overrepresented in PB T cells from HT and showed levels varying by age, thyroid volume and disease severity. *BCL6* transcripts were predominantly enriched in severely affected, hypothyroid cases, both on and off LT4. Increased *EOMES* RNA expression was associated with advancing age, lower thyroid volumes and higher peak adjusted TSH levels over the course of the disease. The body mass-adjusted, steady-state maintenance dose of LT4 increased with *GZMB* and *BCL6* levels in PB T cells of hypothyroid cases, mostly postmenopausal women having long-standing, non-goitrous and atrophic disease form.

Conclusions: Our exploratory results suggest a role for GZMB, EOMES, and BCL6 in the context of HT, thyroid injury, and aggressive/advanced disease forms. Functions enriched within differentially expressed transcripts could be an important new target in understanding the pathogenesis of HT.

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Introduction

Hashimoto's thyroiditis (HT) is a common, multifactorial autoimmune disorder characterized by the presence of thyroid-specific autoantibodies, chronic lymphoplasmacytic infiltration and marked fibrosis that gradually replaces thyroid parenchyma, thereby

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inducing hypothyroidism. B cells, $CD4^+$ T helper (Th)1, Th2, Th17 and regulatory $FoxP3^+$T (Treg) cells play prominent roles (Ajjan and Weetman, 2015); however, few details are available concerning the importance of cytolytic T cells (CTL) in the progression of HT.

Direct T cell cytotoxicity against thyroid epithelial cells (TEC) has been recognized long ago (Del Prete et al., 1986; Iwatani et al., 1993; Sugihara et al., 1995). Activated $CD8^+$ (Del Prete et al., 1986; Sugihara et al., 1995), and to a lesser degree, perforin (PRF)1^+ $CD4^+$ cytolytic T effectors (Wu et al., 1994) make up a considerable proportion of thyroid infiltrating T cells in HT (Iwatani et al., 1993). These thyroid infiltrating CTL are clonally expanded, self-reactive, and recognize TEC through major histocompatibility complex class I molecules on their surface (Sugihara et al., 1995), providing a telltale sign of chronic, antigen-driven proliferation. Further evidence is provided by the restricted diversity of $CD8^+$TCRβ CDR3 spectratypes in blood, particularly in HT patients having disease longer than 5 years and requiring thyroid hormone replacement therapy (Okajima et al., 2009).

Several transcription factors have been implicated in programming the CTL functions (Crotty et al., 2010; Hu and Chen, 2013). Of these, major regulators include a T-box transcription factor (TF) eomesodermin (EOMES), B cell CLL/lymphoma 6 (BCL6), and transcription factor 1 (TCF1, encoded by *TCF7*), which coordinate effector to memory differentiation of T cells (Crotty et al., 2010; Hu and Chen, 2013; Pearce et al., 2003). By contrast, BTB and CNC homology 2 (BACH2) is a key negative regulator of terminal effector differentiation in conventional T cell lineages (Hu and Chen, 2013). Genetic ablation of *BACH2* results in the reduction of $CD8^+$ central memory and $CD4^+$$FoxP3^+$Treg cells, with a concomitant increase in short-lived effector cells and $CD4^+$Th cells, respectively (Roychoudhuri et al., 2016, 2013). EOMES expression is also central to the cytotoxic transdifferentiation of $CD4^+$Th cells and renders $CD4^+$ and $CD8^+$T cells pathogenic by activating granzyme B (GZMB), PRF, and FasL pathways (Eshima et al., 2012). GZMB, an effector serine protease that activates target cell apoptosis through caspase-dependent and independent pathways, is regularly expressed by activated CTL and belongs to cytotoxicity-associated transcripts often used as indicators of CTL presence in tissues (Pearce et al., 2003).

Transcripts expressed in CTL may have mechanistic, diagnostic, and therapeutic importance in HT; however, little is known about their expression and influence on HT course in humans. Identification of such transcripts, which set apart particular disease subgroups or predict a severe clinical outcome, is essential to improve our understanding of HT. To this end, we screened the transcriptional patterns of *EOMES*, *BACH2*, *BCL6*, *TCF7*, and *GZMB* in bulk peripheral blood (PB) T cells across different outcomes of HT in a population of Croatian patients.

