Endokrynologia Polska
DOI: 10.5603/EP.2017.0010
Tom/Volume 68; Numer/Number 3/2017
ISSN 0423–104X

Altered expression of CTLA-4, CD28, VDR, and CD45 mRNA in T cells of patients with Hashimoto's thyroiditis — a pilot study

Zmiana ekspresji mRNA dla CTLA-4, CD28, VDR i CD45 w limfocytach T u osób z chorobą Hashimoto — badanie pilotowe

***Stana Tokić[1, 2 3], *Mario Štefanić[3, 4], Ivan Karner[5], Ljubica Glavaš-Obrovac[2]**

*These authors contributed equally to this work
[1]Department of Molecular Diagnostics and Tissue Typing, Osijek University Hospital, Osijek, Croatia
[2]Department of Medicinal Chemistry, Biochemistry, and Clinical Chemistry, Faculty of Medicine, University of Osijek, Osijek, Croatia
[3]Clinical Institute of Nuclear Medicine and Radiation Protection, Osijek University Hospital, Osijek, Croatia
[4]Department of Nuclear Medicine and Oncology, Faculty of Medicine, University of Osijek, Osijek, Croatia
[5]Department of Pathophysiology, Faculty of Medicine, University of Osijek, Osijek, Croatia

Abstract

Introduction: CD28/T-cell receptor (TCR)/cytotoxic T-lymphocyte antigen 4 (CTLA4) complex controls T-cell tolerance and autoimmunity in Hashimoto's thyroiditis (HT). In addition, CD45 protein tyrosine phosphatase (PTPase) and vitamin D receptor (VDR) cooperatively interact with the TCR complex to affect autoimmune processes central to the pathogenesis of HT. Nevertheless, their role in HT aetiology has been less well established. In this study, we aimed to explore mRNA expression levels of CTLA4, CD28, CD45, and VDR in T-cells, across different outcomes of HT.

Material and methods: The study included 45 HT patients and 13 euthyroid, healthy controls. T-lymphocytes were isolated from peripheral blood mononuclear cells, total mRNA was extracted from T-cells, and gene expression was studied by reverse transcription-polymerase chain reaction (RT-PCR) and ImageQuant method relative to glyceraldehyde-3-phosphate dehydrogenase RT-PCR products.

Results: Nominally higher expression levels of VDR, CTLA4, CD28, and CD45RAB mRNA were found in unsorted T-lymphocytes of healthy controls when compared to the HT patients. No difference was observed between hypothyroid/untreated, spontaneously euthyroid and LT4-treated HT patients. VDR mRNA expression was linked to both T3 levels and CTLA4 gene expression, whilst CD45RB mRNA expression coincided with CTLA4 and CD28 transcript levels. Conversely, older age and lower T3 levels were associated with increased abundance of CD45R0 isoform in HT patients.

Conclusions: The results suggest a cross talk between endocrine and immune functions in HT pathology: an altered peripheral T cell mRNA profile with reduced VDR, CTLA4, CD28, and CD45RAB transcript levels is accompanied by age-related shift from naive to memory/late-differentiated T cell CD45R mRNA signature and associated with thyroid hormone status in the HT patients. **(Endokrynol Pol 2017; 68 (3): 274–282)**

Key words: *Hashimoto disease; CD4 positive T lymphocytes; vitamin D3 receptor; CD28 antigen; cytotoxic T-lymphocyte-associated antigen 4; CD45 antigen*

Streszczenie

Wstęp: Kompleks antygenu CD28/receptora limfocytów T (TCR)/antygenu 4 związanego z limfocytem T cytotoksycznym (CTLA4) reguluje tolerancję limfocytów T oraz autoimmunogenność w chorobie Hashimoto (HT). Ponadto białkowa fosfataza tyrozynowa (PTPase) CD45 oraz receptor witaminy D (VDR) wchodzą w interakcję z kompleksem TCR, modyfikując procesy autoimmunologiczne mające podstawowe znaczenie w patogenezie HT. Jednak rola tych cząsteczek w etiologii HT nie została dokładnie ustalona. Celem autorów badania była ocean poziomów ekspresji mRNA dla CTLA4, CD28, CD45 i VDR w limfocytach T w zależności od różnego statusu HT.

Materiał i metody: Do badania włączono 45 chorych na HT i 13 zdrowych osób z prawidłową czynnością tarczycy. Limfocyty T wyizolowano spośród komórek jednojądrzastych krwi obwodowej, wyekstrahowano z nich całkowity mRNA i określono ekspresję genów za pomocą łańcuchowej reakcji polimerazowej z odwrotną transkryptazą (RT-PCR) i metody ImageQuant związanej produktami reakcji RT-PCR dehydrogenazy aldehydu 3-fosfoglicerynowego.

Wyniki: U osób zdrowych stwierdzono nominalnie wyższy poziom ekspresji mRNA dla VDR, CTLA4, CD28 i CD45RAB w niesortowanych limfocytach T niż u chorych na HT. Nie zaobserwowano żadnych różnic między chorymi na HT z niedoczynnością tarczycy/nieleczonymi, u których samoistnie nastąpiło przywrócenie eutyreozy, i stosującymi leczenie LT4. Ekspresja VDR mRNA była powiązana zarówno ze stężeniem T3, jak i ekspresją genu *CTLA4*, natomiast ekspresja mRNA dla CD45RB wiązała się z poziomami transkryptu CTLA4 i CD28. Z kolei starszy wiek i niższe stężenia T3 wiązały się ze zwiększoną ilością izoformy CD45R0 u chorych na HT.

Stana Tokić M.D., Department of Molecular Diagnostics and Tissue Typing, Osijek University Hospital, J. Huttlera 4, HR-31 000 Osijek, Croatia, phone: +38 531 514 312, fax: +38 531 514 206, e-mail: tokic.stana@kbo.hr

Wnioski: Uzyskane wyniki sugerują interferencje między czynnością wewnątrzwydzielniczą i immunologiczną w patologii HT: zmiana profilu mRNA obwodowych limfocytów T z ograniczeniem poziomu transkryptu białek VDR, CTLA4, CD28 i CD45RAB współistnieje z zależnym od wieku przesunięciem sygnatury mRNA limfocytów CD45R od komórek naiwnych do komórek pamięci immunologicznej/zróżnicowanych i jest powiązana ze stężeniami hormonów tarczycy u chorych na HT. **(Endokrynol Pol 2017; 68 (3): 274–282)**

Słowa kluczowe: *choroba Hashimoto; limfocyty T CD4-dodatnie; receptor witaminy D3; antygen CD28; antygen 4 związany z limfocytem T cytotoksycznym; antygen CD45*

This study was supported by the Croatian Ministry of Science, Education, and Sport (grant No. 219-0982914-2176, 219-2190372-2068, and 0127-0000000-3420).

