

POSSIBLE INVOLVEMENT OF HUMAN HERPESVIRUS-6 U83 GENE EXPRESSION IN AUTOIMMUNE THYROIDITIS DEVELOPMENT

Alina Sultanova^{1,#}, Maksims Čistjakovs¹, Lība Sokolovska¹, Egils Cunskis², and Modra Murovska¹

¹ Institute of Microbiology and Virology, Rīga Stradiņš University, 5 Rātsupītes Str., Rīga, LV-1067, LATVIA

² Rīga East University Hospital, Clinical Centre “Gaiļezers”, 2 Hipokrāta Str., Rīga, LV-1038, LATVIA

Corresponding author, a.sultanova@inbox.lv

Contributed by Modra Murovska

Viral infections have been frequently cited as important environmental factors implicated in autoimmune thyroiditis (AIT) development, although no specific virus has yet been conclusively associated with the disease. Some evidence implicates human herpesvirus-6 (HHV-6) in this disease. The aim of this study was to investigate the role of the HHV-6 U83 gene expression in autoimmune thyroiditis development. Fifty-one patients with AIT following thyroidectomy and a control group of 30 autopsied subjects without thyroid pathologies for comparing virology results and 30 healthy blood donors for comparing serology results were enrolled in this study. HHV-6 U83 gene expression was determined using nested PCR with complementary DNA as the template acquired from thyroid gland extracted RNA. Plasma samples of AIT patients and blood donors were tested for IL-2, IL-4, IL-10, sTNF-RII and IL-1beta levels by ELISA. Virology results were compared with pro- and anti-inflammatory cytokine levels to determine possible interaction of HHV-6 with host immune response. HHV-6 U83 gene expression was found only in 24% (12/49) of AIT patient thyroid gland tissue samples and in none of the control group individuals, showing possible involvement of this gene in AIT development. However, no interaction between HHV-6 and changes in cytokine levels was found.

Key words: HHV-6, cytokines, thyroid gland, autoimmunity.

INTRODUCTION

Autoimmune thyroiditis (AIT) can be considered as one of the most frequent organ-specific autoimmune diseases that affects humans, and its incidence has dramatically increased over time worldwide (Lerner *et al.*, 2015). AIT raises big challenge for research as the aetiological factors are still unknown.

Viral infections have been frequently cited as important environmental factors implicated in AIT development, although no specific virus has yet been conclusively associated with the disease. Some evidence implicates human herpesvirus-6 (HHV-6) in this disease (Chen and Hundall, 2006; Caselli *et al.*, 2012).

HHV-6 is widely distributed in the general population. The primary infection usually occurs in the early years of life and remains latent throughout life (Costa *et al.*, 2011).

HHV-6 is associated with several autoimmune diseases, such as haemolytic anemia (Yagasaki *et al.*, 2011), acute autoimmune hepatitis (Grima *et al.*, 2008), and multiple sclerosis (Tejada-Simon *et al.*, 2003). HHV6 has also been shown to be related to Hashimoto's thyroiditis. HHV6 was observed to be more common in thyroid cells in patients with Hashimoto thyroiditis, than in a control group — 82% vs 10%, respectively. In addition, there are thyroid cells that are infected with both HHV-6A and HHV-6B, which have been shown to be susceptible to natural killer (NK) mediators (Caselli *et al.*, 2012). It is possible that HHV-6A/B can

cause autoimmune diseases by exposing a large amount of normally secreted cell antigens through the infected cell line. Another potential way of development of the disease is via molecular mimicry, the synthesis of viral proteins similar to those of the host cell molecules to avoid the body's immune response. HHV-6 can cause the major tissue compatibility of complex molecules to promote the presentation of autoantigens (Broccolo *et al.*, 2013). Like other herpesviruses, HHV-6 has several biological properties that could explain the ability of the virus to stimulate and modulate the host's immune response, such that the virus is able to avoid specific immune responses and create better conditions for replication (Wang *et al.*, 2014). For instance, the regulation of inflammatory cytokines in peripheral blood mononuclear cells is associated with IL-2 synthesis regulation and subsequent reduction of T-lymphocyte activation. Such effects are specifically related to HHV-6-encoded proteins that act as analogues to human chemokines and chemokine receptors and could modulate cytokine production influencing the immune response. For example, HHV-6 gene *U83* encodes a chemoattractant protein, which is an agonist for several human chemokine receptors (CCRs), and the *U12* and *U51* genes encode chemokine receptors, which are believed to activate and attract immune cells (Agut *et al.*, 2015).