Methods

Subjects

The collection consisted of 30 HT patients (3 male) and 10 healthy controls (HC, all female). Healthy control subjects were euthyroid, thyroid peroxidase antibodies (TPOAb)-negative individuals, with normal ultrasound findings of the thyroid gland and no

evidence or family history of autoimmune and endocrine disorders. All patients were referred to the thyroid clinic of the Osijek University Hospital, Croatia. HT was diagnosed as previously described (Stefanic et al., 2008) and patients were classified as spontaneously euthyroid (euHT, n = 11, 1 male), hypothyroid (hypoHT, n = 9, 2 male), and rendered euthyroid by hormone replacement therapy [substHT, n = 10, median steady-state L-thyroxine (T4) dose 1.06 μg/kg body mass daily, interquartile range 0.85–1.3 μg/kg]. Overt hypothyroidism requiring LT4 treatment was defined by thyroid-stimulating hormone (TSH) >10 mIU/l and/or free-T4 (FT4) <10 pmol/l. A single brand name of LT4 and the morning intake, after an overnight fast, were used. All participants were unrelated Caucasian adults from East Croatia, a region considered iodine sufficient since the year 2003, and had to be free of any obvious medical illness for at least 1 month before the blood withdrawal, including acute infections and allergic reactions. Patients with a fluctuation in body mass of >10% were excluded from the study. None of the participants used immune-modifying drugs. Informed consent was obtained from all individual participants included in the study. The study protocol was reviewed and approved by the institutional ethical committee (Document No. 29-1:9890-11/2007).

Thyroid function measurement

TSH (normal range: 0.46–4.7 mIU/l), free tri-iodothyronine (FT3) (1.9–5.7 pmol/l) and FT4 (10–22 pmol/l) were measured in morning sera, 24 h after the last ingestion of LT4, using immunoassay methods (Vitros FT3 Reagent Pack, Vitros FT4 Reagent Pack and Vitros TSH Reagent Pack, Ortho-Clinical Diagnostics, Amersham, UK) according to the manufacturer's instructions. The overall coefficient of variation for serum TSH was <10%. The maximum baseline serum TPOAb-IgG (negative < 125 kIU/l) levels were measured by ELISA (Anti-TPO Kit, Milenia Biotec, Germany). Peak serum TSH (third generation assay, functional sensitivity 0.01 mIU/l) and the respective FT4 value were extracted from the charts of HT cases, provided TSH/FT4 had been determined since the onset of HT, by identical generation assay, in a single accredited laboratory, prior to any pharmacological intervention.

Thyroid volume measurements

The thyroid volume was sonographically determined as the sum of the volumes of the two lobes according to the ellipsoid formula (10 MHz linear array transducer, Accuson X-150, Siemens, Germany).

Peripheral blood mononuclear cells (PBMC) isolation and cell separations

PBMC were isolated from heparinized blood samples by gradient density centrifugation on LymphoPrep (Axis Shield, Oslo, Norway) according to the instructions in the manufacturer's leaflet. As previously detailed (Tokić et al., 2016), T cells were purified from PBMC (1 x 10^7 cells per 100 μl Isolation buffer with 20% FBS) by negative immunomagnetic selection (Dynabeads Untouched Human T cells Isolation Kit, Invitrogen, Paisley, UK) using a mixture of mouse monoclonal antibodies against CD14, CD16, CD19, CD36, CD56, CDw123 and CD235. The kit depletes B cells, natural killer (NK) cells, monocytes,

platelets, dendritic cells, granulocytes and erythrocytes. Bead-bound cells were captured by placing the polypropylene tubes containing the cell suspension in the DynaMag magnet. The remaining, non-adherent, bead and antibody-free T cells were gently removed by pipetting to a new tube. Immunomagnetic selection and washing steps were repeated twice.

