Title: Lower CD28+ T cell proportions were associated with CMV-seropositivity in patients with Hashimoto’s thyroiditis

Short title: Lower CD28+ T cell proportions in patients with Hashimoto’s thyroiditis

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Abstract

Background: Alterations in the naive T cell subpopulations have been demonstrated in patients with T cell mediated autoimmune disorders, reminiscent of immunological changes found in the elderly during immunosenescence, including the switch from CD45RA+ to CD45RO+ T cells and decreased thymic function with increased compensatory proliferative mechanisms, partly associated with latent Cytomegalovirus (CMV) infection. The present study was aimed to investigate proportions of lymphocytes, their relation to CMV-seropositivity and the replicative history of CD45RA+ expressing T cells in Hashimoto’s thyroiditis (HT, n=20) and healthy controls (HC, n=70).

Methods: Proportions of peripheral T cells were investigated by flowcytometry. The replicative history was assessed by T cell receptor excision circles (TRECs) and relative telomere length (RTL).

Results: Our results demonstrated a significant increase of CD28-negative T cells, associated with CMV-seropositivity in HT patients. HT showed abundant CD45RO+ T cells with peripheral loss of CD62L-expressing CD8+CD45RA+ T cells, the latter mainly depending on disease duration. The diagnosis of HT and within the HT group CMV-seropositivity were the main determinants for the loss of CD28 expression and increase of CD45RO+ T cells. RTL was not different between HC and HT, and physiological shortening of telomeres with advancing age, as found in HC, was not observed in HT patients. HT showed significantly lower TRECs in CD4+CD45RA+ T cells compared to HC.

Conclusions: Patients with HT display a peripheral T cell phenotype reminiscent of findings in elderly people or other autoimmune disorders. Whether these mechanisms are primary or secondary to the immunological alterations of autoimmune conditions should be investigated in longitudinal studies which may open research on new therapeutic regimes for treatment of HT and associated autoimmune diseases.
Background

Naive T cell subpopulations are significantly altered in patients at the onset of T cell mediated autoimmune disorders, such as diabetes mellitus type 1 and juvenile idiopathic arthritis (JIA) [1,2]. These alterations were reminiscent of immunological changes found in the elderly during the aging of the immune system (immunosenescence), such as the switch from the CD45RA+ to the CD45RO+ T cell phenotype and a decrease in thymic function with increased compensatory mechanisms [3,4]. Patients with rheumatoid arthritis and JIA demonstrated increased CD28-negative T cells, an erosion of telomere length as a marker of replicative senescence and a loss of T cell receptor excision circles (TRECs) as a parameter of thymic function and peripheral proliferation of naive T cells [4].

Latent Cytomegalovirus (CMV) infection was shown to be a driving factor for T cell differentiation [5]. The majority of CMV-specific T cells are included within the CD28-negative T cell subpopulation. CMV-seropositive healthy individuals demonstrated accelerated loss of CD28 expression [6], which was not seen in CMV-seropositive JIA patients [7].

Hashimoto’s thyroiditis (HT) is an organ-specific autoimmune disorder of the thyroid gland associated with diffuse lymphoid infiltration, inflammatory destruction of follicular cells and auto-antibodies against thyroid-specific self-antigens [9,10]. However, the etiopathogenesis of HT is far from being clearly understood. Previous studies have shown significant alterations of the peripheral B and T cell subpopulations in HT [11] with a restricted T-cell-receptor repertoire [12] and a suggested role of CD25+ regulatory T cells in balancing immune tolerance [13-17].

Thus, the present study was aimed to assess whether patients with HT show proportional alterations of T cell subpopulations as found in other T cell-mediated autoimmune diseases, to investigate the replicative history of CD45RA+ expressing T cells by assessment of TRECs and relative telomere length (RTL) and the association with CMV-seropositivity.
Methods

Study population

Peripheral blood mononuclear cells (PBMCs) and serological samples were obtained from 20 patients with HT and 70 healthy controls (HC) (table 1). Diagnosis of HT was based on clinical, serologic and ultrasound findings according to the definitions of the American Thyroid Association. Patients were recruited from outpatient clinics at the Departments of Pediatrics, Surgery or Nuclear Medicine, Medical University Innsbruck and Vienna. HC according to WHO definition without any therapy, autoimmune disorders, endocrinologic diseases, cancer or other immunological impairments were recruited at the outpatient clinics at the Departments of Pediatrics or Surgery, Medical University Innsbruck.