Introduction

Hashimoto's thyroiditis (HT) is an organ-specific autoimmune disorder characterised by chronic thyroid inflammation, and subsequently, hypothyroidism [1]. Morphological and functional alterations in HT are predominantly mediated by T-helper (Th) 1 cytokines and cytotoxic T-lymphocytes through apoptotic cell death [2]. Recently, however, both CD4+CD25+ regulatory T-cells (Treg) [3–5] and interleukin IL-23-driven Th17 effectors [6–8] have been independently associated with thyroid inflammation, disease chronicity, and HT destructiveness.

CD28/T-cell receptor/CTLA4 complex controls T-cell homeostasis, tolerance, and autoimmunity in Hashimoto's thyroiditis [9, 10]. In addition, many other molecules, such as CD45 protein tyrosine phosphatase (PTPase) [11, 12] and vitamin D receptor (VDR) [13, 14], cooperatively interact with the TCR complex to affect Th1/Th17/Treg processes central to the pathogenesis of HT. Nevertheless, their role has been comparatively less well established.

CD28 acts as potent regulator of T-cell mRNA splicing [15]. In addition, emerging data suggest that the identification of disease-specific splice variants may provide novel biomarkers and drug targets [16, 17]. CD45 PTPase activity is regulated by isoform-specific differential homodimerisation, and alternative splicing of exons 4–6 (A-C) within the CD45 extracellular domain results in the production of at least five different CD45 isoforms: CD45RABC (exons 4-6 included), CD45RAB, CD45RBC, CD45RB, and CD45R0 (exons 4–6 skipped). Naive T-cells express longer isoforms at higher levels, while activated and memory T-cells express shorter isoforms (CD45R0) [18]. The alternatively spliced CD45 variants differentially homodimerise in primary T-cells, with the smallest isoform (CD45R0) being able to dimerise more efficiently and rapidly than the larger isoforms [19]. The resulting CD45 dimers have significantly reduced phosphatase activity; thus, the splicing switch is potentially critical for preventing prolonged TCR signalling and undesirable tissue injury.

We therefore sought to investigate VDR, CTLA4, CD28, and CD45 mRNA expression profiles in peripheral T-cells of Croatian HT patients in relation to HT susceptibility and phenotypic characteristics of affected individuals.

Material and methods

Subjects

The collection consisted of 45 HT patients (3 males) and 13 healthy controls (2 male, Table I). Control subjects were characterised as euthyroid, thyroid peroxidase autoantibodies (TPOAb)-negative individuals, with normal ultrasound findings of the thyroid gland and no evidence or family history of autoimmune and endocrine disorders. HT was diagnosed as previously described [20], and patients were classified as hypothyroid (hypoHT, n = 15), spontaneously euthyroid

Table I. ***Descriptive analysis of clinical and biochemical characteristics of patients and healthy controls***
Tabela I. ***Analiza opisowa klinicznych i biochemicznych cech pacjentów i osób zdrowych***

Group	Age (years)	fT4 [pmol/L]	fT3 [pmol/L]	TSH [mIU/L]	TPOAb [kIU/L]
euHT	57 (42–62)	11.6 (11.3–13.4)**	2.6 (2.4–3.1)	1.9 (0.96–3.1)**	155 (21–1112)
substHT	50 (29–62)	16 (13.3–16.5)**	2.7 (2.3–3)	2.57 (0.57–3.13)**	368 (112–1253)
hypoHT	46 (40–55)	10 (8.4–11.2)	2.5 (2.3–3)	8.1 (5.9–14.8)	321 (128–1520)
Controls	41 (29–61)	12.1 (10.8–12.7)**	3 (2.7–3.1)	1.6 (1.03–2.52)**	neg.
P*	0.532	7.3 × 10-5	0.136	10-6	–

Data correspond to median with interquartile range (25–75th percentile). The study included 13 healthy controls and 45 HT patients classified as hypothyroid (hypoHT, n = 15), spontaneously euthyroid (euHT, n = 18), and rendered euthyroid by thyroxine (L-T4) replacement therapy (substHT, n = 12). fT4 — free thyroxine, fT3 — free tri-iodothyronine, TSH — thyroid stimulating hormone. *Kruskall-Wallis test; **P < 0.05, Dunn's post hoc test, *vs.* hypoHT group

(euHT, n = 18), and rendered euthyroid by hormone replacement therapy (substHT, n = 12). Overt hypothyroidism requiring L-thyroxine (T4) treatment was defined by thyroid-stimulating hormone (TSH) > 10 mIU/L and/or free-T4 < 10 pmol/L; otherwise, it was considered subclinical if TSH was > 4.7 mU/L and fT4 was ≥ 10 pmol/L on at least two consecutive visits ≥ 3 months apart. All participants were unrelated adults from eastern Croatia and had to be in self-proclaimed good health and free of any obvious medical illness for at least one month before the blood withdrawal, including acute infections and allergic reactions. None of the participants used vitamin D supplements or immune-modifying drugs. Informed consent in written form was obtained from all participants prior to the testing, and the study protocol was reviewed and approved by the institutional ethical committee (Document No. 29-1:9890-11/2007).

Thyroid function measurement

Thyroid stimulating hormone (TSH) (normal range: 0.46–4.7 mIU/L), free tri-iodothyronine (fT3) (1.9–5.7 pmol/L) and free thyroxine (fT4) (10–22 pmol/L) were measured in morning (8–11 a.m.) sera 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. Maximum baseline serum TPOAb-IgG (negative < 125 kIU/L) levels were measured by ELISA (Anti-TPO Kit, Milenia Biotec, Germany).

Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated by density gradient centrifugation on a LymphoPrep (Axis Shield, Oslo, Norway) as described by Böyum [21]. After collection, heparinised blood samples were diluted 1:1 with 0.9% NaCl and following application to the LymphoPrep centrifuged for 20 minutes at 1000 g. Fraction of total lymphocytes and monocytes was harvested and rinsed with saline and then pelleted by centrifugation for 10 minutes at 550 g. The procedure was repeated twice. Cells were resuspended in 1 mL of Isolation buffer (PBS without Ca^{2+} and Mg^{2+} with 0.1% BSA and 2 mM EDTA) and counted after trypan-blue staining using the Bürker-Türk counting chambers and light microscope.