Total RNA extraction

Total RNA from pelleted T cells was extracted using TRI REAGENT (Sigma, USA) solution as described by (Chomczynski and Sacchi, 1987). Quality of RNA samples was verified by ethidium bromide staining in 2% agarose gel electrophoresis and OD_{260}/OD_{280} ratio > 1.8 measurements by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

cDNA synthesis

First-strand cDNA synthesis was performed using PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. Both random hexamer (5.0 µM) and oligo dT (2.5 µM) priming were used for efficient transcription of 400 ng total RNA in a 40 µl final master mix comprising 8 µl of 5× PrimeScript Buffer and 2 µl of PrimeScript™ RT Enyme Mix 1. The reaction mixture was first incubated at 37°C for 15 min, then heated to 80°C for enzyme inactivation and after 5s finally cooled down to 4° C. All cDNA samples were diluted 8-fold to 1.25 ng/µl end-point concentrations and stored in aliquots at −20°C until use.

Quantitative real-time PCR

cDNA was used as a template for quantitative real-time PCR (RT-qPCR) analysis of five target (EOMES, BACH2, BCL6, GZMB, TCF7-long isoform) and one reference gene (TBP) using 96-well format plates and a LightCycler 480 II PCR instrument (Roche). All qRT-PCR measurements of transcript levels were performed in triplicate 10 µl reactions containing 4.5 µl of cDNA, 5.0 µl of TaqMan Universal PCR Master Mix II kit and 0.5 µl of pre-developed individual TaqMan gene expression assay (Applied Biosystems). The cycling conditions were set according to the manufacturer's guidelines and the list of assays and amplicon sizes is given in Table 1.

Table 1. List of TaqMan probes and amplicon sizes of investigated genes.

Assay ID	Gene abbreviation	Gene name	GenBank Accesion Number	Amplicon size (bp)
Hs00172872_m1	EOMES	Eomesodermin	NM_001278183.1	81
Hs00175273_m1	TCF7	Transcription Factor 7	NM_003202.3	105
Hs00222364_m1	BACH2	BTB and CNC homology 2, basic leucine zipper transcription factor 2	NM_021813.2	106
Hs01554355_m1	GZMB	Granzyme B	NM_004131.4	134
Hs00153368_m1	BCL6	B-Cell CLL/Lymphoma 6	NM_001706.4	76
Hs99999910_m1	TBP	TATA box binding protein	NM_003194.4	127

To evaluate the linear range of amplification and the variation in PCR efficiency for all TaqMan assays, 5-point fourfold serial dilutions of the arbitrary standards were included in each experiment. Subsequently, the amplification efficiency and the linear regression coefficient (R^2) determined for all studied genes varied between 94–100% and 0.995–0.999, respectively. Intra-assay variability was less than 0.64% for all investigated transcripts and less than 0.71% between different PCR experiments.

At the end of each run, fluorescence signals in the FAM channel were automatically normalized to ROX intensity, and Cp values were determined by the 2nd derivative maximum method using Advanced Relative Quantification module of the LightCycler® 480 Software. Expression levels of investigated transcripts were normalized relative to previously validated TBP endogenous control (Tokić et al., 2016) and fold difference in mRNA levels in unknown samples versus controls was calculated using ΔΔCt relative quantification method as described by (Livak and Schmittgen, 2001).

Statistical analysis

Normality of distributions was tested by Anderson-Darling test. Continuous data are presented as medians with interquartile ranges. If not otherwise stated, Fisher exact, Mann–Whitney and Kruskall–Wallis test with Bonferroni–Dunn's post-hoc analysis were used for group comparisons. Pairwise and first-order partial correlations were determined by Spearman rank-test. Two-tailed $P < 0.05$ was considered significant. All statistical analyses were performed with NCSS2007 (v07.1.20, NCSS LLC, Kaysville, Utah, USA).

Results

Demographic and biochemical data

Patients' characteristics are detailed in Table 2. Sex composition (Fisher–Freeman–Halton exact $P = 0.162$) and body mass (Kruskall–Wallis $P = 0.92$, data not shown) were similar in all groups. SubstHT cases were older than healthy controls, had longer follow-up than other HT groups and required higher serum FT4 levels to achieve FT3 and TSH levels comparable to healthy T cell donors. The replacement dose of LT4 per unit body mass was a principal determinant of serum FT4 in those patients (Spearman's correlation coefficient $\rho = 0.782$, $P = 0.0075$, $n = 10$). By contrast, a lower median level of FT3, but still within the reference range, was characteristic of euHT and hypoHT when compared to healthy individuals. Four euHT cases had a history of transient, mild subclinical hypothyroidism (peak TSH 5.4–8.75 mIU/l, normal FT4). The patients with the smallest thyroid volumes (lower tertile, ≤12.6 ml, $n = 9$) showed higher peak TSH levels at diagnosis or at follow-up [11.87 (5.85–49.4) vs 5.34 (3.62–13.16) mIU/l, ≤12.6 vs >12.6 ml, $n = 28$, Mann–Whitney $P = 0.039$].