Serological measurements of thyroid-stimulating hormone (TSH) (normal range: 0.35-3.50 µU/ml), free triiodothyronine (fT3) (3.10-6.50 pmol/l), free thyroxine (fT4) (10.30-21.90 pmol/l), thyroid peroxidase antibody (TPOAb), thyrotropin-receptor antibody (TRAb), and Tg antibodies (TgAb) were performed by ELISA (Roche Diagnostics, Vienna, Austria) according to standard laboratory procedures at the Central Laboratory, University Hospital Innsbruck.

As CMV is well known to drive T cell differentiation and alter proportions of peripheral lymphocyte subpopulations [5-7], HC and HT were separated into CMV IgG seropositive and seronegative subgroups. Anti-CMV IgG was measured by ELISA (Enzygnost, Dade Behring, Vienna, Austria) according to standard laboratory procedures at the Department of Hygiene and Medical Microbiology, Medical University Innsbruck.

Fifteen HT patients had treatment with L-thyroxine (dosage 75-150 µg/day). Thyroidectomy was performed in 7 HT patients due to large nodular goiter in 2 cases and suspect inactive nodules in 5 cases. These 7 patients had been treated with L-thyroxine in the last 6 months preceding blood sampling.
All patients or their legal guardians gave their written informed consent to participate in the study. The study was performed according to the Declaration of Helsinki and was approved by the local ethical committee of the Medical University Innsbruck.

**Lymphocyte separation**

PBMCs were isolated by using LymphoPrep™ (Axis Shield, Oslo, Norway) according to manufacturer’s instructions as described previously [2]. CD4⁺CD45RA⁺ (naive) T cells were separated by negative selection using a naive CD4⁺ T cell isolation kit (Miltenyi Biotec, Teterow, Germany), magnetic beads and Auto MACS system with sterile columns (Miltenyi Biotec). Purity of separated CD4⁺CD45RA⁺ T cells was checked using 4-colour flow cytometry (FACS-Calibur flow cytometer; Becton Dickinson, Oxford, United Kingdom) and ranged from 97 to 99%.

**Lymphocyte phenotypes**

Lymphocytes were characterized by staining with monoclonal mouse antibodies (mAbs) specific for CD4, CD8, CD45RA, CD45RO, CD28, CD25 and CD62L labeled with fluorochromes Fluoreszeinisothiocyanat (FITC), Phycoerythrin (PE), Peridinin-Chlorophyll (PerCP) or Allophycocyanin (APC) (all antibodies were purchased from BD Pharmingen, San Jose, California, USA) for 20 min at room temperature in the dark as described previously [2]. Results were expressed as percentage of gated lymphocytes. T cells were characterized by expression of CD3 and either CD4 or CD8, B cells by CD19 and natural killer (NK) cells by CD3-CD16/56+. HLA-DR was used as an activation marker of T cells. CD28 is a co-stimulatory molecule, which is lost through differentiation of T cells. CD45RO mainly defines the memory T cell subset, expression of CD45RA activated naive T cells expressing the CD62L lymphocyte homing factor. High expression of CD25 in combination with CD62L is characteristic for regulatory CD4⁺ T cells, and in low amounts also for naive T cells.
**Quantification of TREC numbers**

TRECs are stable circular DNA fragments that are created during T cell receptor rearrangement in the thymus \([18,19]\) and have been described to be an indicator of thymic production. TRECs are not replicating during mitosis. Therefore, TREC numbers are as much influenced by peripheral cell turnover as by the influx of newly generated TREC-positive T cells from the thymus. In our study, dilution of TRECs was used to estimate the thymus output and the peripheral proliferative history of the CD45RA-expressing T cell pool, which contains recent thymic emigrants (usually high TREC numbers), pre-existing peripheral, naive T cells (usually low TREC numbers due to peripheral replication and dilution of TRECs) and a small amount of differentiated effector T cells (usually TREC-negative). DNA was extracted from separated CD4\(^+\)CD45RA\(^+\) T cells using QIAamp DNA Mini Kit (Qiagen, Chatsworth, California, USA). Signal-joint TREC concentrations were determined by quantitative SYBR-green real-time PCR based on the coding TREC sequence using an iCycler quantitative RT-PCR system (BioRad Laboratories, Hercules, Canada) and log\(_2\) dilutions of an internal standard as described previously \([2,19]\). To avoid bias by different numbers of naive T cells, TRECs were calculated in relation to CD4\(^+\)CD45RA\(^+\) T cell numbers \([20]\).