Lymphocyte separation

T-lymphocytes were separated from PBMC by negative selection using a Dynabeads untouched human T-cells isolation kit (Invitrogen, Paisley, UK) according to the guidelines in the manufacturer's leaflet. Briefly, PBMC (1×10^7) were incubated with FBS and mouse monoclonal antibodies specific for CD14, CD16, CD19, CD36, CD56, CDw123, and CD235 (20 minutes at 2–8°C). Following incubation, cells were washed with Isolation buffer and pelleted by centrifugation (300 g, 8 minutes). The pellet was resuspended and incubated for 15 minutes with pre-washed magnetic beads coated with monoclonal human anti-mouse IgG antibody. The bead-bound cells were subsequently separated on a DynaMag™ magnet, leaving the cell suspension free from B-lymphocytes, NK cells, monocytes, platelets, dendritic cells, granulocytes, and erythrocytes. The remaining untouched T-lymphocytes were transferred to a new tube. Steps involving washing and binding of bead-bound cells to a magnet were repeated twice. Residual Dynabeads were removed by placing the tube in a magnet for two minutes and then transferring the supernatant to a new tube.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from T-lymphocytes using TRI REAGENT (Sigma, USA) solution as described by Chomczinsky and Sacchi [22]. After isolation, RNA integrity was electrophoretically verified by ethidium bromide staining and OD_{260}/OD_{280} nm absorption ratio > 1.9 determined by UV spectrometry using a Shimadzu-UV-1601 instrument (Shimadzu Scientific Instruments, SAD). Total RNA (500 ng) was reverse transcribed to cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA) and the provided oligo(dT) primer mix according to the manufacturer's instructions.

Gene expression profiling

Following the RT step, mRNA expression analysis of target (VDR, CTLA4, CD28, CD45) and reference (GADPH) genes was carried out in a thin-wall PCR tubes using a 96-well Veriti thermal cycler (Applied Biosystem). Sequence-specific PCR primers for all assays were designed using PrimerQuest software (http://eu.idtdna.com/Scitools/Applications/PrimerQuest) and NCBI reference sequences (Table II). All PCR experiments were performed in 25 μl final volume reactions comprising 2 μl of cDNA template, 1.5 mM of MgCl2, 200 μM of each dNTP (Roche Diagnostics, Mannheim, Germany), 0.25 μM of each primer (TIB MOLBIOL, Berlin, Germany), and 1U of EUROTAQ polymerase (EuroClon, Italija, EU). Following initial denaturation (two minutes at 95°C), 35 repeating cycles with the same denaturation (95°C for 30 seconds) and elongation (72°C for 1 minute) steps were performed for all genes. The annealing step for GAPDH was set at 68°C for 90 seconds, for VDR at 56°C for 30 seconds, and for CTLA4, CD28, and CD45 at 55°C for 30 seconds. All reactions were terminally extended at 72°C for 15 minutes. All RT-PCR experiments were performed in duplicate, and no template negative

Table II. *Description of investigated genes, used primers, and amplicon sizes in RT-PCR*
Tabela II. *Opis badanych genów, używanych starterów i wielkości amplikonu w RT-PCR*

Gene abbreviation	Gene names (synonyms)	GenBank# Accession number	Sense, antisense primer	Amplicon size (bp)
VDR	vitamin D receptor	NM_000376.2	5′–gtttgattttagctgcagaacg–3′ 5′–agagacagggtttctccatgtt–3′	277
CTLA4	cytotoxic T-lymphocyte-associated protein 4	NM_005214.4	5′–agtatgcatctccaggcaaagc–3′ 5′–ccagaggaggaagtcagaatctg–3′	316
CD28	T-cell-specific surface glycoprotein CD28	NM_006139	5′–gtttgagtgccttgatcatgtgc–3′ 5′–ggcgatctgcttcaccaaaatc–3′	238
CD45RABC	protein tyrosine phosphatase, receptor type C, PTPRC	NM_002838.4	5′–ggcaaagcccaacacctt–3′ 5′–tgtggttgaaatgacagcg–3′	577
CD45RAB				434
CD45RBC				380
CD45RB				236
CD45RO				95
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_002046.5	5′- ccatcaatgacccctcattgacc–3′ 5′–gaaggccatgccagtgagcttcc–3′	605

#gene sequences available online at www.ncbi.nlm.nih.gov, primers were designed using PrimerQuest software

control was included in each experiment. To check for DNA contamination, the reverse transcription negative controls with no added RT enzyme were run in parallel and did not yield any PCR product.

Relative quantification of gene expression

RT-PCR products (5 μl) were applied to 2% agarose gel, separated in an electric field, and visualised by ethidium bromide staining (Fig. 1). The amount of cDNA present in agarose bands was quantified by measuring fluorescence band intensities using a UV Transilluminator (HVD life sciences, Vienna, Austria), Olympus digital camera, and computer image processing software ImageQuant™. The fluorescent signal ratio of target and GAPDH transcripts in each sample were used for normalisation of the mRNA expression levels according to the formula:

$$normalised\ cDNA\ levels = \frac{florescence\ signal\ gene\ (x)}{fluorescene\ \ signal\ GAPDH} \times 100$$

Each sample was subjected to two cycles of RT-PCR experiments, followed by quantification and normalisation of the results. Collected data were described by arithmetic mean and standard deviation. Finally, the fold difference in mRNA levels in unknown samples was calculated relative to healthy controls.

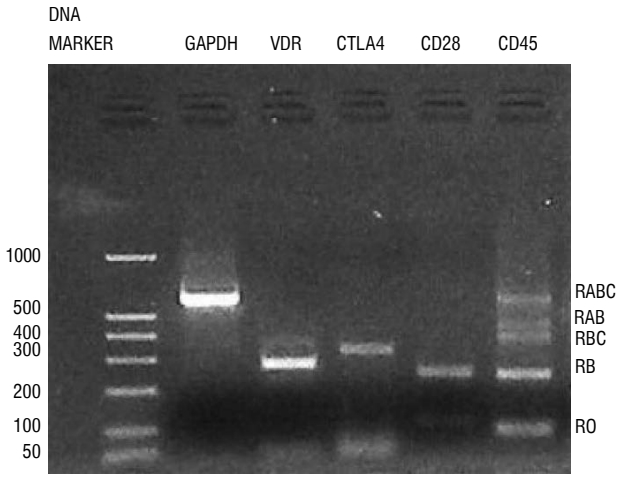

Figure 1. *RT-PCR analysis of GAPDH (605 bp), VDR (277 bp), CTLA4 (316 bp), CD28 (238 bp), CD45RABC (577 bp), CD45RAB (434 bp), CD45RBC (380 bp), CD45RB (236 bp) and CD45R0 (95 bp) gene expression. The total RNA (n = 500 ng) extracted from peripheral CD4+ and CD8+T cell population was reverse transcribed with oligo(dT) primer mix and amplified in the presence of sequence-specific primers*

