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Upregulation of HLA class I and Antiviral Tissue Responses in Hashimoto's Thyroiditis

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Abstract

Background: Hashimoto's thyroiditis (HT) is a common autoimmune disease of unknown origin. However, viral infections have been implicated as triggers for autoimmunity. Human leukocyte antigen (HLA) class I presents antigens to circulating immune cells and plays a crucial role in the defense against viral infections. This study aimed to investigate the presence of enterovirus and HLA class I expression in one of the largest HT thyroid tissue cohorts to date. In addition, viral receptors and viral immune response proteins were examined.

Methods: Thyroid tissue samples from 46 HT patients were obtained using core needle biopsy. Thyroid tissue collected during neck surgery for other reasons than thyroid autoimmunity served as controls. Standard immunohistochemistry on formalin-fixed, paraffin embedded tissue samples were used to detect HLA class I, enteroviral capsid protein 1 (VP1) and coxsackie and adenovirus receptor (CAR) in thyroid cells. A subset of the samples was examined with combined immunofluorescence staining for signal transducer and activation of transcription 1 (STAT1) and protein kinase R (PKR).

Results: Significantly more HLA class I positive samples were found in the HT group (31 out of 46 [67.4%]) than in the control group (5 out of 24 [20.8%]) ($p < 0.001$). Moreover, the semi-quantitative score assessing the grade of HLA class I expression was significantly higher in the HT group (3.9 ± 3.1) than in the control group (0.5 ± 0.9) ($p < 0.001$). In addition, STAT1 was co-localized with HLA class I, and PKR and VP1 were also found and were co-localized together. VP1 was detected in both controls and the HT samples, with slightly more VP1

positive thyroid cells in the HT samples ($20.1\pm16.4\%$) than in controls ($14.9\pm10.5\%$). Finally, the presence of CAR in thyroid cells was confirmed.

Conclusion: The current study confirmed that HLA class I hyper-expression is a defining feature of HT. Thyroid cells express CAR, thus making them susceptible to enterovirus infection. The co-localization of HLA class I with STAT1 and VP1 with PKR indicates an intracellular, antiviral host response. These findings support the concept of a firm link between viral infection and autoimmune thyroid diseases.

Introduction

Hashimoto's thyroiditis (HT) is a common autoimmune disease characterized by the generation of autoantibodies to thyroglobulin and thyroid peroxidase, lymphocytic infiltration of the thyroid gland, and for some patients, overt hypothyroidism. A strong genetic component has been established (1, 2), implicating both thyroid specific susceptibility genes and genes involved in immunoregulatory mechanisms (3, 4). In addition to genetic influences, environmental factors are also believed to play an important part in the development of HT. Despite this, the etiology of HT remains unknown, and the immunological pathway leading to the characteristic lymphocytic displacement of thyroid cells has yet to be fully described. Evidence of an association between autoimmune thyroid diseases (AITD) and viral infections is, however, emerging. (5-12). Still, the evidence linking viral infection to disease development is limited and direct investigation of thyroid tissue for evidence of antiviral responses has rarely been undertaken. Nevertheless, an immunological thyroid tissue response similar to that seen in viral infection has previously been described in the current cohort of 46 HT patients (13). Therefore, this study aimed to augment these findings by further investigation of viral receptors, antigen presentation, and viral immune response proteins.

Coxsackie and adenovirus receptor (CAR) is a transmembrane glycoprotein that serves as the main cellular entry route for Coxsackie B virus and adenovirus. In addition, more complex functions involving endothelial tight junction regulations and epithelial permeability have been identified (14, 15). Four of five known isoforms of CAR bind enterovirus, but only two

of the isoforms (CAR-SIV and CAR-TVV) contain a transmembrane domain, thus making them able to mediate an intracellular infection. RNA sequencing studies recently demonstrated that the SIV-isoform was the most highly expressed CAR isoform in the thyroid (16).