**Telomere length analysis**

Determination of relative telomere length (RTL) was performed to estimate the individual replicative history of CD45RA-expressing T cells by calculating the ratio of a quantitative PCR reaction product from the same sample using specific primers for telomeres and a single copy gene as described previously \([21,22]\). Quantitative PCR is the method of choice for determining telomere length in small extractable quantities of DNA, as is the case in our study. Due to limited sample size not all laboratory investigations regarding quantification of lymphocytes, RTL or TRECs could be performed in all patients.
**Immunohistochemistry**

CD62L-expressing lymphocytes were identified after fixation of thyroid tissue samples from the 7 thyroidectomized HT patients with 4% neutral-puffered formalin by staining with mouse-anti-human-CD62L antibody (MIB-1 clone; DAKO, Glostrup, Denmark) using an automated immunostainer (Nexes, Ventana Medical Systems, Tucson, AZ, USA) with 3,3-diaminobenzidine tetrahydrochloride (DAB) A chromogen and counterstaining with Hematoxylin and Bluing Reagent. Slides were evaluated semiquantitatively by two independent investigators (MP and AB) counting the number of CD62L+ lymphocytes/100 lymphocytes/high power field (HPF) in at least 5 HPFs of inflammatory hot spots.

**Statistics**

The Kolmogorov-Smirnov test was applied to evaluate the normality of distribution of different parameters. Non-parametric Mann-Whitney U test was used to compare HT and HC (SPSS, Version 19.0, Chicago, IL). For multiple comparisons, Bonferroni’s correction was applied. X² test was used to compare dichotome variables between HT and HC, such as sex (male or female) or CMV (IgG seropositive or seronegative). Spearman Rank’s correlation coefficient was used to analyze correlations of T cell subpopulations and age of subjects. To identify independent factors for alterations in T cell proportions, age, disease duration, CMV-seropositivity, sex and HT disease were entered into a multivariate linear regression model. A p<0.05 was defined statistically significant.
Results

Patients

Despite the wide age-range in HT and HC, no significant differences were present between the groups regarding age (table 1). There was no significant correlation between age and disease duration. In HT, 80.0% were female compared to 61.4% in HC. There was no significant difference between HT (40.0%) and HC (30.0%) regarding frequency of CMV-seropositive individuals (table 1). Significantly elevated TSH concentrations were found in patients with HT compared to HC, but no difference was seen in free serum thyroid hormones (table 1).

Lymphocyte subpopulations

Absolute lymphocyte counts were significantly lower in HT patients compared to HC (table 2). In HC, absolute counts of lymphocytes ($R=0.679$; $p<0.0001$) and proportions ($R=-0.556$; $p<0.001$) negatively correlated with age. These correlations were not seen in HT patients.

HT patients showed significantly lower proportions of CD8+ T cells than HC, higher proportions of CD28-negative CD8+ T cells and higher proportions of CD45RO+ T cells in both CD4+ and CD8+ T cells (table 2). Proportions of CD45RA+CD62L+ were lower in HT within the CD8+ T cell pool compared to HC and negatively correlated with disease duration ($R=-0.722$; $p<0.001$). Proportions of CD4+CD25+CD62L+ T cells correlated with age in HC ($R=0.278$; $p<0.05$) and HT ($R=0.710$; $p<0.001$). No correlations between lymphocyte subpopulations and hormone levels or thyroid hormone substitution were seen.

CMV-seropositive HC showed higher proportions of CD4+CD28- T cells compared to CMV-seronegative HC (table 4, figure 1A). CMV-seropositive HT patients had significantly higher proportions of CD8+CD28- T cells compared to CMV-negative HT and CMV-seropositive
Lower CD8+CD45RA+CD62L+ T cells were found in CMV-seropositive HT compared to CMV-seronegative HT (table 4).

Including HT and HC, age, sex, disease duration and CMV-seropositivity into a linear regression model, the absolute lymphocyte counts (R=0.493; p<0.01) and the CD28-negative T cell subpopulations (CD4+: R=0.504; p<0.01; CD8+: R=0.469; p<0.01) were only influenced by having HT. In the HT group, regression analysis revealed an independent influence of disease duration for lower CD8+CD45RA+CD62L+ T cells. CMV-seropositivity was an independent factor for higher proportions of CD28-negative CD8+ T cells (R=0.693; p<0.01), as well as for higher CD4+CD45RO+ proportions (R=0.642; p<0.05). CMV-seropositivity was an independent factor for increase of CD4+CD28- T cells (R=0.449; p<0.01) in HC as well.