Rycina 1. *Analiza RT-PCR ekspresji genów GAPDH (605 bp), VDR (277 bp), CTLA4 (316 bp), CD28 (238 bp), CD45RABC (577 bp), CD45RAB (434 bp), CD45RBC (380 bp), CD45RB (236 bp) i CD45R0 (95 bp). Całkowita wartość RNA (n = 500 ng) pochodzącego z peryferyjnego CD4+ i CD8+T populacji komórek była odwrotnie zapisana z użyciem primera oligo (dT) i zwiększona w obecności primerów o sekwencjach specyficznych*

Statistical analysis

Normality of distributions was tested by Anderson-Darling test. Data are presented as medians with interquartile ranges. Mann-Whitney and Kruskall-Wallis test with Dunn's post-hoc analysis were used for group comparisons. Pair-wise correlations were determined by Spearman rank-test. Two-tailed $P < 0.05$ was con-

sidered significant. Uncorrected P-values are presented throughout the text. For correlation data analysis across eight transcripts, Bonferroni's correction was used. Italicised P-values indicate those that remained significant after sequential Bonferroni's corrections ($P < 0.0063$). All statistical analyses were performed with NCSS2007 (v07.1.20, NCSS LLC, Kaysville, Utah, USA).

Results

Patients

Patients' characteristics are presented in Table I. Median age, sex, thyroid volume, and fT3 levels were similar in all groups. As expected, hypothyroid patients exhibited significantly higher serum TSH levels and lower fT4 levels compared to the untreated, spontaneously euthyroid HT patients, HT patients undergoing LT4 treatment, and healthy controls.

Expression profile of peripheral T cells in HT patients vs. controls

No significant differences in mRNA expression profiles were found among patient subgroups (data not shown), which prompted us to further analyse HT data as one group. Subsequently, a nominally higher VDR [median expression levels (IQR), HT *vs.* controls 46 (30–69) *vs.* 80 (62–105), P = 0.021, Fig. 2a], CTLA4 [96 (59–114) *vs.* 113 (98–132), P = 0.018, Fig. 2b], CD28 [35 (23–49) *vs.* 50 (37–62), P = 0.0074, Fig. 2c], and CD45RAB [10 (8–14) *vs.* 13 (10–19), P = 0.042, Fig. 2e] mRNA expression was observed in unsorted peripheral T-lymphocytes of healthy controls when compared to HT patients. However, no significance persisted upon correction for multiple testing ($P < 0.0063$). Concerning other CD45 mRNA isoforms, no changes across the studied groups [HT *vs.* controls] were observed when CD45RABC [16 (12–19) *vs.* 17 (12–23), P = 0.272, Fig. 2d], CD45RBC [11 (8–14) *vs.* 13 (10-19), P = 0.062, Fig. 2f], CD45RB [45 (36–63) *vs.* 63 (40–74), P = 0.126, Fig. 2g], or CD45R0 transcripts were measured [35 (21–52) *vs.* 23 (13–42), P = 0.168, Fig. 2h].

Correlation analysis

Pair-wise correlation analysis of pooled samples (HT patients + controls, n = 58) was preformed to examine the possible association between mRNA expression profiles, age, and thyroid hormone levels measured at the time of T-cell sampling. Firstly, an inverse relationship between serum fT3 and age was found (Spearman's $\rho = -0.307$, P = 0.018). Furthermore, VDR mRNA expression was related to fT3 levels ($\rho = 0.352$, *P = 0.0062*). A weak, but nominally significant positive correlation was observed between VDR and CTLA4 mRNA expression levels ($\rho = 0.326$, P = 0.012). In contrast, CTLA4 mRNA expression was strongly related to CD28 mRNA abundance ($\rho = 0.425$, *P = 0.00087*). CD45RABC mRNA levels weakly paralleled serum fT4 levels ($\rho = 0.31$, P = 0.021), whilst CD45RB mRNA expression was independently and positively related to CD28 ($\rho = 0.52$, *P = 0.000033*) and CTLA4 mRNA levels ($\rho = 0.583$, *P = 0.000002*). In contrast, CD45R0 mRNA levels were inversely related to both VDR ($\rho = -0.318$, P = 0.017) and fT3 levels ($\rho = -0.433$, *P = 0.0012*). Upon correction for fT3 levels, the association between CD45R0 and VDR mRNA was lost (data not shown). There was no effect of gender, and age did not affect expression of VDR. Subgroup analysis revealed an additional, positive association between CD45R0 mRNA and age in HT patients (n = 45, $\rho = 0.33$, P = 0.025), predominantly in spontaneously euthyroid HT cases (n = 18, $\rho = 0.5$, P = 0.036), but not in healthy controls. Furthermore, a positive association was observed between CD45R0 transcript levels and serum logTSH in untreated, hypothyroid cases (n = 15, $\rho = 0.593$, P = 0.02). Finally, a negative association between transcript levels of CTLA4 and age ($\rho = -0.63$, P = 0.028), between CD45RB mRNA levels and age ($\rho = -0.74$, *P = 0.0056*), and a positive relation between CD45RBC mRNA and serum fT4 levels ($\rho = 0.73$, P = 0.0074) was found in the treated patients group. However, a minority of these correlations would have remained statistically significant after Bonferroni's correction for multiple testing.

Discussion

Vitamin D deficiency is widespread among patients with HT [23, 24], but how vitamin D metabolism affects HT pathophysiology is not well understood. Most effects of vitamin D are mediated by the binding of $1,25(OH)_2D_3$ to the intracellular vitamin D receptor (VDR), which promotes or inhibits transcription of vitamin D responsive genes [25]. In thyroid, VDR ligands directly affect thyroid cell function [26, 27] and suppress tissue inflammatory processes by inhibiting Th1 [28], Th17, Tc17 [14], $CD8^+$Tc, and invariant natural killer T-cells [29]. Despite this, no data currently exist on VDR mRNA expression in target cell populations from HT patients. In the present study, a nominally higher expression level of total VDR mRNA was observed in peripheral T-lymphocytes of healthy controls when compared to the HT patients; however, the mechanisms that mediate the apparent down-regulation of VDR transcript levels in T-cells of HT patients remain to be clarified.