Table 2. Demographic, clinical and biochemical characteristics of patients and healthy controls. Data are presented as medians (interquartile range).

Group	Age (years)	Follow-up time (months)	FT4 (pmol/l)	FT3 (pmol/l)	TSH (mIU/l)	Thyroid volume (ml)	TPOAb (kIU/l)
euHT (N = 11)	54 (38–59)	12 (6–36)	11.4 (11.2–11.9)	2.58 (2.33–3)[b]	3.78 (1.38–4.33)	15 (12.9–23.6)[b]	714 (145–3000)
substHT (N = 10)	63 (61–64)[b]	66 (19–102)	15.4 (12.2–18.8)[c]	3 (2.66–3.54)	2.54 (1.11–2.93)	11.1 (6.8–13.4)[c]	834 (208–1318)
hypoHT (N = 9)	48 (36–63)	6 (6–30)	11.3 (9.4–11.9)	2.55 (2.3–3.14)[b]	11.1 (6.47–17.23)[d]	16.5 (14.5–21.4)[b]	324 (116–1649)
Controls (N = 10)	42 (39–52)	-	12.4 (10.7–13.1)	3.21 (2.97–4.39)	1.5 (0.98–2.43)	11.8 (10.8–13)	neg.
Kruskall–Wallis P	0.013	0.029	0.0015	0.023	0.0001	0.00074	-

FT4: free thyroxine, FT3: free tri-iodothyronine, TSH: thyroid-stimulating hormone, TPOAb: thyroid peroxidase antibodies
[a]Bonferroni–Dunn's post hoc test.
[b]$P < 0.05$ vs control group.
[c]$P < 0.05$ vs euHT group, $P < 0.01$ vs hypoHT group.
[d]$P < 0.05$ vs euHT, $P < 0.01$ vs substHT, $P < 0.0001$ vs control group.