**RTL and TRECs**

Relative telomere length was not different between HC and HT (table 3). Physiological shortening of telomeres with advancing age, as found in HC (R=-0.513; p<0.0001), was not observed in HT patients.

HT showed significantly lower TRECs in CD4+CD45RA+ T cells compared to HC (table 3).

**Expression of CD62L in thyroid glands of HT patients**

Staining of CD62L in the thyroid tissue was positive in 5 of 7 HT patients, with a mean of 1.28 cells/100 cells/HPF, 2.56 cells/100 cells/HPF (figure 2A, B), 0.36 cells/100 cells/HPF, 1.8 cells/100 cells/HPF and 0.08 cells/100 cells/HPF. No difference regarding proportions of peripheral blood T cell subpopulations was found between patients with high or low CD62L+ or CD62L-negative staining.
Discussion

Although HT results from local autoimmune mechanisms in the thyroid gland, changes in the peripheral T cell distribution were detected in our study. Our results demonstrated a significant increase of CD28-negative T cells, associated with CMV-seropositivity in HT patients. HT showed abundant CD45RO+ T cells with peripheral loss of CD62L-expressing CD8+CD45RA+ T cells, the latter mainly depending on disease duration. The diagnosis of HT and within the HT group CMV-seropositivity was the main determinant for the loss of CD28 expression and increase of CD45RO+ T cells.

Our results on CD28 expression differ from a study in 35 children with autoimmune thyroiditis in whom baseline CD28 expression was similar to healthy controls [23]. However, after unspecific stimulation in vitro, CD28 expression decreased in autoimmune thyroiditis patients to a much higher extend as seen in controls in that study [23], which may underline the characteristic down-regulation of CD28 seen in HT patients. A more significant loss of CD28 in our group may be also influenced by the relative higher age of HT patients compared to that study [23], as CD28 expression is dramatically affected by age [3,24] and changes of CD28-expressing T cells may be more pronounced in older HT patients.

CMV is known to accelerate the increase of CD28-negative T cells [6]. A similar association was also seen in our HT group. These findings support the hypothesis of accelerated differentiation of T cells with latent CMV infections and an association with signs of premature immunosenescence known from patients with autoimmune disorders [4,7,25,26]. Loss of CD28 is also seen as a marker of replicative senescence of the immune system [24]. However, the specificity of CD28-negative CD8+ T cell subpopulations for CMV has to be demonstrated in HT patients, as abundance of CMV-specific CD8+ T cells has been so far shown only for healthy elderly people [27]. Thus, causality between CMV and loss of CD28-expression cannot be answered by our study, although regression analysis revealed CMV-
seropositivity as an independent factor for higher proportions of CD28-negative T cells within the HT group.

Regulatory T cells, mostly defined as CD4+ with high expression of CD25, usually increase with age, but obviously have less suppressive function on inflammatory mechanisms in some autoimmune diseases [26]. Higher proportions of peripheral CD25+CD62L+ CD4+ T cells, supposed to be activated when expressing CD62L, could be shown in our HT patients. In our cohort, age was the driving factor for enhancing CD25+CD62L+ T cells. However, CD4+CD25+ T cells include not only regulatory but also naive T cells, which may home to secondary lymphoid organs or inflammation sites. Thus, the role of CD25+ T cells in HT is discussed controversially [28,29]. An increased proportion of CD4+CD25+ cells was also found in patients with autoimmune thyroiditis irrespective of age [28]. In another study, the proportion of CD4+CD25+ T cells was not different between newly diagnosed and untreated autoimmune thyroiditis patients compared to HC [29]. In different studies [12,28,29], HT patients appeared to be heterogeneous regarding age, disease activity and thyroid hormone production which makes comparability difficult.

In our cohort, the regulatory role of these CD25+CD62L+ T cell fraction is unclear as the use of these markers, CD25 and CD62L, cover both the regulatory and the naive T cell phenotypes. In order to further explore the role of CD62L in our HT patients, who demonstrated lower peripheral CD45RA+CD62L+ T cells and increased CD25+CD62L+ T cells, the expression of CD62L was investigated in histological sections of seven HT patients who underwent surgery. Five HT patients displayed significant CD62L expression in follicular nodules within the afflicted thyroid gland, suggesting a role of CD62L in homing of lymphocytes.