A putative positive relation between VDR mRNA expression in T-cells and thyroid functional status has been observed. Nevertheless, no causal inference was possible because several mechanisms, such as

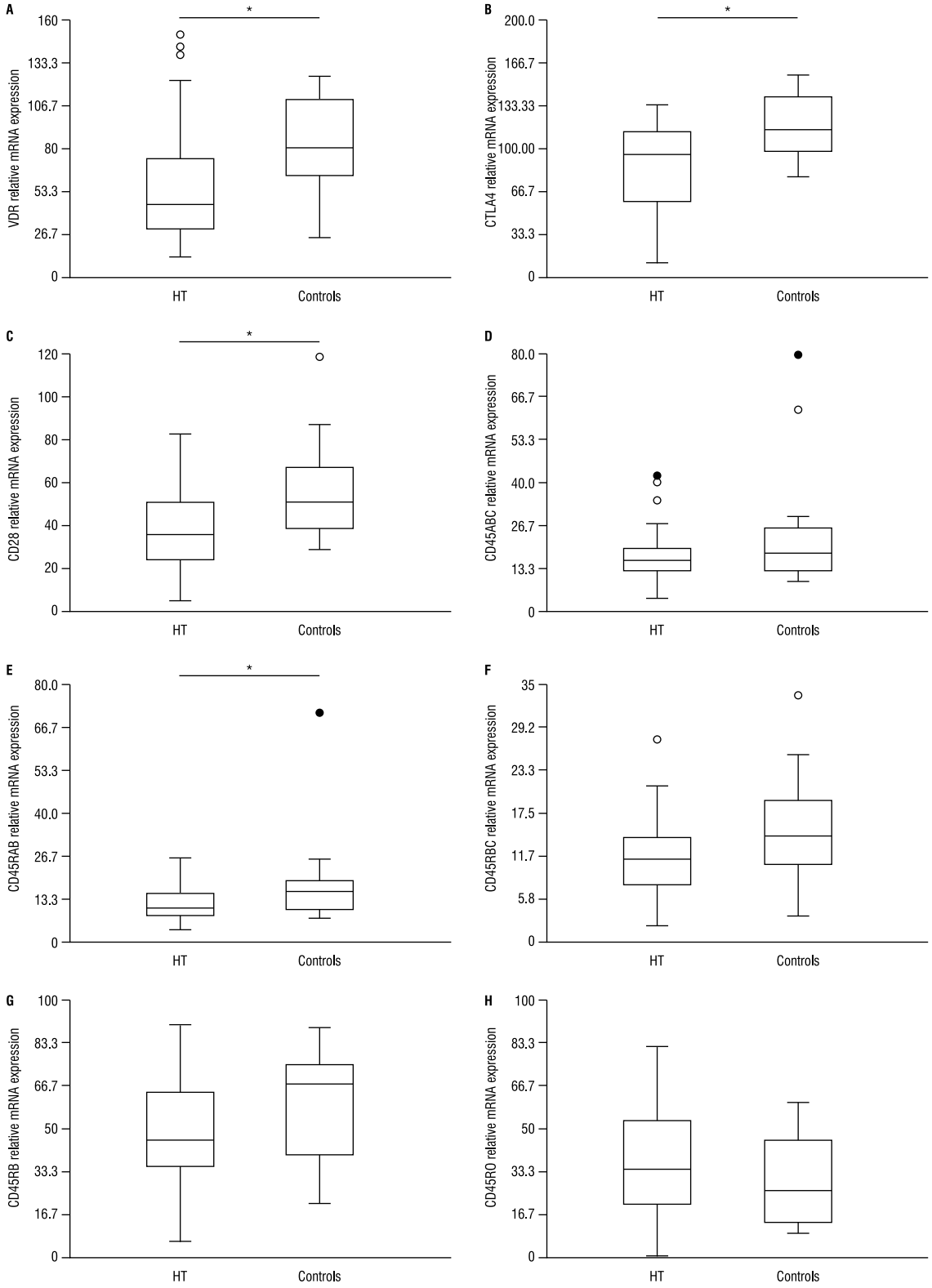

Figure 2. *Relative mRNA levels of VDR, CTLA4, CD28 and CD45 isoforms in HT patients and healthy controls. Compared to healthy controls (n = 13).* ***A.*** *VDR (P = 0.021);* ***B.*** *CTLA4 (P = 0.018);* ***C.*** *CD28 (P = 0.0074) and* ***E.*** *CD45RAB (P = 0.042) mRNA levels were downregulated in peripheral T cells of HT patients (n = 45). No difference was found in* ***D.*** *CD45RABC;* ***F.*** *CD45RBC;* ***G.*** *CD45RB or* ***H.*** *CD45R0 mRNAs levels across the studied groups. Data are presented as median with interquartile range and min and max values are demonstrated by whiskers; *P < 0.05*

Rycina 2. *Względne poziomy mRNA izoformów VDR, CTLA4, CD28 i CD45 u pacjentów z HT i osób zdrowych. W porównaniu z osobami zdrowymi (n = 13).* **A.** *VDR (p = 0.021);* **B.** *CTLA4 (p = 0.018);* **C.** *CD28 (p = 0.0074) and* **E.** *CD45RAB (p = 0.042), poziomy ekspresji genów był zmniejszone na poziomie mRNA w peryferyjnych komórkach pacjentów z HT (n = 45). Nie odnotowano żadnej różnicy w* **D.** *CD45RABC;* **F.** *CD45RBC;* **G.** *CD45RB or* **H.** *CD45R0 na poziomach mRNA w badanych grupach. Dane przedstawione zostały jako rozstęp międzykwartylowy, minimalne i maksymalne wartości przedstawione są za pomocą wąsów; *p < 0,05*

VDR-mediated protection against autoimmune thyroid dysfunction [26, 30], T3-dependent shift in T-cell composition/vitamin D3 metabolism [31], and immune effects on both T3 conversion and thyroid activity [32], may all provide parts of conjecture. Furthermore, a question remains about the exact source of these mRNAs. Peripheral T-cell populations exhibit high levels of cellular complexity; thus, lineage-specific up- or down-regulation, altered composition of T-cell compartments, a developmental shift – from naïve to memory cells — or a combination — may be responsible for observed mRNA data. Finally, the number of VDR binding sites in primary CD4+ cells is strongly correlated with 25 hydroxy vitamin D_3 levels [33]. Thus, it would also be useful to obtain 25(OH)D_3 and 1,25$(OH)_2D_3$ measurements, environmental ultraviolet radiation exposure, and seasonal data for future study.