The major histocompatibility complex (MHC) class I, also called the human leukocyte antigen (HLA) class I in humans, plays a crucial role in the defense against viral infections. Once a virus enters a cell, peptides derived from virally synthesized proteins are presented by HLA class I on the cell surface to circulating CD8⁺ T cells. This leads to an immune response resulting in destruction of the infected cell. Although all nucleated cells express HLA class I, the molecule complex can only be stably expressed on the cell surface when loaded with peptide (17). Additionally, its expression can be affected by viral infections, with some viruses downregulating HLA class I expression, while others cause an upregulation of HLA class I expression (18-25).

Signal transducer and activator of transcription 1 (STAT1) and protein kinase R (PKR) are antiviral proteins activated by interferons and the viral product dsRNA respectively (26-29). Upon activation by interferons, STAT1 translocates from the cytoplasm to the nucleus and initiates transcription of antiviral interferon-stimulated genes (30-33). Indeed, one of the interferon-stimulated genes transcribed in response to activated STAT1 is PKR. PKR modifies and interacts with STAT1, although the effects of PKR on STAT1 are not fully understood. Nevertheless, studies have shown that PKR-deficient cells display reduced and defective STAT1-dependent functions, including transactivation and interferon-stimulated phosphorylation of STAT1 (34). Interestingly, STAT1 activation has been found to underpin HLA class I upregulation by virally induced interferons.

We previously reported an increased expression of the downstream interferon type 1 response protein, myxovirus resistance protein 1 (MxA) as well as increased numbers of CD8+ T cells and plasmacytoid dendritic cells in this collection of HT thyroid tissue and in Graves' disease (13, 35). Furthermore, enteroviral capsid protein 1 (VP1), which is found in all enteroviruses, have been reported in thyroid cells in both Graves' disease (10) and in the current HT cohort (13).

On this basis, we hypothesized that thyroid cells are susceptible to enteroviral infection and that virus elicits an immune response in thyroid tissue that triggers autoimmunity. In the current study of thyroid tissue from HT patients, our aims were to: (i) investigate the presence of CAR-receptor; (ii) assess HLA class I expression; (iii) explore the presence of STAT1 and PKR; and (iv) re-investigate the presence of VP1. Using immunohistochemistry (IHC), we assessed the expression of CAR-SIV, HLA class I, STAT1, PKR and VP1 in thyroid tissue from a large cohort of mainly newly diagnosed HT patients.

Material and Methods

Study participants and thyroid tissue collection

For this study, we used previously collected tissue samples. As reported earlier, thyroid tissue from 46 patients with HT (defined as anti-thyroid peroxidase antibodies (TPO-Ab) >34 kIU/L with or without thyroid dysfunction) and 24 controls were collected at Oslo University Hospital (13). Age, gender, duration from diagnosis to thyroid biopsy, TPO-Ab, TSH and

thyroid function tests (free T4 [FT4] and free T3 [FT3]) were registered (Table1). HT patients were divided into three subgroups according to thyroid function tests: overt hypothyroidism ($\text{TSH} \geq 10 \text{ mIU/L}$), subclinical, non-treated hypothyroidism ($3.6 \text{ mIU/L} \leq \text{TSH} \leq 10 \text{ mIU/L}$ and $\text{FT4 } 8\text{-}20 \text{ pmol/L}$) and non-treated prethyroiditis (presence of TPO-Ab and normal thyroid function tests).

Tissue samples from HT patients were collected by core needle biopsy. Thyroid tissue samples from 24 patients undergoing neck surgery for other reasons than AITD, i.e. thyroid tumors or parathyroid adenomas, served as controls. We insured that all controls had no pre-existing or unrecognized thyroid autoimmunity by measuring serum TPO-Ab and antibodies against TSH-receptor and thyroglobulin.

The thyroid specimens were taken from normal thyroid tissue, adjacent to the pathological lesion. The formalin-fixed, paraffin embedded (FFPE) tissue samples were cut into $3 \mu\text{m}$ slices and mounted on slides for further analysis. The Regional Ethics Committee approved the study and written informed consent was obtained from all participants.