The aim of this study was to investigate the role of the HHV-6 *U83* gene expression in autoimmune thyroiditis development.

MATERIALS AND METHODS

Study groups. Fifty-one patients with autoimmune thyroiditis following thyroidectomy were enrolled in this study, of which three were males (6%) and 48 were females (94%), with median age of 52 (interquartile range [IQR]: 41–60).

The control group included 30 autopsied subjects (26 women and four men; median age 58 IQR: 51–67) without thyroid pathologies, for comparing virology results, and 30 healthy blood donors (25 women and 5 men; median age 33 IQR: 28–45) for comparing serology results, as the plasma samples from autopsied subjects were not available.

Permission to conduct the study was received from the Rīga Stradiņš University (RSU) Ethics Committee and all participants in the study gave their written consent to the examinations. Samples were received from the Rīga Eastern Clinical University Hospital.

Nucleic acid isolation, complementary DNA (cDNA) synthesis and quality determination. DNA from thyroid gland tissues was extracted using the phenol-chloroform method. RNA from thyroid gland tissue and blood specimens was extracted using TRI-reagent (Life Technologies, USA), and cDNA synthesised using the innuSCRIPT One Step RT-PCR SyGreen Kit (Analytik Jena, Germany). The integrity and quality of isolated RNA was tested in denaturing gel electrophoresis using NorthernMax™-Gly Gel Prep/Running Buffer (Thermo according to manufacturer

protocol (Fisher Scientific, USA). The quality of genomic DNA obtained from patients and autopsies and synthesised cDNA was determined by beta (β)-globin polymerase chain reaction (PCR) with the appropriate primers (Vandamme *et al.*, 1995).

Detection of HHV-6 genomic sequences and gene expression using nested PCR (nPCR). The nPCR technique was used to detect viral genomic sequences in DNA isolated from thyroid gland tissue. PCR amplification of viral DNA was carried out in the presence of 1 μ g of thyroid gland tissue DNA. HHV-6 was detected in accordance with Secchiero *et al.*, 1995. Positive controls (HHV-6A and HHV-6B genomic DNA; Advanced Biotechnologies Inc, Columbia, MD, USA and negative controls (DNA obtained from healthy HHV-6 negative blood donors and without template DNA) were included in each experiment.

HHV-6 *U83* gene expression was detected using nPCR with cDNA as the template acquired from thyroid gland extracted RNA with appropriate PCR primers (Sjahril *et al.*, 2009).

HHV-6 load determination using quantitative PCR. AIT patient and autopsied subject thyroid tissue DNA samples that were positive on the presence of HHV-6 genome sequence were used for HHV-6 virus load detection using the HHV-6 Real-TM Quant (Sacace Biotechnologies, Italy) commercial kit in accordance with the manufacturer's instructions.

Determination of IL-2, IL-4, IL-10, sTNF-RII and IL-1beta levels. Plasma samples acquired from AIT patient and blood donor peripheral blood were tested for IL-2 (Aviva Systems Biology, USA), IL-4 (Thermo Scientific, USA), IL-10 (DIAsource, Belgium), sTNF-RII (DIAsource, Belgium) and IL-1beta (Biorbyt, UK) levels by ELISA commercial kits. ELISA reagent kits were used and the tests were carried out in accordance with the manufacturer instructions.

Statistical analysis methods. All statistical calculations and graphs were performed using GraphPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). To test whether the collected data were normally distributed, the D'Agostino and Pearson, Anderson-Darling and Shapiro-Wilk normality tests were applied. Statistical differences in the prevalence of HHV-6 infection were assessed using the Fisher exact test. Statistical differences in viral load and serological results were assessed using the Mann-Whitney test. Because most of the data did not have a normal distributions, results are expressed as median and interquartile range (IQR) as a variability characteristic, and a *p*-value of less than 0.05 ($p < 0.05$) was considered as statistically significant.