Tregs, mostly defined as CD4+CD25+FoxP3+ expressing T-lymphocytes, constitutively express CTLA4 [34] and play an important role in HT aetiology. In the present study, transcript levels of CTLA4, a major gatekeeper of thyroid autoimmunity, have been nominally reduced in T-cells of HT patients. Decreased CTLA4 levels were also detected by Kucharska [9], although another report failed to find a clear decrease in CD4+CTLA4+ lymphocytes [35]. In addition, a weak relation between VDR and CTLA4 mRNA was also seen in our sample set, but no simple explanation could be provided. Both VDR and CTLA4 expression are tightly controlled and rapidly co-induced by T-cell activation [13]; in addition, CTLA4 is constitutively expressed on CD4+CD25+ Treg cells [34], shows higher levels in Th2 [36] and Th17 clones [37], and is expressed significantly higher in the CD4+ than in CD8+ T-cells [38]. Lastly, VDR binding has recently been described within the CTLA4 region in lymphoblastoid cell lines upon ligand stimulation [39], and a vitamin D-dependent increase in CTLA4 expression and the frequency of FoxP3+CTLA4+ T-cells has been reported through direct VDR binding to the *FOXP3* gene [14].

In addition to vitamin D_3, thyroid hormones and TSH are also known to influence the function, composition, and development of lymphoid cells and organs [31, 40]. During these processes, alternative splicing of pre-mRNA encoding the transmembrane phosphatase CD45 marks the transition from naïve to activated T-cells [18]. In the present study, CD45 composition was apparently affected by age, thyroid hormones, and case-control status, suggesting a shift from long/CD45RA+ isoforms to short/CD45R0 transcripts in an inverse, age- and T3-dependent manner. Higher proportions of both CD4+ and CD8+ T cells expressing CD45R0 have previously been detected in HT patients [41]; moreover, these cells have been functionally characterised as memory/effector T-cells and high producers of proinflammatory cytokines TNF and IFN [42]. Here, older age and lower T3 levels have been associated with the accumulation of CD45R0 isoform in HT patients, but not controls, a pattern suggestive of accelerated T-cell ageing in elderly, hypothyroid HT subjects. The characterisation of other functional markers, an aim that seems worth pursuing in future studies, is necessary to delineate the subsequent stage of maturation for various CD45R0+ T-cell compartments in those patients.

A critical phenotypic and genetic change as T-cells becomes late-differentiated and progress to senescence is the loss of gene and surface expression of CD28 [43]. CD28 surface expression in HT is not steady and is influenced by several mechanisms [41]. In this study, nominally reduced levels of CD28 mRNA expression and the peripheral loss of CD45RAB mRNA were associated with HT development. Other analyses support these results: a parallel loss of CD28 expression and the peripheral reduction of CD45RA+ T-cells have recently been described in HT [41]. Several groups have reported an expansion of CD4+ T-cells lacking CD28 in chronic inflammatory disorders [44–46], as well as in some normal-aged subjects [47]. Such CD4+CD28- T-cells display an effector/memory CD45R0+ phenotype with increased replicative history and oligoclonality but reduced apoptosis [48]. An increased proinflammatory and cytotoxic capacity of these cells has been demonstrated [46], suggesting a pathogenic role of CD4+CD28 null T-cells in the development of chronic immune-mediated disorders.

The transitions of CD45R isoforms are rapidly induced upon TCR activation [11, 12]. In the present study, the CD45RB mRNA expression coincided most consistently with CTLA4 mRNA levels, but with wide inter-individual variations. As opposed to CD45R0+ T-lymphocytes, T-cells expressing CD45RB isoform have been shown to be less susceptible to T-cell activation [16]; moreover, different cytokine profiles have been observed between CD4+CD45RB^{low} (IL-4/IL-10 producing) and CD4+CD45RB^{high} (TNF producing) T-cells, independent of the CD45RA or CD45R0 expression [49]. Finally, the CD45RB-inducible regulatory region had been recently discovered within the CTLA4 promoter [17], lending mechanistic support to the tolerance mediated by anti-CD45RB through CTLA4 up-regulation [50]. Nevertheless, the functional significance of these observations for HT and L-T4 treatment remains unknown.

There are several caveats that deserve further attention.

1. Our findings emphasise that examination of transcript abundance alone in unsorted T-cells provides only a partial picture.

2. The correlation between RNA and protein expression levels of the selected target mRNAs is uncertain. Lack of difference in mRNA profiles does not preclude differences in protein expression, protein kinetics, and intracellular trafficking.
3. T-cell activation is followed by complex, context- and lineage-specific dynamics of CD28, CTLA-4, VDR, and CD45R expression. In the present study, expression data from vitamin D-stimulated cells were not available.
4. Studies of peripheral T-cell mRNAs may have been confounded by the composition of T-cell subpopulations and the different roles they play in HT. T-cells exhibit vast cellular complexity and plasticity; thus, only most robust changes are readily detected.
5. Statistical power remains limited in small data sets; thus, the cohort was exploratory in nature. Nevertheless, strict selection criteria were applied, resulting in a well-characterised cohort regarding demographics, treatment exposures, and outcomes. Detailed knowledge of disease status and residual thyroid function is fundamental in order to elucidate any pathogenetic component of HT, and may be an explanation for the contradictory results in several similarly sized but phenotypically mixed studies. Finally, the majority of patients in this study were female Caucasians of European ancestry, thus limiting generalisations to males and individuals of other ethnicities.

In conclusion, an altered peripheral blood T-cell mRNA expression profile, encompassing nominally reduced VDR, CTLA4, CD28, and CD45RAB mRNA levels, occurs in HT. The current study also reports associations between free T3 and VDR/CD45R transcripts, particularly in older individuals, evidence that may contribute to our understanding of senescence and immunoregulation in HT. Our data provide preliminary evidence that CD28/CTLA4-CD45R axis might contain relevant investigational targets whose identification could improve our understanding of thyroid-immune interactions and treatment effects in HT. Nevertheless, methodological refinements, encompassing cell sorting, qPCR, and protein expression data are necessary in the confirmatory step.

Acknowledgments

This study was supported by the Croatian Ministry of Science, Education, and Sport (grant No. 219-0982914-2176, 219-2190372-2068, and 0127-0000000-3420). The authors wish to thank Ms T. Stipanović and Ms. K. Krizmanić for valuable technical assistance.