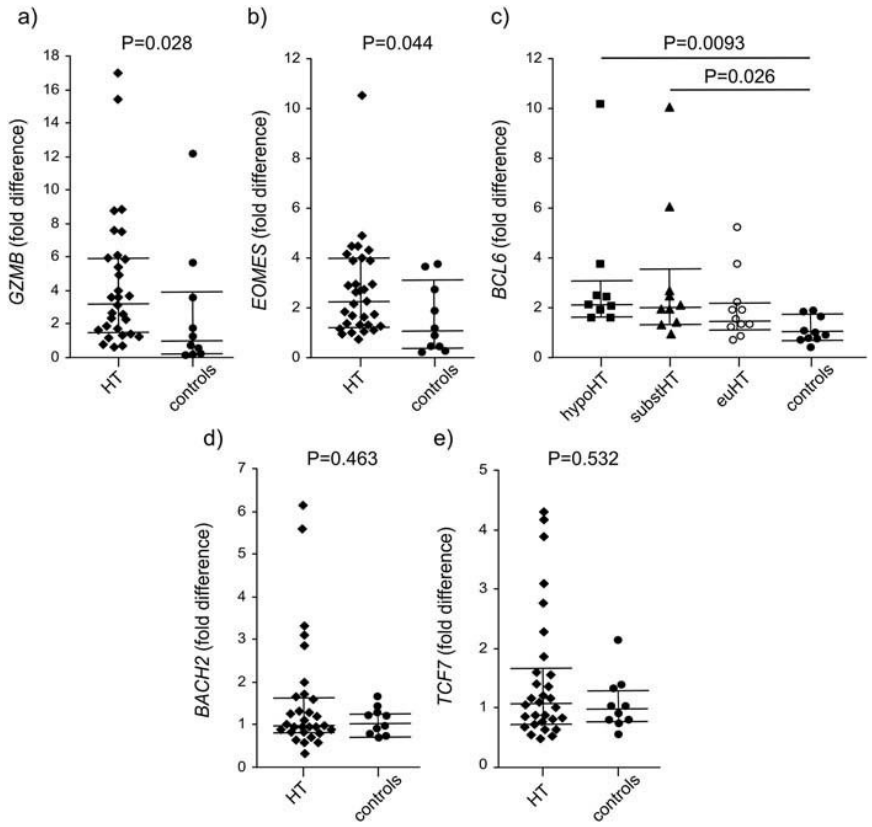

Figure 1. Differential expression of GZMB (a), EOMES (b) and BCL6 (c) mRNA in peripheral blood CD56-/CD16-T cells across the studied groups of HT patients (HT) and healthy controls. Gene expression was measured by RT-qPCR relative to TBP in an independent cohort of 9 untreated (hypoHT), 10 LT4-treated hypothyroid HT patients (substHT), 11 spontaneously euthyroid HT cases (euHT) and 10 healthy controls. P values for each mRNA relative expression are presented [Mann–Whitney test (a–b, d–e); Bonferroni–Dunn's post-hoc analysis following Kruskall–Wallis test (c)]. Horizontal lines represent median with interquartile range.

Expression analysis

Deregulated expression of EOMES, BCL6, and GZMB mRNA in PB T cells from HT

RT-qPCR analysis showed increased RNA levels of several genes in PB T cells from HT patients, including GZMB (Figure 1a), EOMES (Figure 1b) and BCL6 (Mann–Whitney P = 0.001, Figure 1c), but not BACH2 (Figure 1d) or TCF7 (Figure 1e). For EOMES and GZMB, no significant differences in RNA levels were found among the patient subgroups (data not shown); by contrast, BCL6 transcripts were predominantly enriched in severely affected, hypothyroid patients, both LT4-treated and untreated (Kruskall–Wallis P = 0.0026, Figure 1c). The restoration of euthyroidism alone did not prevent PB T cell alterations in LT4-treated versus untreated HT. Age differences did not affect the results (covariate-adjusted general linear model, log2 fold change, $P_{EOMES} = 0.023$, $P_{GZMB} = 0.012$, $P_{BCL6} = 0.0044$).