RESULTS

The presence of persistent HHV-6 infection in AIT patients was significantly higher ($p = 0.0111$) than in the control

group (96% and 77%, respectively). The HHV-6 genomic sequence was found in 49 of thyroid tissue DNA samples of the 51 (96%) patients. In contrast, the HHV-6 genome sequence was found in 23 of the 30 (77%) DNA samples isolated from autopsy material of the control group (Fig. 1).

No HHV-6 *U83* gene expression was observed in thyroid gland tissue samples from the control group. In contrast, HHV-6 *U83* mRNA was identified in thyroid tissue samples of 12 of 49 (25%) AIT patients, which was significantly higher than in the control group ($p = 0071$) (Fig. 1).

The HHV-6 load was determined for the patient and control group thyroid gland tissue samples positive after nPCR. Median HHV-6 load was found higher in AIT patient thyroid gland tissue samples (582.7 IQR: 168.4–2191.0) in comparison to that in the control group (361.0 IQR: 110.0–1029.0); however, the Mann-Whitney test showed no significance ($p = 0.2209$) (Fig. 2).

Comparison of results on HHV-6 load found in patient thyroid gland samples positive for the presence and absence of HHV-6 *U83* mRNA showed a higher median value in the former (913.0 IQR: 248.0–2049.0 vs 486.8 IQR: 168.4–2191.0), but without statistical significance (Mann-Whitney test, $p = 0.4820$) (Fig. 3).

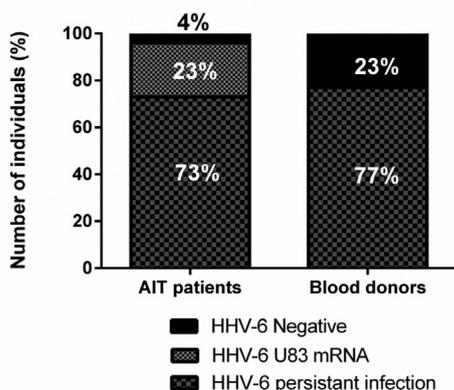


Fig. 1. Presence of HHV-6 genomic sequence and HHV-6 *U83* mRNA in thyroid gland tissues acquired from AIT patients and autopsied individuals (control group).

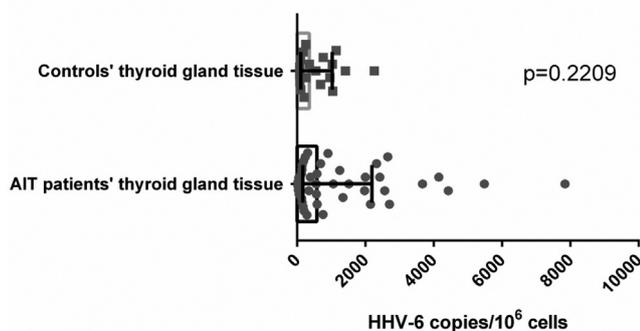


Fig. 2. Comparison of HHV-6 load between AIT patient and control thyroid gland samples. Statistical differences in viral load comparison were tested using the Mann-Whitney test. As most of the data lacked normal distribution, results are expressed as median and interquartile range (IQR) as a variability characteristic, and $p < 0.05$ was considered as statistically significant. Each point represents one individual.

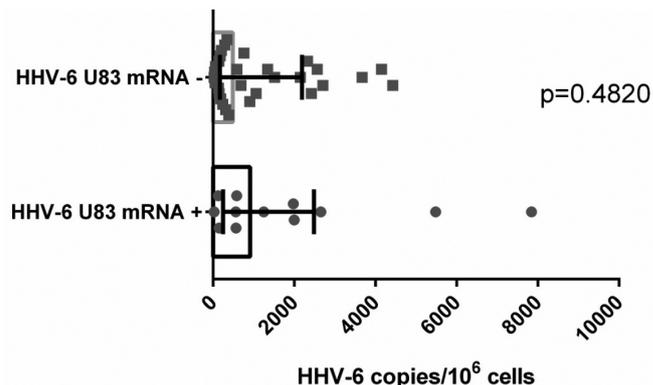


Fig. 3. Comparison of HHV-6 load in samples of the patient thyroid gland with presence and absence of HHV-6 *U83* gene expression. Statistical differences in viral load comparison were tested using the Mann-Whitney test. As most of the data lack normal distributions, the results are expressed as median and interquartile range (IQR) as a variability characteristic, and $p < 0.05$ was considered as statistically significant. Each point represents an individual.