Nine (19.6%) HT patients had other autoimmune diseases (*rheumatoid arthritis* $n=2$, *celiac disease* $n=2$, *type 1 diabetes* $n=1$, *type 1 diabetes and celiac disease* $n=1$, *pernicious anemia* $n=1$, *ankylosing spondylitis* $n=1$, *systemic lupus erythematosus and Sjogren's syndrome* $n=1$). Only one HT patient received immunosuppressive therapy (steroids) before inclusion in the study. Two patients in the control group (8.3%) had an additional autoimmune disease (ulcerous colitis $n=1$ and psoriasis $n=1$). None of the control patients received immunosuppressive therapy before study inclusion.

Immunohistochemistry

HLA class I and VP1 immunostaining was performed with a standard IHC protocol. Antigens were unmasked by heating in 10mM citrate buffer pH6.0, in a pressure cooker in a microwave oven on full power (800W) for 20 minutes, followed by 20 minutes of cooling at room temperature. Sections were blocked with 10% goat serum and primary antibodies (supplemental data) diluted in Dako REAL antibody diluent (Agilent S202230). The Dako anti-enteroviral VP1 (5D8/1 clone) was used at a dilution of 1:1400 (55ng/ml) for 30 minutes. The HLA class I primary antibody (Abcam class I HLA [EMR8/5] Ab70328) was used at a dilution of 1:1500 for one hour. CAR-SIV (Abcam Ab100811 antibody) was used at a dilution of 1:1000 for two hours. Primary antibodies were visualized using the Dako REAL EnVision Detection system (Agilent K5007).

Combined Immunofluorescence

A subgroup of samples were immunostained (supplemental data) sequentially with STAT1 (Ab109320, dilution 1:500, overnight), followed by HLA class I (1:1000, 1hour). The same subset of cases were stained simultaneously for PKR (Abcam ab32052 at a dilution of 1:700, overnight) and VP1 [Dako anti-enteroviral VP1 (5D8/1 clone), 1:1000 dilution, overnight primary incubation]. Primary antibodies were detected using species-specific secondary antibodies conjugated to either AlexaFluor® 488 or AlexaFluor® 555 as appropriate (Invitrogen, UK).

Image Acquisition and Analysis

Bright-field image acquisition and analysis were performed on a Nikon 50i Microscope fitted with a DS-Fi camera and a DSL2 camera control unit. Images were captured and analyzed using the ImageJ platform. Immunofluorescence image collection and processing were achieved using a Leica DM4000 B LED upright fluorescence microscope and Leica Image analysis software (LASX).

Immunohistochemical analysis

The slides were analyzed by light microscopy on 400X magnification by two independent scientists (TW and SJR). First, samples were classified as positive or negative for HLA class I. Only immunostaining of thyroid follicular cells (thyrocytes) was evaluated. Next, HLA class I immunoreactivity was graded according to the semi-quantitative Allred scoring system. The Allred scoring system takes both intensity (0 to 3) and proportion (0 to 5) into account, with eight being the maximum score possible and 0 being the lowest score. This system is used commonly in clinical settings to assess the immunostaining of pathological specimens (36).

We assessed VP1 staining by counting all positively stained thyrocytes alongside the total number of thyrocytes in ten consecutive counting grids (0.058 mm²) on 400X magnification, thus yielding a percentage of positively stained thyrocytes. The immunostaining and interpretation were both performed in a blinded fashion.

Statistics

Results are presented as mean and standard deviation, or as numbers and percentages. We calculated statistical significance of differences between mean values with the independent samples T-test if fulfilling criteria of normality, and with the Mann-Whitney U test if not. Pearson's chi-square test was used to determine the statistical significance of differences in proportions. Associations were explored using binary logistic regression and Spearman's correlation. All analyses were performed using IBM SPSS Statistics version 25 and GraphPad Prism 7.02. We considered p values less than 0.05 significant.

Results

Few studies have characterized the immunological responses in thyroid tissue from HT patients. In the present study, we found immunologically active HLA class I and several antiviral immune response proteins. Moreover, we confirmed that thyrocytes are susceptible to enterovirus infection.