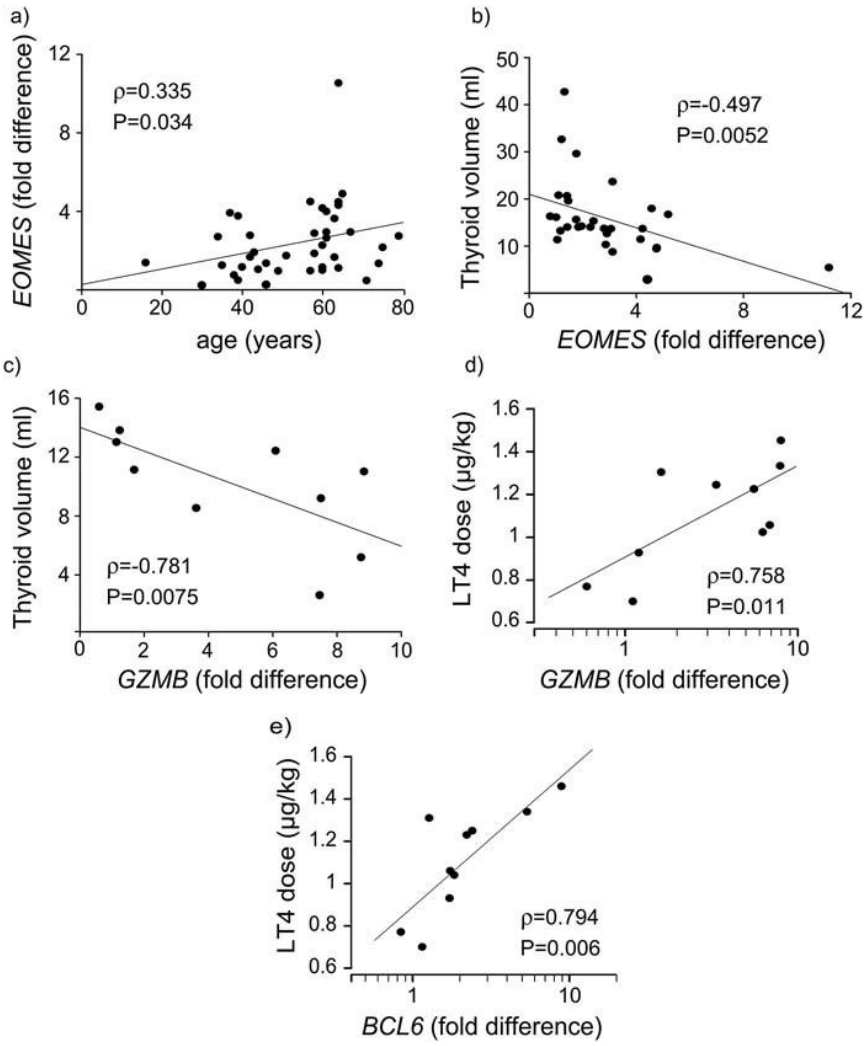

Figure 2. Association of EOMES (a) gene expression with age in peripheral blood CD56-/CD16-T cells, by combining 10 healthy controls and 30 HT patients. Thyroid volume was inversely associated with the expression profiles of EOMES (b) and GZMB (c) transcripts in combined HT patients (n = 30) and 10 LT4-treated hypothyroid HT cases, respectively. The body mass-adjusted maintenance dose of LT4 in hypothyroid HT patients requiring hormone replacement therapy is related to GZMB (d) and BCL6 (e) mRNA expression in peripheral T cells (Spearman rank test, solid line: the least square estimate). Note that x-axes (d–e) are log scales.

Selected PB T cell transcripts are associated with clinical covariates of HT

EOMES transcript abundance increased with age (Figure 2a). Increased T cell EOMES mRNA expression was moderately associated with decreasing residual thyroid volume in the HT group (Figure 2b), particularly among the LT4-treated cases ($\rho = -0.758$, $P = 0.011$, $n = 10$). A strong inverse association between the GZMB mRNA levels and the thyroid volume was observed in the latter (Figure 2c). Age, the

level of TSH stimulation and body mass did not affect the volumetric associations (partial correlations, data not shown).

In the HT group, the peak serum TSH was independently associated with interindividual variations in expression levels of *EOMES* mRNA (partial $\rho = 0.49$, $P = 0.013$, $n = 26$) after adjustment for the respective FT4 value.

A significant positive association was also noted between the *GZMB*, *BCL6* transcript expression and the body mass-adjusted replacement dose of LT4 in similarly aged substHT patients (Figure 2d,e). The target serum TSH did not affect the results (partial correlations, data not shown). TPOAb levels were not associated with any transcript (data not shown).

Transcriptional cross-correlations

Several between-mRNA correlations were observed, all broadly consistent with the previous reports from perturbation studies (Hu and Chen, 2013; Pearce et al., 2003): *GZMB* transcript abundance was tightly linked to *EOMES* mRNA expression levels ($\rho = 0.788$, $P < 10^{-6}$). *EOMES* ($\rho = 0.348$, $P = 0.028$) and *BCL6* levels ($\rho = 0.476$, $P = 0.0019$) were related to *TCF7*; in addition, *BCL6* levels were also related to *BACH2* transcript expression ($\rho = 0.41$, $P = 0.0087$, all $n = 40$).

Discussion

Using RT-qPCR profiling of HT participants, we detected that transcripts encoding EOMES, its canonical target GZMB, and a B/T cell TF BCL6 are I) overrepresented in PB T cells of HT patients, and II) show substantial interindividual variations in affected individuals, partly in relation to known clinical phenotypes.