To examine possible immunomodulating interactions of HHV-6 *U83* gene all patient peripheral blood plasma samples were tested for IL-1 β , IL-2, IL-4, IL-10 and sTNF-RII using ELISA commercial kits. To determine overall differences in cytokine levels between AIT patients and healthy individuals, cytokine levels were also measured in plasma samples acquired from the blood donors.

The IL-1 β level in all AIT patient and blood donor plasma samples was < 1 pg/ml. No difference in IL-2 levels was found between the patient and control groups (median 1.503 U/ml IQR: 1.379–1.689 against 1.503 U/ml IQR: 1.410–1.907, respectively). Likewise, there was no significant difference in sTNF-RII levels between patient and control groups (median 6.037 ng/ml IQR: 4.402–8.234 against 5.523 ng/ml IQR: 4.213–7.206, respectively). In contrast, a significant difference ($p = 0.0033$) was found in the IL-4 levels. The median amount of IL-4 was lower in AIT patient plasma samples in comparison to that of control groups (10.62 pg/ml IQR: 8.31–12.15 vs 12.15 pg/ml IQR: 10.62–17.15, respectively). Also, the level of IL-10 was significantly ($p = 0.0022$) higher in AIT patient plasma samples than in the control groups (median 52.00 pg/ml IQR: 37.00–104.50 against 24.50 pg/ml IQR: 12.00–114.50, respectively) (Fig. 4).

A closer analysis of cytokine levels in the patient group showed no significant difference between cytokine levels in HHV-6 *U83* mRNA positive and negative samples. The corresponding medians for cytokine levels in the AIT patients with and without HHV-6 *U83* mRNA were: IL-2 1.472 (IQR: 1.332–1.658) U/ml against 1.503 (IQR: 1.441–1.752) U/ml, IL-4 10.62 (IQR: 7.923–15.04) pg/ml against 11.38 (IQR: 9.269–13.69) pg/ml, IL-10 50.75 (IQR: 38.25–94.5) pg/ml against 54.5 (IQR: 37–104.5) pg/ml, sTNF-RII 6.037 (IQR: 3.981–7.953) ng/ml against 6.084 (IQR: 4.402–8.234) ng/ml, respectively (Fig. 5).