References

1. Weetman AP. Cellular immune responses in autoimmune thyroid disease. Clin Endocrinol 2004; 61:405-413. doi: 10.1111/j.1365-2265.2004.02085.x.
2. Wang SuHe, Bretz JD, Phelps E, et al. A unique combination of inflammatory cytokines enhances apoptosis of thyroid follicular cells and transforms nondestructive to destructive thyroiditis in experimental autoimmune thyroiditis. J Immunol. 2002; 168(5): 2470–2474, indexed in Pubmed: 11859140.
3. Horie I, Abiru N, Sakamoto H, et al. Induction of autoimmune thyroiditis by depletion of CD4+CD25+ regulatory T cells in thyroiditis-resistant IL-17, but not interferon-gamma receptor, knockout nonobese diabetic-H2h4 mice. Endocrinology. 2011; 152(11): 4448–4454, doi: 10.1210/en.2011-1356, indexed in Pubmed: 21862617.
4. Xue H, Yu X, Ma L, et al. The possible role of CD4 CD25(high)Foxp3 / CD4 IL-17A cell imbalance in the autoimmunity of patients with Hashimoto thyroiditis. Endocrine. 2015; 50(3): 665–673, doi: 10.1007/s12020-015-0569-y, indexed in Pubmed: 25771887.
5. Liu Y, Tang X, Tian J, et al. Th17/Treg cells imbalance and GITRL profile in patients with Hashimoto's thyroiditis. Int J Mol Sci. 2014; 15(12): 21674–21686, doi: 10.3390/ijms151221674, indexed in Pubmed: 25429429.
6. Figueroa-Vega N, Alfonso-Pérez M, Benedicto I, et al. Increased circulating pro-inflammatory cytokines and Th17 lymphocytes in Hashimoto's thyroiditis. J Clin Endocrinol Metab. 2010; 95(2): 953–962, doi: 10.1210/jc.2009-1719, indexed in Pubmed: 20016049.
7. Horie I, Abiru N, Nagayama Y, et al. T helper type 17 immune response plays an indispensable role for development of iodine-induced autoimmune thyroiditis in nonobese diabetic-H2h4 mice. Endocrinology. 2009; 150(11): 5135–5142, doi: 10.1210/en.2009-0434, indexed in Pubmed: 19797122.
8. Li D, Cai W, Gu R, et al. Th17 cell plays a role in the pathogenesis of Hashimoto's thyroiditis in patients. Clin Immunol. 2013; 149(3): 411–420, doi: 10.1016/j.clim.2013.10.001, indexed in Pubmed: 24211715.
9. Kucharska AM, Gorska E, Wasik M, et al. Expression of CD152 (CTLA-4) in children with autoimmune thyroiditis and +49 A/G polymorphism of exon 1 of the CTLA-4 gene. J Physiol Pharmacol. 2009; 60 Suppl 5: 77–80, indexed in Pubmed: 20134044.
10. Kucharska AM, Gorska E, Wasik M, et al. Altered expression of T lymphocyte surface markers in children with chronic autoimmune thyroiditis. J Physiol Pharmacol. 2008; 59 Suppl 6: 375–382, indexed in Pubmed: 19218661.
11. McNeill L, Salmond RJ, Cooper JC, et al. The differential regulation of Lck kinase phosphorylation sites by CD45 is critical for T cell receptor signaling responses. Immunity. 2007; 27(3): 425–437, doi: 10.1016/j.immuni.2007.07.015, indexed in Pubmed: 17719247.
12. Seki I, Suzuki M, Miyasaka N, et al. Expression of CD45 isoforms correlates with differential proliferative responses of peripheral CD4+ and CD8+ T cells. Immunol Lett. 2010; 129(1): 39–46, doi: 10.1016/j.imlet.2009.12.020, indexed in Pubmed: 20093141.
13. von Essen MR, Kongsbak M, Schjerling P, et al. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. Nat Immunol. 2010; 11(4): 344–349, doi: 10.1038/ni.1851, indexed in Pubmed: 20208539.
14. Jeffery LE, Burke F, Mura M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. J Immunol. 2009; 183(9): 5458–5467, doi: 10.4049/jimmunol.0803217, indexed in Pubmed: 19843932.
15. Butte MJ, Lee SJ, Jesneck J, et al. CD28 costimulation regulates genome-wide effects on alternative splicing. PLoS One. 2012; 7(6): e40032, doi: 10.1371/journal.pone.0040032, indexed in Pubmed: 22768209.
16. Parikh K, Poppema S, Peppelenbosch MP, et al. Extracellular ligation-dependent CD45RB enzymatic activity negatively regulates lipid raft signal transduction. Blood. 2009; 113(3): 594–603, doi: 10.1182/blood-2008-04-150987, indexed in Pubmed: 18840711.
17. Jen KYu, Campo M, He H, et al. CD45RB ligation inhibits allergic pulmonary inflammation by inducing CTLA4 transcription. J Immunol. 2007; 179(6): 4212–4218, indexed in Pubmed: 17785861.
18. Tchilian EZ, Beverley PCL. Altered CD45 expression and disease. Trends Immunol. 2006; 27(3): 146–153, doi: 10.1016/j.it.2006.01.001, indexed in Pubmed: 16423560.
19. Xu Z, Weiss A. Negative regulation of CD45 by differential homodimerization of the alternatively spliced isoforms. Nat Immunol. 2002; 3(8): 764–771, doi: 10.1038/ni822, indexed in Pubmed: 12134145.
20. Stefanić M, Papić S, Suver M, et al. Association of vitamin D receptor gene 3'-variants with Hashimoto's thyroiditis in the Croatian population. Int J Immunogenet. 2008; 35(2): 125–131, doi: 10.1111/j.1744-313X.2008.00748.x, indexed in Pubmed: 18279374.
21. Böyum A. Separation of leukocytes from blood and bone marrow. Introduction. Scand J Clin Lab Invest Suppl. 1968; 97: 7, indexed in Pubmed: 5707208.