Noteworthy, high *EOMES* mRNA expressors were characterized by higher peak adjusted TSH levels over the course of the disease and also by lower thyroid volumes, which, in turn, may contribute to the progression of thyroid insufficiency and the development of hypothyroidism in untreated HT (Carlé et al., 2009). TSH, in its own turn, can play an important role in T cells (Hodkinson et al., 2009; Wu et al., 2017) and thymus (Stefan et al., 2014; Wu et al., 2017), and regulates their functions via TSH receptor. Combined, these results suggest that the inflammatory process of HT and the subsequent thyroid immune injury, transient or permanent, could lead to or result from deregulation of EOMES-driven T cell networks. There is also evidence that *EOMES* expression is a heritable and longitudinally stable phenotype in PBMC (McKay et al., 2016), so it is possible that the observed result is not a disease effect, but the net effect of genetic or epigenetic upregulation, the specific phenotype of HT being one possible consequence of it. Accordingly, both *EOMES* and $EOMES^{+}$T cells have been already associated with multiple sclerosis (McKay et al., 2016; Raveney et al., 2015), rheumatoid arthritis (RA, Kim et al., 2015) and dysthyroid orbitopathy (Khong et al., 2015).

EOMES is a paralogue of another differentially expressed T-box TF in HT, T-bet (Peng et al., 2015; Tokić et al., 2016). Both T-bet and EOMES have been reported to inhibit peripheral FOXP3 induction in murine $CD4^{+}$T cells (Lupar et al., 2015). The two TF also cooperate to create and arm short-lived $CD8^{+}$ effector CTL (Pearce et al., 2003); thenceforth, high EOMES expression promotes effector to memory cell transition (Knox et al.,

2014). Ageing and antigen persistence increase further the percentage of EOMES$^+$CD8$^+$ cells in CD8$^+$T cells (Dolfi et al., 2013; Moskowitz et al., 2017), providing an explanation for the progressive enrichment of *EOMES* transcripts in PB T cells from aged subjects. Aside from CD8$^+$T cells, EOMES is expressed in several other cytotoxic effector subsets that could mediate the damage of TEC and affect the development of HT, like CD3$^-$CD56$^+$/CD16$^+$NK cells (depleted in our study samples), invariant (i)NKT, CD4$^+$CTL αβ and CD3$^+$CD4$^-$CD8$^-$ (DN) γδ T cells (Knox et al., 2014). In support, patients with HT show variable expansion of CD8$^+$Tc17 (Liu et al. 2016b), CD3$^+$CD56$^+$NKT (Guo et al., 2014) and CD28$^-$/CD45RO$^+$ memory T cells in both CD4$^+$ and CD8$^+$ compartments of circulating blood (Nanba et al., 2009; Prelog et al., 2013). Of these, CD28$^-$T cells display features of effector/memory cells with increased replicative history and an intense, GZMB/PRF cytotoxic profile, a pattern that increases further during aging (Maly and Schirmer, 2015). High expression of EOMES, T-bet, and FasL is another feature that separates CD28$^-$ from CD28$^+$CD8$^+$T cells (Fann et al., 2005). A numerically sparse set of γδ and DN αβ T cells has been also described within the Hashimoto's thyroids (Iwatani et al., 1993; Liu et al. 2016a; Paolieri et al., 1995); however, the scarcity of peripheral EOMES$^+$γδ and iNKT cells (Knox et al., 2014) somewhat limits their competitiveness under current experimental conditions favoring the bulk of CD56$^-$/CD16$^-$T cells. Thus, a more precise analysis of T cell subpopulations would offer a better definition of EOMES activity in PB and affected thyroids. Also, the inclusion of more aged control subjects may be needed to see whether the observed overexpression of *EOMES* transcripts is related to immunosenescence of T cells in HT.