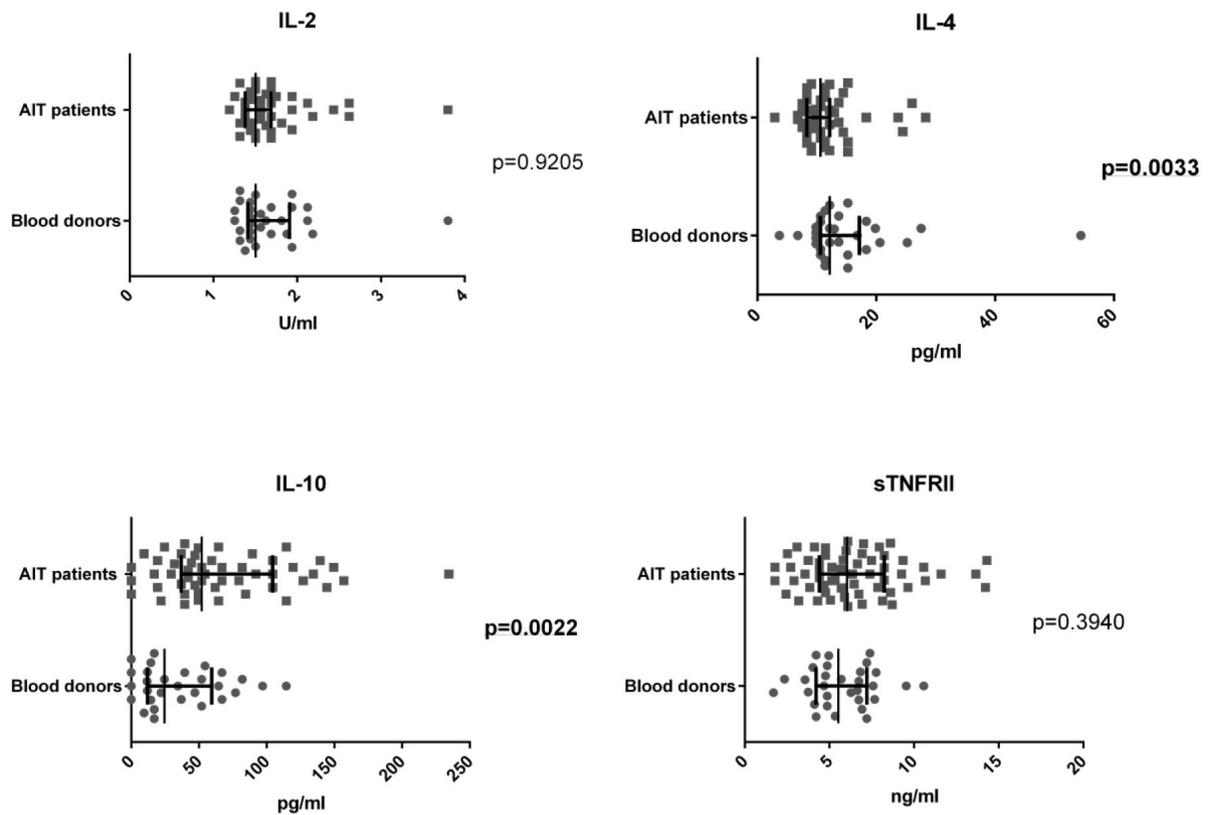


Fig. 4. Levels of IL-2, IL-4, IL-10 and sTNF-RII with median and IQR in AIT patient and donor plasma. Statistical differences were assessed using the Mann-Whitney test and $p < 0.05$ was considered as statistically significant. Each point represents an individual.