Coxsackie and adenovirus receptor is expressed in thyrocytes

In order to explore whether thyrocytes might be susceptible to enterovirus infection, we analyzed the expression of CAR. We chose to examine the SIV-isoform, since this is known to mediate intracellular infection and was recently shown to be the most highly expressed CAR

isoform in the thyroid by RNA sequencing (16). We found CAR-SIV in thyrocytes in all ten samples studied. CAR-SIV had a distinct granular expression (**Fig.1A**).

HLA class I is upregulated in HT

HLA class I presents both endogenously and exogenously derived antigens to CD8+T cells of the immune system, and plays a vital role in the body's defense against virus. In the present study, we demonstrate that compared to controls, HLA class I is upregulated in the thyrocytes of HT patients (**Fig.1B** and **Fig.1C**). We found HLA class I positive thyrocytes in 31 out of 46 (67.4%) thyroid tissue samples in the HT group and in five out of 24 (20.8%) thyroid tissue samples in the control group ($p<0.001$). All three patient subgroups had a significantly higher number of HLA class I positive samples than controls. Among the subgroups, thyroid tissue from patients with overt hypothyroidism had the highest proportion of HLA class I positivity with 11 out of 15 (73.3%) being positive (**Fig.2A**). In the subclinical hypothyroidism group, nine out of 14 samples were positive (64.3%) and in the prethyroiditis group, 11 out of 17 samples were positive (64.7%) (**Fig.2A**). There were no statistically significant differences between the numbers of HLA class I positive samples among the three subgroups.

The mean HLA class I expression score (Allred score) was 3.9 ± 3.1 in HT patients and 0.5 ± 0.9 in controls ($p<0.001$) (**Fig.2B**). Each subgroup had a significantly higher HLA class I expression score compared to the control group: 4.7 ± 3.3 in the subgroup with overt hypothyroidism, 3.0 ± 2.6 in the prethyroiditis group, and 4.1 ± 0.8 in the subclinical

hypothyroidism group. There were no statistically significant differences in HLA class I expression score between the three subgroups.

When considering only the HLA class I positive samples (HLA class I expression score ≥ 1), there was still a significant difference in HLA class I expression score between HT patients (5.7 ± 1.8) and controls (2.2 ± 0.4) ($p < 0.001$, 95% CI 2.8, 4.3). Moreover, there were no samples with HLA class I expression score > 3 in the control group. There was a significant positive correlation between TSH and HLA class I expression score ($\rho = 0.458$, $p = 0.001$) and a significant negative correlation between FT4 and HLA class expression score ($\rho = -0.379$, $p = 0.009$). However, when using binary logistic regression TSH level and FT4 level did not influence HLA class I positivity.

TPO-Ab is the hallmark antibody in HT, and higher TPO-Ab titer levels have been associated with an increased likelihood of developing manifest hypothyroidism (37). There was a wide range of TPO-Ab levels within the HT group, ranging from 53 kIU/L to 22795 kIU/L (1320.7 ± 3330.0 kIU/L) (**Table1**). TPO-Ab levels were higher (7708 kIU/L ± 4012) in HLA class I positive HT patients, than in HLA class I negative HT patients (520 kIU/L ± 356), however the difference did not reach statistical significance ($p = 0.077$) (**Fig.3**). There was, a significant correlation between TPO-Ab and HLA class I expression score ($\rho = 0.358$, $p = 0.015$). Assessed with binary logistic regression, TPO-Ab, TSH, FT4 and age did not significantly influence HLA class I positivity.

Antiviral tissue response proteins

STAT1 and PKR are both antiviral proteins induced by interferons or viral products. The antiviral enzyme Protein kinase R (PKR) was detected in three out of six HT samples and in one out of four control samples. In two of the cases, PKR was co-localized with VP1 (**Fig.4A and Table 2**). STAT1 is a cytoplasmic protein, which upon activation relocates to the nucleus and initiates transcription of antiviral response factors. We analyzed STAT1 in a subset of samples (six HT and four controls). We found STAT1 in five out of six HT samples and in two out of four control samples (**Table 2**). STAT1 expression was co-localized with HLA class I (**Fig.4B**), and both cytosolic and nuclear STAT1 expression was observed.