22. Chomczynski P, Sacchi N, Chomczynski P, et al. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987; 162(1): 156–159, doi: 10.1006/abio.1987.9999, indexed in Pubmed: 2440339.
23. Bozkurt NC, Karbek B, Ucan B, et al. The association between severity of vitamin D deficiency and Hashimoto's thyroiditis. Endocr Pract. 2013; 19(3): 479–484, doi: 10.4158/EP12376.OR, indexed in Pubmed: 23337162.
24. Tamer G, Arik S, Tamer I, et al. Relative vitamin D insufficiency in Hashimoto's thyroiditis. Thyroid. 2011; 21(8): 891–896, doi: 10.1089/thy.2009.0200, indexed in Pubmed: 21751884.
25. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008; 8(9): 685–698, doi: 10.1038/nri2378, indexed in Pubmed: 19172691.
26. Wang SuHe, Koenig RJ, Giordano TJ, et al. 1Alpha,25-dihydroxyvitamin D3 up-regulates Bcl-2 expression and protects normal human thyrocytes from programmed cell death. Endocrinology. 1999; 140(4): 1649–1656, doi: 10.1210/endo.140.4.6659, indexed in Pubmed: 10098499.
27. Borgogni E, Sarchielli E, Sottili M, et al. Elocalcitol inhibits inflammatory responses in human thyroid cells and T cells. Endocrinology. 2008; 149(7): 3626–3634, doi: 10.1210/en.2008-0078, indexed in Pubmed: 18372324.
28. Cippitelli M, Santoni A. Vitamin D3: a transcriptional modulator of the interferon-gamma gene. Eur J Immunol. 1998; 28(10): 3017–3030, doi: 10.1002/(SICI)1521-4141(199810)28:10<3017::AID-IMMU3017>3.0.CO;2-6, indexed in Pubmed: 9808170.
29. Cantorna MT, Snyder L, Lin YD, et al. Vitamin D and 1,25(OH)2D regulation of T cells. Nutrients. 2015; 7(4): 3011–3021, doi: 10.3390/nu7043011, indexed in Pubmed: 25912039.
30. Liu S, Xiong F, Liu Em, et al. [Effects of 1,25-dihydroxyvitamin D3 in rats with experimental autoimmune thyroiditis]. Nan Fang Yi Ke Da Xue Xue Bao. 2010; 30(7): 1573–1576, indexed in Pubmed: 20650769.
31. Hodkinson CF, Simpson EEA, Beattie JH, 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, indexed in Pubmed: 19398496.
32. Klein JR. The immune system as a regulator of thyroid hormone activity. Exp Biol Med (Maywood). 2006; 231(3): 229–236, indexed in Pubmed: 16514168.
33. Handel AE, Sandve GK, Disanto G, et al. Vitamin D receptor ChIP-seq in primary CD4+ cells: relationship to serum 25-hydroxyvitamin D levels and autoimmune disease. BMC Med. 2013; 11: 163, doi: 10.1186/1741-7015-11-163, indexed in Pubmed: 23849224.
34. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008; 322(5899): 271–275, doi: 10.1126/science.1160062, indexed in Pubmed: 18845758.
35. Marazuela M, García-López MA, Figueroa-Vega N, et al. Regulatory T cells in human autoimmune thyroid disease. J Clin Endocrinol Metab. 2006; 91(9): 3639–3646, doi: 10.1210/jc.2005-2337, indexed in Pubmed: 16804051.
36. Alegre ML, Shiels H, Thompson CB, et al. Expression and function of CTLA-4 in Th1 and Th2 cells. J Immunol. 1998; 161(7): 3347–3356, indexed in Pubmed: 9759851.
37. Brucklacher-Waldert V, Stuerner K, Kolster M, et al. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. Brain. 2009; 132(Pt 12): 3329–3341, doi: 10.1093/brain/awp289, indexed in Pubmed: 19933767.
38. Chan DV, Gibson HM, Aufiero BM, et al. Differential CTLA-4 expression in human CD4+ versus CD8+ T cells is associated with increased NFAT1 and inhibition of CD4+ proliferation. Genes Immun. 2014; 15(1): 25–32, doi: 10.1038/gene.2013.57, indexed in Pubmed: 24173147.
39. Ramagopalan SV, Heger A, Berlanga AJ, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome Res. 2010; 20(10): 1352–1360, doi: 10.1101/gr.107920.110, indexed in Pubmed: 20736230.
40. Montufar-Solis D, Klein JR. Splenic Leukocytes Traffic to the Thyroid and Produce a Novel TSH Isoform during Acute Listeria monocytogenes Infection in Mice. PLoS One. 2016; 11(1): e0146111, doi: 10.1371/journal.pone.0146111, indexed in Pubmed: 26771831.
41. Prelog M, Schönlaub J, Würzner R, et al. Lower CD28+ T cell proportions were associated with CMV-seropositivity in patients with Hashimoto's thyroiditis. BMC Endocr Disord. 2013; 13: 34, doi: 10.1186/1472-6823-13-34, indexed in Pubmed: 24006909.
42. Dawes R, Petrova S, Liu Z, et al. Combinations of CD45 isoforms are crucial for immune function and disease. J Immunol. 2006; 176(6): 3417–3425, indexed in Pubmed: 16517710.
43. Chou JP, Effros RB. T cell replicative senescence in human aging. Curr Pharm Des. 2013; 19(9): 1680–1698, indexed in Pubmed: 23061726.
44. Martens PB, Goronzy JJ, Schaid D, et al. Expansion of unusual CD4+ T cells in severe rheumatoid arthritis. Arthritis Rheum. 1997; 40(6): 1106–1114, doi: 10.1002/1529-0131(199706)40:6<1106::AID-ART14>3.0.CO;2-#, indexed in Pubmed: 9182921.
45. Markovic-Plese S, Cortese I, Wandinger KP, et al. CD4+CD28- costimulation-independent T cells in multiple sclerosis. J Clin Invest. 2001; 108(8): 1185–1194, doi: 10.1172/JCI12516, indexed in Pubmed: 11602626.
46. García de Tena J, Manzano L, Leal JC, et al. Active Crohn's disease patients show a distinctive expansion of circulating memory CD4+CD45RO+CD28null T cells. J Clin Immunol. 2004; 24(2): 185–196, doi: 10.1023/B:JOCI.0000019784.20191.7f, indexed in Pubmed: 15024186.
47. Vallejo AN. Aging-related Deficiency of CD28 Expression in CD4+ T Cells Is Associated with the Loss of Gene-specific Nuclear Factor Binding Activity. Journal of Biological Chemistry. 1998; 273(14): 8119–8129, doi: 10.1074/jbc.273.14.8119.
48. Maly K, Schirmer M. The story of CD4+ CD28- T cells revisited: solved or still ongoing? J Immunol Res. 2015; 2015: 348746, doi: 10.1155/2015/348746, indexed in Pubmed: 25834833.
49. Ten Hove T, The Olle F, Berkhout M, et al. Expression of CD45RB functionally distinguishes intestinal T lymphocytes in inflammatory bowel disease. J Leukoc Biol. 2004; 75(6): 1010–1015, doi: 10.1189/jlb.0803400, indexed in Pubmed: 15020649.
50. Ariyan C, Salvalaggio P, Fecteau S, et al. Cutting edge: transplantation tolerance through enhanced CTLA-4 expression. J Immunol. 2003; 171(11): 5673–5677, indexed in Pubmed: 14634073.