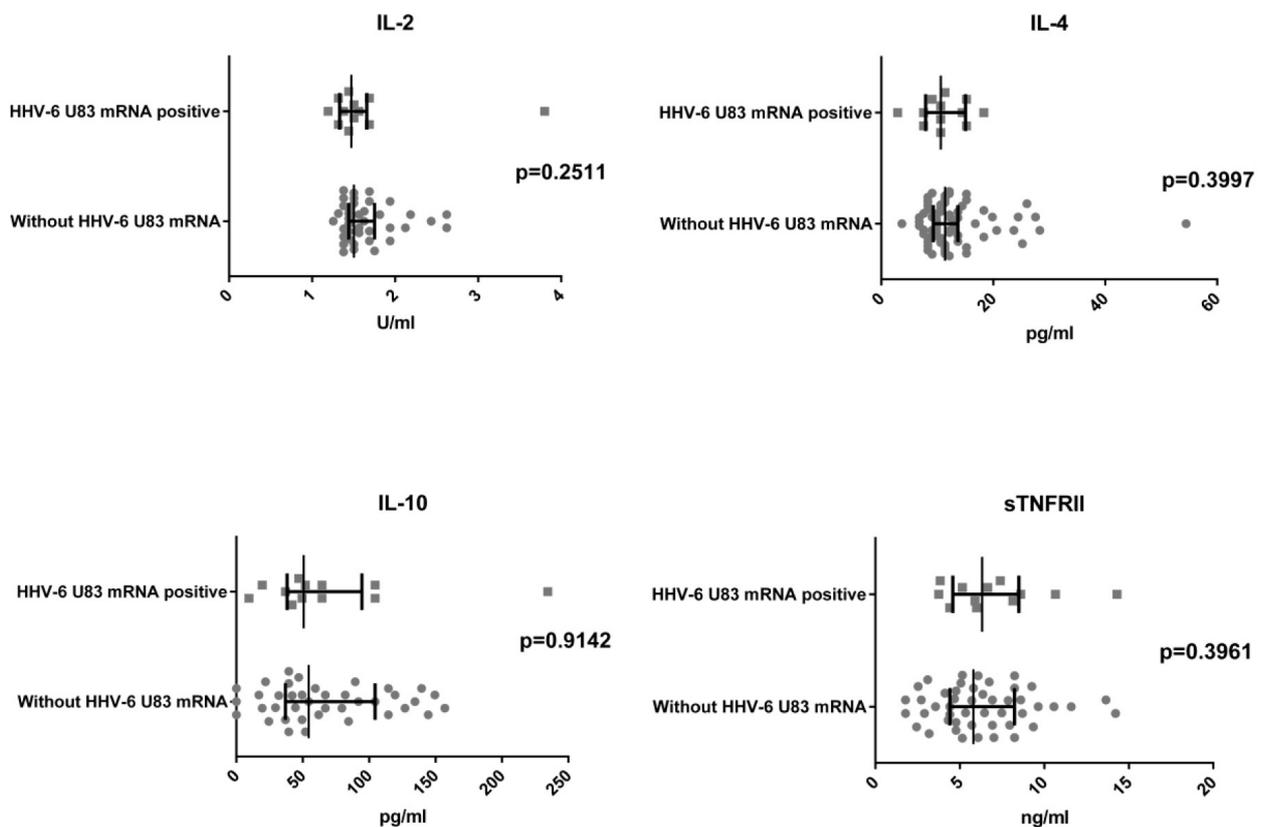


Fig. 5. Levels of IL-2, IL-4, IL-10 and sTNF-RII in AIT patients' peripheral blood plasma (median with IQR) with and without HHV-6 U83 mRNA in thyroid gland. In acquirement of statistical differences was used Mann-Whitney test and $p < 0.05$ was considered as statistically significant. Each point represents each individual's result.

DISCUSSION

While autoimmune thyroiditis is one of the most frequent autoimmune diseases, its precise prevalence in Latvia is unknown. Moreover, diagnosis and treatment of AIT in Latvia is associated with many issues and one of them is related to the lack of differential diagnosis. Early differential diagnosis could allow to apply required treatment and to avoid progression of diseases with subsequent surgical interference (thyroidectomy). Identification of potential pathogens and interaction mechanisms could help in diagnosing of autoimmune disease in the early stages of development as well as in choosing appropriate treatments to avoid the removal of the thyroid gland when unnecessary.

Viral infections have been considered as possible disease-promoting factors in various studies (Caselli *et al.*, 2012). Our recent study showed a very high frequency rate (99%) of the HHV-6B genomic sequence in AIT patient thyroid gland tissues and 41% of tissues contained markers of active HHV-6 infection (presence of HHV-6 U79/80 mRNA) (Sultanova *et al.*, 2017). These results corroborate the results of this study, where 96% of thyroid gland tissue samples were HHV-6 positive. This could serve as evidence for thyroid gland tissues as persistence site of HHV-6 and indicate the possible involvement of HHV-6 in AIT development. This supports other studies, but the exact mechanism of possible interactions of HHV-6 in AIT development is not yet clear.

Some studies have reported that specific viruses are involved in molecular piracy and mimicry mechanisms, in this way acquiring host genes within virus genomes, which makes them able to produce proteins capable of interfering with the normal host defence response (Wells and Schwartz, 1997). HHV-6 is an immunomodulating virus and encodes one viral chemokine (*U83*) and two chemokine receptors (*U12* and *U51*); however, the role of these genes in viral pathogenesis is not well understood (Isegawa *et al.*, 1998; Zou *et al.*, 1999; Menotti *et al.*, 1999). One study has shown that HHV-6 viral chemokine *U83* has the ability to promote the migration of monocytes/macrophages (Zou *et al.*, 1999). Therefore, this viral chemokine might be involved in induction of autoimmune processes. The aim of this study was to investigate the possible interaction between HHV-6 *U83* gene expression and some pro-inflammatory and anti-inflammatory cytokines.

HHV-6 *U83* gene expression was found only in 24% (12/49) of AIT patients' thyroid gland tissue samples and in none of the control group individuals, showing possible involvement of this gene in AIT development. However, there was no significant difference in IL-4 and IL-10 levels between the AIT patient and control group. Within the patients' group there was no association between cytokine level and presence and absence of HHV-6 *U83* gene expression in thyroid gland. As the HHV-6 *U83* gene encodes a putative chemokine that could modulate immune responses, our main hypothesis was to test for differences in cytokine level. However, the results showed lack of effect

of this gene on cytokine levels, which can have several explanations. Firstly, only a small part of the group was positive for HHV-6 *U83* gene expression, indicating that the studied group was too small. Another issue was the use of ELISA kits from the different manufactures with different sensitivities, which could have been resolved by using a more sensitive method like suspension multiplex immunoassay for Luminex 200 system application. This method allows simultaneous measuring and detecting more than one cytokine in a sample.