VP1 detection

We confirmed earlier findings indicating the presence of VP1 in HT, with no significant difference between patients and controls (10). However, there were more, albeit not significantly, VP1 positive thyrocytes in the HT samples (20.1 ± 16.4) than in the control samples (14.9 ± 10.5) (**Fig.5A**). VP1 immunoreactivity was found in 10 out of 46 samples (21.7%) in the HT group, and in 10 out of 24 samples (41.7%) in the control group. Eight of 46 (17.3%) HT samples had both HLA class I positivity and VP1 immunoreactivity, whereas only two out of 24 (8.3%) of the controls had both HLA class I and VP1 immunoreactivity ($p=0.304$) (**Fig.5B**). There was a significantly higher proportion of HT samples (23 out of 46, 50.0%) that were VP1 negative and HLA class I positive, compared to controls (3 out of 24, 12.5%) ($p=0.002$) (**Fig.5B**). There was no significant correlation between HLA class I expression score and the number of VP1 positive thyrocytes.

Myxovirus resistance protein and CD8+ T cells

We previously reported an increased expression of the type 1 interferon downstream response protein MxA and an increased density of CD8+ T cells in this HT cohort (13). When combining previous MxA results with the current HLA class I findings, we found a significant positive correlation between HLA class I score and number of MxA positive cells per mm² thyroid tissue ($p = 0.572$, $p=0.001$). Moreover, upon examination of HT samples only, we found significantly more MxA positive cells in the HLA class I positive samples (112.8 ± 177.7) than in the HLA class I negative cases (5.0 ± 5.7) ($p=0.030$) (**Fig.6A**). Additionally, there was a significant correlation ($p=0.605$, $p<0.001$) between HLA class I expression score and CD8+Tcells per mm². Moreover, CD8+T cells were found in the HLA class I positive HT samples, but not in the HLA class I negative HT samples (**Fig.6B**).

Discussion

Our main findings were that (i) the CAR-SIV isoform is present in thyrocytes; (ii) HLA class I is upregulated in thyrocytes of HT thyroid tissue; (iii) both STAT1 and PKR are present in thyrocytes from HT patients; and lastly, (iv) that VP1 is present in thyroid tissue from both HT patient samples and controls.

We report an upregulation of HLA class I in all three clinical stages of HT, in a large collection of thyroid tissue samples from mainly newly diagnosed HT patients (**Fig.1B** and **Fig.2A**). Our results support previous findings of upregulated HLA class I in HT (38, 39). There was a

significant correlation between thyroid function and HLA class I expression; lower thyroid function levels were associated with higher HLA class I expression scores. However, TSH level and FT4 level did not influence HLA class I positivity.

Thyroid cell lines have previously been successfully infected by enterovirus (40). We detected a granular distribution of CAR-SIV in all investigated thyroid samples, thus confirming that thyroid cells are potentially susceptible to infection by enteroviruses (**Fig.1A**). Interestingly, a granular pattern of the specific SIV-isoform of CAR detected in our samples was recently detected in abundance in pancreatic beta cells where it was mainly associated with insulin secretory granules, suggesting a novel role for the receptor in granule maturation and trafficking (16). In thyroid tissue, it remains to be explored whether CAR-SIV serves this and/or other functions separate from mediating viral entry. VP1 was found in both HT patient samples and control samples, thus adding to the evidence of thyroid tissue being susceptible to enterovirus.

STAT1 expression was monitored using an antiserum, which detects both phosphorylated and non-phosphorylated forms and was present in both the cytosol and nucleus of thyrocytes, and its expression correlated with HLA class I (**Fig.4B**). Viral infection induces production of type 1 interferons by plasmacytoid dendritic cells, and HLA class I is upregulated in response to interferon- α or interferon- γ in a manner, which can be dependent on STAT1 activation (41). Our results support these findings, as HLA class I was found in the same thyroid cells as STAT1 (noting that we were not able to distinguish between phosphorylated STAT1 and total STAT1). Moreover, both plasmacytoid dendritic cells and MxA were previously found in the same thyroid tissue samples as those used in this

study (13). In addition, we demonstrate the presence of PKR, an antiviral enzyme that is known to interact with STAT1. Finally, we found co-localization of PKR and VP1, implying that enteroviral infection may lead to PKR induction (**Fig. 4A**).