Within the T cell compartment, TCF1 and BCL6 support the formation of self-renewing (Crotty et al., 2010; Pearce et al., 2003), stem-like CD8$^+$ memory precursor cells (Wu et al., 2016). TCF1–BCL6 axis also directs the acquisition of a T follicular helper (Tfh) cell phenotype in both CD4$^+$ precursors (Pratama and Vinuesa, 2014) and exhausted CD4$^+$/CD8$^+$ effectors (Tex) (Crawford et al., 2014; Wu et al., 2016). In mice, a role for BCL6 in controlling Th2, Th17 and Treg cells has been also reported (Crotty et al., 2010). The tight regulation of *BCL6* expression is thus essential, as an aberrant acquisition of Tex or Tfh phenotype affects disease severity, autoantibody levels and end-organ injury in several autoimmune disorders (McKinney et al., 2015; Pratama and Vinuesa, 2014). In this context, it is relevant that both thyroid-resident, germinative center (GC) CD4$^+$Tfh and PB CD4$^+$CXCR5$^+$PD1$^+$Tfh-like cells (Liu et al. 2016b; Zhu et al., 2012) are expanded in HT; of these, only GC Tfh cells, but not their circulating counterparts, express BCL6 (Pratama and Vinuesa, 2014). Herein, we found a stepwise increase of *BCL6*, but not *TCF7* expression from HC to euHT and severe HT *in vivo*, with the highest levels in PB T cells from hypothyroid HT, both on and off LT4. In LT4-treated cases, mostly postmenopausal women having long-standing HT and non-goitrous/atrophic disease form, the body mass-adjusted LT4 dose necessary to maintain mid-normal TSH values escalated with *BCL6* expression in PB T cells. The latter finding is at odds with two earlier HT studies (Liu et al. 2016b; Zhu et al., 2012), in which circulating cells of HT cases lacked increased amounts of *BCL6* mRNA, a difference that perhaps reflects cell isolation (PBMC (Liu et al. 2016b) and CD4$^+$T (Zhu et al., 2012) vs CD56$^-$T cells), selection of housekeeping genes, divergent ancestry, environmental heterogeneity, variations in disease duration, statistical power, differentiation states of T cells and the severity of HT. Therefore, *BCL6* is an interesting, but little understood aspect of HT.

The presence of PRF/GZMB-secreting T cells has been detected in Hashimoto's thyroids (Kie et al., 2001; Wu et al., 1994), but the functional relevance of GZMB remains unknown. A prominent intraglandular expression of GZMB is a hallmark of interferon-α induced inflammatory thyroiditis, leading to profound hypothyroidism in genetically susceptible mice (Akeno et al. 2011). In addition to having a cytolytic effect, GZMB, which functions downstream of EOMES and T-bet, may be also important for homeostasis of $CD8^+$T cells in the periphery, homing of primed CTL (Mollah et al., 2012) and killing of Treg cells by $GZMB^+CD4^+$ effectors (Ashley and Baecher-Allan, 2009). CTLA-4, a major gatekeeper of thyroid autoimmunity, reduces the frequency of GZMB-expressing $CD8^+$T cells by selectively inhibiting the accumulation of EOMES (Hegel et al., 2009). The number of these $GZMB^+CD8^+$T cells in PB correlates with disease activity in systemic lupus erythemathosus (Blanco et al., 2005), whereas the circulating GZMB has been associated with the severity of erosions in RA (Goldbach-Mansky et al., 2005). In our study, the overall distribution of *GZMB* transcripts in PB T cells from HT patients showed upregulation, at least partially in an EOMES-related fashion. Moreover, a cytotoxic signature, consisting of *EOMES* and/or *GZMB* upregulation, was associated with smaller thyroid remnants and higher body mass-adjusted maintenance doses of LT4 in similarly aged, hypothyroid cases undergoing replacement therapy, respectively. The exact mechanism behind these co-occurrences is uncertain but remains interesting because it could point, among other factors, to the origins of hypothyroidism and potentially identify mechanistic and therapeutic targets in susceptible individuals.

Several issues remain to be addressed. I) In activated T cells, protein levels are often controlled by translational, rather than transcriptional mechanisms. Multiparametric labeling, longitudinal samples, and functional assays are necessary to establish the stability and cellular sources of target protein expression over disease course. II) PB T cells may not be relevant to the control of HT *in situ*. Another question relates to the T cell clonality and reactivity against thyroid self-antigens in expanded PB populations. III) A larger sample would improve power and effect size estimation across patient subgroups. IV) Obesity, different salt iodization ordinances, and genetic backgound may confound the results of thyroid volume and TSH measurements (Brčić et al., 2017; Taylor et al., 2015).

In closing, our exploratory study suggests that *EOMES*, *BCL6* and *GZMB* gene expression are aberrant within the PB T cell transcriptome of HT patients. The association of this transcription signature with the heterogeneity of HT and disease control is suggested. Further work is necessary to confirm and expand the functions of identified targets across the spectrum of HT.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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