To measure peripheral turn-over of CD45RA-expressing T cells, RTL and TRECs were assessed in some patients in whom DNA of separated CD4+CD45RA+ and CD8+CD45RA+ T cells was available. HT showed lower TREC numbers in CD4+CD45RA+ T cells, with an
additional trend towards lower TREC numbers in CD8+CD45RA+ T cells and shorter telomere length in CD8+CD45RA+ T cells. TREC numbers are influenced by thymic output of recent thymic emigrants and peripheral dilution by proliferation of naive T cells [20]. Since the peripheral CD45RA+ T cell pool consists of predominantly naive T cells but also small proportions of differentiated CD28-negative T cells [2], reduction of TREC may be mostly caused by peripheral replication of CD4+CD45RA+ T cells. However, these findings together with a trend to shorter RTLs and abundance of CD28-negative T cells may support the hypothesis of increased peripheral T cell turn-over and differentiation of peripheral T cells in HT. Whether other mechanisms, such as accelerated switching from CD45RA+ to CD45RO+, apoptosis of CD45RA+ T cells or distribution of specific T cell subpopulations to secondary lymphatic organs or the thyroid gland (e.g. mediated by homing factors such as CD62L), play a role in diminishing TREC and in causing accumulation of CD45RO+ memory and effector T cells in the periphery remains to be investigated.
Conclusions

Patients with HT display a peripheral T cell phenotype reminiscent of findings in elderly people or other autoimmune disorders, such as rheumatoid arthritis [3,30] or JIA [4,26], with increased CD45RO+ memory and CD28-negative T cells, increased peripheral replication and altered distribution of T cell proportions, such as CD62L-expressing T cells with accumulation of CD62L+ lymphocytes in the thyroid gland. CMV seems to accelerate T cell differentiation by influencing CD28-negative T cell proportions. In our study, age appears to promote CD25+ T cells. Whether these mechanisms are primary or secondary to the immunological alterations of autoimmune conditions should be investigated in longitudinal studies which may open research on new therapeutic regimes for treatment of HT and associated autoimmune diseases.

Competing interests

There are no competing interests.

Authors’ contributions

Martina Prelog designed the study and wrote the manuscript.

Jörn Schönlaub recruited the patients, prepared the lymphocytes and performed the flowcytometry analysis.

Reinhard Würzner performed the CMV ELISAs and interpreted the CMV data.

Christian Koppelstätter performed the telomere length analysis and interpreted the relative telomere length data.

Giovanni Almanzar performed the interpretation of the flowcytometry data.
Andrea Brunner performed the immunhistochemistry and the interpretation of immunhistochemical data.

Rupert Prommegger was involved in patient recruitment and performed the thyreoidectomies.

Gabriele Häusler performed the recruitment of patients at the University of Vienna and helped in interpretation of clinical data.

Klaus Kapelari recruited patients and helped in interpretation of clinical data.

Wolfgang Högler designed the study, interpreted the data and critically discussed the manuscript.

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Legend to figure

Figure 1. Percentage of CD28-negative CD4+ (A) and CD8+ (B) T cells in CMV-negative (CMV-) and CMV-positive (CMV+) Hashimoto Thyreoiditis (HT) patients and healthy controls (HC).

* p<0.05, ** p<0.02.

Figure 2. CD62L-expression in Hashimoto's thyroiditis.

(A) Representative example of CD62-expression and a characteristic lymphoid follicle in the thyroid gland of a patient with Hashimoto’s thyroiditis (HT). 10x magnification.

(B) 40x magnification.
Table 1. Characteristics of the study populations.