With the methodology provided in this study, we did not find convincing evidence of current enterovirus infection in most of the samples. However, as previously reported by our group, tissue destruction, lymphocyte infiltration and few remaining thyroid cells were found in HT thyroid specimens, even in early stages of the disease (13). Therefore, we cannot exclude that enterovirus infection occurred earlier.

We hypothesize that the increased HLA class I expression is due to an upregulated immune response following viral infection. MHC class I, Natural Killer (NK) cells and interferons are all essential for antiviral defense. MHC class I presents viral fragments to CD8⁺ T cells, which upon activation, initiate cell destruction by releasing interferons. In contrast to CD8⁺ T cells, NK cells are not dependent on the MHC complex to identify and destroy infected cells. On the contrary, the “killing” action of NK cells is inhibited by self-MHC class I, because it activates an inhibitory receptor on NK cells. Thus, expression of MHC class I is fundamental for evading NK-mediated destruction. A number of viruses have developed immune escape strategies in order to establish themselves within the host. Several of these strategies are directed against antigen presentation by MHC class I. Some viruses such as cytomegalovirus, downregulate MHC class I to avoid CD8⁺ T cell mediated attacks (18). However, common viruses such as hepatitis C virus, flavivirus, herpesvirus, rhinovirus, and hantavirus induce upregulation of MHC class I (19-25).

Aberrant expression of HLA class II in AITD thyroid tissue is reported, arguing that thyroid cells are complicit in inducing the autoimmune response (42). Moreover, several HLA class II genes are correlated with autoimmune susceptibility. On the other hand, the role of HLA class I in autoimmune diseases is less firmly established. Nevertheless, it is shown that specific HLA class I alleles are associated with type 1 diabetes (43). As far as we know, this association has not been studied in autoimmune thyroiditis.

Viral infections have been implicated as triggers for various autoimmune diseases; type 1 diabetes being the most studied. Emerging evidence points to enteroviruses as being infective agents that can give rise to persistent infection in the pancreas, and ultimately lead to autoimmune destruction of beta cells (44-48). Enterovirus, hepatitis C virus, herpesvirus, parvovirus and many other viruses have all been associated with development of AITD (5-9, 12). HT and type 1 diabetes share many common features; for example, active endocrine cells are destructed in both diseases, and the absence of the hormone normally produced by the destroyed cells, ultimately causes disease. Interestingly, HLA class I upregulation in islet cells from patients with type 1 diabetes has recently been confirmed at both the protein and RNA levels (49-51). Five controls had an increased HLA class I expression, one of which had a preexisting autoimmune disease. However, the intensity and proportion of HLA class I staining was lower in the controls (HLA class I score <3) compared to HT patients. We cannot exclude that the five controls with increased HLA class I are at risk for developing autoimmune diseases.

We demonstrate not only increased HLA class I expression, but also the presence of the virus receptor CAR-SIV (**Fig.1A**) and the viral response proteins STAT1 (**Fig.4B**) and PKR (**Fig.4A**) in

thyroid tissue from HT individuals. Moreover, there was a strong correlation between previously found CD8+ T cells and HLA class I in this cohort (**Fig.6B**). This suggests that the upregulated HLA class I found in our samples may present antigen to CD8+ T cells and thereby prompt an immunological response. Overall, this can be interpreted as part of the immune response to viral infection. Interestingly, a recent study showed that transfection of a thyroid cell line with dsRNA caused upregulation of HLA class I , PKR, STAT1, toll like receptor 3, and other immune response proteins associated with viral infections (52), thus confirming that viral products can cause a response in thyroid cells similar to our findings. Nevertheless, there is still a lack of substantial evidence of viral presence in thyroid tissue.