The observed significant difference between patient and control groups in IL-4, and IL-10 levels showed a shift in humoral immune response as these both cytokines are stimulating B lymphocyte antibody production (Hershey *et al.*, 1997; Aster *et al.*, 2009). The presence of IL-4 might promote alternative activation of macrophages into M2 cells and inhibit classical activation of macrophages into M1 cells. An increase in repair of macrophages (M2) is coupled with secretion of IL-10 and TGF- β , which results in a diminution of pathological inflammation (Aster *et al.*, 2009). This mechanism should be studied much closer in conjunction with HHV-6 interference, as it might provide new evidence of involvement of this virus in AIT development. Nevertheless, due to complex ethiopathogenesis of AIT, other various factors that might cause immunomodulation could not be excluded.

Another way to expand this study is to add HHV-6 *U12* and *U51* encoded chemokine receptors similar to G protein coupled receptor (GPCR) in the study. It has been shown that proteins encoded by HHV-6 *U12* and *U51* genes can be expressed on the surface of epithelial and some peripheral blood mononuclear cell populations, which might make them the potential cause for evoking autoimmunity and make host GPCRs as targets for auto-reactive T and B lymphocytes (Isegawa *et al.*, 1998; Menotti *et al.*, 1999; Milne *et al.*, 2000). Also, it has been shown *in vitro* that HHV-6 specific GPCRs can interact with cytokine signalling pathways by down-regulating RANTES (Milne *et al.*, 2000). On the basis of this knowledge, both *U12* and *U51* proteins are ideal objects for study of HHV-6 involvement in autoimmune thyroiditis development, in contrast to the approach in the present study.

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CILVĒKA HERPESVĪRUSA-6 *U83* GĒNA EKSPRESIJAS IESPĒJAMĀ LOMA AUTOIMŪNĀ TIREOIDĪTA ATTĪSTĪBĀ

Vīrusu infekcija bieži tiek minēta kā nozīmīgs faktors autoimūnā tireoidīta (AIT) attīstībā. Lai gan līdz šim konkrētais vīruss nav pārliecinoši sasaistīts ar AIT, ir pierādījumi, ka cilvēka herpesvīrusam-6 ir nozīme slimības attīstībā. Pētījuma mērķis bija izpētīt HHV-6 gēna *U83* ekspresijas iespējamo lomu autoimūnā tireoidīta attīstībā. Pētījumā tika iekļauts 51 pacients ar autoimūno tireoidītu pēc tireoidektomijas, 30 autopsijas paraugi bez vairogdziedzera patoloģijām virusoloģisko rezultātu salīdzināšanai un 30 veseli asins donori seroloģijas rezultātu salīdzināšanai. HHV-6 *U83* gēna ekspresija tika noteikta ar polimerāzes ķēdes reakciju ar iekšējo praimēšanu, kā matricu lietojot komplementāru DNS, kas ar attiecīgiem praimeriem sintezēta no vairogdziedzera audiem izdalītās RNS. Lietojot ELISA, AIT pacientu un asins donoru plazmā tika noteikts IL-2, IL-4, IL-10, sTNF-RII un IL-1beta līmenis. Lai novērtētu iespējamo mijiedarbību starp HHV-6 un organisma imūnsistēmas atbildi, virusoloģiskie rezultāti tika salīdzināti ar iekaisuma un pretiekaisuma citokīnu līmeņiem. *HHV6* gēnu ekspresija tika detektēta 24% (12/49) no AIT pacientu vairogdziedzera audu paraugos un nevienā no kontroles grupas indivīdu paraugiem, kas norāda uz šī gēna iespējamu iesaisti AIT attīstībā. Taču saistība starp HHV-6 *U83* ekspresiju un citokīnu līmeņu izmaiņām netika atrasta.