<table>
<thead>
<tr>
<th>Groups (number; male/female)</th>
<th>Age (years)</th>
<th>Duration of disease (years)</th>
<th>TSH (µU/ml)</th>
<th>fT3 (pmol/l)</th>
<th>fT4 (pmol/l)</th>
<th>TPOAb/TRAb/TgAb positive (number)</th>
<th>CMV IgG positive/negative (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC (70; 27/43)</td>
<td>13.7 ± 4.0</td>
<td>n. a.</td>
<td>0.9 ± 0.3</td>
<td>5.4 ± 1.0</td>
<td>14.6 ± 1.9</td>
<td>0/0/1</td>
<td>21/49</td>
</tr>
<tr>
<td></td>
<td>(14.6; 5 – 28.5)</td>
<td></td>
<td>(0.8; 0.7 – 1.2)</td>
<td>(4.9; 4.7 – 6.6)</td>
<td>(15.5; 12.3 – 15.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT (20; 4/16)</td>
<td>20.2 ± 16.2</td>
<td>9.7 ± 8.2 (7.5; 1.0–33.0)</td>
<td>9.1 ± 13.6</td>
<td>5.5 ± 1.5</td>
<td>14.4 ± 3.7</td>
<td>20/17/19</td>
<td>8/12</td>
</tr>
<tr>
<td></td>
<td>(14.1; 4.2 – 67.3)</td>
<td></td>
<td>(2.6; 0.01 – 13.7) *</td>
<td>(5.4; 1.0 – 8.2)</td>
<td>(7.1 – 21.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are given in mean ± standard deviation (median; range). Abbreviations: Healthy controls (HC); Hashimoto’s thyroiditis (HT); thyroid peroxidase antibody (TPOAb); thyrotropin-receptor antibody (TRAb); Tg antibody (TgAb); not applicable (n. a.). Difference between HT and HC: * p<0.05.
Table 2. Comparison of lymphocyte subpopulations in HT and HC.

<table>
<thead>
<tr>
<th>Lymphocyte subpopulation</th>
<th>Healthy control (HC)</th>
<th>Hashimoto’s thyroiditis (HT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes absolute per µl</td>
<td>2508 ± 763 (2550; 960 – 4200)</td>
<td>1940 ± 609 (1809; 832 – 3264)**</td>
</tr>
<tr>
<td>Lymphocytes (% of white blood cells)</td>
<td>33.6 ± 10.9 (33.3; 15.0 – 62.0)</td>
<td>33.8 ± 10.1 (32.4; 15.0 – 54.0)</td>
</tr>
<tr>
<td>CD3+ (% of total lymphocytes)</td>
<td>68.3 ± 6.9 (68.0; 47.8 – 82.5)</td>
<td>70.8 ± 6.2 (72.0; 60.7 – 84.7)</td>
</tr>
<tr>
<td>CD4+ (% of total lymphocytes)</td>
<td>39.1 ± 6.9 (39.6; 25.8 – 51.1)</td>
<td>42.7 ± 7.9 (15.6; 7.8 – 27.1)</td>
</tr>
<tr>
<td>CD8+ (% of total lymphocytes)</td>
<td>27.8 ± 6.4 (26.5; 13.7 – 44.3)</td>
<td>23.5 ± 5.0 (23.2; 14.5 – 35.6)**</td>
</tr>
<tr>
<td>CD19+ (% of total lymphocytes)</td>
<td>16.1 ± 4.3 (15.3; 9.2 – 25.9)</td>
<td>16.8 ± 5.1 (15.6; 7.8 – 27.1)</td>
</tr>
<tr>
<td>CD3-CD16/56+ (% of total lymphocytes)</td>
<td>10.9 ± 5.9 (9.7; 3.8 – 33.7)</td>
<td>7.5 ± 3.4 (6.4; 3.3 – 15.0)</td>
</tr>
<tr>
<td>HLA-DR+ (% of CD3+)</td>
<td>5.9 ± 2.8 (5.2; 1.8 – 12.4)</td>
<td>6.8 ± 2.4 (6.8; 2.8 – 11.3)</td>
</tr>
<tr>
<td>CD28- (% of CD4+)</td>
<td>1.5 ± 1.3 (1.3; 0.09 – 8.4)</td>
<td>3.1 ± 5.1 (1.2; 0.1 – 20.9)</td>
</tr>
<tr>
<td>CD28- (% of CD8+)</td>
<td>11.1 ± 13.7 (3.9; 0.15 – 61.7)</td>
<td>21.9 ± 12.5 (18.5; 7.7 – 52.3)***</td>
</tr>
<tr>
<td>CD45RO+ (% of CD4+)</td>
<td>35.9 ± 12.9 (36.4; 1.4 – 59.4)</td>
<td>46.9 ± 11.9 (47.5; 23.2 – 67.3)**</td>
</tr>
<tr>
<td>CD45RO+ (% of CD8+)</td>
<td>19.7 ± 10.6 (17.8; 0.07 – 48.3)</td>
<td>30.4 ± 12.5 (28.4; 11.9 – 57.8)***</td>
</tr>
<tr>
<td>CD45RA+CD62L+ (% of CD4+)</td>
<td>61.1 ± 14.4 (60.9; 9.9 – 88.2)</td>
<td>52.1 ± 16.3 (52.9; 10.8 – 79.8)</td>
</tr>
<tr>
<td>CD45RA+CD62L+ (% of CD8+)</td>
<td>61.6 ± 14.9 (64.2; 16.7 – 93.3)</td>
<td>52.7 ± 18.7 (55.1; 18.2 – 81.8)*</td>
</tr>
<tr>
<td>CD25+CD62L+ (% of CD4+)</td>
<td>22.8 ± 11.8 (20.8; 4.5 – 78.1)</td>
<td>27.4 ± 11.8 (23.4; 14.2 – 56.8)</td>
</tr>
</tbody>
</table>