We were not able to detect enterovirus with the limited methods used in this study. However, the infection might occur on such a modest scale, that it largely escapes detection with antibody-based techniques. It is difficult to provide unequivocal proof of the presence of virus in tissue samples using indirect methods. In addition, extensive fibrosis, tissue destruction and lymphoid displacement in thyroid tissue limit the possibility of finding virus. Our group previously reported the results for EV RNA by *in situ* hybridization with similar results to that for VP1. (13). Finally, the timeline of disease progression in HT is difficult to establish (for example, some patients have high titers of TPO-Ab but never develop overt hypothyroidism). We argue that viral infection might initiate the autoimmune process, but that the primary infection is cleared or undetectable by the time of clinically significant disease. A recent study found that patients with a known genetic predisposition for AITD increased their expression of another AITD susceptibility gene when exposed to products of microbial infection (53). This interplay of genetic predisposition and infectious insults could

explain why common infections give rise to autoimmunity in some but are negligible in others.

Our study has several limitations. Due to limited resources, we did not analyze PKR, STAT1 or CAR-SIV in all samples. Moreover, the size of the control group was limited.

In conclusion, we report the presence of the viral receptor CAR-SIV, in thyroid tissue. We also confirm that HLA class I upregulation is a significant feature of Hashimoto's thyroiditis. In addition, we demonstrate that STAT1 is co-localized with HLA class I in thyrocytes and that PKR is co-localized with VP1, which is potentially indicative of an intracellular host antiviral response. Taken together with the previously reported increase in MxA, plasmacytoid dendritic cells and CD8+ T cells in the same cohort, our results support the hypothesis of an association between enteroviral infections and HT. Our study implies that antigen presenting HLA class I elicits a CD8+ T response, and activates interferon downstream responses such as MxA, PKR and STAT1 induction in HT thyroid tissue. However, the pathogenesis of HT is complex and our knowledge of the numerous immunological pathways leading to lymphocytic displacement of thyroid cells is still limited. Hence, we call for further clarification of the pathogenesis and final confirmation of viral presence in Hashimoto's thyroiditis.

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Author Contributions

T.W. contributed to IHC examinations, data collection, analysis, and interpretation; and drafting of the manuscript. S.S.H. contributed to all parts of the study; study design, clinical

coordination and patient recruitment; data collection, analysis, and interpretation; and drafting of the manuscript. T.P. contributed to the surgery and writing of the manuscript. S.J.R. and N.G.M., contributed to the IHC analysis, data analysis and interpretation and writing the manuscript. K.D.-J., as the principal investigator of the study, had the initial idea of the study and contributed to the study design; funding; regulatory issues; international collaboration; data collection, analysis, and interpretation; and writing of the manuscript. T.W., S.S.H. and K.D.-J. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Author disclosure statement

The authors have nothing to disclose, and no competing financial interests exist.

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Table 1. Patient characteristics

	Prethyroiditis (n=17)	<i>p</i> <i>Value</i>	Subclinical hypothyroidism (n=14)	<i>p</i> <i>Value</i>	Hypothyroidism (n=15)	<i>p</i> <i>Value</i>	Control group (n=24)	<i>Reference range</i>
Duration (months)	0.0 (24)	-	1.2 (48)	-	3.0 (180)	-	-	-
Female n(%)	18 (88.2)	0.715	12 (85.7)	0.564	15 (100)	0.251	22 (91.7)	-
Age (years)	45.3±10.7	0.115	41.7±17.5	0.059	42.9±10.1	0.041	52.0±14.4	-
TSH (mIU/L)	1.9±0.9	0.218	5.3±1.2	<0.001	29.6±36.0	<0.001	1.5±0.9	0.5-3.6
FT4 (pmol/L)	13.7±1.7	0.185	12.4±1.5	0.282	11.3±5.5	0.078	13.2±2.0	8-20
TPO-Ab (kIU/L)	903.8±997.7	<0.001	596.1±514.4	<0.001	2469.5±5666.6	<0.001	<35	<35

Duration is the time from diagnosis to biopsy. Values are presented as mean±SD , numbers (%) or median and range (duration) ; FT4, free T4; TPO-Ab, thyroid peroxidase antibody. P values compared to controls and tested with Student's T-test, Mann-Whitney U test or Pearson's chi-square test.