Values are given in mean ± standard deviation (median; range).
Difference between HT and HC: *** p<0.001, ** p<0.01, * p<0.05.
Table 3. Relative telomere length (RTL) and T cell receptor excision circles (TRECs).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy control (HC)</th>
<th>Hashimoto’s thyroiditis (HT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number</td>
<td>mean ± standard deviation (median; range)</td>
</tr>
<tr>
<td>RTL in CD4+CD45RA+</td>
<td>59</td>
<td>1.5 ± 0.9 (1.0; 0.03 – 4.3)</td>
</tr>
<tr>
<td>RTL in CD8+CD45RA+</td>
<td>20</td>
<td>1.4 ± 0.6 (1.4; 0.6 – 2.9)</td>
</tr>
<tr>
<td>TRECs/1000 CD4+CD45RA+</td>
<td>11</td>
<td>2299 ± 205 (2278; 2105 – 2514)</td>
</tr>
<tr>
<td>TRECs/1000 CD8+CD45RA+</td>
<td>16</td>
<td>1206 ± 1633 (541; 164 – 2615)</td>
</tr>
</tbody>
</table>

Numbers of investigated samples from HC and HT are given in separate columns. Difference between HT and HC: ** p<0.01.
Table 4. Comparison of proportions of lymphocyte subpopulations in CMV-seropositive HC and HT.

<table>
<thead>
<tr>
<th>Lymphocyte subpopulation</th>
<th>Healthy controls (HC)</th>
<th>Hashimoto’s thyroiditis (HT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMV negative</td>
<td>CMV positive</td>
</tr>
<tr>
<td>CD28 ( % of CD4+)</td>
<td>1.0±0.8 (0.8; 0.1-3.0)</td>
<td>1.9±1.6 (1.9; 0.2-8.4)##</td>
</tr>
<tr>
<td>CD28 ( % of CD8+)</td>
<td>9.0±9.4 (5.3; 0.5-32.2)</td>
<td>7.8±14.1 (2.2; 0.2-61.7)</td>
</tr>
<tr>
<td>CD45RO+ ( % of CD4+)</td>
<td>32.5±12.1 (31.5; 4.7-59.4)</td>
<td>30.7±16.9 (38.4; 1.4-41.9)</td>
</tr>
<tr>
<td>CD45RO+ ( % of CD8+)</td>
<td>18.7±10.4 (15.7; 0.5-48.3)</td>
<td>14.3±8.6 (15.7; 0.07-21.6)</td>
</tr>
<tr>
<td>CD45RA+CD62L+ ( % of CD4+)</td>
<td>61.8±17.6 (65.8; 9.9-88.2)</td>
<td>62.3±12.6 (62.4; 27.5-80.8)</td>
</tr>
<tr>
<td>CD45RA+CD62L+ ( % of CD8+)</td>
<td>64.2±15.0 (68.5; 16.7-83.6)</td>
<td>61.9±15.5 (61.8; 32.2-93.3)</td>
</tr>
<tr>
<td>CD25+CD62L+ ( % of CD4+)</td>
<td>22.4±11.8 (20.5; 4.5-75.5)</td>
<td>23.7±14.5 (19.7; 10.8-78.1)</td>
</tr>
</tbody>
</table>

Values are given in mean ± standard deviation (median; range).
Difference between HT and HC in the CMV-positive or CMV-negative groups: ** p<0.01, * p<0.05.
Difference between CMV-positive and CMV-negative individuals in the HC or HT groups: # p<0.05; ## p<0.01.
Figure 2.