Table 2. Sequential immunofluorescence in a subset of the samples

	HLA class I	STAT 1	PKR	VP1
Case 1	Negative	Negative	Negative	Negative
Case 2	Positive	Positive	Negative	Negative
Case 3	Positive	Positive (low)	Positive	Positive
Case 4	Positive	Positive	Positive	Negative
Case 5	Positive	Positive (low)	Positive	Positive
Case 6	Positive	Positive	Negative	Negative
Control 1	Negative	Negative	Negative	Negative
Control 2	Negative	Negative	Negative	Negative
Control 3	Positive	Positive	Positive	Positive
Control 4	Positive	Positive	Negative	Negative

Ten samples were stained sequentially with immunofluorescence for HLA class I, STAT1, PKR and VP1. The table shows that some samples were positive for HLA class I, in addition to the antiviral proteins STAT1 and PKR. A few samples were even positive for HLA class I, STAT1, PKR and the viral capsid protein VP1.

Fig.1.

Fig.2.

Fig.3

Fig.4

Fig.5.

Fig.6

HLA class I in HT - Figure Legends

Fig.1. Immunohistochemical staining of CAR and HLA class I of thyroid tissue samples from HT patients and controls

CAR-SIV was found in thyrocytes of all samples studied, both HT and controls. (A) An example of staining of CAR-SIV in normal thyroid tissue. The inset represents higher magnification (of the area outlined by the black box) and clearly shows an intracellular, granular staining pattern. Scale bar 25 μ m. (B) Positive HLA class I staining (brown color) in thyroid follicular cells in an HT sample. (C) Negative HLA class I staining in a control sample. All samples stained with a standard horseradish peroxidase immunohistochemistry protocol (see supplemental data for antibodies).

Fig.2. HLA class I immunodetection and HLA class I expression score

All three clinical subgroups showed a significantly higher number of HLA class I positive samples compared to controls. (A) Proportion of samples with HLA class I positivity within the three clinical subgroups of HT. (B) HLA class I expression score (Allred score), taking both immunostaining intensity and proportion of stained tissue into account (maximum score 8, and lowest score 0). Bars represent median and 95% CI. ** $p \leq 0.01$, *** $p \leq 0.001$.

Fig.3. HLA class I and association with TPO-Ab

TPO-Ab titers were higher in HLA class I positive HT patients than in HLA class I negative HT patients. However the difference did not reach statistical significance. Bars represent median and 95% CI.

Fig.4. Combined immunofluorescence of PKR/VP1 and HLA class I/STAT1

(A) An example of PKR (red) and VP1 (green) immunofluorescence staining in HT thyroid tissue. The insets represent higher magnification (of the area outlined by the white and yellow boxes) and shows co-localized VP1 and PKR. (B) An example of co-localized HLA class I (red) and STAT1 (green) immunofluorescence staining in HT thyroid tissue. (C) HLA class I (red) and STAT1 (green) immunofluorescence staining in thyroid tissue from a control. Merged images with blue nuclear DAPI staining, all scale bars at 25 μm .

Fig. 5. VP1 assessment in HT samples and controls

(A) Number of VP1-positive thyrocytes in HT group and controls. There were more VP1 positive thyrocytes in the HT samples than in the control samples. (B) Proportion of double positive samples (VP1+/HLA I+) and VP1-negative and HLA I positive samples in HT (VP1-/HLA I +) and controls. There was a significantly higher proportion of HLA I positivity in VP1 negative HT samples compared to VP1 negative controls. Bars represent mean and 95%CI. ** $p \leq 0.01$.

Fig.6. HLA class I positivity and associations with previously analyzed MxA and CD8+ T cells

(A) Number of MxA-positive cells per mm^2 in HLA class I positive and HLA class I negative HT samples. The number of MxA positive cells was significantly higher in the HLA class I positive samples than in the HLA class I negative samples. (B) Number of CD8+ T cells per mm^2 in HLA class I positive and HLA class I negative HT patients. CD8+T cells were only found in the HLA class I positive HT samples. Bars represent median and 95% CI. * $p \leq 0.05$, *** $p \leq 0.001$.